

Associations between altered vitellogenin concentrations and adverse health effects in fathead minnow (*Pimephales promelas*)

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Abstract

Mechanism specific biomarkers are used in ecotoxicology to identify classes of chemicals and to inform on their presence in the environment, but their use in signalling for adverse effects has been limited by a poor understanding of their associated links with health. In this study an experimental analysis was undertaken to investigate how induction or suppression of an estrogen-dependent biomarker, vitellogenin (VTG), related to health effects in fathead minnow (*Pimephales promelas*, FHM). Exposure to an oestrogen agonist, estradiol (29 and 60 ng/L), resulted in rapid induction of VTG (elevated plasma concentrations within 2 days of exposure) in male FHM that was subsequently slow to clear from the plasma (concentrations remained elevated 70 days after cessation of exposure). The induction of VTG to concentrations of 0.5 mg/mL, however, and its continued presence in the plasma were not associated with any overt adverse health effects to the males. In contrast, induction of higher concentrations of VTG (>1 mg/mL) in reproductively active FHM exposed to estrone (307 and 781 ng/L), were associated with impacts on male survival (>33% male mortality) and an inhibitory effect on egg production in females (>51% decrease in egg number). Exposure of reproductively active FHM to a chemical that disrupts estrogen biosynthesis (an aromatase inhibitor; fenarimol 497 µg/L) also reduced reproductive success (40% decrease in egg number), and this was associated with a reduction in plasma VTG concentrations in females (36% decrease). These findings show that high level induction or suppression (in females) of plasma VTG are associated with alterations in health status and reproductive fitness. VTG, therefore, has the potential to act as a health measure as well as a biomarker for exposure, for chemicals that alter the oestrogen signalling pathway. © 2007 Elsevier B.V. All rights reserved.

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1. Introduction

It is now well established that effluents from waste water treatment works (WwTW) are estrogenic to fish and may impair reproduction with potential detrimental consequences for fisheries (Harries et al., 1996; Jobling et al., 1998, 2002a,b; Ma et al., 2005; Martinovic et al., 2007). The chemicals implicated as causative agents, the natural (estradiol [E2] and estrone [E1]) and synthetic (ethinylestradiol [EE2]) steroidal estrogens (Desbrow et al., 1998), were not previously subject to routine monitoring due to their low toxicity and environmental concentrations. The realisation, however, that these estrogens are prevalent in the

aquatic environment has highlighted the need for their inclusion in environmental monitoring programmes. Traditional methods of environmental monitoring focus on the measurement of concentrations of individual chemicals, however, steroidal estrogens in particular are difficult to detect analytically at the low biologically active concentrations found in complex chemical matrices (e.g. WwTW effluents). Also, such methods do not adequately address the issue of interactions between the individual steroidal estrogens, that are known to occur as mixtures in the environment, or other estrogenic and non-estrogenic chemicals that have the potential to modify their biological response. The focus has therefore shifted to the development of short-term biological tests that employ mechanism-specific biomarkers to identify estrogenic chemicals and to quantify their biological activity. In this regard, the estrogen-dependent induction of vitellogenin has been widely employed as a biomarker for chemicals that induce an estrogenic response (Sumpter and Jobling, 1995).

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Vitellogenin (VTG) is an egg yolk precursor that is produced at high concentrations in mature female fish in response to increased concentrations of endogenous estrogens (Van Bohemen et al., 1982; Ng and Idler, 1983; Tyler, 1991). Males and immature females possess very little VTG, but exposure to exogenous estrogen receptor agonists has been shown to result in high concentrations in their plasma (Sumpter and Jobling, 1995; Van Bohemen et al., 1982). There is some evidence that excessive production of VTG increases mortality in males and immature females, as a consequence of renal failure (Herman and Kincaid, 1988; Kramer et al., 1998; Schwaiger et al., 2000; Zillioux et al., 2001; Folmar et al., 2001; Seki et al., 2002; Kang et al., 2003), but the implications of lower more environmentally relevant levels of VTG induction are less clear. Even less is known on the consequences of disruptions in VTG dynamics in females and although an association between VTG induction and reduced egg production has been reported (Kramer et al., 1998), the effects on egg production were only observed at concentrations that were toxic to males. It is possible that altered VTG concentrations in adult females have consequences for reproductive output affecting either the number of eggs and/or the quantity of VTG sequestered into each individual oocyte, and thus supply of yolk for the subsequent embryo. Yolk proteins provide the majority of the nutrients for the developing embryo (cleavage products from VTG contribute >80–90% of the dry weight of the egg; Wallace, 1985) and therefore altered VTG concentrations in mature females could potentially impact on viability of the offspring.

