Pak. J. Agri. Sci., Vol. 55(2), 417-422; 2018
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI: 10.21162/PAKJAS/18.2829
http://www.pakjas.com.pk

EFFECT OF METHYL TESTOSTERONE ON THE BODY COLOR OF CHINESE BITTERLING (*Rhodeus sinensi*)

Xiao-jiang Chen[1], Wei Li[1], Yong-yong Zhu[2] and Quan Wang[1,*]

[1]Jiangsu Agri-Animal Husbandry Vocational College, Taizhou, 225300, Jiangsu Province, China;
[2]Chongqing University of Education, Chongqing, 400067, China
***Corresponding author's e-mail: cq_cxj@126.com**

In order to investigate the effect of methyl testosterone on the body color, *Rhodeus sinensis* of juveniles (fifty days post-fertilization, 50-DPF) and adults (eighty days post-fertilization, 80-DPF) were divided into six groups, each treatment group cultured in duplicate, randomly and separately, with 80 ind each. All juveniles and adults were raised, respectively, in the recirculation aquarium tanks with 500 liters separately. Five treatment groups (TG_1, TG_2, TG_3, TG_4, and TG_5) were dieted with 17α-methyl testosterone (17α-MT) at different concentration of 10, 20, 30, 40 and 50 mg/kg, while the control group was dieted without 17α-MT. During the 56 days of test duration, the carotenoid content of the test fishes and the carotenoid deposited in the muscle were researched comparatively. The results showed there was significant pigmentation for 50-DPF *Rhodeus sinensis* dieted with 17α-MT at concentration of 40 mg/kg and 50 mg/kg ($p < 0.05$). When *Rhodeus sinensis* dieted with 17α-MT at concentration of more than 30 mg/kg, the body color of 50-DPF *Rhodeus sinensis* started to change obviously at the 28th day ($p < 0.05$), then sustained coloring to the maximum at the 56th day. While for 80-DPF *Rhodeus sinensis*, there was no significant differences between each treatment group and control group ($p > 0.05$)
Keywords: 17α-methyl testosterone; body color; carotenoid; *Rhodeus sinensis*

INTRODUCTION

Androgen analogous, which were of natural or synthetic, were a typical endocrine disruptor (Lee *et al.,* 2007; Zheng *et al*., 2008). Natural androgens include testosterone and its derivatives, such as androsterone, androstenedione etc. Environmental androgens would disturb the normal aquatic endocrine, that would result in higher proportion of male, male-secondary-sexual-characteristics in females, occurrence of ovaries and testes, suppressed the induction of vitellogenin, a decrease of reproductive capacity (Soto *et al*., 2004; Orlabdo *et al*., 2004; Örn *et al*., 2006) and appearance of masculinization characteristics in mammals (Hotchkiss *et al*., 2002; Hotchkiss *et al*., 2007a, b) were proved.

17α-MT was a synthetic androgen of white crystalline powder, which was used to instead of androgen. It would promote and maintain the maturation of male with male-secondary-sexual-characteristics. 17α-MT at different concentration had different biological effects on different fishes (Zhang *et al*., 2001). Lower concentrations of 17α-MT were supposed to promote the growth of fishes, while higher concentrations would cause deformities and deaths of fishes (Chu *et al*., 2006). Rivero-Wendt's researches on endocrine disrupting effects of 17α-MT showed that methyl testosterone would significantly inhibit the development of mature oocytes, and changes of gender (Rivero-Wendt *et al*., 2013). *Perca fluviatilis*, *Paralichthys olivaceus*, *Epinephelus*, *Oncorhynchus mykiss*, *Gadus morhua* and other fishes would be functional male with genomic female, for a few months of feeding or implanting exogenous androgen (Glamuzina *et al*., 1998; Kitano *et al*., 2000; Rougeot *et al*., 2002; Atar *et al*., 2009; Haugen *et al*., 2011). So far, more than 47 species of fishes in 15 families were tried in ideal results.17α-MT has the best effect in the induction of female into male, among the 31 kinds of tested hormones (Pandian *et al*., 1995). It was reported that *Xiphophorous helleri*, *Molliensia Veliferia* and *Brachydanio rerio* would be with a brighter body color by feeding with 17α-MT (Li *et al*., 1999; Zhou *et al*., 2000; Huang, 2008).

