ORIGINAL ARTICLE

Induction of vitellogenin and gonadal impairment in chub (*Leuciscus cephalus L.*) after exposure to 17β-estradiol and testosterone

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Abstract

OBJECTIVES: A controlled laboratory study was carried out to quantify vitellogenin (VTG) concentrations in a common cyprinid freshwater fish, the chub (*Leuciscus cephalus* L.), exposed to steroid hormones.
DESIGN: The effect of 17β-estradiol, testosterone and testosterone-estradiol mixture was investigated on vitellogenin induction. Gonad status was also determined.
RESULTS: Oral exposure to estradiol and a testosterone-estradiol mixture increased ($p < 0.01$) blood plasma concentrations of VTG in blood plasma of both sexes. The testosterone-estradiol mixture had a negative effect on the investigated chub gonads. The effects were signified by histological changes when compared to control fish.
CONCLUSION: Our results showed a significant VTG increase in blood plasma of both sexes, indicating that vitellogenic response in the chub is sensitive to steroid hormones.

INTRODUCTION

A number of studies have shown that some populations of freshwater fish are being exposed to hormone-like chemicals resulting in disruption of the reproductive physiology of the organism. Anthropogenic chemicals, including synthetic and natural hormones, can disrupt the endocrine systems of wildlife species (Tyler *et al.* 1998; Kime *et al.* 1999; Arukwe, 2001). In fish, hormones play an essential role in gonad development, and display unique seasonal cycles. A study to investigate reproductive parameter responses after exposure to steroid hormones would yield valuable knowledge. Effects of particular chemical substances on aquatic organisms often overlap and it's difficult to understand consequential synergistic and antagonistic effects. Biochemical markers and haematological parameters are valuable indicators in such cases (Blahova *et al.* 2008; Modra *et al.* 2008).

Unfortunately, field study results are often misleading to interpret. Limited data is available reporting on the effect of environmental endocrine disrupting chemicals (EDCs) using chub (*Leuciscus cephalus*) as a model organism. Chub is a fish routinely used to assess the quality of surface water (Agtas *et al.* 2007; Christoforidis *et al.* 2008; Hajslova *et al.* 2007; Krcca *et al.* 2007; Stachel *et*

Abbreviations & units

eDCs – endocrine Disrupting Chemicals
E_2 – 17β-estradiol
T – testosterone
VTG – vitellogenin
GSI – gonadosomatic index
HSI – hepatosomatic index
ELISA – enzyme-linked immunosorbent assay
MJ – mega joule

al. 2007). However, there is a lack of fundamental data from the controlled laboratory studies for evaluation of field biochemical monitoring of surface water pollution. Presently the effect of steroid hormones exposure on vitellogenin induction in chub is limited to only two studies (Flammarion *et al.* 2000; Zlabek *et al.* 2009).

To date, there is missing data regarding the effects of endocrine disruptors (EDCs) on vitellogenin synthesis and gonad development in chub, which is essential for following successful reproduction processes. First, the effect of chemicals must be studied under controlled conditions. Laboratory studies of individual chemical effects on fish are essential to understand its impact on aquatic animals living in the natural environment. Fish exposure to chemicals, which are considered to be the standards for estrogenic and androgenic modulation in laboratory conditions, deliver valuable data about the effect of a single chemical and their mixtures on the physiological status of the fish.

In the present study, estrogenic (17β-estradiol; E_2) and androgenic chemical (testosterone; T) were chosen as test compounds. In female teleosts, 17β-estradiol and testosterone are the dominant sex steroids in plasma during oogenesis, while T and 11-ketotestosterone are elevated during spermatogenesis in males (Skjæraasen *et al.* 2004). The sex steroid 17β-estradiol activates the production of vitellogenin (VTG) by the liver. This protein is then incorporated into maturing oocytes (Kime *et al.* 1999). Vitellogenin synthesis normally takes place only in mature females, but it is possible to induce it in males by exposing them to estrogens or estrogenically active chemicals. Since estrogen receptors are present in the liver of male fish, non-physiological induction of vitellogenesis caused by exogenous steroids may occur. Therefore, vitellogenin induction in males has been shown to be a specific biomarker for exposure to endocrine disrupting chemicals (Örn *et al.* 2003; Holbech *et al.* 2006). Both endogenous and synthetic estrogens are suspected to play a key role in the field observations of elevated vitellogenin concentrations and intersex in fish (Jobling & Tyler 2003). The major part of the estrogens detected in fresh water systems originates from women who excrete conjugated estrogens that are deconjugated in sewage treatment plants and from livestock operation (Holbech *et al.* 2006). Finally, the structural integrity of gonads can be altered by xenobiotics. Abnormal gonadal development, such as delayed maturation, high levels of atresia or intersexuality may also be detected by histological analysis. Such parameters are frequently investigated in fish exposed to anthropogenic chemicals or living in contaminated environments (Jobling & Tyler, 2003; Bateman *et al.* 2004; Mikula *et al.* 2006).

