Oestrogenic Activity of Tropical Fish Food Can Alter Baseline Vitellogenin Levels in Male Fathead Minnow (*Pimephales promelas)*

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Abstract–Vitellogenin (VTG) is a precursor of egg-yolk protein and is therefore present at high concentrations in the plasma of female fish. In male fish, VTG concentrations are usually undetectable or low, but can be induced upon exposure to oestrogenic substances either via the water or the diet. This work was carried out to determine the reason for apparently elevated VTG concentrations in unexposed stock male fathead minnow maintained in our laboratory. The results showed clearly that some of the food given to the fish was oestrogenic and that replacement of this with non-oestrogenic food led to a significant reduction in the basal VTG levels measured in male fish after a 6 month period. This reduction in male VTG concentrations drastically increased the sensitivity of the VTG test in further studies carried out with these fish. Moreover, a review of published concentrations of VTG in unexposed male fathead minnow suggests that this problem may exist in other laboratories. The fathead minnow is a standard ecotoxicological fish test species and so these findings will be of interest to any laboratory carrying out fish tests on endocrine disrupting chemicals.

Keywords–Vitellogenin, Fathead minnow, Fish diet, Oestrogenic food

INTRODUCTION

Vitellogenin (VTG) is a precursor of egg-yolk protein, present in high concentrations (typically from 1 – 15 mg/ml [1-3]) in the plasma of sexually mature female fish. It is produced in the liver in response to oestrogens from the ovaries. Under normal circumstances, VTG levels in male fish are either undetectable or low (approximately 10,000 to 100,000 times lower than typical female concentrations [4,5]). However, when male fish are exposed to oestrogenic substances, they are capable of producing a large amount of VTG and blood concentrations can reach values similar to those in females. Thus VTG induction in male fish is commonly used as a specific biomarker for the detection of oestrogenic endocrine disruptors in the environment and in Organisation for Economic Cooperation and Development tests [6] of suspected oestrogenic chemicals [4,7].

A number of direct and indirect methods exist for measuring VTG; enzyme-linked immunosorbent assays (ELISAs) are most commonly used nowadays because they are easy to perform, highly sensitive, and amenable to high throughput. However, because laboratories often use slightly different ELISAs, results are not always comparable between laboratories [8]. The need for this investigation arose when we noticed an increase in the VTG concentrations measured in unexposed male fathead minnows maintained in our laboratory. The increase coincided with a change from using a heterologous carp competitive ELISA employing a polyclonal antibody to a commercial fathead minnow sandwich ELISA employing a monoclonal antibody. Independent analysis of the samples with a fathead minnow ELISA utilizing a polyclonal antibody [9], however, produced the same elevated results (Fig. 1A).

Moreover, the concentrations of VTG found in our male stock fish further increased with time (see Fig. 1B). Taken together, these results raised concern about the sensitivity of our ecotoxicity test in which VTG induction in male fish is used as a biomarker of exposure to oestrogenic chemicals. The presence of higher than normal basal levels of VTG in male fish may mask the effects of weakly oestrogenic compounds on VTG induction, and thus, desensitise any assay used to measure these effects [10].

A review of the literature revealed that published basal concentrations of VTG in fathead minnow vary greatly; from undetectable/very low levels [11,12] to levels in the μg/ml range [3,13]. There is also disagreement over the levels of VTG that one would expect to find in unexposed male fathead minnow, and some researchers believe that where VTG is detected in male fish, this is due to an exposure to oestrogens via the water or food [14]. Vitellogenin is also highly fragile, and physical or proteolytic degradation can lead to an overestimation of the amount of VTG present in stored samples due to breakdown of the molecule and an increase in the number of antigenic sites [15]. Other researchers argue that the VTG levels determined using heterologous ELISAs are too low compared with those data obtained using homologous ELISAs because the antibodies are not specific enough [16,17], yet they have been shown to have good cross-reactivity with VTG in other species [18,19].

When taken together, the review of the literature and the results of our investigations suggested that VTG concentrations found in our male stock fathead minnows may be abnormal and could have arisen due to oestrogenic contamination

of either the food or the water, or both. Contamination of the water could occur via exposure to xenoestrogens (e.g., from plastic pipe work and tubing [20]) or via exposure to natural oestrogens released by female fish also present in the same water as the males [21]. This may be more apparent when fish are kept at high density with a recirculating water supply (e.g., in stock tanks) or in experiments with slow flow rates and thus long water replacement times [22]. This is further exacerbated by the fact that males lack the ability to readily eliminate VTG. Hence, concentrations can remain elevated for a period of weeks after exposure to an oestrogen and may at that time not reflect the effect of treatment [23].