Rhodeus sinensis is one of famous native fishes in China. In 1960s, it was introduced into Europe as aquarium fish, which was known as China rainbow. Researches on *Rhodeus sinensis* were focused on biological habit (Pateman-Jones *et al*., 2011; Akai *et al*., 1998; Zhang *et al*., 2002; Guan *et al*., 1991; Shen, 2000; Zeng *et al*., 2006; Wang *et al*., 2012) and molecular biology (Ueda *et al*., 1997; Saitoh *et al*., 2000; Yang, 2013). Researches on the body color changes of *Rhodeus sinensis* induced by adding methyl testosterone in diet were rare. In this paper, diets of different levels of methyl testosterone was fed to *Rhodeus sinensis* of juveniles (fifty days post-fertilization,50-DPF) and adults (eighty days post-fertilization, 80-DPF) to investigate the effects on the body color of *Rhodeus sinensis*, which were scaled by carotenoid content, to provide a theoretical basis for the development of ornamental *Rhodeus sinensis*.

MATERIALS AND METHODS

***Culture conditions and treatment periods*:** This study was carried out in Jiangsu Agri-animal Husbandry Vocational College. The parents of *Rhodeus sinensis* was selected from Lixiahe region. Fishes, which were of the same size, same color, were transferred into indoor water recirculation system (Fig. 1), with river water which was 24-25℃, with dissolved oxygen concentration of 6.0-8.0 mg l^{-1}, pH level of 7.5-8.0 (Chen *et al*., 2013a and b). The treatments were taken at juveniles and adults (50-DPF and 80-DPF). At each stage, samples were divided into six groups, randomly and separately, with 80 ind each. Five groups (G_1, G_2, G_3, G_4 and G_5) were dieted with 17α-MT at different concentration of 10, 20, 30, 40 and 50 mg per kg, while one group (Ctrl), which was taken as the control, and were dieted without 17α-MT. The fishes were fed regularly at 9:00 am and 5:00 pm daily according to their weight (Hussain *et al*., 2010). After 56-days' diets, fishes were ended and tested. All the test procedures complied with the legal requirements of People's Republic of China.

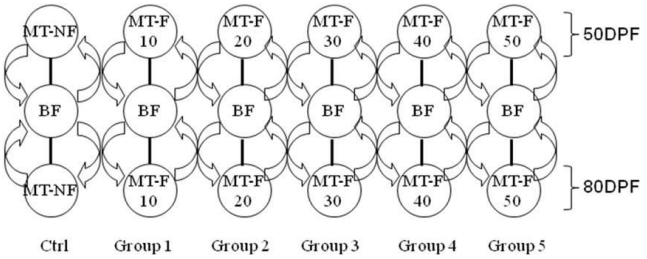

Figure 1. Schematic drawing of experimental design. Each unit represents a water recirculation system (Ctrl, TG_1, TG_2, TG_3, TG_4 and TG_5).

Each system consisted of two 500-L aquarium tanks connect with the biological filter (BF). The treatments were taken at juveniles (50-DPF) and adults (80-DPF), each treatment group be cultured in duplicate. MT-NF: Food without 17α-MT as control group (Ctrl); MT-F 10, 20, 30, 40 and 50: 17α-MT at different concentration of 10, 20, 30, 40 and 50 mg per kg. Arrows indicate water flow in the recirculation systems.