Initially we investigated whether chub (*Leuciscus cephalus*) is suitable to indicate the presence of endocrine-disrupting chemicals. The aim of the present study was to evaluate the impact of estrogenic and androgenic model substances on vitellogenin induction and gonad status in chub. Additionally, a mixture of steroid hormones with different modes of action was used to simulate effects of pollutant "cocktails" present in the aquatic environment.

Table 1. The main characteristics of sampled fish (n = number of fish, mean ± standard deviation)

Group	Sex	n	Total Length (mm)	Weight (g)
Control	♀	14	226 ± 68.6	140 ± 168.5
	♂	8	213 ± 41.0	101 ± 72.8
T	♀	11	220 ± 36.4	106 ± 57.4
	♂	11	244 ± 41.2	146 ± 74.7
E_2	♀	10	202 ± 24.8	88 ± 30.1
	♂	11	211 ± 23.9	95 ± 31.3
T+E_2	♀	14	223 ± 50.3	115 ± 97.1
	♂	8	205 ± 36.1	83 ± 44.5

MATERIAL AND METHODS

Compound and treatment preparation. The test chemicals, 17β-estradiol (E_2) and testosterone (T) were purchased from Sigma Aldrich Chemical Company, and were dissolved in 99.5% ethanol. The ethanol solutions were mixed with the feed resulting in an E_2 concentration 20 mg kg^{-1} feed, T concentration (0.1 g kg^{-1} feed) and E_2+T mixture (20 mg E_2 + 0.1 g T kg^{-1} feed) diets. The control diet was treated with ethanol only. After thorough mixing, the ethanol was evaporated from the feed. An Ecolife 15 (Biomar, 3 mm, 45 % protein, 16% fat, energy 20.8 MJ kg^{-1}) fish feed was used. The dose was calculated at the end of exposure, based on total weight of fish from each group and amount of feed consumed. The estimated dose of 17β-estradiol was 9.1 mg kg^{-1} fish, the testosterone dose was 39.3 mg kg^{-1} fish and the mixture dose was 8.9 mg kg^{-1} and 44.6 mg kg^{-1} of 17β-estradiol and testosterone, respectively.

Animals and study design. The fish from pond aquaculture were transferred to experimental 200-liters aquariums for a 7 day adaptation period. The main characteristics of the 3 years old fish are shown in ***Table 1***. The fish were exposed to the chemicals through *per-oral* route for 30 days; under semi-static water conditions. Aerated tap water was used daily for the renewal of the test volume. The water temperature was 18-21°C with

a pH range of 6.9-7.8 throughout the test procedure. Experimental diets were fed *ad libitum* three times a day. All exposures, including controls, were performed in duplicates with 11 fish per aquarium.

After 30 days of exposure, the fish were collected for VTG analyses from each duplicate aquarium. Blood samples were collected from the caudal vein into heparinized tubes. Samples were centrifuged and blood plasma samples were frozen in liquid nitrogen and stored at -80°C until analysis. The length and body weight were recorded. The gonads and livers were dissected from the fish, and the gonadosomatic index (GSI) as well as the hepatosomatic index (HSI) were calculated as described by Hecker *et al.* (2002):

GSI (%) = [gonad weight / body weight (without viscera) × 100]
HSI (%) = [liver weight / body weight (without viscera) × 100]

The measurements of VTG in the blood plasma samples were performed using a pre-coated ELISA kit (Biosense laboratories® Norway). The procedure was implemented in compliance with the manufacturer's instructions. The use of carp vitellogenin ELISA for determination of vitellogenin in chub was validated by Flammarion *et al.* (2000). The absorbance was measured using a SLT Spectra (A5082) instrument set at wavelength 492 nm. Twenty individuals from each group were sampled for histological analysis. The gonads were fixed in phosphate-buffered formalin and embedded in paraffin. The sex was confirmed by light microscopic evaluation of hematoxylin–eosin stained sections. Gonads with the presence of both previtellogenic oocytes and testicular tissue were classified as intersex.