In this study, three experiments were conducted to investigate the health effects of altered concentrations of blood VTG in fish. In the first experiment the rate of induction and clearance of the VTG protein and the associated health consequences were determined in males exposed to a potent natural estrogen (E2). In the second experiment, the health effects (survival, egg production) of VTG induction in estrogen (E1)-exposed reproductively active adult fish were investigated and the ability of the exposed adults to produce viable offspring (embryo hatching success, survival, growth and development to 80 dph) assessed. E1 is a less potent estrogen than E2, but is of considerable environmental relevance and was therefore used in the second experiment to expand on the earlier work of Kramer et al. (1998) who investigated for association between VTG induction and reduced reproductive success in fathead minnow exposed to E2. In the third experiment, the effects of a reduction in VTG concentrations for reproduction (survival, egg production and viability) were determined in fathead minnow exposed to the aromatase inhibitor, fenarimol. The collective data from these experiments were used to evaluate the potential health effects of altered VTG concentrations for the reproductive health of fish populations.

2. Materials and methods

2.1. Test organisms

The adult male fathead minnow (FHM; mean \pm S.E.M. body weight 5.59 ± 0.30 g, $n = 360$) used in experiment I were bred

at AstraZeneca's Brixham Environmental Laboratory (BEL). The FHM used for experiment II (males 4.19 ± 0.10 g, females 2.20 ± 0.05 g, $n = 44$), were originally supplied as juveniles by Osage Catfisheries Inc. (Missouri, USA) and held for 3 months at BEL, until they reached sexual maturity. The FHM used for experiment III (males 3.14 ± 0.16 g, females 1.31 ± 0.07 g, $n = 48$) were bred at BEL. Two weeks prior to the start of each study, males and females were separated and acclimated to the test conditions; de-chlorinated water at 25 ± 1 °C, with a 16 h light:8 h dark photoperiod. Fish were fed twice daily with frozen brine shrimp (Tropical Marine Centre, Hertfordshire, UK) and once daily with Ecostart 17 pelleted fish food (BIOMAR, Houghton Springs Fish Farm, Dorset, UK).

2.2. Water quality

The supply of de-chlorinated water was monitored daily for conductivity, weekly for alkalinity, hardness and free chlorine, and monthly for total ammonia. Dissolved oxygen and pH levels were determined in the individual tanks on days 0 and 1 and then twice weekly. Throughout all studies the conductivity remained between 204 and 282 μ S/cm, alkalinity between 17.4 and 35.8 mg/L and the hardness between 38.3 and 53.3 mg/L (as CaCO₃). Free chlorine remained below 2.0 μ g/L and ammonia (as N-NH₃) was below 10 μ g/L. The dissolved oxygen concentration remained >70% of the air saturation value and pH values ranged from 6.7 to 8.0. Water temperatures were monitored constantly and were between 24.3 and 25.2 °C.

2.3. Experimental design: experiment I

Male FHM were exposed for 14 days to a dilution water control (DWC), ethanol solvent control (SC) and nominal concentrations of E2 at 10, 32 and 100 ng/L ($n = 72$ fish/treatment). Subgroups of fish were randomly sampled on day 0 ($n = 16$) and on days 2, 4, 7 and 14 ($n = 8$) of the exposure. After the 14 days exposure, all remaining fish were transferred to clean aquaria receiving dilution water only and further subgroups ($n = 8$) randomly sampled on days 28, 42, 55 and 85.

Stock solutions of E2 (98% purity; Lot 70K1206; Sigma) were prepared weekly in HPLC grade ethanol (Fisher Scientific) and dosed to glass mixing vessels via a syringe pump (2.0 μ L/min), to mix with the dilution water flowing at a rate of 750 mL/min. The SC vessel received the same rate of addition of ethanol, such that the water in all test vessels, except the DWC, contained 0.003 μ L ethanol/L. The flow rates provided a 99% tank replacement time of 4 h. For measurement of E2, water samples (50 mL) were collected from each tank on days 0, 7 and 14 of the exposure, extracted onto preconditioned solid phase extraction columns and the columns eluted using 5 mL methanol. The methanol was removed under nitrogen and the extracts re-suspended in 1 mL ethanol for analysis using a commercially available enzyme immunoassay for E2 (Cayman Chemical Company, USA).