Commercial fish diet is a known source of oestrogens. Matsumoto et al. [24] surveyed fish foods for oestrogenic activity using a yeast oestrogen screen and found all the foods tested to have activity. Commercial fish diets contain high levels of protein, in the form of fish meal and soybean. Fish meal, which usually represents 15 to 40% of the diet [21], is prepared from several sources, including whole fish and fish viscera. Depending on the fish species and the sexual stage of the fish available, fish meal can contain high levels of sex steroid hormones, and Feist and Schreck [25] found that sex steroid levels in fish diets were correlated with the amount of fish by-products incorporated into them. Fish diets are also prepared using soybean (up to 20-30%), which is known to contain several kinds of phytoestrogens, such as genistein and daidazein, and these have been shown to have oestrogenic activity [24,26,27]. Furthermore, Miyahara et al. [26] tested a variety of animal diets using an in vitro yeast 2 hybrid assay, and found a correlation between the phytoestrogen content and oestrogenic activities of the diets.

Pelissero et al. [28] observed that both a commercial and a soya-based synthetic diet stimulated significant levels of plasma VTG in Siberian sturgeon compared with a diet based on casein. Other species of fish have also been shown to experience diet-induced increases in the plasma VTG concentration, caused by sex steroids and/or phytoestrogens in the diet, including tilapia [10], goldfish [29,30], medaka [31], and carp [32]. No studies have, however, been carried out on commonly available tropical fish diets or with fathead minnows. Although our tropical fish food had tested negative for environmental contaminants, oestrogenic activity could also have arisen from natural hormones present in fish meal or phytoestrogens.

In this study, we aimed to determine whether either endogenous sex steroids in the water or sex steroids/phytoestrogens in the fish diet were responsible for the raised VTG levels in our unexposed male fathead minnow.

MATERIALS AND METHODS

Test organisms

Fathead minnows were hatched and reared at Brunel University. Adult fish (12±1 months, unless otherwise stated) were maintained in dechlorinated tap water at 25±1°C and with a photoperiod of 16:8 h light:dark. Fish were fed frozen brine shrimp twice daily and flake food once daily. All pipe work and casings were glass, silicone or Durapipe ABS (Durapipe UK, Cannock, UK).

Experiment 1: effect of natural steroids in the water system on VTG levels

Experimental design. At time zero, 16 male fish were sampled. Granulated activated charcoal was then added to the recirculating water system (Tropical Marine Centre, Chorleywood, UK) to remove any organic chemicals, including steroid oestrogens, and the charcoal in this system was renewed every 2 weeks. Fish were placed in 25L mixed sex tanks (8 males and 8 females) and 25L male only tanks (16 males) at flows of 40L/h. In addition to the tanks set up with recirculating water, tanks were similarly set up under flow-through conditions. Male fish only were sampled after 6 weeks and 12 weeks.

At each sampling point, 1L tank water samples were collected from all tanks for determination of oestrogenic activity. 5ml methanol was added to each water sample to prevent bacterial growth and aid subsequent solid-phase extraction [33] prior to storage at 4°C.

Preparation of water samples for the analysis of oestrogenic activity. After overnight storage at 4°C, the 1L water samples were extracted by passing them through preconditioned Sep-Pak C_{18} cartridges (Waters Ltd., Elstree, UK). The extract was then eluted with 5ml methanol, dried under nitrogen and dissolved in 0.5ml ethanol. Negative controls and oestradiol-spiked positive controls were included with every set of extractions. Samples were stored at 4°C until tested for oestrogenicity using the yeast oestrogen screen (YES assay; [34]). Each sample was tested in duplicate and every assay was carried out at least twice. The water samples had been concentrated 2000 times, and the resulting detection limit for oestradiol in these water samples was 0.01ng/L.

Experiment 2: effect of fish diet on VTG levels

Preparation of fish food samples for the analysis of oestrogenic activity. Seven commercial fry/juvenile diets and 7 adult diets were tested for oestrogenic activity. The fry/juvenile diets were as follows: Tetramin Baby (Tetra, Southampton, UK), ZM100, ZM200, ZM300, ZM400 (Zebrafish Management, Winchester, UK), Liquifry No. 1 (Interpet Ltd., Dorking, UK) and Liquifry Baby Plus (Interpet Ltd.). The adult diets tested were Brine shrimp (San Francisco Bay Brand, Newark, CA, USA), Ecostart 17 1mm pellets (Biomar, Brande, Denmark) and the following tropical flake foods: Tetramin (Tetra), King British (King British, Gainsborough, UK), Aquarian (Mars Fishcare Europe, Southall, UK), Nutrafin Max (Rolf C. Hagen, Castleford, UK) and New Era (New Era Aquaculture, Thorne, UK). In addition, for two adult diets (King British flake food and Tetramin flake food), 4 different batches of each were tested.

One gram of each fish food was mixed with 25ml double glass-distilled water in a 50ml polypropylene tube. This was homogenised for a total of 4 mins using a Polytron (Kinematica AG, Lucerne, Switzerland) electronic homogeniser at setting 25. Then 20ml ethyl acetate was added, and the samples were stored overnight at 4°C with periodic mixing. Tubes were centrifuged for 1 h at 4°C and 700rpm. The extract was then removed to a 15ml polypropylene tube, dried under vacuum centrifugation, and dissolved in 1ml ethanol. A negative control without the addition of fish food and an oestradiol-spiked positive control were also included. Samples were stored at 4°C until tested for oestrogenicity using the YES assay [34]. Each sample was tested in duplicate and every assay was carried out at least twice.

