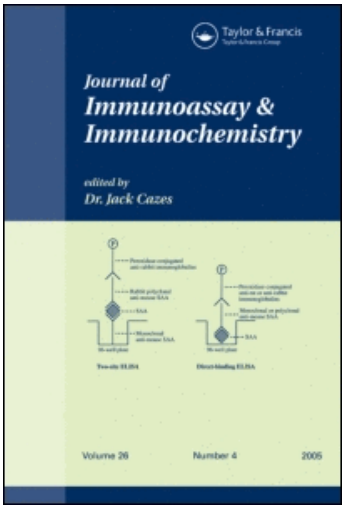

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The Development and Validation of a Quantitative ELISA for *in vivo* and *in vitro* Synthesized Vitellogenin from Mossambicus tilapia (*Oreochromis mossambicus*)

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Abstract: The induction of vitellogenin (VTG), an egg yolk precursor found in most oviparious vertebrates, has been well established as a valuable biomarker for assessing estrogenic endocrine disruption. *Oreochromis mossambicus* is a tilapiine species indigenous to Southern Africa and, therefore, represents a potential African bio-indicator for the development of *in vitro* and *in vivo* screens for estrogenicity using VTG as biomarker. However, few ELISAs have been developed to quantify tilapia VTG protein levels. In the present study, commercially available VTG antiserum that shows cross reactivity with tilapia VTG was used to set up and validate a quantitative competition ELISA for tilapia VTG. This ELISA has a broad detection range between 80 ng/ml–5.4 μg/ml VTG and is able to detect both *in vivo* and *in vitro* synthesized VTG. This ELISA is highly repeatable with intra- and inter-assay variations less than 3.4% at the lowest detection limit.

Keywords: Biomarker, ELISA, Endocrine disruption (EDC), Estrogenicity, *Oreochromis mossambicus*, Vitellogenin

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INTRODUCTION

Several toxicants found in the environment act by disrupting the endocrine systems of animals and humans. Traditional chemical monitoring techniques have become very expensive, are time consuming, and require a high level of skill. Moreover, since it is not yet known which of the myriad of substances in the environment and combinations of them may cause endocrine problems, the measurement of biological effects seems to be the more appropriate way to go. A number of studies have concluded that, in terms of cost effectiveness and to allow for screening of a large number of compounds, *in vitro* test methods are more appropriate.[1,2]

Vitellogenin has become a very popular biomarker for environmental estrogen induced toxicity and is used in both *in vitro* and *in vivo* screening assays.

Vitellogenin (VTG) is a high molecular weight phospholipoglycoprotein (250–600 kDa) that is produced and secreted by the liver of oviparious vertebrates as the precursor to several egg yolk proteins.[3–5] During sexual maturation, the female fish synthesizes 17β-estradiol (E2) in the gonads, and the circulating E2 is subsequently taken up by hepatocytes where it binds to estradiol receptors (ER), leading to the transcription of the VTG gene. The VTG protein is then secreted from the hepatocytes, enters blood circulation, and is taken up by growing oocyte through receptor-mediated endocytosis.[6] Within the oocyte, VTG is degraded to the yolk proteins, lipovitellin 1, lipovitellin 2, and phosphitin.[7,8] These yolk proteins serve as a nutrient store for developing embryos.[9]

Investigations have shown that, although VTG is normally expressed in female fish only, E2 and a number of synthetic chemicals (e.g., ethinyl estradiol, nonylphenols) are capable of inducing VTG production in male fish.[10–12] This endocrine disruption phenomenon is due to the fact that these xenoestrogens may interact with estrogen receptors by mimicking natural estrogens.[13] Exposure of fish to estrogenic sewage effluent may lead to reproductive impairment.[14,15] Spermatogenesis is inhibited by estrogens and some exposed male fish have testisova.[15] Because of the sensitivity of this response, VTG detection in blood and/or liver from male oviparous verterbrates has become a common biomarker for monitoring environmental estrogen contamination.[16–20]