2.4. Experimental design: experiments II and III

The basic design for experiments II and III was the same. To initiate each reproduction test, males and females were wet weighed, and placed as pairs into replicate ($n=6$ replicates/treatment in experiment II and $n=8$ replicates/treatment in experiment III) glass test vessels (12 L working volume) containing a spawning substrate (PVC half guttering tile placed above a stainless steel mesh screened glass egg collection tray; Thorpe et al., 2007). The fish were acclimated to the test conditions for 10 days, and the spawning substrates checked daily (at 10:30 a.m.) for the presence of eggs to confirm spawning activity. Egg number was then determined daily for each pair of fish, over a pre-exposure period of 3 weeks, to provide pair-specific data. After determination of egg number, on the final day of the pre-exposure period, dosing of the test chemical to the individual tanks was initiated and the number of eggs spawned by each pair of fish determined daily, over a further 3 weeks exposure period. All adult fish were sampled at the end of the exposure period.

To determine whether altered VTG concentrations in the adult fish were associated with altered embryo viability, eggs were collected from a minimum of three spawning events for each individual pair of fish, during both the pre-exposure and exposure periods. Fifty eggs were collected at each spawning, rinsed in de-chlorinated water and transferred to replicate incubation cups (25 eggs/cup). The incubation cups were suspended from an oscillation unit into tanks receiving a continuous supply of dilution water. Embryo viability was assessed daily until hatch and any dead eggs noted and discarded each day.

For experiment II only, hatched fry from the last spawning event for each pair of adult fish, were maintained until 80 days post hatch (dph) to determine whether VTG induction in adults affected the survival, growth and sexual differentiation of the offspring. A total of 20 fry were selected at random from the final hatching trial for each adult pair and released into glass tanks receiving dilution water. A separate tank was assigned for the fry from each adult pair. Survival was assessed daily and at 80 dph all fish were sacrificed using the method described below and body weights and lengths determined. The sex of each fish was identified by gross examination of the gonads. Where the sex could not be easily identified macroscopically the fish were fixed in Bouin's solution and later sectioned and examined histologically. Additional fish, selected at random, were also fixed for histological examination to confirm the accuracy of the macroscopic determinations.

For experiment II, daily stocks of E1 (99% purity; Lot 30K1168; Sigma) were prepared by spiking 1 L de-chlorinated water with E1 (prepared as a concentrate in HPLC grade methanol). The stocks were dosed to glass mixing vessels using a peristaltic pump (0.375 mL/min), to mix with the dilution water flowing at 750 mL/min. The SC vessel received the same rate of addition of methanol, such that the water in all test vessels contained 0.9 μ L methanol/L. Nominal concentrations of E1 were 0, 32, 100, 320 and 1000 ng/L. For measurement of E1 concentrations, water samples were collected from one tank per treatment on days 1, 4, 7, 14 and 21 of the exposure; 500 mL (control and 32 ng/L), 250 mL (100 ng/L) and 100 mL (320 and

1000 ng/L), spiked with d_2 -estradiol (Aldrich; 98% D atom); 0.05 ng/L (control and 32 ng/L), 0.2 ng/L (100 ng/L), 2.5 ng/L (320 ng/L) and 5 ng/L (1000 ng/L) and extracted onto 47 mm C_{18} ENVI-18TM solid phase extraction disks (Supelco). The disks were dried and eluted with 15 mL HPLC grade methanol. The methanol was removed under nitrogen and the sample extracts derivatised by adding 200 μ L of pyridine (Aldrich) and 300 μ L of bis(trimethylsilyl)trifluoroacetamide with 10% trimethylchlorosilane (Aldrich) and heating the sample to 60 °C for 30 min. The reagents were removed under nitrogen and the sample residues re-suspended in 250 μ L (control, spike, 32 and 100 ng/L), 500 μ L (320 ng/L) and 1000 μ L (1000 ng/L) dichloromethane (HPLC grade; Rathburn). The final extracts were analysed using tandem GCMS; sample volume, 1.0 μ L; GC column, HP5 MS 30 m \times 0.25 mm (i.d.) fused silica with 0.25 μ m film thickness; injector temperature, 280 °C; column program (1) 75 °C for 5 min, (2) increase to 300 °C at 10 °C/min. The MS was operated in the electron impact ionisation mode (70 eV) with selected reaction monitoring. Parent ions for the E1 derivative (m/z 324), and for deuterated E2 (m/z 418) were stored and fragmented to give daughter ions (m/z 257 for E1 derivative and m/z 287 for deuterated E2).