***Experimental diet*:** In this experiment, artificial formulated feed was prepared with 20% of fish meal, 17% of dry tenebrio molitor, 13% of flour, 5% of pumpkin powder, 20% of corn flour, 10% of wheat bran, 2% of kelp powder, 0.45% of *Calendula officinalis*, 0.5% of brewer's yeast, 1% of calcium biphosphate, 0.5% of betaine, 0.5% of spirulina powder, 0.5% of red chili powder, 0.5% of garlic powder, 4% of red worms powder, 2% of fish oil, 3% of soybean oil, and 0.05% of fungicide. 17α-MT stock solution of 1mg ml^{-1} was prepared in ethanol, and then mixed with diet by dilution, according to 0, 10, 20, 30, 40, 50 mg per kg.

***Drugs and equipment*:** 17α-methyltestosterone (17α-MT, with purity greater than 98%) was purchased from Sigma. Acetone, petroleum ether, and anhydrous sodium sulfate were all of analytical grade. The main instruments were UV1902 UV-Vis spectrophotometer, UV756 scanning UV spectrophotometer, TE3102S electronic balance, THZ-92B Water-bathing Constant Temperature Vibrator.

***Sample collection methods*:** Four fishes were randomly taken from each aquarium at 0, 14, 28, 42, 56 day, which were euthanized on absorbent paper and dried in a 4℃ frost-free refrigerator for 24 hours after removing the internal organs. After 24 hours, the carotenoid content of fish body was measured and hyperchromic effect was recorded. Each experiment was duplicated. The method of carotenoid extraction as follow: Fishes after freeze-dried were shredded, accurately weighed 0.4 g, and homogenized with 28 milliliter mixture of acetone and petroleum ether (2: l). Then, the homogenate was poured into a stoppered test tube, and placed in water-bathing constant temperature vibrator to extract for 8 hours. Then centrifuged at 4000 r/min for 10 min, and the supernatant was transferred to a separatory funnel, washed with 5% sodium sulfate solution repeatedly, with about 15 milliliter each time, until the lower aqueous layer became translucent. The extract was leaked into l0-mL brown volumetric flask through a small funnel containing 10% anhydrous sodium sulfate, and then washed the separatory funnel and anhydrous sodium sulfate layer with a small amount of petroleum ether several times, and the washing liquid was incorporated into the brown volumetric flask, finally added petroleum ether to 10 milliliter. The absorbance value was measured at 448 nm wavelength with petroleum ether as blank by 1 cm quartz cuvette.

***Calculation of total carotenoids*:**

$$X(mg/100g) = \frac{A \times y(ml) \times 10^5}{A_{1cm}^{\%} \times 1000 \times g}$$

X, the carotenoid content; *A*, the maximum absorbance measured at 448 nm; *y,* the volume of the extract used; $A^{\%}_{1cm,}$ the average absorption coefficient of carotenoids (2500); *g*, the weight of the sample analyzed (Gao, *et al*., 2005).

***Data processing*:** SPSS 14.0 statistical software was used for data analysis and statistics. The data was firstly analyzed with one-way analysis of variance (one-way ANOVA), if a significant difference between treatments, which analyzed with Ducan's multiple comparisons, in a differences level of 0.05.

RESULTS

***Effects of different additive amounts of 17α-MT on carotenoid content of Rhodeus sinensis*:** The total carotenoid content of each test group was shown in Table 1. After 56-days' diet, the total carotenoid content in the body of 50-DPF *Rhodeus sinensis* all have increased. The control group, 1.9217±0.2461 mg per 100g, There was no significant

difference with the treatment group 1 (TG_1), treatment group 2 (TG_2), and treatment group 3 (TG_3) ($p>0.05$), while treatment group 4 (TG_4), 3.3578±0.2215 mg/100g and treatment group 5 (TG_5), 3.4345±0.1219 mg/100g, which were significantly higher than the other treatment group ($p<0.05$). The total carotenoid content in the body of 80-DPF *Rhodeus sinensis* in the control group was 2.3241±0.3421 mg/100g, and the treatment group 4 (TG_4) had a highest total carotenoid content, 3.1812 ± 0.1825 mg/100g, but there was no significant differences between each treatment group and control group ($p> 0.05$).

Table 1. Effect of different additive amounts of 17α-MT on carotenoid content of *Rhodeus sinensis*.