Experimental design. At time zero, the fish diet was changed from King British tropical flake food to Tetramin tropical flake food. These fish were maintained in a

recirculating water system. Adult male fish only (*n*=8) from 100L mixed sex tanks were sampled after 3 months and 6 months on the new diet. In addition, six month old fish (*n*=8) fed all their lives on Tetramin flake food, were sampled from a 100L mixed sex tank.

Fish sampling

Fish were sacrificed with a lethal dose of buffered MS-222 (Sigma-Aldrich, Poole, UK) and blood was collected from the caudal vein/artery using a heparinised microhaemocrit tube. Plasma was collected by centrifugation at 7000*g* for 5mins at 4°C, and stored at -80°C until use. Fork length and wet weight were measured and the condition factor was calculated by expressing the cube of the length as a percentage of the weight. The gonads were dissected and weighed, and gonadosomatic indices (GSIs) calculated by expressing the gonad weight as a percentage of the total weight.

Vitellogenin analysis

Plasma VTG concentrations were measured using a homologous VTG kit designed specifically for fathead minnow (Biosense Laboratories AS, Bergen, Norway). The detection limit for this assay was 5ng/ml (Experiment 1) and 2.5ng/ml (Experiment 2). Plasma samples were diluted 1:50, 1:5000 and 1:500,000 and assayed in duplicate according to the manufacturer's protocol. Interassay variation was 15.6% (R^2 values for the standard curves in Experiment 1 were greater than 0.99 and for Experiment 2, 0.995.).

Statistical analyses

Data sets were examined for normal distribution and homogeneity of variance, and vitellogenin data sets were $\log_{10}$ transformed. One-way analysis of variance was then carried out, followed by Tukey's pairwise comparisons, which made it possible to identify differences between groups. Statistical analyses were carried out with the program SigmaStat 3.5 (Systat Software, USA). Differences were considered significant at $p \leq 0.05$.

RESULTS

Experiment 1: effect of natural steroids in the water system on VTG levels

Water analysis. The positive control recoveries were ≥90% and the detection limit for the yeast oestrogen screens was 21ng/L (data not shown). No oestrogenic activity was detected in any of the water samples from the fish tanks.

Biological results. There were no significant differences between any of the biological measurements (fork length, wet wt, condition factor, gonad wt, and GSI) in any of the groups of fish, at each time point.

When charcoal was added to the recirculation water system, there was no reduction in the basal VTG concentrations with time, and these remained very high for the 12 week duration of the study (mean values remained between 10,000 and 100,000ng/ml; only 100 to 1000 times less than typical female VTG concentrations [2]). Similarly, there was no difference between the VTG concentrations in the fish kept under recirculating water conditions and those in a flow-through system, or

between those kept in single sex tanks and those kept in tanks with females (data not shown).

Experiment 2: effect of fish diet on VTG levels

Food analysis. For the fish food extractions, the positive control recoveries were ≥83% and the detection limit for the yeast oestrogen screens was 21ng/L. Of the juvenile fish diets, only the Liquifry had any oestrogenic activity (Fig. 2A). At the higher Liquifry concentrations, there was a reduction in the absorbance (apparent reduction in oestrogenic activity) measured in the YES assay, which was concomitant with a reduction in the yeast turbidity and it is therefore difficult to determine whether the reduction in absorbance was due to toxicity or to anti-oestrogenicity. Three of the adult fish foods (King British, Nutrafin and Ecostart) had oestrogenic activity, with the most oestrogenic being the 'King British' flake food (Fig. 2B). The different batches of Tetramin flake food were all non-oestrogenic (Fig. 3A), whereas an equal number of batches of King British flake food were consistently oestrogenic (Fig. 3B).

Biological results. There were no treatment-related changes in the weights or lengths of the fish, although the fish only ever fed Tetramin were smaller than the other groups of fish at the end of the study because they were 6 months younger than these groups at the start of the study. The condition factor of the fish initially fed King British flake increased slightly 3 months after the change in diet to Tetramin (p=0.038), but by 6 months the condition factor was not significantly different. There

were no significant differences in the gonad weight or GSI between the different groups of fish.

Fathead minnow fed on King British flake food until the start of this experiment had mean and median VTG concentrations of approximately 100,000 and 10,000ng/ml, respectively (Fig. 4). Three months after the change in diet to Tetramin, VTG concentrations had reduced to a mean value of 1970ng/ml and a median value of 621ng/ml, although this reduction was not statistically significant at the 95% level (p=0.249). After 6 months on Tetramin flake food, VTG concentrations had significantly reduced to mean and median values of 4507 (this higher mean VTG value was due to one fish with a level of 34,550ng/ml) and 114ng/ml, respectively (p=0.006). Fish that hatched after the change in diet, and therefore had only ever been fed Tetramin, had even lower mean and median concentrations, of 172 and 41ng/ml (p<0.001), than those fish that had only ever been fed on King British flake food.

