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Determination of vitellogenin kinetics in male fathead minnows (*Pimephales promelas*)

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Abstract

A lack of knowledge persists concerning the combination of kinetics on protein and mRNA levels of the most commonly used biomarker for estrogenic influences—vitellogenin (VTG). Consequently, male fathead minnows were exposed to 17α-ethinylestradiol (EE_2) for 35 days, followed by an equally long depuration period in a flow-through system. VTG mRNA levels reached a plateau after 3 days of exposure, which remained stable until 3 days after EE_2 removal. Control levels were re-attained within 7 days of the depuration phase. VTG protein accumulated in the plasma following a two-phased model. The first phase depicting an exponential increase lasted 15 days and was followed by a saturation phase approaching a plateau of approximately 47 mg VTG/ml plasma. Clearance kinetics could be described by a two-compartment open model, with half-lives of 2.17 and 21.32 days for the α- and β-phases, respectively. In addition, a high VTG protein synthesis rate seemed to adversely affect fitness and mortality of the fish.

Keywords: Vitellogenin; Half-life; Kinetics; Fathead minnow; Ethinylestradiol; Fitness

1. Introduction

Currently, one of the most frequently used endpoints to detect effects mediated by estrogenic substances, is the measurement of the induction of hormone-dependent protein synthesis, i.e. proteins which are synthesized under the (strict) control of specific hormones. Vitellogenin (VTG), a prominent member of this class of biomarkers (Sumpter and Jobling, 1995), is under estrogenic control. It is a glycoprotein which is synthesized in the liver of female oviparous vertebrates (Wahli et al., 1981), released into the blood, sequestered from the blood by the growing oocyte and cleaved there to yield the main egg yolk proteins—phosvitin and lipovitellin (Mommsen and Walsh, 1988). VTG levels in the plasma of female fish undergo seasonal variations reaching concentrations of up to several milligrams per milliliter in some species during oocyte development (Tyler et al., 1996). The VTG gene is also present in male fish, but under normal conditions is not ex-

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pressed, possibly due to low concentrations of estrogens in the blood. Nevertheless, upon stimulation with estrogenic substances, blood plasma VTG protein levels in male fish can attain the same range as in mature females (Purdom et al., 1994; Korte et al., 2000). As methods to detect VTG synthesis both at the protein (Mourot and Le Bail, 1995; Monteverdi and Di Giulio, 1999; Parks et al., 1999; Tyler et al., 1999; Folmar et al., 2000) and at the mRNA level (Flouriot et al., 1996; Folmar et al., 2000; Korte et al., 2000) have been developed in recent years, this model offers a sensitive and specific means to detect estrogenic influences in male fish.

However, no satisfactory characterization of the accumulation and especially the clearance kinetics in male fish has been carried out yet. Most current studies involve short-term exposure regimens of a maximum duration of 21 days without examining the depuration. This allows neither the accurate determination of the accumulation kinetics, including the possibility of a plateau being approached, nor does it give any information concerning possible elimination mechanisms. These parameters are, however, indispensable to the determination of the value of this biomarker, with particular reference on how long influences of estrogenic substances are sustained and detectable following the actual exposure. This might be even more important for male fish, since they do not have the ability to remove VTG protein from their blood via uptake into the oocytes and until now no specific metabolic pathways for VTG protein have been found. Thus, the elimination of this rather large protein (approximately 156000 Da in fathead minnows) (Parks et al., 1999) could prove difficult for the male organism.

Another mostly neglected aspect of VTG protein synthesis in male fish is the correlation of this 'unphysiological' process with an actually harmful outcome of an estrogenic exposure on the organism. The main focus so far has been the possible reproductive impairment leaving possible adverse effects on the health of the animal largely unnoticed. Such findings would put the reproductive effects in perspective, since the most important effect would be, of course, the direct reduction of the fitness of the individual.