A number of methods have been developed for the quantification of VTG in blood plasma, liver tissue, or whole-body homogenates from several fish species. These methods include radioimmunoassay (RIA), enzyme linked immuno sorbent assay (ELISA), single radial immunodiffusion,[5] and real time PCR.[21] These various methods differ in sensitivity, specificity, and technical difficulty. Currently, the most popular approach to measure VTG is some form of an ELISA, although RIAs

may occasionally be employed.[22] Fish VTG was quantified for the first time by ELISA in *Solea vulgaris*[23] and, since then, in various other species. The anti-VTG antibodies are very specific for each individual fish species and often require the production of specific antibodies for the selected species.[24] These antibody based procedures for VTG quantification may use antisera prepared against VTG from the fish species being studied. Although antisera can cross react with VTG from multiple fish species, the affinity can vary substantially.[25]

The development of screening and testing programmes for endocrine disrupting effects of new chemicals are one of the priority focus areas of major organisations such as the US Environmental protection Agency (US-EPA) and Organization for Economic Cooperation and Development(OECD).[22] The focus of these organizations are the use of small fish test species such as fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), and Japanese medaka (*Oryzias latipes*) as bio-indicators. These fish species share several characteristics that make them ideal test species for reproductive toxicity testing such as small size at maturity, relatively short generation times, asynchronous spawning, and overall ease of culture. Southern Africa, like most areas in the world, is also facing major aquatic pollution problems and these are posing a threat to human and animal health. Tilapia (*Oreochromis mossambicus*) also shares the same characteristics as the above mentioned fish species. Tilapia is found in the natural environment throughout Southern Africa and can potentially be used as an indigenous indicator species for this region. The aim of this study was to use commercially available antibodies to set up, validate, and implement a competition ELISA to quantify *Oreochromis mossambicus* VTG.

EXPERIMENTAL

Experimental Animals

Tilapia were obtained from the breeding stock of the Welgevallen experimental farm from the University of Stellenbosch, Stellenbosch, South Africa. Tilapia juveniles were four weeks old, whereas grown fish used for all experiments weighed in the order of 100 g.

Chemicals

All auxiliary enzymes, cofactors, and substrates used in this study were from either Sigma Chemical Company (St. Louis, MO, USA) or Roche (South Africa). Sea Bream Vitellogenin polyclonal antibody

(Anti-VTG) were supplied by Cayman Chemical Co. Anti-rabbit IgG (whole molecule) horseradish peroxidase conjugate (HRP) and Hybond-C Extra nitrocellulose were supplied by Amersham International. All other protein blotting detection reagents were supplied by Roche International. The standard protein molecular mass markers were from Sigma Chemical Company. All solvents and biochemicals were of analytical grade.

Juvenile Diethylstilbestrol (DES) Exposure

Four-week-old juvenile Tilapia fishes were obtained from the Welgevallen experimental farm and brought back to the lab. Juvenile tilapias were exposed to DES for seven days under static conditions in two-liter glass containers containing one liter of water. Water was not changed during the exposure period. DES was dissolved in dimethyl sulfoxide (DMSO) and did not exceed 0.1 mL per liter as per OECD, 2004 protocol agreement. Two treatment groups received 25 ng/L DES ($n = 20$) and 250 ng/L DES ($n = 20$), respectively. An additional group of 20 fish were kept without DES and served as a control group.

Tissue Culture

The medium used for tissue culture was RPMI 1640 containing L-glutamine, $NaHCO_3$, and 25 mM Hepes (Highveld Biologicals, South Africa). A mixture of penicillin, streptomycin, and fungizone (Highveld Biologicals) was added to the RPMI medium according to manufacturer's instructions. The RPMI medium containing the antibiotics will subsequently be referred to simply as "medium."

Fish were decapitated and placed in 70% ethanol for three minutes in order to decontaminate the skin. All subsequent procedures were carried out aseptically in a laminar flow cabinet. Cubes of liver tissue, measuring approximately 1 mm^3, were prepared and put into high quality 1.5 mL Eppendorphs at one cube per tube. Four cubes were prepared for each DES treatment exposure. Cubes were covered with medium an incubated in the dark at a constant 27°C in a water bath. Cubes were incubated for four days and the medium was not changed during the exposure time. Aspirant medium, for the determination of VTG concentration, were stored at −80°C until use.

