Bull Environ Contam Toxicol (2009) 82:280–284
DOI 10.1007/s00128-008-9579-2

Sex Differentiation and Vitellogenin and 11-Ketotestosterone Levels in Chub, *Leuciscus cephalus* L., Exposed to 17 β-Estradiol and Testosterone During Early Development

Vladimír Žlábek · T. Randák · J. Kolářová · Z. Svobodová · H. Kroupová

Received: 13 August 2007 / Accepted: 8 October 2008 / Published online: 25 October 2008

Abstract The effects of 17 β-estradiol (E_2) and testosterone (T) singly and in combination were tested on juvenile chub (*Leuciscus cephalus* L.). Vitellogenin (VTG) and 11-ketotestosterone (11-KT) were determined by ELISA in whole body homogenates and the gonads were examined histologically. Testosterone and estradiol, in combination, significantly increased whole body VTG ($p < 0.01$), but not 11-KT, compared to controls and the T treated groups. The only intersex observed (1/80) was in the combined treatment group. We suggest that VTG measured in whole body homogenates could be used to determine the effects of exogenous steroids in juvenile chub.

Keywords Fish · Endocrine disruptors · Gonads · Hormone

Chemical analysis of heterogeneous substances in fish is a reliable method of evaluation of aquatic ecosystem pollution. However, effects of chemical substances on aquatic organisms often overlap, making it difficult to isolate effects of individual pollutants, so field study results are often difficult to interpret. Biochemical markers are beneficial indicators in such cases.

Limited data are available on the effects of environmental endocrine chemicals. Anthropogenic chemicals, including synthetic and natural hormones, can disrupt the endocrine systems of wildlife species (Kime et al. 1999). The effects of endocrine disrupting chemicals (EDCs) on steroid hormone levels and vitellogenin (VTG) synthesis have not been studied clinically in juvenile chub. Furthermore, there is limited information on the effects of hormone disturbances on early gonad development. Studies under controlled conditions are essential to understanding the effects of individual chemicals on fish. Experimental exposure to chemicals considered to be the standards for estrogenic and androgenic modulation will provide valuable data about the effect of chemicals, singly and in combination, on the physiological status of fish.

17 β-estradiol (E_2) and testosterone (T) were chosen as test compounds. In female teleosts, E_2 and T are the dominant plasma sex steroids during oogenesis, while T and 11-ketotestosterone (11-KT) are elevated during spermatogenesis in males (Skjæraasen et al. 2004). Both estrogen and androgen receptors have been identified in developing fish gonads, providing a mechanism for the action of exogenous steroids on gonad differentiation. If sufficient amounts of sex steroids are administered to fish, particularly at stages of development when endogenous pathways are not fully established, altered sex differentiation and endocrine disruption may occur (Devlin and Nagahama 2002).

The effects of xenobiotics are generally evaluated using plasma sex steroid concentrations (McMaster et al. 1995; Janssen et al. 1997) or VTG (Kime et al. 1999; Örn et al. 2003; Holbech et al. 2006). The plasma sex steroids most often measured are T, E_2, and 11-KT. Both endogenous

V. Žlábek (✉) · T. Randák · J. Kolářová · Z. Svobodová · H. Kroupová
Research Institute of Fish Culture and Hydrobiology, University of South Bohemia in České Budějovice, Zatisi 728/II, 389 25 Vodnany, Czech Republic
e-mail: zlabek@vurh.jcu.cz

V. Žlábek
Department of Food Science, Swedish University of Agricultural Sciences, 750 07 Uppsala, Sweden

Z. Svobodová
University of Veterinary and Pharmaceutical Sciences, Palackého 1-3, 612 42 Brno, Czech Republic

and synthetic estrogens are believed to play a key role in the elevated VTG concentrations and intersex seen in fish in field observations (Jobling and Tyler 2003). Abnormal gonadal development, such as delayed maturation, high levels of atresia or intersexuality may also be detected histologically. Such parameters are frequently investigated in fish from contaminated environments or those exposed to anthropogenic chemicals (Jobling and Tyler 2003; Bateman et al. 2004).

Chub (*Leuciscus cephalus* L.) are routinely used in the Czech Republic to assess the quality of surface water (Hajšlová et al. 2007). However, data from controlled laboratory studies validating field biochemical monitoring of surface water pollution are lacking. The aim of the present study was to evaluate the impact of estrogenic and androgenic substances on early stage gonad development and sex differentiation, and VTG and 11-KT induction in juvenile chub.