To elucidate these issues, it was decided to expose fathead minnows to 17α-ethinylestradiol (EE_2) at a concentration of 50 ng/l. Fathead minnow was chosen as test species, since it is one of the recommended test species for long-term exposure experiments in the testing of pesticides (OECD, 1992; EPA, 1996b,a,c; OECD, 1996). The concentration used is rather high, though still within the range found in some German rivers (Stumpf et al., 1996) and even lower than that used in recent studies, which claimed a concentration of 100 ng/l to be 'on the high end of levels found in the environment' (Bowman et al., 2000; Denslow et al., 2001a,b). This concentration was chosen mainly for two purposes: firstly, to achieve a rapid increase in VTG levels and secondly, if possible, to attain a plateau level of the protein in the plasma. In contrast to previous studies, the exposure phase was extended to 35 days with a subsequent depuration phase of 35 days, which again is in accordance with guidelines for bioaccumulation studies (EPA, 1996a; OECD, 1996). This time-frame was chosen to allow elucidation of both the accumulation as well as the clearance pattern of the VTG protein. A plateau of VTG protein in the plasma was aimed at, since this would allow for a proper analysis of the depuration kinetics, which were intended to be the main focus of this study. According to previous studies, VTG mRNA levels were expected to require a somewhat shorter time frame to attain plateau and reattain control levels (Bowman et al., 2000). The results obtained should allow for a further validation of the biomarker qualities of VTG in the assessment of estrogens in male fish. In addition, monitoring of the mortality during the study as well as the fitness levels of sampled fish should allow for further predictions concerning the correlation between VTG levels and adverse affects on the male organism induced by estrogenic influences.

2. Materials and methods

2.1. Test organisms

Sexually mature, male fathead minnows

(*Pimephales promelas*) were obtained from Osage Beach Catfisheries (Missouri, USA). Prior to and during the experiment they were maintained under standardized conditions (16 h light, 8 h dark, 30 min transition periods; active-charcoal filtered, dechlorinated water). Controlled parameters included temperature (24 ± 1 °C), oxygen saturation ($95 \pm 15\%$) and pH (8.2 ± 0.2). Feeding consisted of frozen artemia larvae at a rate of 10–15% of the bodyweight per day in two portions.

2.2. Test substance

EE_2 was obtained from Sigma-Aldrich (Buchs, Switzerland). It was shown to be pure with respect to the presence of other estrogens (estrone (E_1) and 17β-estradiol (E_2)) by comparing the GC-MS analysis to that of a certified batch of EE_2 kindly provided by Schering AG (Berlin, Germany) and batches of E_1 and E_2 obtained from Sigma-Aldrich.

2.3. Chemicals

N,*N*-dimethylformamide (DMF), obtained from Fluka (Buchs, Switzerland), was used as vehicle. Chemicals for reverse transcription (RT) were obtained from Perkin-Elmer (Weiterstadt, Germany) and chemicals for polymerase chain reaction (PCR) were purchased from Roche Molecular Biochemicals (Rotkreuz, Switzerland). All other chemicals used unless otherwise stated were of the highest quality commercially available.

2.4. Experimental design

Fish were exposed in a flow-through system to either 50 ng EE_2/l (0.0001% DMF used as vehicle), 0.0001% DMF or filtered water only. At the beginning, 144 fish were kept in each of the three 92 l tanks. The exposure lasted 35 days followed by a depuration phase of another 35 days during which all fish received filtered water.

The concentration of EE_2 was determined via comparison of the pump rates of water and substance pumps, which were measured daily (Monday to Friday) and corrected if necessary. The mean concentration was determined to be 48.3 (± 6.3) ng/l.

Fish samples ($n \geqslant 9$) were taken on days 3, 7, 14, 21, 28, 35, 36, 38, 42, 49, 56, 63 and 70 from each tank. Fish were terminally narcotized using a 100 μg/l MS222-solution (Fluka). Before blood samples were taken, length and weight of each fish were measured. Fitness was calculated using the following equation:

$$\text{Fitness factor} = 100 \times \text{weight (g)} \times (\text{length (cm)})^{-3}$$

Mortality was monitored daily and added to a 7-day-accumulative mortality for each sampling day. Subsequently, blood samples were taken by cardiac puncture with a sterile, heparinized syringe. The plasma was gained by centrifugation at $3000 \times g$ for 30 min and thereafter, stored at -20 °C. Liver samples for the determination of the VTG mRNA content were stored at -20 °C in RNAlater™ (purchased from Ambion, Austin, USA) upon sampling.