Tissue Sampling

Tilapias were anesthetized using 100 mg/L Benzocaine. Organs used for experiments were dissected out and placed in extraction buffer (saline

containing 0.01% (w/v) Phenylmethylsulphonylfluoride with a weight to volume ratio of 1:10. Samples were then sonicated at 14 watts (Virsonic-60) for six bursts of ten seconds each. Samples were allowed to stand for one-minute intervals on ice between bursts. Samples were then centrifuged at 12,000 × g for 10 min at 4°C. Blood was collected using capillary tubes and dispensed into 1.5 mL tubes. After clotting, the samples were centrifuged at 12,000 × g for 10 minutes at 4°C. The supernatants from both the blood and organ samples were aliquoted and stored at −80°C for analysis.

Protein Determinations

Protein contents of samples were measured according to the method of Bradford[26] using bovine serum albumin (BSA) as a standard protein (Sigma).

SDS PAGE and Protein Blotting

Proteins were resolved in a 10 % (w/v) polyacrylamide gel. Gels were prepared using a SDS-PAGE gel preparation kit supplied by Sigma Chemical Company (St Louis, MO, USA). Gels and solutions were prepared according to the manufacturer's instructions. Polyacrylamide gels were run at 120 V in a slab electrophoresis unit.

Separated polypeptides were transblotted onto nitrocellulose in Towbin Buffer (25 mM Tris-HCl, 192 mM glycine, and 20% (v/v) methanol) at 15 Volts for 1 hour and dried between filter papers. Following transfer, the blots were stained in Ponceau-S (0.2 % [m/v] Ponceau-S, 3% [m/v] trichloroacetic acid), to be able to mark the standard molecular weight bands. The blots were then thoroughly rinsed in saline and blocked for 1 hour at room temperature in blocking solution containing 1% (w/v) human serum albumin (HSA) in saline. All subsequent steps in the procedure were carried out at room temperature. Blots were incubated in saline containing 0.1% (w/v) BSA, 0.01% (v/v) Tween and 1/2,000 anti-VTG. Unbound antibody was removed by washing the nitrocellulose 4 times for 5 minutes in wash solution containing saline and 0.01% (v/v) Tween. The nitrocellulose was then incubated for 1 hour in wash solution containing anti-rabbit horse radish peroxidase conjugate (1/2,500), 0.1% BSA, and saline. The nitrocellulose was washed as before and, finally, it was stained for horseradish peroxidase binding using BM Blue POD precipitating substrate. After staining the nitrocellulose, it was washed with distilled water, dried, and stored in an aluminium foil envelope.

Tilapia Vitellogenin Competition ELISA

Nunc-Immuno Maxisorp® plates (Nalge Nunc, Denmark) were used for all ELISA assays. Plates were coated overnight at 4°C with 50 μL per well of 1/2,000 diluted ovary homogenate in saline. At the end of the incubation period, plates were washed four times with saline. Following the wash procedure, remaining adsorption sites on the plate were blocked by dispensing 0.2 mL of block solution (1% w/v HSA in saline) per well and incubating the plate for one hour at room temperature. The same wash procedure was followed where samples for analysis and the VTG standards were dispensed at 50 μL per well. Anti-VTG was diluted 1/1,000 with saline containing 1% (w/v) HSA and were also dispensed at 50 μL per well. Samples were incubated for three hours at room temperature and the same wash procedure was followed again. Anti-rabbit horse radish peroxidase conjugate was diluted 1/2,500 with saline containing 1% (w/v) HSA and dispensed at 50 μL per well. Plates were incubated for one hour at room temperature after it was washed eight times with saline. BM Blue POD soluble substrate was heated to 37°C and dispensed at 50 μL per well. Plates were incubated at room temperature for thirty minutes followed by addition of 50 μL per well of stop solution (0.5 M H_2SO_4). The optical density was lastly determined at 450 nm.

Expression of Results and Statistical Analysis

The percentage of maximum (percentage binding) in each standard or sample dilution was calculated using the following relationship: % of maximum = [(standard or sample absorbance)/(maximal binding absorbance)] × 100. Results were analyzed using analysis of variance (ANOVA). Tuckey's HSD test was used for all pairwise multiple comparisons.