DISCUSSION

The results of this study confirmed our hypothesis that the VTG concentrations in male fish at the start of our study were abnormally high as a result of the ingestion of commercial fish diet contaminated with oestrogenic substances. Considering that the fish receive the food continuously, any oestrogenic activity of the fish diet is likely to affect the baseline VTG concentration measured in male fish and reduce the sensitivity of experiments designed to assess the oestrogenicity of chemicals. As male fish lack the ability to remove VTG from their blood via uptake into oocytes, VTG concentrations remain high in the plasma for many weeks after exposure [1,35,36]. This probably explains why there was only a very slow reduction in the

plasma VTG concentration after ceasing to feed the fish with oestrogenic food. As mentioned briefly in the introduction, the King British fish food did not contain detectable levels of over 200 environmental contaminants, from numerous groups including those from organochlorine, organophosphate, organonitrogen, triazine, carbamate, triazole, dicarboximide, strobiluron, and pyrethroid groups, but this does not rule out the possibility of additive effects of undetectable levels of contaminants. Moreover, the King British flake food was the most oestrogenic, but as this food contains higher amounts of both fish meal and soya, it is unclear whether one or both of these components were the reason for this activity, and identifying the oestrogenic culprit(s) remains outside the scope of this present study.

Our data provide evidence to refute the theory that the presence of VTG in the plasma of our unexposed male fish was a result of method artefacts. After 6 months on a non-oestrogenic diet, the VTG concentrations in our stock fish were approximately 100ng/ml, and comparable to many of the lower levels reported in the literature. We cannot say, however, that these levels are now normal, as there are very few baseline data to know how our present levels would compare with those in wild fathead minnows or whether they may still be elevated compared with concentrations determined using a more sensitive assay, e.g., a luminometric immunoassay [37].

The VTG concentrations measured in the fish, even within the same treatment group, were highly variable. Other researchers have also reported large individual variation in the VTG concentration of control fish [38] and in the VTG response following oestrogen exposure [39]. This variation in plasma VTG concentration

between fish seems unlikely to be due to the physiological status of the fish, as all fish within one tank were of the same age and kept under identical conditions. Also, it is not likely to be due to different amounts of oestrogenic activity in fish diet consumed by individual fish, as even after the change to non-oestrogenic food, the VTG concentrations remained variable. Therefore, it could be due to genetic variation in the degree of the response of the fish. Biales et al. [40] examined the transcriptional responses of the VTG gene in fathead minnows and found high biological variability between identically treated individuals, even under controlled laboratory conditions. The variability was not seen in other genes from the same RNA preparations, suggesting that it was specific to the VTG response.

The drastic reduction in the plasma VTG concentrations of unexposed male fathead minnows led to an increase in the sensitivity of further assays that used VTG concentrations as an endpoint. For example, we carried out an exposure study 7 months after our feeding regime changed and, in that study, control male plasma VTG concentrations were found to be 130±258ng/ml (see Table 1) when the fish were maintained in single sex tanks (8 male fish per 20L tank), and 1482±1513ng/ml when male fish were paired with female fish in 6L tanks. These results further confirmed that the raised concentrations of VTG seen previously were not an artefact of the commercial ELISA kit that we had used. With this increased sensitivity, it was possible to measure statistically significant higher VTG concentrations in male fish kept in tanks with female fish (these were mirrored by the higher levels of steroids in these tanks; see Table 1) than in those maintained only with other males.

The results of this work will be relevant to any laboratory that carries out exposure studies with any species of fish. Contaminated food may be the reason for other laboratories reporting VTG concentrations in control male fish in the µg/ml range. Our study strongly suggests that such concentrations are abnormal, and perhaps not due to technical problems or artefacts with the assays used to measure VTG.

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Legends

Figure 1. (**A**) Plasma vitellogenin (VTG) concentrations in male fathead minnow determined using the Biosense fathead minnow VTG enzyme-linked immunosorbent assay (ELISA) (black bars) and a non-commercial fathead minnow VTG ELISA (grey bars). Baseline fish were sampled prior to a 3-week exposure to no chemical (control tank 1 and control tank 2) or to oestradiol at 100ng/L (E2 tank 1 and E2 tank 2). Values are expressed as means ± standard deviation (n=16). Pearson's product moment correlation coefficient was r=0.766. (**B**) Plasma VTG concentrations in control male fathead minnow determined using the Biosense fathead minnow VTG ELISA. The data are control values from exposure studies carried out at Brunel University between 2003 and 2008. Many of these control values are solvent controls, and the solvent used is indicated in the parentheses (etOH = ethanol; DMF = dimethylformamide). The box represents the 25th and 75th percentiles, the whiskers the 5th and 95th percentiles, and the dots are the outliers.