2.5. mRNA analyses

Liver samples were homogenized in the frozen state in liquid nitrogen using a mortar and pestle. Total RNA was extracted using the Perfect RNA™ Eukaryotic extraction kit (mini scale) from Eppendorf (Schönenbuch, Switzerland). After extraction, the samples were diluted to 0.5 μg RNA/μl in DMPC-treated, sterile water. RT was carried out with 0.5 μg of RNA from each sample. PCR was performed using carp α-actin primers (Watabe et al., 1995), which were responsive in fathead minnow as well, and fathead minnow VTG primers (Korte et al., 2000) in a LightCycler™ from Boehringer (Mannheim, Germany). The LightCycler™ PCR was always performed until all samples reached a plateau level of the amplified DNA, thus the number of cycles used differed among runs. Individual samples were evaluated for their VTG mRNA content with respect to a serial dilution of one sample of an exposed fish, sampled on day 35, i.e. the values given in the results are always relative to this

individual sample, which, thus, was assigned a value of 1 (= 100%). This was done because no standard cDNA was commercially available and samples of this day were assumed to have VTG mRNA levels equal or close to the maximum beforehand because of the longest exposure time. α-actin was used to verify that the same amount of cDNA was used for each sample in the PCR.

The identity of the amplified fragment as VTG was verified by agarose gel separation of the PCR products and subsequent extraction of the desired fragment, followed by sequencing of this product, performed by GATC GmbH (Konstanz, Germany). The sequence was identified using FASTA3 of the EMBL Outstation of the European Bioinformatics Institute (Pearson and Lipman, 1988).

On the basis of the protein data, only mRNA from the water and solvent control fish of days 0, 35 and 70 was analyzed.

2.6. Protein analyses

VTG protein was analyzed using the preliminary version of the carp-VTG enzyme-linked immunosorbent assay (ELISA) No. 103 from Biosense (Bergen, Norway), which was developed on the basis of the assay described by Tyler et al. (1999). This assay was performed as a competitive ELISA. For this purpose, plasma samples were thawed on ice and diluted in blocking buffer. The dilution factor for the control samples (DMF- or water-exposed animals) which was dependent on the amount of plasma gained from the individual animal was kept at a minimum and lay between 1:20 and 1:100. Plasma from exposed fish was diluted between 1:100 and 1:1000000, depending on the stage of the study.

2.7. Statistics

Statistical analyses were performed with GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). Protein and mRNA levels were compared using a one-way ANOVA with Tukey-Kramer's post-test. Protein kinetics were calculated and statistically evaluated using non-linear regression with a Run's test.

3. Results

3.1. Fitness status and mortality

Fish from control tanks yielded fitness values between 0.78 and 0.93 g/cm^3 with an average of 0.87 ($\pm$0.04) g/cm^3. In the EE_2-exposure tank, the values decreased to an average of 0.69 g/cm^3 at the end of the exposure phase (35 days), re-attaining control values of 0.88 g/cm^3 at day 70 of the study (Fig. 1). The decrease in the fitness of the exposed fish in comparison to the mean of the control fish proved to be statistically significant only for fish sampled on days 14, 35, 36, 38, 42, 49 and 56 (Fig. 1).

Mortality accumulated to a total of 3.5 and 2.8% in the control and the solvent control, respectively. In the EE_2 exposure tank total mortality reached 12.5%, with a peak mortality of 13.3% per preceding 7 days at day 36 (Fig. 2), values dropping to 0% within 2 weeks following removal of the test substance. All mortalities in this tank occurred between days 20 and 36.

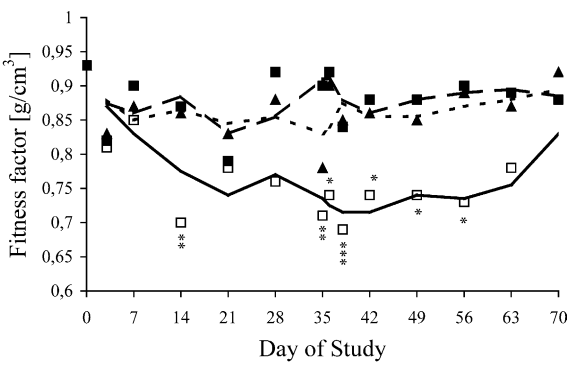

Fig. 1. Fitness factors of the sampled fish from the control (■ and dashed line), the solvent control (▲ and dotted line) and the EE_2 exposure tanks (□ and solid line). The asterisks indicate that values are significantly different compared to the other values obtained on the same day as determined using a one-way ANOVA with Tukey-Kramer's post test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $n \geqslant 9$ for each data point). The lines represent the moving average value for each treatment.