Statistical analysis. Measured parameters were analysed using Kruskal-Wallis test. All analyses were performed using Statistica for Windows 7.1 (Statsoft Inc., 2005), with a significance of $p < 0.01$.

RESULTS

VTG in Females. The VTG concentrations given in ng ml^{-1} blood plasma of females are shown in ***Fig.1***. Significant vitellogenin induction ($p<0.01$) was registered in the dietary group exposed to E_2 (555 ± 35.7 μg ml^{-1}) and the E_2+T (511 ± 64.0 μg ml^{-1}) mixture group compared to the control group (468 ± 354.1 ng ml^{-1}). No significant differences were found between the control and the experimental group exposed to testosterone; however, T group showed a higher vitellogenin concentration (1135 ± 553.8 ng ml^{-1}).

VTG in Males. Exposure of males to only testosterone resulted in increased induction of VTG (72.1 ± 34.8 ng ml^{-1}) compared to the control group (13.2 ± 4.4 ng ml^{-1}), but was not significant. A highly significant vitellogenin induction ($p<0.01$) was registered in the group exposed to E_2 (487 ± 80.2 μg ml^{-1}) and the E_2+T (446 ± 131.2 μg ml^{-1}) mixture group. Blood plasma concentrations of VTG after exposure to estradiol and a testosterone-estradiol mixture surprisingly reached the levels of VTG in exposed females**.**

GSI and HIS. The gonadosomatic index and the hepatosomatic index are presented in ***Fig. 3***. The highest GSI of males was found in the control group. The males from all exposed groups showed lower GSI although not significantly different from the control group. Females did not show any differences in GSI. The lowest HSI was found in both sexes from the control group. The fish from all exposure groups expressed higher HIS although not significantly different from the control.

Histology. Early vitellogenic oocytes were characteristic for ovaries of females from the control group (***Fig. 4 A***). Oocyte degeneration was observed in testosterone treated females (***Fig. 4B***). Perinucleolar oocytes predominated in ovaries of females after exposure to 17β-

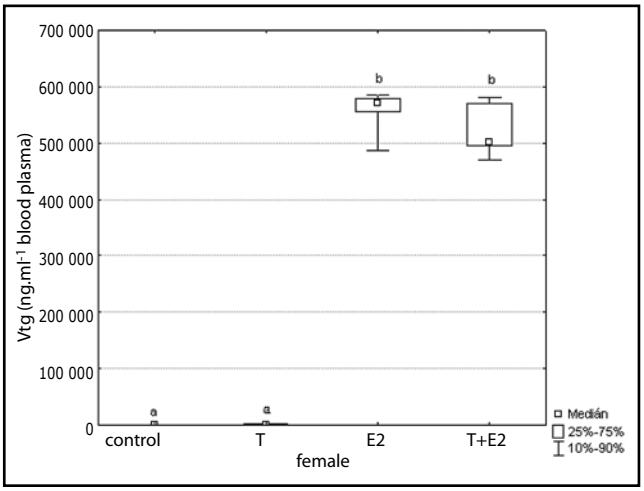

Figure 1. The blood plasma concentrations of vitellogenin (VTG) in female chub after the exposure to testosterone (T), 17β-estradiol (E_2) and their mixture T+E_2. Different superscript letters indicate significant differences (Kruskal-Wallis, $p < 0.01$) between groups.

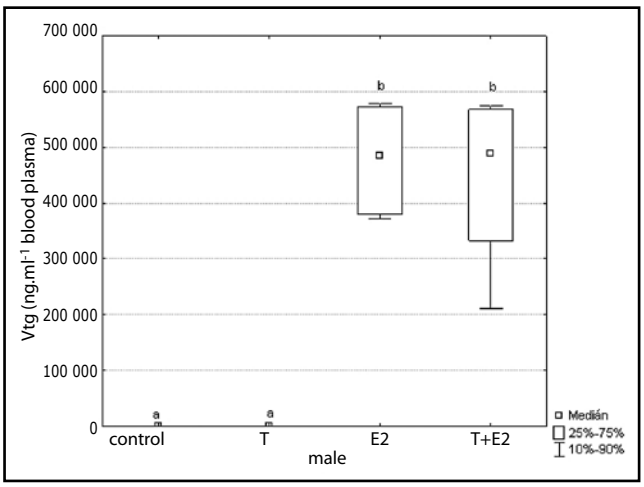

Figure 2. The blood plasma concentrations of vitellogenin (VTG) in male chub after the exposure to testosterone (T), 17β-estradiol (E_2) and their mixture T+E_2. Different superscript letters indicate significant differences (Kruskal-Wallis, $p < 0.01$) between groups.