Figure 2. Oestrogenic activities of (**A**) Juvenile fish foods, and (**B**) Adult fish foods. The inset on 2B shows in more detail, the lower end of the scale of the same adult food plots. Each point is the mean of duplicate values and the data are representative of the experiments carried out.

Figure 3. Oestrogenic activities of four different batches of (**A**) Tetramin tropical flake food, and (**B**) King British tropical flake food. Each point is the mean of duplicate values and the data are representative of the experiments carried out.

Figure 4. Plasma vitellogenin (VTG) concentrations in male fathead minnow after a change in diet from King British flake food to Tetramin flake food. The boxes represent the 25th and 75th percentiles, the whiskers the 5th and 95th percentiles, and the dots are the outliers. Significantly different from the VTG levels in the fish fed only King British flake food at $p<0.01$ (**) and $p<0.005$ (***).

Table 1. The data are from an exposure study carried out after the change in diet to non-oestrogenic Tetramin flake food. The first three rows show concentrations of oestrone (E1), 17 β-oestradiol (E2) and oestradiol equivalents (E2EQ, measured by the yeast oestrogen screen) in the water of control fish tanks containing either male or mixed sex fish. The final row shows plasma vitellogenin (VTG) concentrations in control male fathead minnow from these tanks, determined using the Biosense fathead minnow VTG enzyme-linked immunosorbent assay (n=8).

Figure 1A.

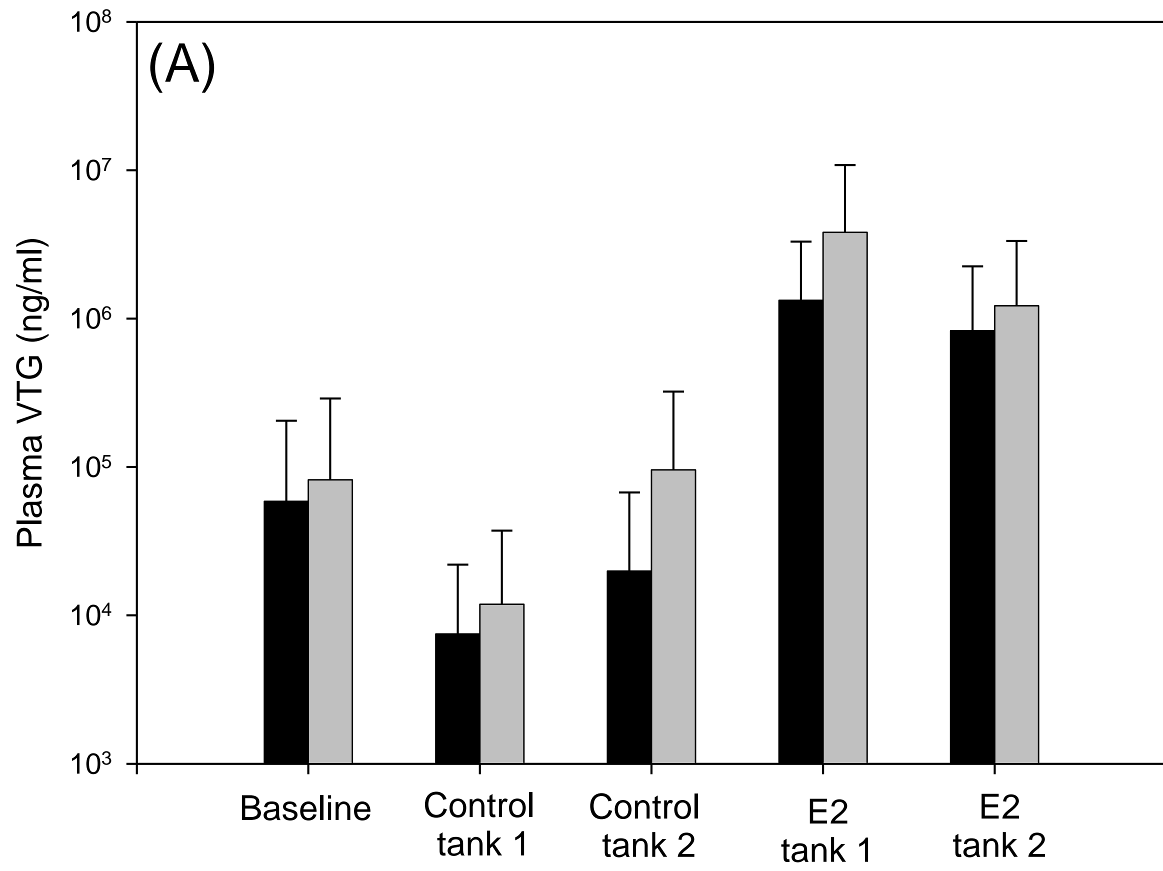

Figure 1B.