Group	Additive Amount of 17α-MT (mg/kg)	Total Carotenoid Content after 56 days of test (mg/100g)	
		50-DPF *Rhodeus sinensis*	80-DPF *Rhodeus sinensis*
Ctrl	0	1.9217±0.1461[a]	2.3241±0.1421[a]
TG_1	10	2.0345±0.1212[a]	2.4223±0.1812[a]
TG_2	20	2.3121±0.1231[a]	2.6243±0.1211[a]
TG_3	30	2.5853±0.1214[a]	2.8217±0.1514[a]
TG_4	40	3.3578±0.1215[b]	3.1812±0.1825[a]
TG_5	50	3.4345±0.1219[b]	3.1513±0.1216[a]

Note: A different lowercase letter in the same column indicates significant difference ($P <0.05$), while same lowercase letters in the same column indicates no significant difference ($p> 0.05$)

***Effects of changes in feeding time of 17α-MT on carotenoid content of juveniles and adults*:** The change of total carotenoid content of 50-DPF *Rhodeus sinensis* in each treatment group was showed in Figure 2. The total carotenoid content in different treatment groups varied with the time of feeding. The treatment groups showed a similar change trend with the control group, the total carotenoid content increased with the feeding time and reached the maximum at 56 days, TG_1, 2.0345±0.1212 mg/100g, TG_2, 2.3121±0.1231 mg/100g, TG_3, 2.5853±0.1214 mg/100g, TG_4, 3.3578±0.1215 mg/100g, TG_5, 3.4345±0.1219 mg/100g. There was no significant difference between the TG_1, TG_2, TG_3 and the control group ($p> 0.05$), but the TG_4 and TG_5 showed significant difference with the other group ($p <0.05$). TG_5, showed the best coloring effect. The total carotenoid content increased rapidly at 28 days and then with a slower growth until the end of treatment. The change of total carotenoid content of 80-DPF *Rhodeus sinensis* in each treatment group was showed in Figure 3. The total carotenoid content of each group showed similar change, which increasing gradually with feeding time and reached the maximum at 56 days, TG_1, 2.4223±0.1812 mg/100g, TG_2, 2.6243±0.1211 mg/100g, TG_3, 2.8217±0.1514 mg/100g, TG_4, 3.1812±0.1825 mg/100g, TG_5, 3.1513±0.1216 mg/100g, respectively. TG_4, showed the best coloring effect, but there was no significant difference between the treatment groups and the control group ($p> 0.05$).

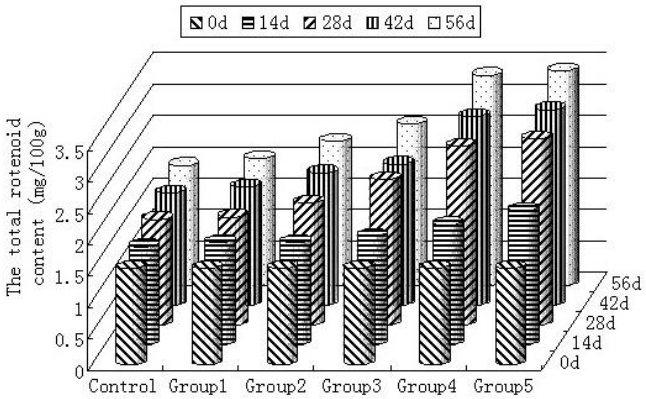

Figure 2. Dynamic changes of the total carotenoids contents under different experimental foods on 50-DPF *Rhodeus sinensis.*

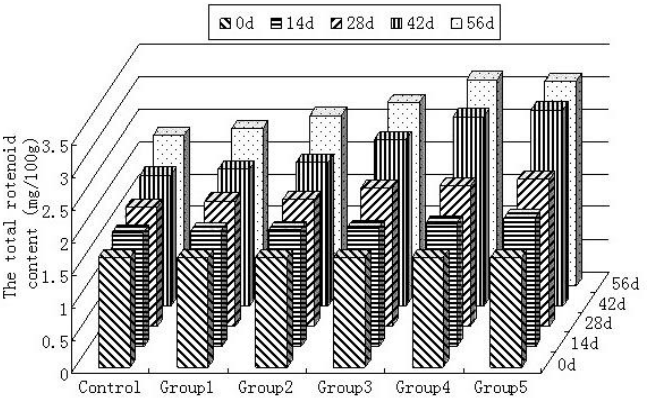

Figure 3. Dynamic changes of the total carotenoids contents under different experimental foods on 80-DPF *Rhodeus sinensis*.