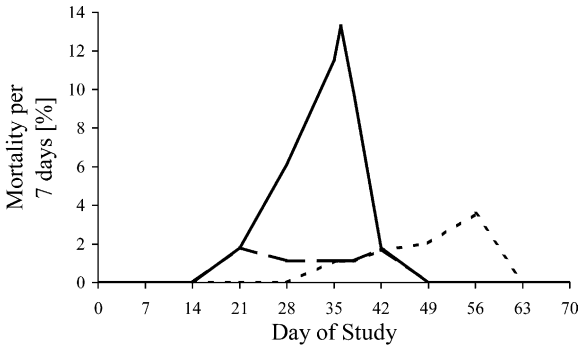

Fig. 2. Mortality per 7 days in control (dashed line), solvent control (dotted line) and EE_2-exposure tanks (solid line), as a percentage of the remaining fish in the tanks.

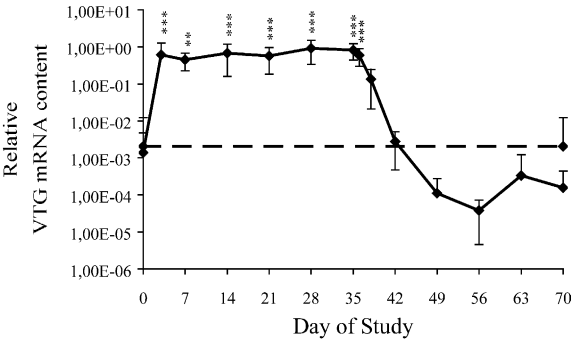

Fig. 3. Relative VTG mRNA content in pooled liver samples from control and solvent control fish (dashed line), as compared to EE_2 exposed fish (solid line). Values are given relative to a self-defined 'standard sample', which was set as 1. The asterisks indicate that values are significantly different to the pooled control as determined using a one-way ANOVA with Tukey-Kramer's post test (**$P < 0.01$; ***$P < 0.001$; $n \geqslant 9$ for each data point).

3.2. VTG mRNA development

α-Actin mRNA levels of all samples showed similar results, thus the total mRNA used for the determination of VTG mRNA was deemed to be equal for all samples. All VTG mRNA values (from exposed and control animals) are given relative to the VTG mRNA level of a randomly selected standard sample from an EE_2-exposed fish taken on day 35 of the exposure phase which was assigned a value of 1 (Section 2.5).

Since the protein levels did not vary to any significant degree between all control samples, VTG mRNA levels were just determined for water and solvent control fish from days 0, 35 and 70. No significant changes in VTG mRNA levels could be detected when comparing these control values, from either the solvent or the water control. Thus, all control values were pooled to yield a more representative control level, which was determined to be 2.05×10^{-3} (as compared to the defined standard sample).

Fish exposed to EE_2 showed a rapid increase in VTG mRNA within 3 days, from the control level to a level of 6.11×10^{-1}. The level of VTG mRNA did not vary to any significant degree until day 1 following cessation of EE_2 exposure (= day 36) having maximal levels of 9.16×10^{-1} at day 28. The VTG mRNA values from exposed fish decreased significantly to 1.33×10^{-1} on day 38 and on day 42 reached levels similar to the control value of 2.7×10^{-3} (Fig. 3). From day 42 onwards, no significant difference between control values and values from the EE_2-exposed animals could be detected.

Following the PCR reaction, the amplified fragments were separated on an agarose gel and subsequently the main fragment was sequenced and thereby, confirmed to be 98.587% identical to the gene used for the alignment of the primer. Thus, it was deemed to be the desired VTG mRNA.

3.3. VTG protein development

Samples from control animals (water and solvent) from all time-points consistently displayed VTG protein levels at or below the detection limit, which varied between 0.5 and 250 ng VTG/ml. The measurements could not be carried out to any more accurate degree, since the samples had to be diluted at least 20-fold due to their small volumes. The variation in the detection limit accounts for the strong differences within the control groups on individual sampling days. The VTG protein concentration in control fish remained at an average level (including all control fish of all time-points) of 175 ($\pm$156) ng/ml plasma for the entire duration of the experiment. No significant differences were observed between the water control and the vehicle control, thus in

contrast to previous reports (Ren et al., 1996) no estrogenic influence of DMF could be demonstrated.