Materials and Methods

Artificially spawned chub from pond aquaculture were hatched in June 2006 and adapted to laboratory conditions in October. After adaptation, 20 juvenile fish were transferred to each of eight 20 L aquaria containing aerated tap water. Water in the aquaria was renewed daily. Water temperature was 17–20°C with a pH range 7.5–8.2 maintained throughout the experimental procedure.

Experimental exposure began 5 months post-hatching. Fish weight was 0.3–1.2 g and total length 35–52 mm. Fish were fed on Bio Optimal Start (Biomar, 0.5 mm, 58% protein, 15% fat, 17.1 MJ/kg). The test chemicals, E_2 and T (Sigma Aldrich Chemical) were dissolved in 99.5% ethanol and the solution mixed with the feed to concentrations of 20 mg kg^{-1} for E_2 and 0.1 g $kg^{-1}$for T. An E_2 + T combination was formulated at 20 mg kg^{-1} E_2 + 0.1 g kg^{-1} T. Feed for controls was treated with ethanol only. After thorough mixing, the ethanol was evaporated from the feed. Fish were fed *ad libitum* three times a day for 30 days.

All exposure groups, including controls, were duplicated. The mean estimated amount of chemical consumed was calculated on the basis of total weight of fish for each group and the amount of feed consumed. The estimated mean for E_2 was 10.3 mg kg^{-1} fish, for T, 50.8 mg kg^{-1} fish. The combination amount was estimated as 10.2 mg kg^{-1} of E_2 and 50.0 mg kg^{-1} of T.

After 30 days, 20 fish (10 fish from each duplicate aquarium) from each treatment group were taken for VTG and 11-KT analysis. The fish were frozen in liquid nitrogen and stored at −80°C until analysis. The frozen whole fish was weighed and homogenized individually using homogenization buffer (12 mL of Tris–HCl + 2 mg of aprotinin + 120 μL of PMSF). The homogenate was centrifuged at 15,000*g* for 1 h and the supernatant beneath the fat layer was collected. Measurement of VTG and 11-KT in the supernatant of each fish was performed using a precoated ELISA kit (Biosense laboratories® Norway), according to the manufacturer's instructions. The use of carp VTG ELISA for determination of VTG in chub was validated by Flammarion et al. (2000). Absorbance was measured using a SLT Spectra (A5082) instrument at 492 nm and 420 nm for VTG and 11-KT detection, respectively. The final concentrations related to whole body homogenate were normalized to the weight (g) of the corresponding sample, and were expressed as ng VTG and pg 11-KT g^{-1} body weight.

Twenty fish (10 from each duplicate aquarium) for each treatment group were fixed in phosphate buffered formalin and embedded in paraffin for histology. Sex was confirmed by light microscopic evaluation of hematoxylin and eosin stained sections. Gonads with the presence of both previtellogenic oocytes and testicular tissue were classified as intersex.

Measured parameters were analysed using Kruskal–Wallis test. Comparison of sex differences from controls was performed using Pearson χ-square test. All analyses were performed using Statistica for Windows 7.1 (Statsoft Inc., 2005), with significance at 0.05.

Results and Discussion

Published data on the effects of steroid hormones on reproductive function are sometime contradictory. One of the most sensitive responses to estrogens in fish is the induction of vitellogenesis (Arukwe and Goksoyr 2003). Immunological assay was used to identify VTG induction in juvenile chub. The effect of steroid hormone on VTG induction and concentration of 11-KT in juvenile chub has not been characterized previously. However, when juvenile salmon were fed diets containing 15 and 30 mg 17 β-estradiol kg^{-1} food for 4 weeks after yolk-sac resorption, significant overrepresentation of phenotypic females occurred (Norrgren et al. 1999). Therefore, high estrogenic potential was expected for an E_2 concentration of 20 mg kg^{-1} feed.