DISCUSSION

***Mechanism of the effect of 17α-methyl testosterone on the body color of Rhodeus sinensis*:** The body color of fish mainly depends on the pigment cells, which was under the control of the nervous and endocrine systems in skin. Carotenoid was a kind of fat-soluble pigment, which was consist of carrot glycol, astaxanthin, lutein, zeaxanthin, etc. it would endow the fish with color from yellow to red (Biacs *et al*., 1989). Carotenoid plays an important role in maintaining the characteristic color of muscle, fin, skin, gonads, crustaceans and other tissues of aquatic animals. For example, the yellow, orange, pink, deep red body color of salmon and trout would come out depends on the deposition of carotenoid, which was absorbed from food directly or transformed into the right proform (Bell *et al*., 1998). ß-carotenoid taken from diets by female was mostly deposited as pigment in the ovaries, egg yolk, with little deposition in the body surface and muscle. With the maturation of male gonad, the β-

carotenoid was mainly deposited in the body surface and muscle, to present a colorful marriage color. That is to say the male always showed a more colorful body than the female (Li *et al*., 1999).

The development of fish gonad sex is controlled by sex hormones. Fish with little gonad differentiation dieted with 17α-MT, the gonad medulla would differentiate into testis tissue, while it would be in inhibited that the gonad cortex should differentiate into ovarian tissue (Cao *et al*., 1994). In the process of transforming female into male, the gonad cortex developed into a functional ovarian preferentially, while the spermatogonia sac still existed in the gonad medulla. When dieted with 17α-MT, the spermatogonia sac would be promoted to gradually proliferate and differentiate into sperm cells of levels, and it would turn out a gynandromorphy, with the degeneration and atrophy of oocyte as well as the development of spermatid, it would develop into a male with normal physiological function (Zheng *et al*., 1997). The lower degree of gonad differentiation, the higher effect of masculinization and better conducive to the coloring effect of carotenoid, which was consistent with this experiment results.

Effect of 17α-methyl testosterone content and feeding time on the body color of *Rhodeus sinensis*: During the 56 days of test duration. The results showed there was significant pigmentation for 50-DPF *Rhodeus sinensis* dieted with 17α-MT at concentration of 40 mg/kg and 50 mg/kg ($p < 0.05$). While for 80-DPF *Rhodeus sinensis*, there was no significant between each treatment group and control group ($p > 0.05$). Li-yun's researches about the effect of 17α-MT on body color of 3-month-old and 5-month-old *Xiphophorus helleri*, the results showed that the 3-month-old *Xiphophorus helleri* dieted with 17α-MT at concentration of 10 mg/kg deposited pigment better than that of 5-month-old *Xiphophorus helleri* (Li *et al*., 1999). Huang's researches about the effect of 17α-MT at concentration of 10, 20, 30, 40, 50mg/kg on the body color of 1-month-old and 3-month-old *Brachydanio rerio* (Huang, 2008), the results showed that the 1-month-old *Brachydanio rerio* deposited pigment better than that of 3-month-old *Brachydanio rerio*, and the lower gonad differentiation, the better coloring effect, which was agree with this experiment.