EE_2-exposed fish showed an increase in plasma VTG protein over control levels beginning on day 3 of exposure (= first sampling). The most pronounced increase was observed between days 14, 21 and 28 of the study. Starting on day 28, a plateau appeared to have been reached with a level of approximately 47 mg VTG/ml plasma. This level persisted until day 38 (= 3 days following cessation of EE_2 exposure). Thereafter, VTG protein levels were observed to decrease, reaching a value of 4.6 ($\pm$ 1.8) mg VTG/ml plasma on day 35 of the clearance phase (= day 70 of the study) (Fig. 4). The increase in VTG protein concentrations in the exposed animals as compared to the control fish was significant ($P < 0.001$) for fish sampled between days 21 and 42 and for those sampled on day 49 of the study ($P < 0.01$).

The increase in plasma VTG protein concentration from days 0 to 38 could be described by a two-phased accumulation model (Fig. 4A). The first phase was calculated using the data from days 0 to 14 and consisted of an exponential increase of the protein in the blood following the equation:

$$C_T = C_0 \times e^{(KT)}$$

C_T, concentration of VTG in the plasma (mg/ml) at time T; C_0, concentration of VTG in the plasma (mg/ml) at $T = 0$; K, rate constant of the exponential increase (per day); T, time (days).

Calculation of the initial accumulation phase yielded a concentration of 10.74 mg/ml being reached on day 15. The starting concentration C_0 was calculated to be 4000 ng/ml and the rate constant to be 0.5256 per day, resulting in the doubling of VTG protein every 1.319 days.

The second accumulation phase was calculated for the data from days 14 to 38 using a one-compartment saturation model with the following equation:

$$C_T = C_{15} + C_{sat}(1 - e^{(-KT)})$$

C_T, concentration of VTG in the plasma (mg/ml) at time T; C_{15}, concentration of VTG in the plasma (mg/ml) at day 15 (= start); C_{sat}, concentration of VTG in the plasma (mg/ml) at saturation; K, rate constant of the increase (per day); T, time (days).

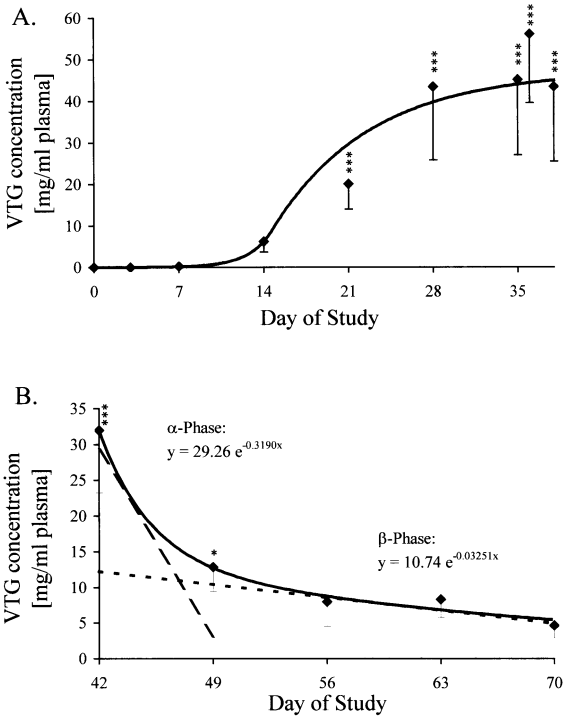

Fig. 4. Development of VTG protein plasma levels ($\pm$ S.D.) in fish sampled from EE_2-exposure tank (dots). (A) Accumulation phase and proposed kinetics model (solid line). (B) Depuration phase including a proposed kinetics model (solid line). In addition, linear regression analyses of the two elimination phases (α-phase: dashed line; β-phase: dotted line) obtained by assuming a two compartment model are given. The numerical values calculated for the model parameters were: $A = 29.26$ mg VTG/ml; $\alpha = 0.3190$; $B = 10.74$ mg VTG/ml; $\beta = 0.03251$. Control values were not included in the graph, since they were too low (mean 175 ($\pm$ 156) ng VTG/ml) to illustrate any additional information. The asterisks indicate that values are significantly different to the pooled control as determined using a one-way ANOVA with Tukey-Kramer's post test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $n \geqslant 9$ for each data point).

