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METABOLIC CHANGES ASSOCIATED WITH 17ALPHA-ETHINYLESTRADIOL-EXPOSURE IN THE PREGNANT TELEOST *ZOARCES VIVIPARUS* (L)

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Abstract: Females of the eelpout *Zoarces viviparus* were exposed in the ambient seawater to the synthetic estrogen 17α-ethinylestradiol at different doses, 5, 10, 25, 50, 500 ng/L during early pregnancy and the effect on the maternal metabolism was studied. A significant dose-related increase in the level of calcium and the yolkprecursor-protein vitellogenin was observed in plasma of the exposed motherfish. The increased synthesis of vitellogenin could also be observed in the liver of the exposed motherfish by PCR and indirectly by the increase in the hepato-somatic index. No significant effects were observed in the concentration of glucose in plasma; the concentrations of amino acids in plasma, however, showed a significant decrease in the groups exposed to the higher doses of EE2. A general decrease in plasma osmolarity and chloride-concentrations were found in the exposed groups compared to controls. A dose-related decrease was observed in the activity of glutamate pyruvate-transaminase (GPT). The EE2- induced changes in the hepatic activity of the glycolytic enzyme pyruvate kinase (PK) and the gluconeogenetic enzyme PEPCK as well as of glucose-6-phosphate dehydrogenase (G6PDH), an indicator for oxidative stress response, were less consistent. The activity of these 3 enzymes was observed to decrease significantly when expressed per unit liver wet weight but to increase in the high dose groups when activity was expressed normalized to the hepatosomatic index.

Key words: xenoestrogens, exposure, metabolism, eelpout, viviparous teleost

Introduction

The synthetic estrogen 17α-ethinylestradiol (EE2), used in contraceptive pills, is known to enter the aquatic environment via seawage effluents, thereby posing a threat to aquatic wildlife. (Tyler et al., 1998). EE2 has been measured in the effluents at concentrations at the low ng per L range. EE2 has been shown to induce the synthesis of the yolk-precursor protein vitellogenin in male rainbow trout at a concentration as low as 0.1 ng/L (Purdom et al. 1994). The natural steroid 17ß-estradiol has been observed at concentrations up to 80 ng/l in British rivers (Tyler et al, 1998), a concentration at which vitellogenin synthesis may be induced in fish in their ambient environment and which may eventually lead to changes in growth, development and reproductive performance.

Very few investigations have been made on metabolic processing in relation to xenoestrogenic exposure. Estrogen mimics may affect metabolism the same way as the natural endogenous estradiol in relation to the increased demand of metabolites associated with the intensive synthesis of vitellogenin (Mommsen and Walsh, 1988), but may also exert dose-dependent stress-like effects on the general metabolic performance of the exposed animal. Steroid hormones including estradiol have been shown to inhibit xenobiotic biotransformation by downregulating the induction of CYP450 (Winzer et al, 2001b). Previous experiments have shown that estradiol-treatment also significantly

alters metabolic flux concurrent with the hepatic induction of vitellogenin synthesis. During prolonged estradiol-treatment of rainbow trouts, carbohydrate metabolism is generally affected by marked reductions in liver glycogen with concomitant changes of enzymes involved in the glycolytic and gluconeogenic metabolism of the liver **(**Washburn et al.,1993; Korsgaard and Mommsen, 1993). Such changes in carbohydrate metabolism have also been observed during normal endogenous vitellogenesis in turbot females (*Scophtalmus maximus)* **(**Soengas et al**,** 1995) and during normal vitellogenesis and pregnancy in a viviparous teleost, the eelpout *Zoarces viviparus* (Korsgaard and Petersen, 1979**).**

Of particular concern are the effects which environmental estrogens may impose on embryonic development. In the eelpout the maternal-fetal trophic relationship has been observed to be affected by dose-related concentrations of octylphenol or EE2 (Rasmussen et al., 2002; Korsgaard *et al.,* 2002). Thus different compounds in ovarian fluid, the ambient medium of the developing embryos, such as calcium, glucose and amino acids, believed to be of nutritional importance for normal growth of the embryos during their intraovarian development, were found to be affected by the EE2 exposure (Korsgaard et al. 2002**)**.