VTG concentrations in whole body homogenate of juvenile chub after 30 days in-feed exposure to E_2, T, and an E_2 + T combination are shown in Fig. 1. VTG induction (7,360 ± 11,014 ng g^{-1}) was observed in the E_2 group, although it did not differ significantly from the control group. The E_2 + T group showed the highest VTG concentration (31,947 ± 27,733 ng g^{-1}) with highly significant ($p < 0.01$) VTG induction compared to controls (451 ± 276 ng g^{-1}). No significant differences were found

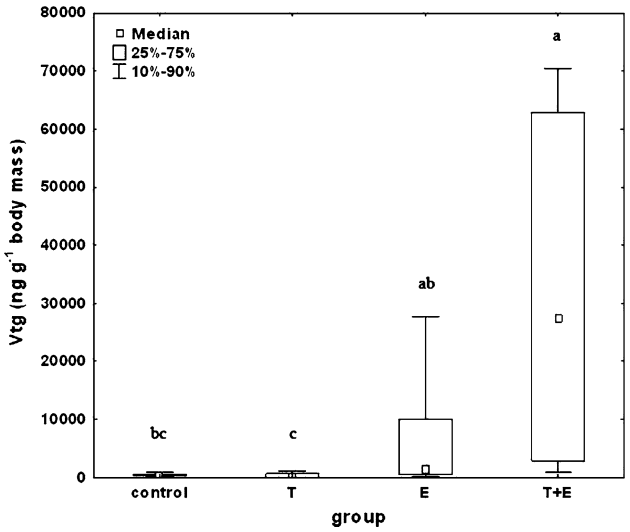

Fig. 1 The whole-body concentrations of VTG in juvenile chub after exposure to T, E_2 or a mixture T + E_2. Different letters indicates significant differences ($p < 0.01$) between the groups (n = 20 in each group)

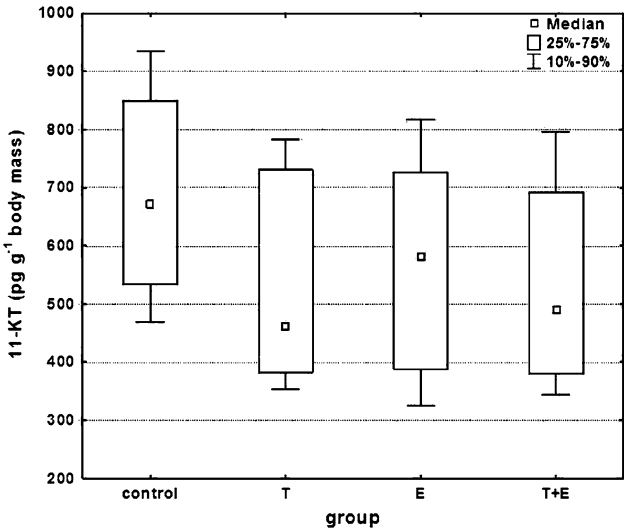

Fig. 2 The whole-body concentrations of 11-KT in juvenile chub after exposure to T, E_2 or a mixture T + E_2; (n = 20 in each group)

between the T group (377 ± 428 ng g^{-1}) and the controls. These results indicate that the vitellogenic response in chub was sensitive to estrogens and was dramatically increased when estrogen was combined with T. The effect of T may be due its aromatization to estrogen. Iwamatsu et al. (2006) reported that aromatizable T in high concentrations may induce a significant increase in E_2 content in embryos of medaka (*Oryzias latipes*) and also a paradoxical sex reversal. Similar observations were made in zebrafish (Örn et al. 2003).

Published data referring to the content of VTG and 11-KT in body mass are limited; therefore, original values related to milliliter of whole body homogenate were used for comparison with previous studies. Measured VTG concentration in whole body homogenate of fish from the control group were comparable to values found by Flammarion et al. (2000) in plasma of control chub following E_2 exposure. They found plasma VTG levels to be 27.0 and 4.0 ng mL^{-1} for control females and males, respectively. On the other hand, reported plasma VTG concentrations in male and female chub reached more than 1 mg mL^{-1} in exposed groups (Flammarion et al. 2000), while exposed juvenile fish in our study reached concentrations of only μg mL^{-1} in whole body homogenate. This might be due to differences in the intraperitoneal and oral exposure methods. The use of immature fish in our experiment may also result in lower VTG induction. Holbech et al. (2006) found VTG concentration below 100 ng mg^{-1} fish in control zebrafish, while VTG response after exposure to 54 ng L^{-1} E_2 in water exceeded 728 ng mg^{-1} fish.