The saturation concentration C_{sat} was kept at a constant value of 47 mg/ml, which was the mean plateau concentration, and the starting concentration C_{15} was set to 10.74 mg/ml. This resulted in an increase rate-constant K of 0.1233 per day.

The clearance kinetics of the protein were calculated using a two-compartment open model.

The concentrations of the protein in the plasma from days 42 to 70 were fitted to a biexponential expression (Klaassen et al., 1995), added by a final plateau level being approached by the decay (Fig. 4B):

$$C_p = A e^{(-\alpha T)} + B e^{(-\beta T)} + \text{Plateau} \quad (4)$$

C_p, concentration of VTG in the plasma (mg/ml) at time T; A, B, y-intercepts; α, elimination constant I ($\alpha = \ln 2/t_{1/2}$); β, elimination constant II ($\beta = \ln 2/t_{1/2}$); T, time (days); Plateau, concentration approached through the decay.

The plateau was normalized to the control level of 175 ng VTG/ml plasma, resulting in y-intercepts of $A = 29.26$ and $B = 10.74$. The elimination constants were calculated to be $\alpha = 0.3190$ and $\beta = 0.03251$. This yielded half-lives of 2.17 days for the first and 21.32 days for the second phase of the elimination phase, respectively.

The fixed plateau level yielded a curve, fitting the actual data with $R^2 > 0.99$ and a P-value of 1.00 for the Run's test.

4. Discussion

Our results concerning the VTG mRNA levels suggest a rapid induction of gene expression and attainment of a plateau level in response to EE_2 exposure. The existence of a plateau level is in accordance with previous reports (Folmar et al., 2000) and may be due to an initial boost of VTG gene expression followed by a period where almost no additional mRNA synthesis occurs and the plateau level observed could be attributed to an extended half-life of VTG mRNA. Previous studies have reported an extension of the half-life of VTG mRNA under estrogenic influences (Brock and Shapiro, 1983; Blume and Shapiro, 1989) from 16–33 h under normal conditions to about 500 h in the presence of E_2 for *Xenopus laevis* hepatocytes in culture. In our study, the mRNA levels decreased to control level within 7 days of EE_2 removal yielding a half-life of 20–30 h. Similar results have been reported for other endocrine disrupters (EDCs) such as nonylphenol (Lech et al., 1996; Ren et al., 1996).

Although the time-course experiment described in this study was only carried out once, the resulting model most likely represents the true situation due to the very large number of samples taken. It was possible to describe the development of VTG protein concentrations in the plasma by a biphasic accumulation model. The first phase was characterized by an exponential increase in VTG concentrations and the second phase followed a saturation kinetics model. Although days 36 and 38 were actually part of the depuration phase, they were included in the accumulation kinetics, because they still displayed strongly elevated VTG mRNA levels, resulting in relatively constant protein levels. Because of the elevated VTG mRNA levels on the first 7 days after removal of the inducing substance from the water, these timepoints were not included in the determination of the depuration kinetics for the protein. Thereafter, the depuration phase yielded a two-phased decreasing curve of VTG plasma concentrations. The first phase of depuration yielded a half-life for VTG of 52.1 h, the second phase, 511.7 h. This two-phased clearance curve might be explained by a primary elimination phase, during which the VTG excretion is still under the influence of the metabolizing enzymes of the liver which have been induced by EE_2. Steroids are known to induce the activity of certain members of the cytochrome P450-dependent monooxygenase system (e.g. the NADPH cytochrome P450 reductase) (Stegeman and Woodin, 1994; Solé et al., 2000). This highly unspecific elimination system might, even if not directly induced by VTG, also be responsible for the elimination of this protein. The subsequent second phase would then represent the pure elimination phase via yet unknown mechanisms without an additional induction of the cytochrome P450-system by EE_2. This decrease kinetics might also be speculated to be caused by resorption mechanisms, i.e. VTG protein might be filtered and thus, excreted via the kidney and resorbed again. These mechanisms might either be more effective at lower concentrations of VTG protein or they are already saturated at the high levels observed during the first elimination phase. Thus, the second phase would describe the VTG excretion under physiological

conditions. In both cases, the second phase could be used to estimate the actual half-life of VTG in the male organism. The rather long half-life of approximately 21 days in this phase confirms the proposed lack of specific excretion mechanisms in male fish. An even longer half-life of VTG in male fathead minnow of more than 40 days has been proposed by Korte et al. (2000), which is similar to the half-life of 40 days described by Tata (1976) for male *Xenopus laevis*. On the other hand, Allen et al. (1999) reported a linear elimination of VTG from the blood with a calculated half-life of 13.5 days in male flounder (*Platichthys flesus*).