Previous work in our laboratory has shown that not only vitellogenesis but also other physiological processes may be affected by xenoestrogens. Treatment with estradiol or 4-nonylphenol was shown to significantly affect the osmoregulatory performance in the atlantic salmon (*Salmo salar)* during smoltification **(**Madsen et al,. 1997**).** Cortisol is an important hormone in osmoregulation and may antagonize the negative effect of estradiol on the osmoregulatory response of the fish (Madsen and Korsgaard, 1991).

The present work was initiated to elucidate how the pregnant female fish responds to exposure to different doses of EE2 under flow-through conditions. As the liver is the major synthesizing organ for vitellogenin it was relevant to study how the xenobiotic exposure affects metabolism and osmoregulation of the pregnant female fish in relation to hepatic synthesis of vitellogenin or as a stress inducer. In addition to vitellogenin, enzymes indicative of glycolysis and gluconeogenesis were measured to investigate effects of xenobiotic exposure on metabolic performance. The enzyme glucose-6-phosphate dehydrogenase was included in the investigation, as this particular enzyme has been shown to be very sensitive to inactivation by chronic exposure to polluted marine habitats (Van Noorden et al, 1997). The enzyme is important as the major provider of NADPH, required for detoxification pathways (Winzer et al., 2002) and may thus be regarded as a stress indicator.

Material and Methods

Zoarces viviparus (L) females were caught during early pregnancy (October) by fyke nets at Dalby Bugt, Funen, Denmark and transferred to the Marine Biology Research Center in Kerteminde to large indoor tanks with aerated running seawater pumped from the Great Belt of Denmark. The fish were acclimated for about one week before experiment and held at a 12L:12D cycle. The fish were exposed under flow through conditions (20 ‰SW) in 50 L aquaria to 17α-ethinylestradiol (0, 5, 10, 25, 50, 500 ng/l) using estradiol-17ß (500 ng/l) as positive control. The compounds were dissolved in 100% isopropanol and applied directly to the experimental aquaria at a rate of 72 ml per day. Each aquarium was fitted with a circulation pump to assure uniform mixing. The continuous flow-through system used in the present study was identical to the system used and validated in previous experiments (Korsgaard et al. 2002), in which the actual water concentration of EE2 was kept constant throughout the experiment at approx. 80% of the nominal concentrations. The experiment lasted 32

days and the fish were not fed during the experimental period to avoid erratic feeding due to the xenobiotic exposure. By sampling the fish were anaesthetized in 0.2‰ phenoxyethanol. Fish were weighed and blood was collected from the caudal vein into heparinized eppendorf tubes. Plasma was collected after centrifugation. Fish were killed by decapitation and the livers were carefully removed, weighed and frozen in liquid N2 and stored at –80^{o}C until use.

Vitellogenin in plasma was measured by ELISA as described by Korsgaard and Pedersen, (1998).

Plasma total calcium was measured by atomic absorption spectrophotometry, plasma glucose by the glucose oxidase method using a commercial kit (Boehringer Mannheim) and total amino acids by the Ninhydrin method (Moore and Stein, 1948). Total osmotic concentration of plasma was measured on a Knauer automatic osmometer using the freezing point depression method and the plasma chloride concentration by a Radiometer chloride automatic titrator.

The hepatic enzymes, GPT (glutamat pyruvate transaminase, PEPCK (phosphoenolpyruvate carboxykinase) and PK (pyruvate kinase) were measured according to methods described by Mommsen et al. (1980**)**, using a 50 mM imidazole buffer and following the appearance or disappearance of NADH at 340 nm. For detemination of G-6-PDH (glucose-6-phosphate dehydrogenase) activity liver was homogenized in 0.66 mM EDTA buffer (pH 7.5) and assayed in a medium containing 50 mM triethanolaminbuffer, 30 mM NADP+ and 40 mM glucose 6-phosphate. The total volume of the reaction mixture was 1 ml. The reaction was started by substrate addition and the increase in absorbance monitored at 340nm.