For experiment III, solvent-free stock solutions of fenarimol (99.8% purity; lot no. 3072X; Sigma) were generated using liquid–solid saturator columns. A U-shaped glass column (1.2 m long, 25 mm i.d.) packed with glass wool, was coated with 10 g of fenarimol, dissolved in acetone. The solvent was removed under vacuum and 0.45 μ m filtered de-chlorinated water pumped through the system at a rate of approximately 20 mL/min. Concentrations of fenarimol eluting from the saturation column dropped from 50 mg/L during the first day of use to 12 mg/L on day 4, therefore, new saturation columns were introduced every 3 days. Mean measured concentrations of the fenarimol stocks were 37 ± 3.4 mg/L over the 21 days of study. The stocks were dosed at a rate of 0, 2.2, 4.8, 10 and 20 mL/min, to glass mixing vessels receiving the dilution water at a rate of 800 mL/min, to give nominal exposure concentrations of 0, 100, 220, 460 and 1000 μ g/L. For measurement of fenarimol, water samples were collected from the centre of each tank on days 1, 2, 7, 10, 14, 17 and 21 of the exposure. All samples were diluted 50:50 with acetonitrile prior to analysis. The actual concentrations of fenarimol were verified using liquid chromatography. A Jasco PU1580 LC pump was used to isocratically pump 65:35 acetonitrile:water stationary phase at 1 mL/min. Separation was achieved on a Hypersil H5BDS-C18 250 mm \times 4.6 mm (i.d.) column. The compound was detected using a Jasco UV-975 ultraviolet spectrometer at 220 nm.

2.5. Fish sampling

Fish were sacrificed in a lethal concentration (500 mg/L) of buffered MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt; Sigma). Total wet body weight was recorded to the nearest 0.01 g. Total body length was measured, using digital callipers, to the nearest 0.1 mm. Condition factor (CF) was derived by expressing the cube of the total fish length as a percentage of the body weight. Blood was collected by cardiac

puncture, using a heparinised syringe (1000 Units heparin/mL), centrifuged ($7000 \times g$; 5 min, 4°C) and the plasma removed and stored at -80°C . Plasma samples were assayed for VTG using a carp ELISA (Tyler et al., 1999). Gonads were removed and wet weighed to the nearest 0.01 mg to determine the gonadosomatic index (GSI).

2.6. Statistical analyses

All biological results are expressed as mean \pm standard error of the mean (S.E.M.). Strengths of association between plasma concentrations of VTG and body weight, length, CF, GSI and egg number and spawning frequency were measured using the Pearson product moment correlation coefficient. Effects of the chemical exposure for each endpoint, relative to the controls, were analysed using one way analysis of variance (ANOVA), where data met the assumptions of normality and homogeneity of variance, followed by a pair-wise multiple comparison procedure (Tukey test). Data which failed to meet these assumptions were analysed using a Kruskal–Wallis ANOVA on Ranks, followed by a pair-wise multiple comparison procedure (Dunnnett's method for equal sample sizes and Dunn's Method for unequal sample sizes). Effects on egg number were determined by comparing mean cumulative egg production over the 21 days pre-exposure and exposure periods, for each treatment group, using the Kolmogorov–Smirnov test.

3. Results and discussion

The results of the first experiment demonstrate some of the features of VTG induction that have contributed to the widespread use of this biomarker for the detection of estrogenic chemicals, namely its sensitivity to exogenous estrogen exposure and the rapidity of the response. Significant increases in VTG concentrations in males were observed after only 2 days (60 ng/L ; $P < 0.05$; Fig. 1). The VTG response occurred at a lower estrogen concentration with increased exposure duration, with a 2-fold decrease in the lowest observed effect concentra-

tion (LOEC) from 60 ng/L on day 2 of the exposure to 29 ng/L on day 4. The LOEC remained unchanged between days 4 and 14 of the exposure, but the concentration of plasma VTG in exposed males continued to increase with the increased duration of exposure (and with chemical concentration). On the final day of exposure (day 14) plasma VTG concentrations in males exposed to 0, 13, 29 and $60\text{ ng E}_2/\text{L}$ were 0.033 ± 0.006 , 0.054 ± 0.018 , 41 ± 11 and $595 \pm 129\ \mu\text{g/mL}$, respectively. This increase in VTG concentrations with time is consistent with reports from earlier investigations using estrogenic chemicals (Thorpe et al., 2000) and effluents (Rodgers-Gray et al., 2000) and indicates that the rate of VTG production significantly exceeds the rate of clearance in males.