Maximum VTG protein levels reported for plasma of fish exposed to EDCs are relatively consistent for various studies and lie within a range of 1–120 mg/ml, depending on species (Jobling et al., 1996; Allen et al., 1999; Harries et al., 1999; Parks et al., 1999; Folmar et al., 2000; Korte et al., 2000). All of the mentioned studies employed exposure scenarios of 16–21 days. There are however, contrary opinions concerning the existence of a relationship between exposure concentration of the EDC and VTG protein 'signal' (Panter et al., 1998; Folmar et al., 2000). The data actually suggest that a concentration dependence exists as long as the VTG level does not attain maximum levels. After reaching a certain induction level this dependence ceases, resulting in the aforementioned relatively consistent maximum plasma VTG concentrations, which seem to be independent of the strength of the stimulus. This threshold value might be due to the fact that male fish possibly die at higher VTG plasma levels. The fact that mortality in the EE_2 exposure tank occurred entirely between days 20 and 36 of the exposure phase suggests a connection with the exposure to the test substance or even the high VTG levels observed. This is supported by the fact that 35 days were needed after the exposure to completely recover the fitness status of the exposed fish. The main difference between EE_2 exposed and control fish was the possible induction of the synthesis of estrogen controlled proteins, the main one being VTG. Since VTG is not synthesized under normal conditions in male fish, it could conceivably cause negative effects such as kidney dysfunction leading to death (Herman and Kincaid, 1988). Another factor which might be involved in the decreased fitness status are metabolizing enzymes in the liver such as the cytochrome P450-dependent monooxygenase system. These enzymes play a key role in the oxidative metabolism of steroids. Therefore, an increased activity of these enzymes would be expected following exposure to EE_2 (Pajor et al., 1990; Snowberger et al., 1991). A strong induction of these enzymes might cause an increased basic turnover and thereby lead to a loss of weight, i.e. a decreased fitness status of the fish. A similar trend has been described by Korsgaard and Mommsen (1993), who observed a strong decline in gluconeogenesis starting after 1 week of exposure to E_2 and reaching a maximum after 2 weeks. In addition, high plasma concentrations of a large protein might cause a blocking of small capillaries, as present in the glomeruli of the kidney and also in the liver. This would explain the histopathological changes of the main excretion (kidney) and metabolizing (liver) organs following the exposure to EDCs described elsewhere (Nicholls et al., 1968; Lewis et al., 1976; Hori et al., 1979; Schweinfurth et al., 1997; Schwaiger et al., 2000), and could also contribute to the observed mortality. The slightly increased mortality in the solvent control (between days 35 and 56) lies within the natural variability. The level remained well below 5% which is a mortality considered to be still acceptable by other studies (Zerulla et al., submitted for publication).

In conclusion, the results of the present study suggest that there is a connection between increased VTG protein concentrations in the plasma, or an increased synthesis of VTG protein, with decreased fitness values and increased mortality in male fathead minnows.

The kinetics of VTG protein versus mRNA, suggest large differences between these parameters in respect to their biomarker qualities. The slow clearance of VTG protein offers the possibility to detect even influences that occurred long before the measurement. This long half-life, on the other hand, does not allow to delimit an estrogenic influence to a specific time point. VTG mRNA measurement, however, provides a method which allows for faster detection of estrogenic influences

and moreover, to delimit the possible exposure to a relatively short period of time. This is due firstly to the shorter time (<3 days) needed for a detectable increase to occur and secondly to the fast return to control levels after removal of the EDC ($\leqslant 7$ days). An observation of both mRNA and protein levels offers the possibility to monitor longer time-frames (protein) and at the same time, allows the determination of if an exposure occurred more recently or even if the exposure is continuing at the moment of sampling (mRNA).

VTG has been implied to be a sensitive and specific biomarker for estrogenic influences. This study gives more information on its applicability for future studies. The information might prove to be of particular interest for the design of field studies and studies used for registration purposes.

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