PCR was carried out as described by Andreassen *et al.*, (2002). Briefly, RNA was extracted from the liver by TRIzol and 1 µg of total RNA was reverse-transcribed in 20µl reactions followed by amplification of reverse-transcribed Vtg mRNA by use of the primers described by Andreassen et al., (2002). ß-actin was amplified as the reference gene. Values are expressed as means ± SEM. Statistics were performed as one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests and significance level was chosen at the 0.05 level.

Results

Dose-response effects of waterborne 17α-ethinylestradiol on the maternal metabolism of the eelpout *Zoarces viviparus* were investigated during early pregnancy. Estradiol-17ß was used as a positive control for estrogenic effects of the xenobiotic compound. During the 32 day period of exposure to EE2 the yolk-precursor protein vitellogenin (Vtg) and total plasma calcium increased in a dose dependent manner, as did the hepatosomatic index (Figure 1).

Vtg mRNA was induced in the liver samples from EE2- and E2 (not shown) exposed pregnant female fish (Figure 2A). Induction of Vtg mRNA was also observed in the embryos during their intraovarian development (Figure 2B). However, Vtg cDNA was only amplified in the embryos of the EE2-group at the high dose (500 ng/l). Glucose levels in plasma remained fairly stable in the different groups, whereas the level of plasma amino acids (NPS) decreased significantly in the groups exposed to the higher concentrations of EE2. (Figure 3). Interestingly the total osmotic concentration and the concentration of chloride in plasma appeared to decrease significantly in the groups exposed to the higher doses of EE2 (Figure 4), thus indicating an effect of the estrogenic compound on the osmoregulatory performance of the pregnant fish. Cortisol levels in plasma (not shown) were not significantly different in the exposed groups when compared to controls.

The activity of enzymes related to amino acid and glucose metabolism was measured and evaluated as activity per unit liver WW, or expressed normalized to the hepatosomatic index (HSI) of the maternal liver (Figure. 5A-H). When evaluating effects of xenobiotics on hepatic metabolism in general it should be taken into consideration that a decrease in enzyme activity