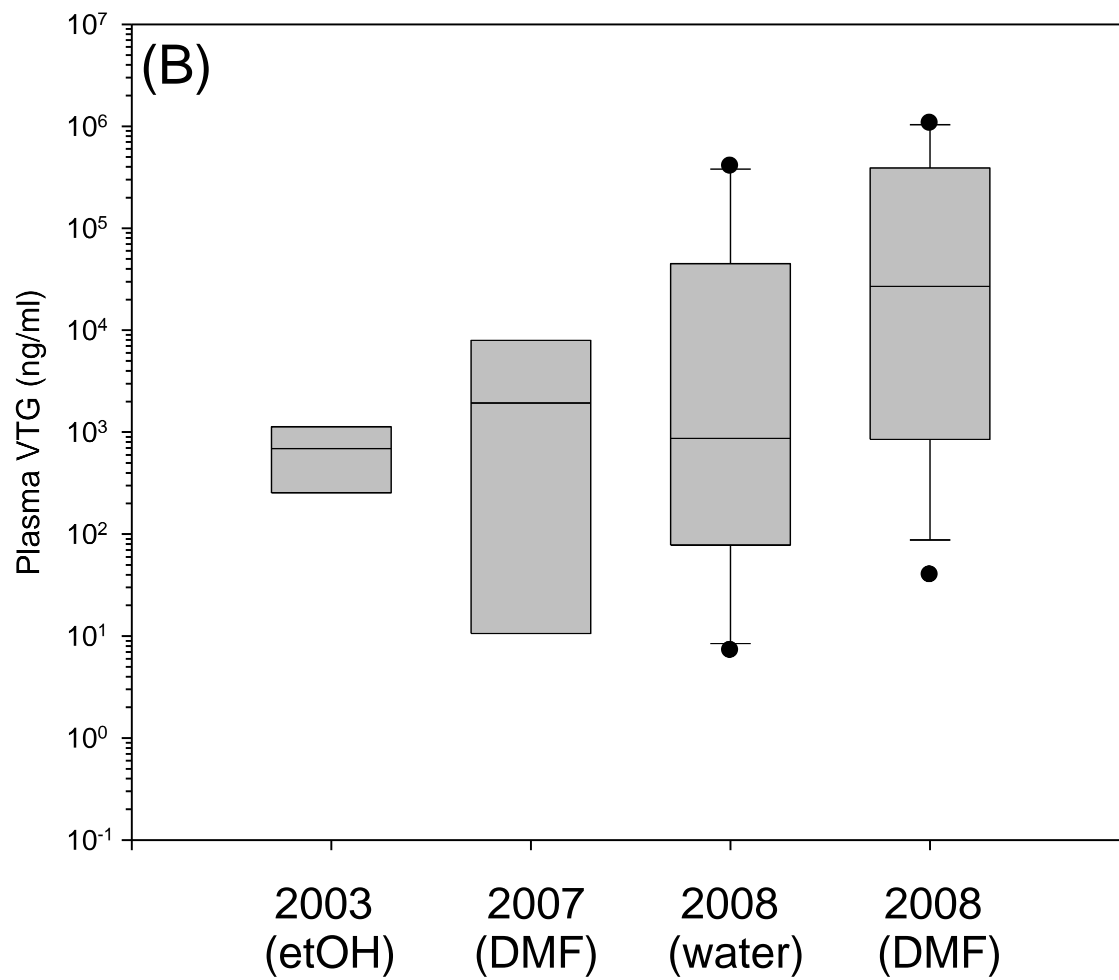

Figure 2.