Different concentrations of 17α-MT have different biological effects on a variety of fish; 12.5 mg/kg of 17α-MT can induce a complete sex reversal in *Epinephelussp* (Fang *et al*., 1992), while low concentrations of 17α-MT (l-10 mg/kg) would affect the growth and sex reversal of *Common carp* (Hulak *et al*., 2008). In this study, 40 mg/kg and 50 mg/kg of 17α-MT have an obvious coloring effect on 50-DPF *Rhodeus sinensis*. TG_5, showed the best effect of coloring. The effects of 17α-MT called for a response time to reach the maximum after dieting, which was related to the additive amount of 17α-MT and the age of the fish. In this study, the efficacy change of 17α-MT could be seen from the change of total carotenoid content. After dieting with 17α-MT at concentration of more than 30 mg/kg for 50-DPF *Rhodeus sinensis*, the body color started to change obviously at the 28th day, and sustained coloring to the maximum until the end of experiment, this was the opposite to Huang's researches, who sustained that diet with 17α-MT on the body color at 1-month-old *Brachydanio rerio*'s total carotenoid content would reach the maximum at 14 days, then fall back.

***Conclusion*:** The authors speculate that this phenomenon may be related to the gonad development of *Rhodeus sinensis*. 17α-MT additive at concentration of 40 mg/kg have the best coloring effect on 80-DPF *Rhodeus sinensis* at the 56th day. However, there was no significant difference between each treatment groups, and the carotenoids deposition was not positively correlated with the content of 17α-MT, within 56 days. Compared with 50-DPF *Rhodeus sinensis*, 80-DPF *Rhodeus sinensis* showed a lower coloring effect. The authors speculate that the response time of methyl testosterone was different on different degrees of gonad differentiation, the earlier feeding, the lower gonad differentiation, the less response time and the better coloring effect.

***Acknowledgments*:** The authors are thankful to the Jiangsu Agri-animal Husbandry Vocational College for providing infrastructural facility and financial support (Grant NO: YB1209; Grant NO: NSFYB1407; Grant NO: NSF201711). It is grateful to Ocean and Fishery Bureau of Jiangsu Province for financial support (Grant NO: PJ2011-56; 00000215010). We would like to thank the professor Wu Zheng-li's revision. Technical support of Huang Yong-zheng and the laboratory members are duly acknowledged.

REFERENCES

Akai, Y and R. Arai. 1998. *Rhodeus sinensis,* a senior synonym of *R. lighti* and *R. uyekii* (*Acheilognathinae, Cyprinidae*). Ichthyol. Res. 45:105-110.

Atar, H.H., S. Bekcan and L. Dogankaya. 2009. Effects of different hormones on sex reversal of rainbow trout (*Oncorhynchus mykiss.* Walbaum) and production of all-female populations. Biotechnol. Biotec. Eq. 23:1509-1514.

Bell, J.G., J. McEvoy, J.L. Webster, F. McGhee, R.M. Millar and J.R. Sargent. 1998. Flesh lipid and carotenoid composition of Scottish farmed Atlantic salmon (*Salmo salar*). J. Agri. Food Chem. 46:119-127.

Biacs, P.A., H.G. Daood, A. Pavisa and F. Hajdu. 1989. Studies on the carotenoid pigments of paprika (*Capsicum annuum* L. var Sz-20). J. Agri. Food Chem. 37:350-353.

Cao, L.Q., Y.G. Tao and H.Z. Tan. 1994. Studies on unisexualization in red tilapia. J. Fish. China. 18:75-79.

Chen, X.J., Y.G. Zhu and Z.M. Zhao. 2013. The influence of micropore oxygen aeration on the pond water quality

environment. J. Food Sci. Tech. 5:1478-1482.

Chen, X.J., J. Wang, Y.Y. Zhu and J.S. Ye. 2013. Test of intercropping *Myxocyprinus asiaticus* in the *Eriocheir sinensis* Pond. J. Food Sci. Tech. 5:1361-1366.

Chu, P.S., M. Lopez, S. Serfling, C. Gieseker and R. Reimschuessel. 2006. Determination of 17α-methyltestosterone in muscle tissues of tilapia, rainbow trout, and salmon using liquid chromatography-tandem mass spectrometry. J. Agri. Food Chem. 54:3193-3198.