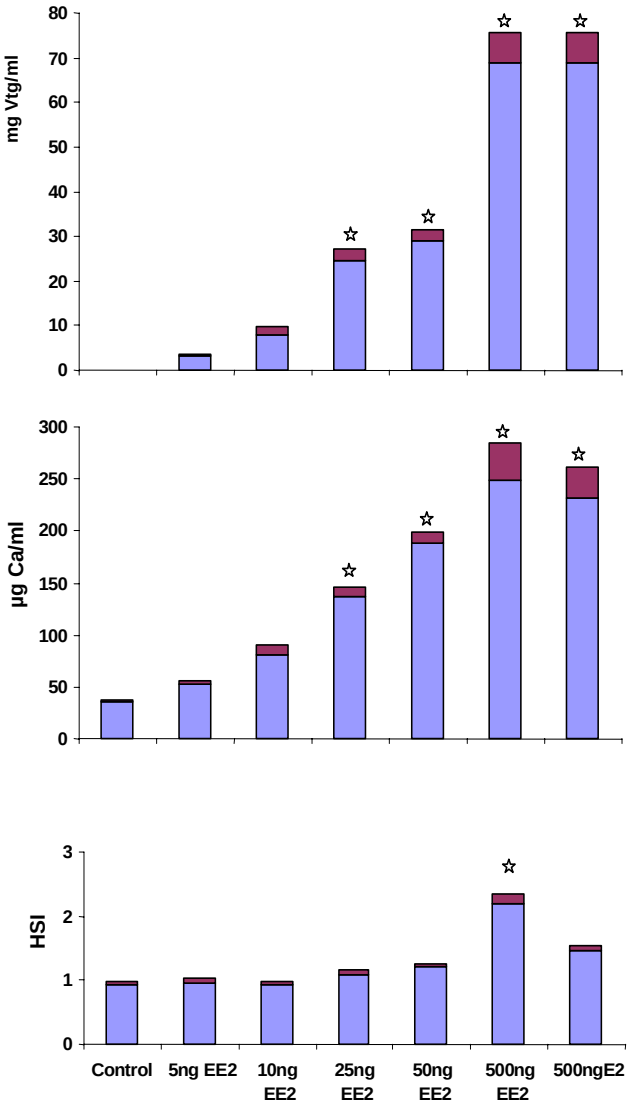

Figure 1: The effect of a 32 day exposure to 17α-ethinylestradiol on the concentration of vitellogenin and calcium in plasma and the hepatosomatic index in pregnant female eelpout.
***indicates significant difference (P<0.05) from control values. N=6 in each group.**

expressed per unit liver wet weight may be compensated for by an induced overall increase in the total hepatic wet weight. A significant dose-related decrease was observed in GTP activity by the two different expressions. The activity of the glycolytic enzyme pyruvate kinase (PK) and the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) expressed per unit liver weight decreased significantly. However a nonsignificant increase in activity of PK and PEPCK was observed in the high dose group (500ng) when expressed in total hepatic units.

As the major provider of NADPH acting as a reductant in various detoxification pathways, G6PDH is a biomarker enzyme for oxidative stress (Winzer et al. 2002). The results show that the xenoestrogenic compound EE2 reduced the activity of this enzyme in a dose dependent way expressed per unit liver wet weight with significant decreases in activities in the groups exposed to the higher doses of the EE2.

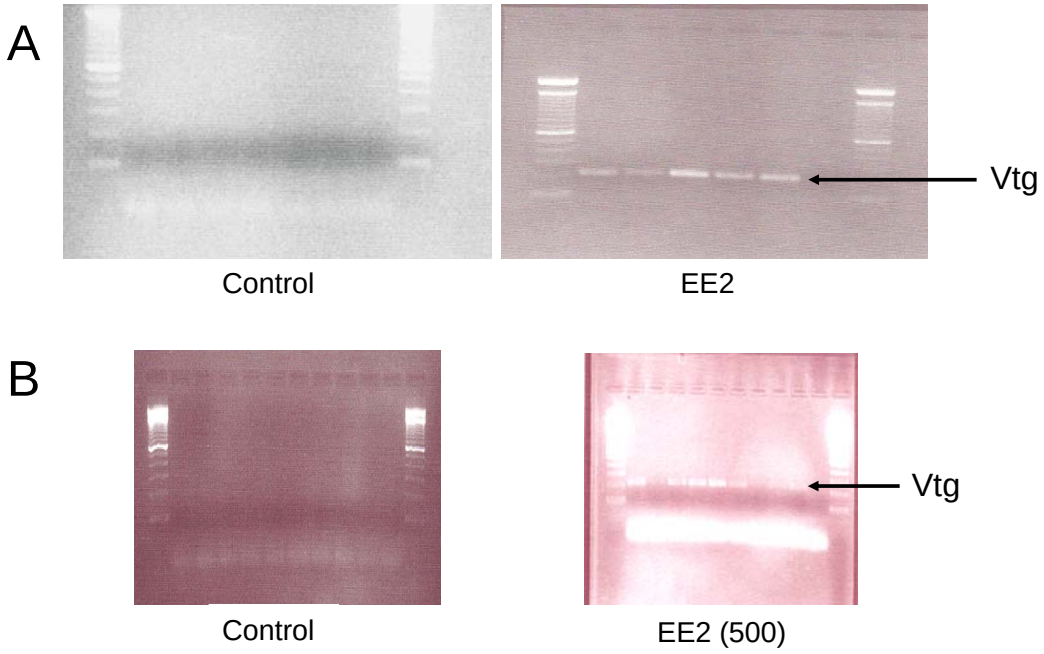

Figure 2: The effect of 32 days exposure to17α-ethinylestradiol on the VtgmRNA in pregnant females (A) and her embryos (B) measured by PCR. Vtg cDNA was only amplified in the embryos at the high dose of EE2.