Results of 11-KT analyses are shown in Fig. 2. No significant differences were found between any of the experimental groups (T = 541 ± 184 pg g^{-1}, E_2 = 565 ± 208 pg g^{-1}, and T + E_2 = 536 ± 177 pg g^{-1}) and controls (698 ± 190 pg g^{-1}). The concentrations were approximately equal, although experimental groups showed lower concentrations of 11-KT in comparison to the control group. Altered concentration of steroid hormones is a biomarker for endocrine disruption in fish (Jobling and Tyler 2003). Results of recent field studies showed altered levels of sex steroids in wild freshwater fish exposed to a wide range of chemicals (Hecker et al. 2002; Mayon et al. 2006). In general, we assume low concentration of 11-KT in juvenile fish. Mean concentrations in all experimental groups were registered in hundreds of pg g^{-1} body mass. Values of 11-KT were similar among control and all experimental groups. No significant depression or induction of 11-KT was found after exposure to T, E_2, or the combination T + E_2. Hecker et al. (2002) found median plasma concentration of 11-KT to be 20,000 pg mL^{-1} and 2,154 pg mL^{-1} in a control group of mature male bream (*Abramis brama* L.) during spawning and winter season, respectively. Our results at 45 ± 26.9 pg mL^{-1} whole body homogenate, were much lower than those reported by Hecker et al. (2002). The use of immature fish with little gonadal development may explain this relatively low concentration. Our result do not support the hypothesis that exposure to steroid hormones causes alteration of 11-KT synthesis.

Sex of juvenile chub is presented in Table 1. All fish had immature gonads and the developmental characteristics of the gonads in both males and females were typical of the stage. Pre-vitellogenic oocytes in the stage of primary growth were characteristic for ovaries. Testicular tissue was dominated by the presence of spermatogonia, typical for the pre-spermatogenic stage of development. There

Table 1 Sex ratio of juvenile chub after 30 days exposure to testosterone (T; 0.1 g kg^{-1} feed), 17 β-estradiol (E_2; 20 mg kg^{-1} feed) or a T + E_2 mixture (20 mg E_2 kg^{-1} feed + 0.1 g T kg^{-1} feed)

Group	Fish n	Females (%)	Males (%)	Intersex n	Sex determined (%)
Control	20	42	58	0	95
17 β-estradiol	20	50	50	0	50
Testosterone	20	47	53	0	85
T + E_2	20	57	36	1	70

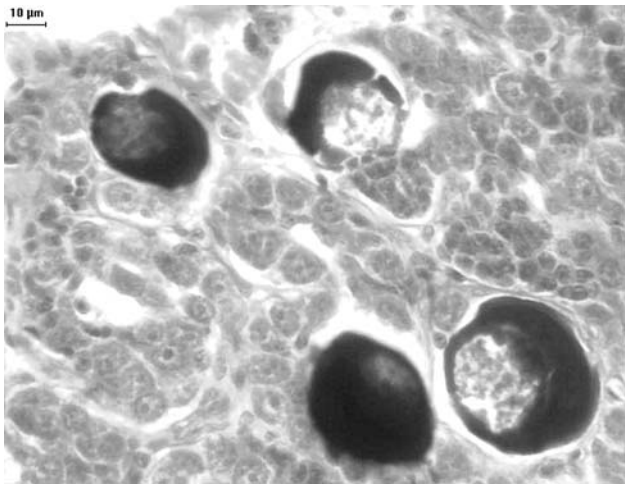

Fig. 3 Light micrograph showing intersex in juvenile chub after exposure to T + E_2 (20 mg E_2 + 0.1 g T kg^{-1} feed). Previtellogenic oocytes are dispersed among spermatogonia

were no significant variations in sex ratio. The control group had the lowest proportion of females (42%). The fish in the T + E_2 group showed an alteration in the sex ratio, with 57% being female. One intersex individual (Fig. 3) was found in T + E_2 exposure group. No evidence of ovotestes was present in the E_2, T or controls. The group which was fed the E_2 treated diet showed a 50:50 sex ratio. In all groups there were fish for which sex could not be determined due to the absence of differentiated gonads. This was especially evident in the E_2 group, where the sex of only 50% of the fish could be determined. Absence of differentiated gonads might be a sign of developmental disorder or the result of inhibition of sexual development.

In the present study, steroid hormones were used to simulate the effect of endocrine disruptors on fish under controlled conditions. Positive VTG induction and histological changes in the T + E_2 exposed group demonstrated the sensitivity of juvenile chub to chemicals with endocrine disrupting properties. The exposure of juvenile chub to steroid hormones at the tested concentrations had no measurable effect on levels of 11-KT when compared to the controls. We suggest that VTG measured in whole body homogenate could be used to determine the effects of exogenous steroids in juvenile chub.

Acknowledgments This study was supported by the USB RIFCH MSM6007665809 and the Czech Science Foundation 525/06/P234.

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