Fang, Y.Q., Q.M. Lin and X. Qi. 1992. Effect of 17*α*-methyltestosterone on sex inversal in *Epinephelus Akaara*. J. Fish. China 16:171-174.

Gao, G.Z., X.M. Wu, G.Y. Lu, B.Y. Chen, K. Xu and X.Z. Li. 2005. Study on mensuration of total carotenoids content in rapeseeds. J. Plant Genet. Resour. (China) 6:414-417.

Glamuzina, B., GlaviÓN and B. Skaramuca. 1998. Induced sex reversal of dusky grouper, *Epinephelus marginatus* (Lowe). Aquac. Res. 29:563-567.

Guan, T.L and B.A. Afzelius. 1991. The spermatozoon of the Chinese bitterling, Rhodeus sericeus sinensis (*Cyprinidae, Teleostei*). J. Submicrosc. Cytol. Pathol. 23:351-356.

Haugen, T., E. Andersson, B. Norberg and G.L. Taranger. 2011. The production of hermaphrodites of Atlantic cod (*Gadus morhua*) by masculinization with orally administered 17-alpha-methyltestosterone, and subsequent production of all-female cod populations. Aquaculture 311:248-254.

Hotchkiss, A.K., J.S. Ostby, J.G. Vandenburgh and L.E. Gray. 2002. Androgens and environmental antiandrogens affect reproductive development and play behavior in the Sprague-Dawley Rat. Environ. Health. Persp. 110:435-439.

Hotchkiss, A.K., J. Furr, E.A. Makynen, G.T. Ankley and L.E. Gray. 2007. In utero exposure to the environmental androgen trenbolone masculinizes female Sprague-Dawley rats. Toxicol. Lett. 174:31-41.

Hotchkiss, A.K., C.S. Lambright, J.S. Ostby, L. Parks-Saldutti, J.G. Vandenbergh and L.E. Gray. 2007. Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. Toxicol. Sci. 96:335-345.

Huang, Y.Z. 2008. Effects of 17α-methyltestosterone and carotinoids on the pigmentation of aquarium fish. Master thesis, Southwest University of China.

Hulak, M., M. Paroulek and P. Simek. 2008. Water polluted by 17 alpha-methyltestosterone provides successful male sex inversion of common carp (*Cyprinus carpio* L.) from gynogenetic offspring. J. Appl. Ichthyol. 24:707-710.

Hussain, S.M., M. Javed, S. Asghar, M. Hussain, S. Abdullah, S.A. Raza and A. Javid. 2010. Studies on growth performance of metals mixture stressed *Cirrhina mrigala* in earthen ponds. Pak. J. Agri. Sci. 47:263-270.

Kitano, T., K. Takamune and Y. Nagahama. 2000. Aromatase inhibitor and 17*α*-methyltestosterone cause sex-reversal from genetical females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (*Paralichthys olivaceus*). Mol. Reprod. Dev. 56:1-5.

Lee, L.S., N. Carmosini, S.A. Sasman and H.M. Dion. 2007. Sepulveda, M.S. Agricultural contributions of antimicrobials and hormones on soils and water quality. Adv. Agron. 93:2-67.

Li, Y., Y. W. Li, X. Xiang, X.H. Zhou and J.W. Wang. 1999. The effects of carotenoid and 17α-MT on the color of ornamental fishes. J. Southwest Agri. Univ. 21:270-273.

Orn, S., A. Svenson, T.Viktor, H. Holbech and L. Norrgren. 2006. Male-biased sex ratios and vitellogenin induction in Zebrafish exposed to effluent water from a Swedish pulp mill. Arch. Environ. Con. Tox. 51:445-451.

Orlando, E.F., A.S. Kolok, G.A. Binzcik, J.L. Gates, M.K. Horton, C.S. Lambright and L.J. Guillette Jr. 2004. Endocrine-disrupting effects of cattlefeedlot effluent on an aquatic sentinel species, the fathead minnow. Environ. Health Perspect. 112:353-3583.