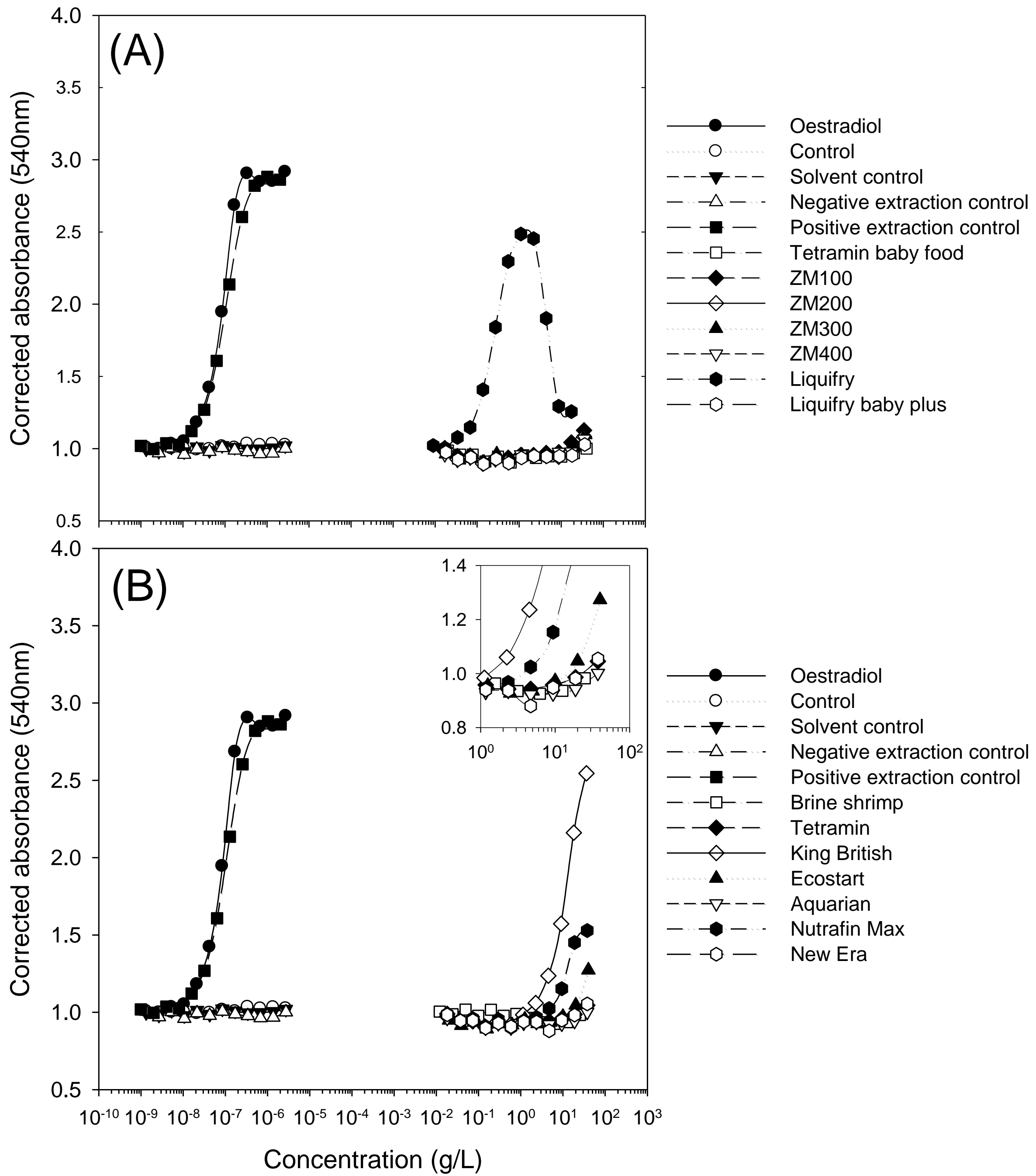

Figure 3.