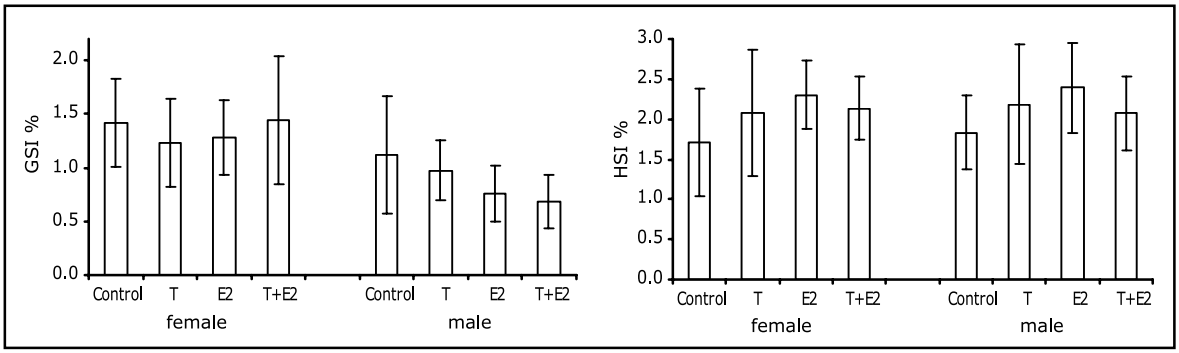

Figure 3. The gonadosomatic index (GSI) and the hepatosomatic index (HSI) of chub after the exposure to steroid hormones.

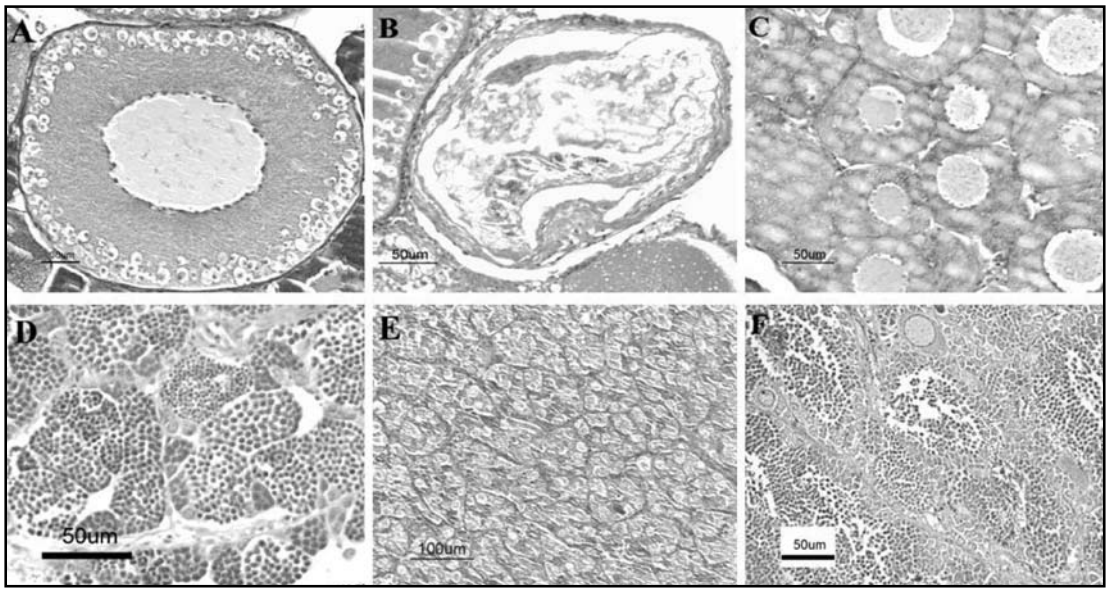

Figure 4. Light micrograph showing: (A) an early vitellogenic oocyte in ovary of female chub from the control group, (B) a degenerating oocyte in female chub after exposure to testosterone, (C) perinucleolar oocytes in female chub after exposure to 17β-estradiol (D) testes of male chub from the control group, containing spermatocytes and spermatids. (E) Regressed testes containing only spermatogonia in male chub after the exposure to 17β-estradiol, (F) intersex in chub after the exposure to T+ E_2 mixture. Perinucleolar oocytes are dispersed in testicular tissue. H&E stain.