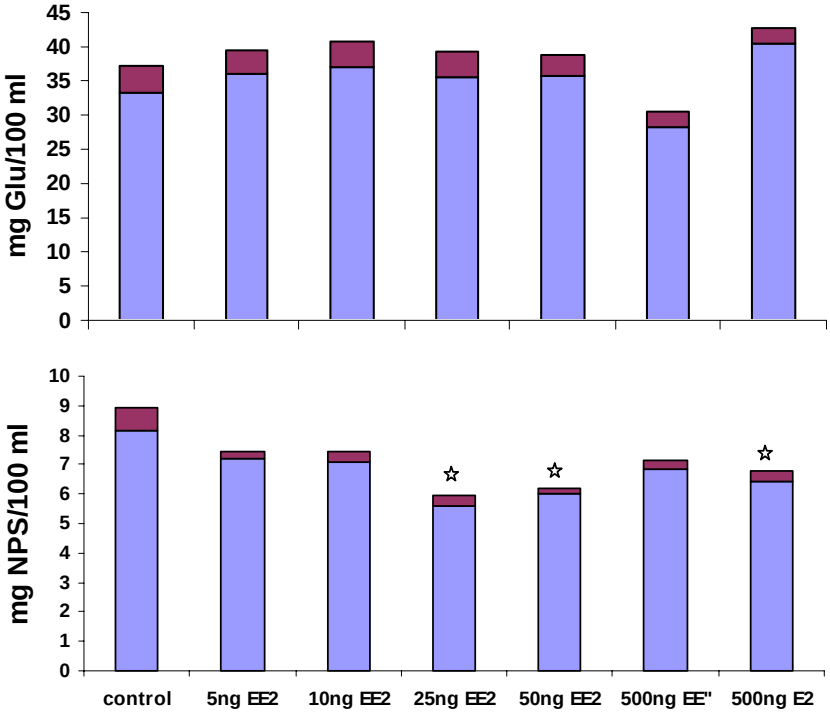

Figure 3: The effect of a 32 day exposure to 17α-ethinylestradiol on the concentration of glucose and amino acids (NPS) in plasma.
*** indicates significant difference (P<0.05) from control values. N=6 in each group.**