VTG concentrations in males stabilised at concentrations of $500\ \mu\text{g/mL}$ after the removal of the estrogen stimulus, and there was no evidence of a significant reduction until day 41 of depuration. Even after 70 days, plasma VTG concentrations remained elevated (in males previously exposed to both 29 and $60\text{ ng E}_2/\text{L}$) when compared with controls (controls, $0.031 \pm 0.014\ \mu\text{g/mL}$; 29 ng/L $0.393 \pm 0.164\ \mu\text{g/mL}$; 60 ng/L $8.85 \pm 1.57\ \mu\text{g/mL}$; $P < 0.05$; Fig. 1). This supports the results of an earlier investigation reporting a persistence of VTG in the plasma of male rainbow trout, following a single intra-arterial injection of EE2 (Schultz et al., 2001). Males lack a natural repository for VTG (ovaries) and it has been hypothesised that the protein is sequestered or concentrated in peripheral tissues such as the kidney, where its accumulation to high concentrations is thought to result in renal failure (Herman and Kincaid, 1988; Schwaiger et al., 2000). Indeed, a number of investigations have reported toxic effects in males following short-term exposure (< 21 days) to oestrogenic chemicals, which are hypothesised to result from the induction of excessive concentrations of VTG (Kramer et al., 1998; Ankley et al., 2001; Länge et al., 2001; Seki et al., 2002; Kang et al., 2003). In this first experiment, the concentrations of VTG observed were not associated with adverse health effects in the males during the 14 days exposure, and furthermore there was no evidence that the persistence of VTG (at concentrations of up to 0.5 mg/mL) in the plasma of males adversely affected their health; survival, total wet body weight, CF and GSI did not differ significantly from the control fish at each sampling point during the exposure or depuration period ($P > 0.05$). Our data from this first experiment thus suggests that although males are slow to eliminate VTG from their plasma, they are able to tolerate elevated concentrations of VTG (to concentrations of 0.5 mg/mL) without subsequent adverse health effects (at least in the short-term).

Exposure of adult pairs of FHM to E1 (mean measured concentrations of 34, 98, 307 and 781 ng/L) highlighted the sensitivity of males to exogenous oestrogen exposure, compared with females. Concentrations of E1 required to induce a statistically significant VTG response (Fig. 2) were 10-fold higher for females (LOEC 307 ng/L ; $P < 0.05$) compared with males (LOEC 34 ng/L ; $P < 0.05$). In females exposed to both 307 and 781 ng/L of E1, VTG concentrations increased 2-fold from 1.4 mg/mL (controls) to 3.0 mg/mL , with no adverse effects on survival. In males, VTG concentrations increased from 160 ng/mL (controls) to $0.019 \pm 0.011\text{ mg/mL}$ ($34\text{ ng E}_1/\text{L}$),

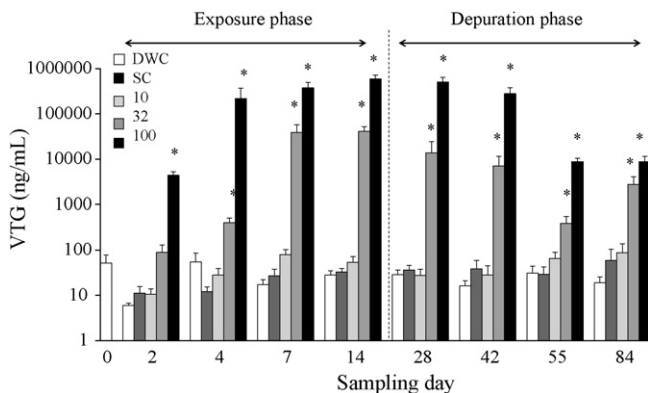


Fig. 1. Plasma vitellogenin (VTG) concentrations in male fathead minnow exposed to a dilution water control, solvent control, and 10, 32 and 100 ng/L of estradiol. Each column represents the mean \pm standard error of the mean (S.E.M.). Significant differences from solvent control values at each sampling point are denoted as $*P < 0.05$ (Dunnnett's test).