Pandian, T.J and S.G. Sheela. 1995. Hormonal induction of sex reversal in fish. Aquaculture. 138:1-22.

Pateman-Jones, C., M.B. Rasotto, M. Reichard, M. Reichard, C. Liao, H. Liu, G. ZIĘBA and C. Smith. 2011. Variation in male reproductive traits among three bitterling fishes (*Acheilognathinae: Cyprinidae*) in relation to the mating system. Biol. J. Lin. Soc. 103:622-632.

Rivero-Wendt, C.L., A.L. Miranda-Vilela, M.F. Ferreira, A.M. Borges and C.K. Grisolia. 2013. Cytogenetic toxicity and gonadal effects of 17 α-methyltestosterone in *Astyanax bimaculatus* (Characidae) and *Oreochromis niloticus* (Cichlidae). Genet. Mol. Res. 12:3862-3870.

Rougeot, C., B.S. Jacob and P. Kestemont. 2002. Sex control and sex determinism study in *Eurasian perch*, *Perca fluviatilis*, by use of hormonally sex-reverses male breeders. Aquaculture. 211:81-89.

Saitoh, K., T. Ueda and R. Arai. 2000. Highly repetitive elements from Chinese bitterlings (*genus Rhodeus, Cyprinidae*). Genes. Genet. Syst. 75:349-355.

Shen, J.Z. 2000. Experimental Investigations in to Some Reproduction Aspects of Chinese Bitterling, *Rhodeus sinensi*. J. Huazhong Agri. Univ. 19:494-496.

Soto, A.M., J.M. Calabro, N.V. Prechtl, A.Y. Yau, E.F. Orlando, A. Daxenberger and C. Sonnenschein. 2004. Sonnenschein androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in eastern Nebraska USA. Environ. Health Perspect. 112:346-352.

Ueda, T., N. Mashiko and H. Takizawa. 1997. Ag-NOR variation in chromosomes of Chinese bitterlings, *Rhodeus lighti* and *Tanakia himantegus* (*Cypriniformes, Cyprinidae*). Ichthyol. Res. 44:302-305.

Wang, J.G., Q. Wang, Q. Feng, A.J. Huang, G.L. Zhu, L.W.

Xiong, X.J. Chen and Y.G. Zhu. 2012. Acute toxicity of glutaraldehyde and benzalkonium bromide on *Rhodeus sinensis*. J. Guizhou Agri. Sci. 40:157-159.

Yang, X., Z. Ma and L. Xie. 2013. Complete mitochondrial genome of the Chinese bitterling *Rhodeus sinensis* (*Cypriniformes: Cyprinidae*). Mitochondr. DNA. pp.1-2.

Zeng, Y., H.Z. Liu and J.Z. Shen. 2006. Choice of oviposition sites on the gills of mussels by bitterling. Curr. Zool. 52:272-278.

Zhang, L.H., W.M. Zhang and H.R. Lin. 2001. Effects of 4-androstene-3,17-dine and 17α-methyl-testosterone on testosterone and 17ß-estradiol levels in the serum of *Anguilla japonica*. J. Fish .China. 25:107-111.

Zhang, T and Z. Li. 2002. Age, growth, and reproduction of the bitterling (*Paracheilognathus imberbis*) in a shallow Chinese lake. J. Freshwater. Ecol. 17:501-505.

Zheng, S.M and Z.B. Chen, 1997. Studies on the sex inversion of *Poecilia reticulata* and *Poecilia latipinna* by 17*α*-methyltestosterone. J. Sichuan. Inst. Anim. Husb. Vet. Med. 16:183-185.

Zheng, W., S.R. Yates and S.A. Bradford. 2008. Analysis of steroid hormones in a typical dairy waste disposal system. Environ. Sci. Technol. 42:530-535.

Zhou, X.H., X. Xiang and J.W. Wang. 2000. A preliminary study on the effects of methyl testosterone on sex reversal and body colour in *Sailfin Molly* and *Red Sword-tall*. Fish. Sci. Tec. Inf. 27:99-101.