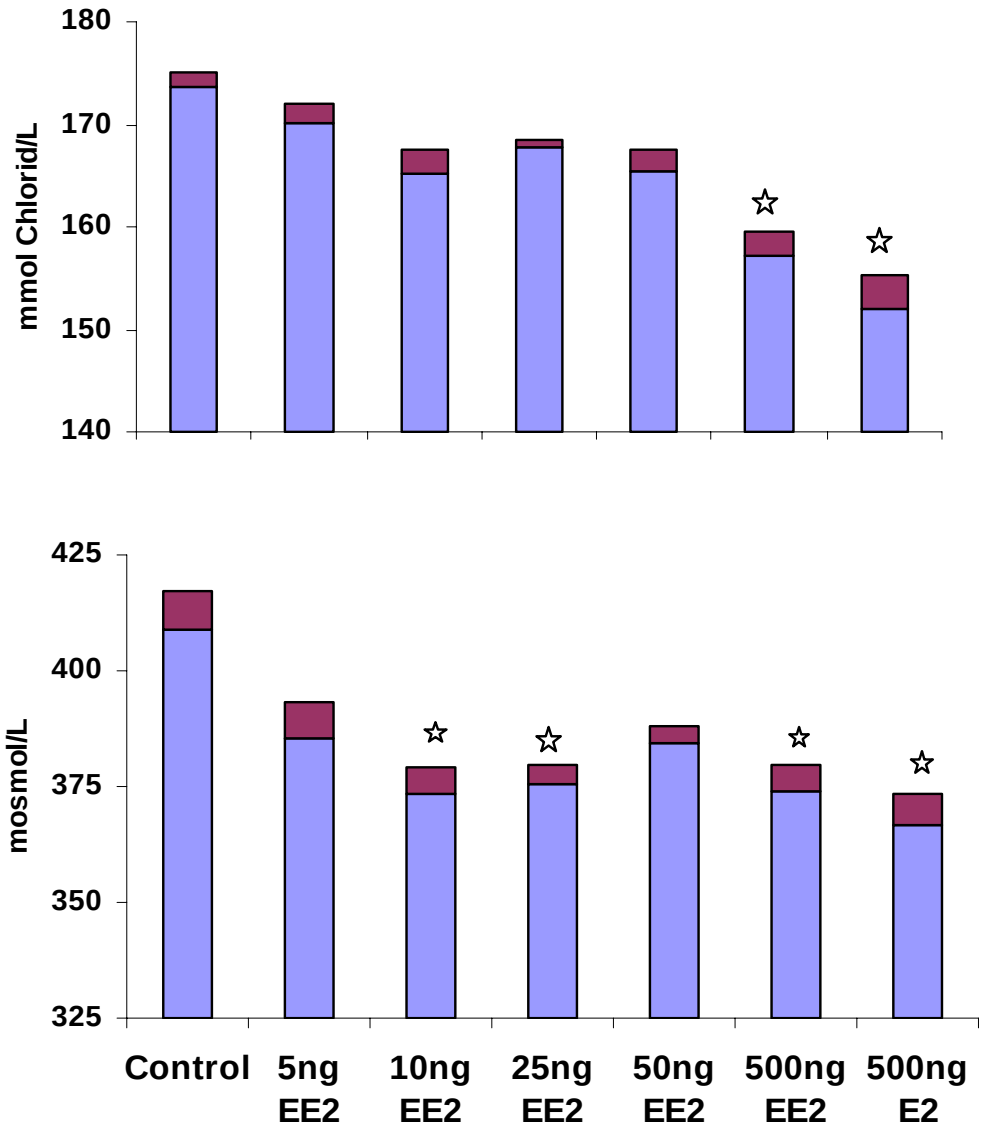

Figure 4: The osmotic concentration and the concentration of chloride in plasma of eelpout females after a 32 day exposure to 17α-ethinylestradiol. *indicates significant difference (P<0.05) from control values. N=6 in each group.