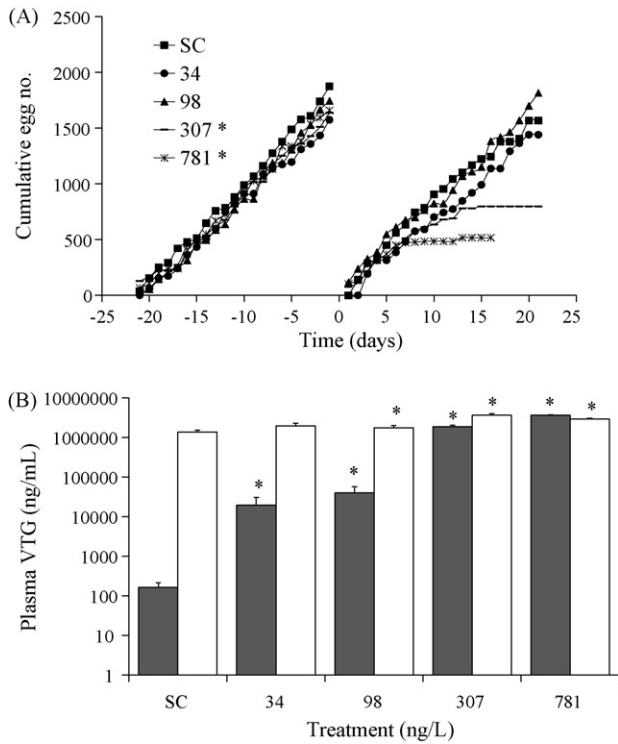


Fig. 2. The effect of estrone on egg production (A) and VTG concentrations (B) in adult fathead minnow. Graph A (adapted from Thorpe et al., 2003) shows the mean cumulative egg number (as assessed daily) for each treatment group over a pre-exposure period (days -21 to 0) and an exposure period (days 0 to 21). Significant differences between the pre-exposure period and the exposure period for each treatment group are denoted as * $P < 0.05$ (determined using the KS-test). Graph B shows the mean VTG concentrations in males (black bars) and females (white bars) for each treatment group. Significant differences between the control and exposure groups are denoted as * $P < 0.05$.

0.039 ± 0.017 mg/mL (98 ng E1/L), 1.9 ± 0.2 mg/mL (307 ng E1/L) and 3.6 ± 0.1 mg/mL (781 ng E1/L). The induction of VTG to concentrations in the mg/mL range in males were associated with adverse effects on survival; two out of six males from the 307 ng E1/L treatment and three out of six males from the 781 ng E1/L treatment died during the exposure. This compares with previous observations where induction of high concentrations of VTG (resulting from exposure to estrogenic chemicals) were linked with toxic effects in males but not in females (Kramer et al., 1998; Ankley et al., 2001; Länge et al., 2001; Seki et al., 2002; Kang et al., 2003).

In the exposures to 307 ng/L and 781 ng E1/L exposures, the high concentrations of VTG were associated with an inhibitory effect on reproduction, with reductions in the number of eggs spawned during the 21 day exposure period by 51% ($P < 0.05$) and 66% ($P < 0.01$), respectively, compared with the pre-exposure period (Fig. 2). This effect on egg production was the result of a decrease in the number of spawning events over the 21 day exposure period ($P < 0.05$). The number of eggs per spawning did not change. There was an exponential relationship between induction of VTG and a reduction in both total egg production ($P < 0.001$; Fig. 3A) and the number of spawning events ($P < 0.001$; Fig. 3B); in males VTG was induced at very low concentrations of E1, but reproduction was inhibited only when VTG concentrations were increased by more than 10000-fold to reach mg/mL concentrations in the plasma of the males. No effects on reproduction were observed when VTG concentrations in males remained below this level. It has previously been demonstrated that the induction of VTG to concentrations that are associated with male toxicity results in an accumulation of VTG in the testis vasculature leading to an increase in testicular atrophy, impaired spermatogenesis and reduced GSI (Folmar et al., 2001). The observation of a 23 and 30% decrease in GSI for males exposed to 307 and 781 ngE1/L, respectively, in our study is consistent with these earlier findings, indicating that