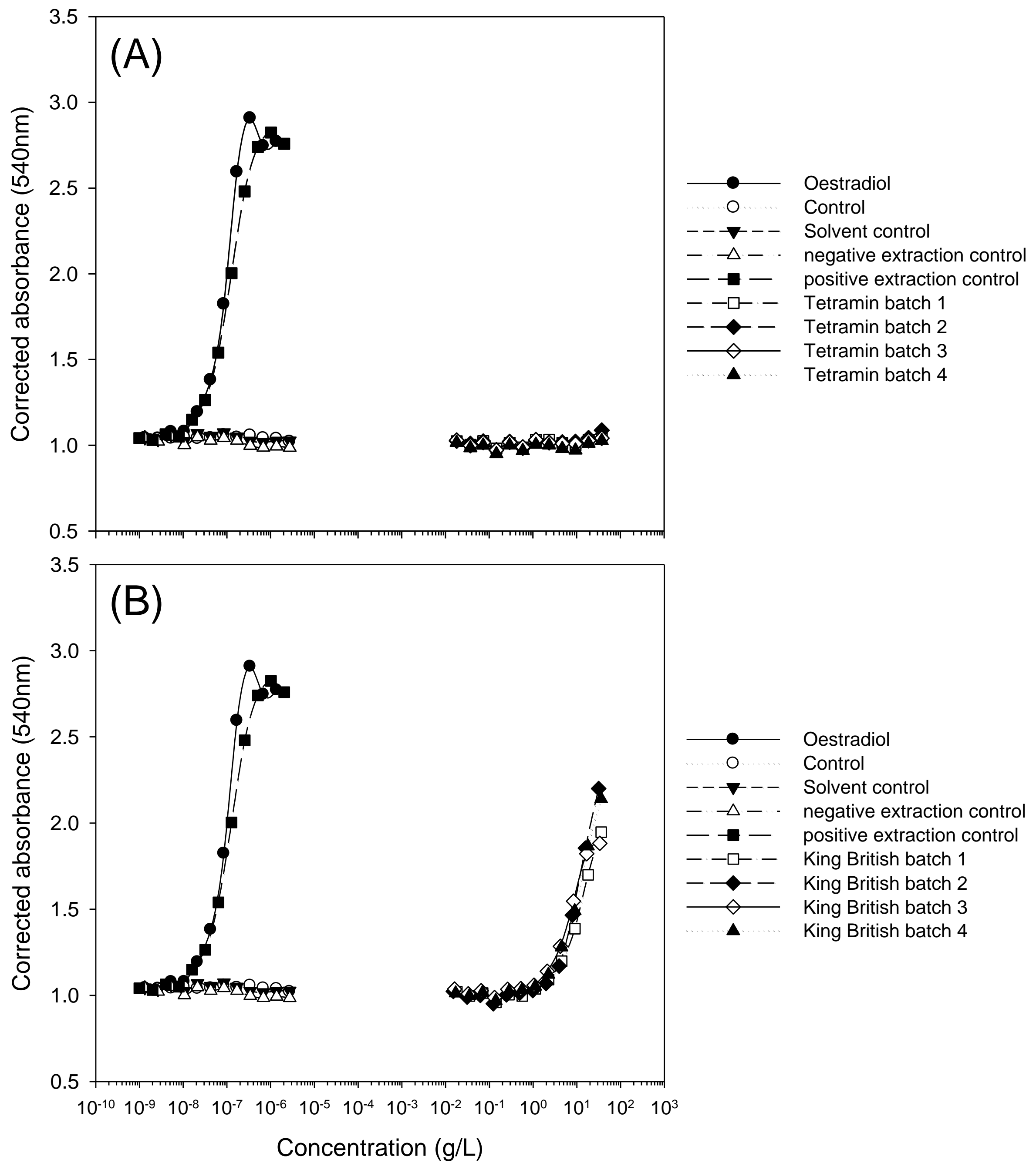

Figure 4.

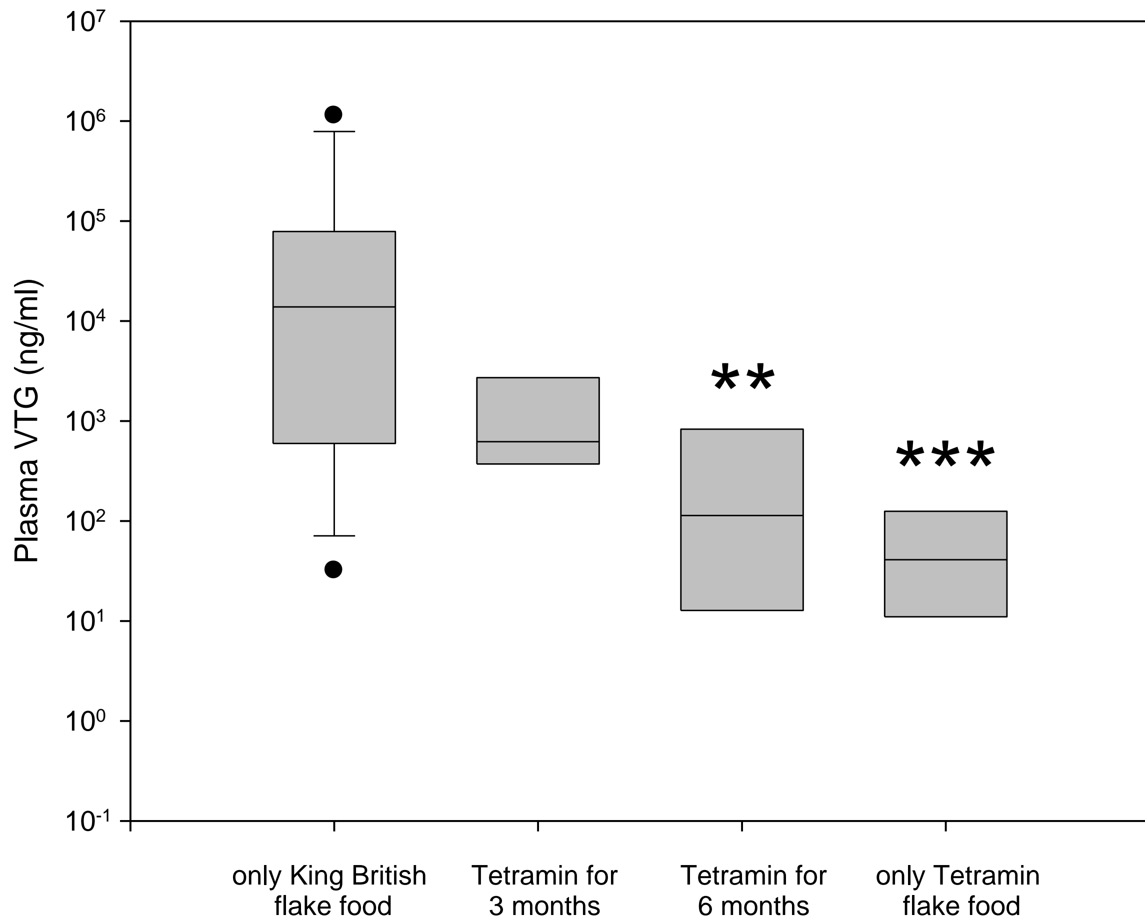

Table 1.

		Male only tanks	Mixed sex tanks
Oestrogen concentration (ng/L)	E1	0.12±0.14	0.4±0.16
	E2	0.06±0.10	0.12±0.08
	E2EQ	0.31±0.2	0.6±0.29
Vitellogenin (ng/ml)		130±258	1482±1513