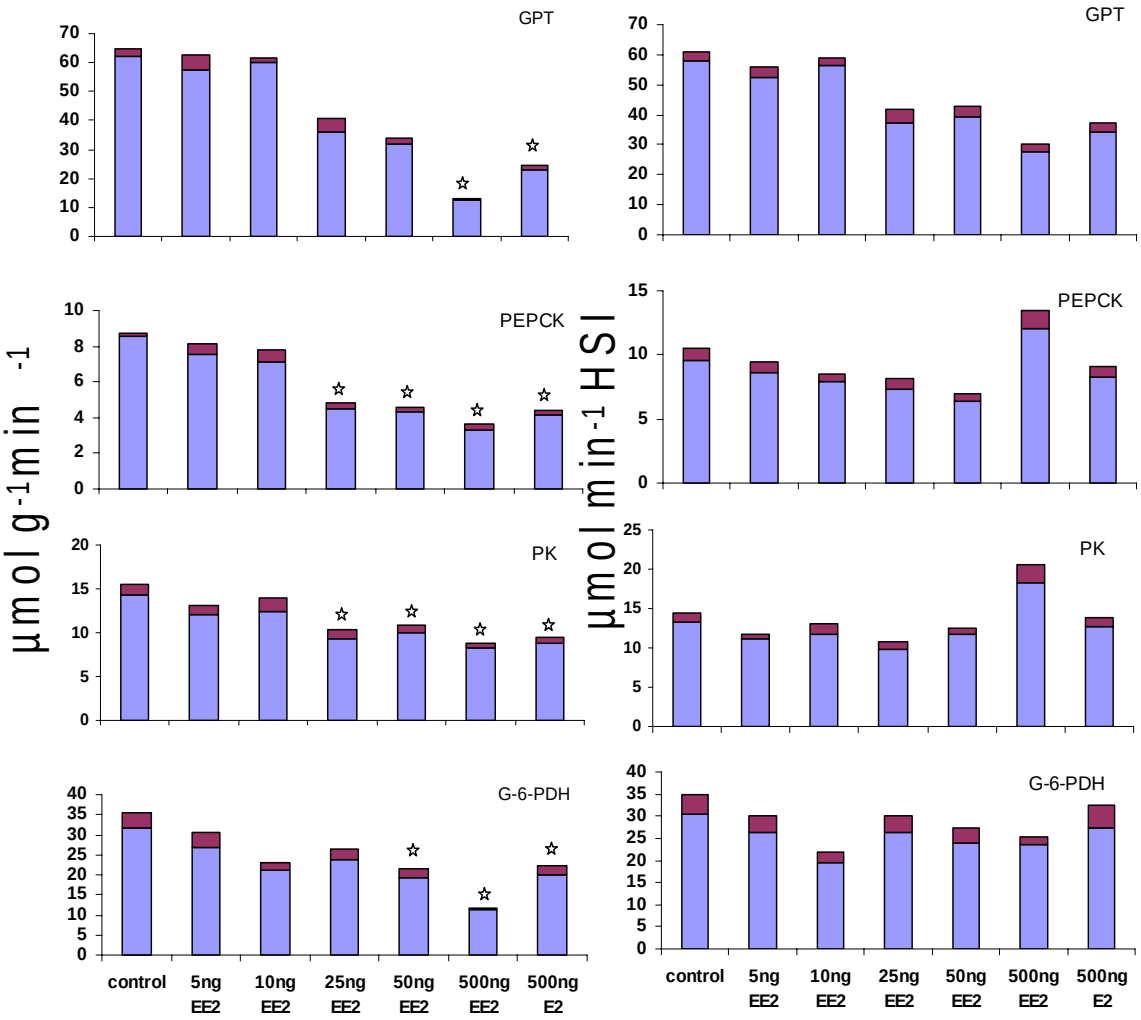

Figure 5: Activities expressed in µmol g^{-1} min^{-1}(A) or relative to the hepatosomatic index, HSI (B) of enzymes in glycolysis, gluconeogenesis or oxidative stress response (G6PDH) in livers of pregnant female eelpout after 32 days of exposure to EE2. *indicates significant difference (P<0.05) from control values. N=6 in each group.

Discussion

The results show that, overall, the maternal metabolism is affected in a dose-dependent manner by exposure to the synthetic estrogen 17α-ethinylestradiol. The concentration of vitellogenin is the common used end point biomarker for estrogenic exposure and the marked dose-related response in the present indicated that the female fish also responds to the estrogenic compound during pregnancy, when Vtg is no longer synthesized in the liver due to a marked decrease in estrogen levels during pregnancy (Korsgaard 1994). The Vtg response was also verified by the PCR-analysis showing that Vtg mRNA was induced in all liver samples from the exposed groups but not in the controls. This effect of EE2 was also observed in the embryos *in ovario,* responding to the high dose of EE2 by VtgmRNA induction. VtgmRNA induction is regarded a very sensitive indicator for estrogenic exposure as observed in several fish species (Jobling et al. 1996; Lech et al. 1996, Rasmussen et al. 2002). Exogenous induction of vitellogenin synthesis in male and immature females may cause serious metabolic stress due to the drain on energy reserves diverting amino acids, lipids, glucose and calcium from their respective target tissues. Increases in cortisol and glucose concentrations in plasma in response to stress factors have been reported by Mommsen et al. (1999). In the present, plasma glucose remained fairly stable in the EE2-exposed groups compared with the control and cortisol levels in plasma of the exposed groups were not significantly different from that of controls.

The present results indicate that the osmoregulatory performance of the exposed pregnant fish is affected by the estrogenic compounds as expressed by a decrease in plasma osmolarity. Vijayan et al. (2001) observed that estradiol treatment of the euryhaline tilapia, *Oreochromis mossambicus*, prevented recovery of plasma osmolality in 50% seawater with no significant effect on plasma cortisol or glucose. The E2 treatment of tilapia, however, was shown to decrease the metabolic capacity of the liver, lowering the activity of key enzymes in the liver.