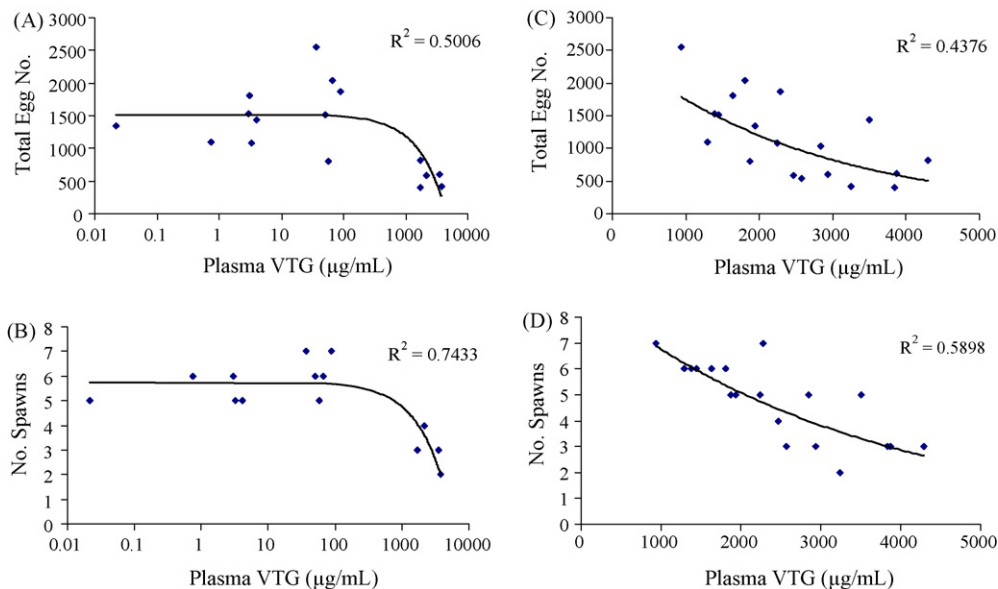


Fig. 3. Assessment of the relationship between male (A and B) and female (C and D) plasma VTG concentrations and total egg number (A and C) and number of spawnings (B and D) in adult fathead minnow exposed for 21 days to estrone (concentration range 34–781 ng/L).

the reduced spawning frequency was the result of impairment in spermatogenesis.

In E1-exposed females, there was an inverse relationship between VTG concentrations and total egg production ($P < 0.001$; Fig. 3C) and the number of spawning events ($P < 0.001$; Fig. 3D). However, it is not possible to determine if the increased VTG concentrations were responsible for the reduced egg production or, as is perhaps more likely, whether the increased VTG concentrations resulted from the lower egg production (and thus a lower rate of VTG clearance from the blood). Irrespective of whether the increased VTG resulted in reduced egg production or the reverse, a demonstration of a link between these two parameters does indicate that in environments where both males and females are exposed simultaneously, increases in VTG concentrations can be associated with a reduced reproductive success.

There was no evidence that exposure of the adult fish to E1 adversely affected gamete viability; mean hatching success ($91 \pm 2.8\%$) did not vary between embryos collected during the pre-exposure and exposure periods for pairs of exposed fish ($P > 0.05$). In addition, there were no differences in the survival ($87 \pm 4.4\%$), total wet weight (1.44 ± 0.04 g), condition factor (1.08 ± 0.01) or sex ratio ($57 \pm 4.3\%$ males) of the offspring hatched from the control adults, compared with those from the E1-exposed adults. These results demonstrate that exposure of the adult females to E1 does not have any subsequent effects for the offspring and further indicates that a 2-fold increase in the circulating VTG concentrations in adult females does not confer any fitness advantages or disadvantages to the offspring (in terms of the fitness parameters measured here).

In experiment III, exposure of adult pairs of FHM to fenarimol (mean measured concentrations of 0, 51, 94, 178 and $497 \mu\text{g/L}$) resulted in a concentration-related decrease in plasma VTG concentrations in females ($P < 0.01$), with a significant effect relative to the controls at the highest exposure concentration (Fig. 4; $497 \mu\text{g/L}$; $P < 0.05$). There was no evidence of an effect on plasma VTG concentrations in males. The observed decrease in female plasma VTG concentrations here is consistent with a previous investigation (Ankley et al., 2005) and with the proposed mechanism of action for fenarimol, which is hypothesized to reduce endogenous estrogen activity through either acting as an inhibitor of aromatase activity or through directing binding the estrogen-receptor as an antagonist. The decrease in female VTG concentrations was associated with a 40% decrease in total egg production (reduced