estradiol (***Fig. 4C***). Testes of male chub from the control group contained both spermatocytes and spermatids. Testicular tissue of males exposed to E_2 was dominated by the presence of spermatogonia, typical for the pre-spermatogenic stage of testes development (***Fig. 4 E***). One intersex individual (***Fig. 4 F***) was found in T+E_2 exposure group. No evidence of ovotestes was present in either the E_2, T or the control group.

DISCUSSION

Published data on the effects of steroid hormones on reproductive functions are sometimes contradictory. One of the most sensitive responses for estrogens in fish is the induction of vitellogenesis (Arukwe & Goksoyr, 2003). An immunological assay was used to identify vitellogenin induction in chub to provide evidence for the endocrine disruption in our study.

Our results showed a significant VTG increase in blood plasma of both sexes, indicating that vitellogenic response in the chub is sensitive to estrogens. Significant induction of vitellogenin was also found after exposure to combination of estradiol and testosterone. Females and males showed 1000 and 10000 fold increase, respectively. Registered VTG concentrations in blood plasma of males from the control group are comparable with the values found by Flammarion *et al.* (2000) in control male chubs. On the other hand, concentrations of VTG in females are more than 10 times higher than expected in mature female fish. 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 fish in our study reached slightly lower concentrations close to 0.5 mg ml^{-1}. This might be due to different exposure methods. Even higher peroral dose in our experiment resulted in lower VTG induction when compared to intraperitoneal application of 2mg E_2 kg^{-1} (Flammarion *et al.* 2000). A similar result was also found in our previous study with juvenile chub (Zlabek *et al.* 2009). Exposure of fish only

to testosterone resulted in the increased induction of VTG compared to the control group, but was not significant. Considering the above fact, the effect observed in the group, which was exposed to testosterone, may be explained by a possible conversion of testosterone into estrogens. Iwamatsu *et al.* (2006) reported that aromatizable testosterone in high concentrations may induce a significant increase in E_2 content in embryos of medaka (*Oryzias latipes*) and also paradoxical sex reversal. In general, it is believed that the paradoxical effect of androgens on sex differentiation results from the conversion of androgens into estrogens (Örn *et al.* 2003). Contrary to our previous study (Zlabek *et al.* 2009) no significant effect of testosterone was found in the T + E2 exposed group.

The GSI in fish from the control group were somewhat lower than expected of mature fish (Flammarion *et al.* 2000), but comparable with GSI reported for wild roach (Hecker *et al.* 2002) in the autumn period. Lower GSI in males from all exposed groups reflects a negative effect of the tested hormones on normal development of gonads.

The developmental stages of gonads in both males and females from the control group were typical for maturing fish. Histological analyses of the testes from the males of the control group confirmed active spermatogenesis. On the contrary, exposure of males to E_2 resulted in regressed testes containing only spermatogonia. Similar effects were also registered in males exposed to testosterone and T+ E_2 mixture. Additionally, one intersex individual was found in the T+E_2 exposure group. Presence of intersex after exposure to steroid hormones is surprising for an already differentiated gonad; however, this was a single finding**.** Lower maturation stages of ovaries were found in females exposed to E_2 compared to the control fish. Absence of fully matured gonads in fish from hormone exposed groups is a sign of disruption of pituitary-gonadal axis in both sexes. Moreover, high incidence of degenerating oocytes reflects developmental disorder in females after exposure to testosterone.

CONCLUSIONS

In the present study, steroid hormones were used as standards to simulate the effect of endocrine disruptors on fish organism under controlled conditions. Positive vitellogenin induction in the E_2 and the T+E_2 exposed groups is indicating that vitellogenic response in the chub is sensitive to steroid hormone exposure. Negative histological changes of fish gonads in exposed groups proved the sensitivity of chub to chemicals with endocrine disrupting properties. Endocrine-disrupting chemicals may adversely affect the hormone systems and thereby the homeostasis, development and reproduction of exposed organisms. Such observations provide important insight into potential impacts from endocrine disruptors, and can provide useful monitoring tools.

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