A general dose-related decrease was observed in the present study in the activities per unit liver WW of all four enzymes (GTP, PEPCK , PK and G6PDH) in the groups exposed to EE2 as well as to E2. These overall changes in the glycolytic and gluconeogenic key enzymes in the exposed groups indicate that major changes occur in carbohydrate and protein metabolism. Such changes have been observed in previous experiments in which fish have been treated with estradiol-17ß (Washburn et al. (1993); Korsgaard & Mommsen (1993). Washburn et al (1993) observed that in male rainbow trout, implanted with estradiol-17ß, the process of gluconeogenesis by isolated hepatocytes was significantly depressed resulting in lower glucose concentration in plasma. Similar observations were made in immature rainbow trout after intraperitoneal injection of estradiol-17ß one or two weeks after the injection. After 6 weeks however, *de novo* glucose synthesis was significantly higher in the estradiol-treated group compared to controls when expressed normalized to the hepatosomatic index (Korsgaard and Mommsen, 1993). In the present experiments the fish were not fed and as the experiment lasted 32 days the circulating glucose would be expected to derive from glucose synthesis from non-carbohydrate precursors rather than from the process of glycogenolysis. Previous experiments have shown that estrogen treatment first mobilizes liver glycogen then leads to enhanced gluconeogenesis (Korsgaard and Mommsen, 1993; Whiting and Wiggs, 1978.). Accordingly, the liver depots are expected to be gradually exhausted in relation to the steady increase in vitellogenin synthesis by the prolonged exposure to xenobiotic compounds, thereby increasing the need for extra-

hepatic precursors such as amino acids. Amino acids enter the liver to be incorporated into vitellogenin or to be shunted into the gluconeogenic pathways by the respective transaminases. The transaminase GTP decreased in the groups exposed to the higher doses of EE2, but this decrease did not seem to have an effect on the circulating glucose, which was found to be at similar (nonsignificant) levels in the various groups. The circulating amino acids (NPS), however, showed significant decreases in the groups exposed to the higher doses of EE2. This decrease in amino acids in plasma may well be due to the dose-related increase in the synthesis of protein (vitellogenin), but may also reflect the enhanced shunting into the gluconeogenic process. However, the observed activity of the gluconeogenic key enzyme PEPCK did not provide any clear indication for this possibility, as it shows a dose-related decrease in activity in the liver. This decrease, however, could have been compensated for by an increase in liver mass, as observed in the high-dose group or by an increased gluconeogenic activity of the muscular mass. Estradiol-treatment of fish has been reported to induce increases in liver wet weight by hypertrophic or hyperplastic growth (Korsgaard and Emmersen, 1976**).** Expressed as total metabolic potential of the liver (multiplied by the hepatosomatic index) activities of GTP were shown to decrease significantly in the high dose groups, while PEPCK and PK activities were observed at an increased level in the same group.

A decrease in activity of the enzyme G6PHD of the flounder (*Platichthys flesus)* has been shown to be one of the short-term responses of the liver to oxidative stressors **(**Winzer et al, 2002). The authors suggest that the observed inhibition of G6PDH may reflect early cellular imbalances due to xenobiotic stress in direct relation to the limited availability of NADPH. They also observed this inhibition of G6PDH to occur in a more robust way in the female flounder. In the present experiment a dose-related decrease was observed in the activity of this enzyme in the groups exposed to the higher doses of EE2. A reduction in G6PDH activity in the liver was also observed in bullhead (*Cottus gobio)* exposed to paper mill effluents (Bucher et al. 1993**).** Thus the observed decrease in hepatic activity of G6PDH in the present may reflect metabolic imbalance after xenoestrogenic stress in the pregnant fish and may indicate that susceptibility for xenobiotic toxicity may increase by long term exposure or by increasing concentrations of xenoestrogens in the environment.

In conclusion the present experiments show that long-term exposure to EE2 has a marked dose-related effect on the overall metabolic and osmoregulatory performance of the pregnant female eelpout with the PCR-results indicating that the xenoestrogenic exposure may also affect the embryos during their development *in ovario*

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