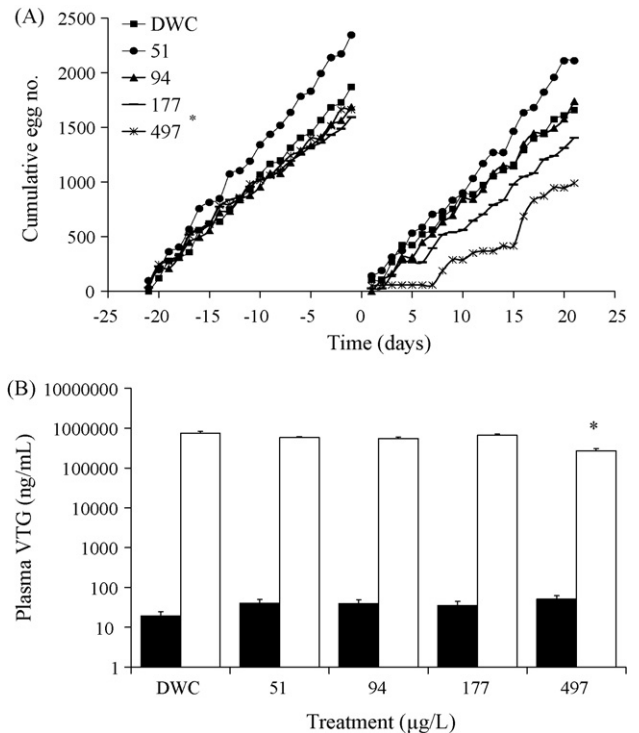


Fig. 4. The effect of fenarimol on egg production (A) and VTG concentrations (B) in adult fathead minnow. Graph A shows the mean cumulative egg number (as assessed daily) for each treatment group over a pre-exposure period (days -21 to 0) and an exposure period (days 0 to 21). Significant differences between the pre-exposure period and the exposure period for each treatment group are denoted as $*P < 0.05$ (determined using the KS-test). Graph B shows the mean VTG concentrations in males (black bars) and females (white bars) for each treatment group. Significant differences between the control and exposure groups are denoted as $*P < 0.05$.

number of spawning events) at the highest concentration tested ($497 \mu\text{g/L}$; $P < 0.01$; Figs. 4 and 5). There was a complete inhibition of spawning during the first week of exposure in the highest treatment group ($497 \mu\text{g/L}$), but by day 15 all pairs of fish had resumed reproductive activity, although the spawning patterns were altered. Specifically, the intervals between spawns increased resulting in reduction in the number of spawning events from 6 ± 0.3 to 2 ± 0.6 spawns/21 days ($P < 0.05$) and the number of eggs per spawn increased from 261 ± 30 to 431 ± 95 eggs/spawn ($P < 0.05$). This may reflect alterations in the timing of oocyte maturation in the females, due to a disruption in the balance of the sex steroid biosynthesis pathway

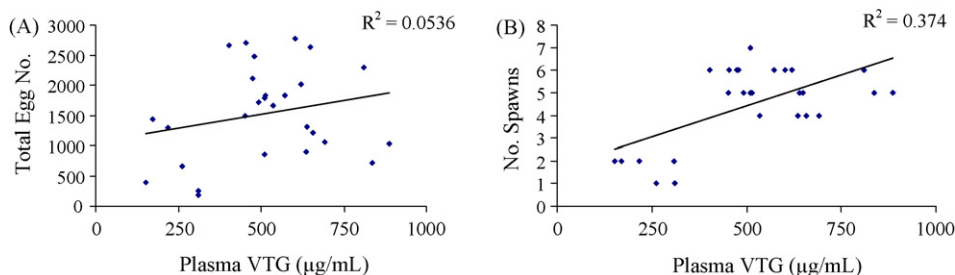


Fig. 5. Assessment of the relationship between plasma VTG concentrations and total egg number (A) and number of spawnings (B) in adult female fathead minnow exposed for 21 days to fenarimol (concentration range 51 – $497 \mu\text{g/L}$).

and supports the earlier work of Ankley et al. (2005) who showed altered timing of oocyte maturation through histological examination of gonads from fenarimol-exposed females.

The decrease in circulating concentrations of VTG in the adult females did not appear to affect embryo viability; percentage hatch ($94 \pm 1.5\%$) did not vary between the exposure and pre-exposure periods for embryos collected from the individual pairs ($P > 0.05$). This suggests that through altering the timing of oocyte maturation, the females are able to compensate for the reduced VTG production to provide the required nutrient levels for maintenance of high survivorship in embryos.

4. Conclusion

The collective results from these investigations support an earlier investigation (Kramer et al., 1998) in demonstrating that the induction of VTG to mg/mL concentrations, signals for adverse reproductive impact in males. Induction of lower levels of VTG (up to concentrations of 0.5 mg/mL), however, did not adversely affect the health of males in the short-term exposure described (21 days). Furthermore, although VTG persisted in the plasma of males, following removal of the estrogen stimulus, there was no evidence for adverse health effects of elevated plasma VTG (up to 0.5 mg/mL) for a period of up to 70 days. The poor ability of males to clear VTG, however, could lead to an accumulation to concentrations that have adverse health effects during prolonged exposures, as can occur in the environment, and should be considered further (Nash et al., 2004; Parrott and Blunt, 2005). In mature females, both an increase and a decrease in VTG concentrations were associated with a reduced reproductive capacity (a reduction in the frequency of spawning that led to an overall reduction in the number of eggs spawned). This further supports the work of Kramer et al. (1998) and Miller et al. (2007) in demonstrating that alterations in the dynamics of the normal cycle of VTG concentrations in mature females could potentially be used to signal for adverse reproductive health effects. The natural variation in VTG concentrations between females with differing stages of sexual maturity, however, is likely to restrict the use of VTG as a biomarker for the reproductive health of females to laboratory test scenarios only, rather than for natural populations.

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