RESULTS

Specificity of the Anti-VTG

Polyclonal anti-VTG was bought from Cayman Chemical Co (Catalog Number: 170150). This antibody was produced from purified VTG of E2 treated Sea Bream (*Sparus* aurata). According to the manufacturer, this antibody recognized VTG from several fish species, including (*Oreochromis niloticus*). The specificity of this antibody was tested on *Oreochromis mossambicus* using protein blotting (Fig. 1). The antiserum immunostained both the plasma of DES treated females as well as ovary

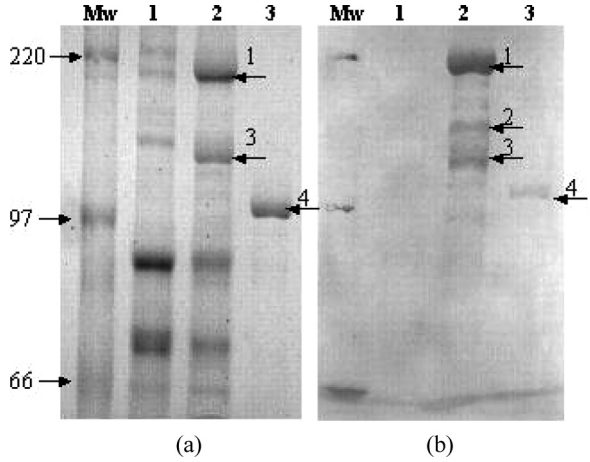

Figure 1. Specificity of the Anti-VTG antiserum. SDS-Page (a) and Protein Blotting (b) of: Male plasma (1), DES treated female plasma (2) and Ovaries (3). SDS-Page gel was stained with Coomassie Blue. Plasma samples were diluted 1:100 and 20 µl of sample were used for SDS-Page and protein blotting.

extracts, while there was no reaction with the control male plasma. The anti-VTG recognized peptides with apparent molecular weights of approximately 200 kDa (protein 1), 140 kDa (protein 2), and 130 kDa (protein 3) in the treated female serum. In the ovary extracts, a protein with an apparent molecular weight of 110 kDa (protein 3) was immunodetected.

ELISA Validation

Anti-VTG and VTG Antigen Titration

The optimal assay concentrations for VTG antigen and antibody were determined to obtain a maximum absorbance value near 1, since most plate readers have a linear response in the range 0.1–1.1 optical density units. The two-way titration of the VTG antiserum and coating antigen is displayed in Figure 2. A double dilution series of both VTG antiserum and antigen were prepared to obtain optimal ELISA assay concentrations. The VTG coating antigen dilution started at 1:200, whereas VTG antiserum started at 1:500. VTG antigen dilution of 1:2,000 and VTG antiserum dilution of 1:1,000 were chosen for setting up the competition ELISA. These assay concentrations gave an absorbance value near 1, which is midway between the linear and plateau area of the graph. At

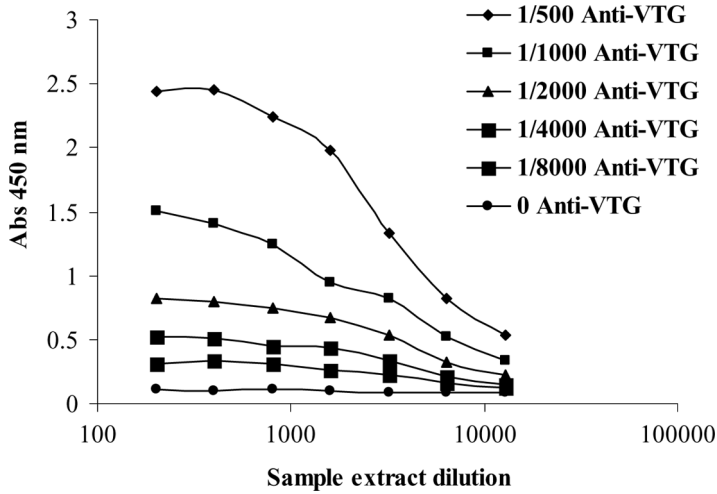

Figure 2. Determination of optimal concentrations of tilapia vitellogenin and antiserum for the validation of the tilapia ELISA. Tilapia ovary extracts were used as the VTG antigen, whereas seabream anti-VTG was used as the primary antibody. Abs = absorbance.

these assay concentrations, very low background readings were obtained (Abs = 0.097).

VTG Antiserum Specificity

Blood from DES treated female tilapia and control untreated males were screened for VTG induction using the optimal ELISA assay concentration (Fig. 3). At a blood dilution factor of 1:2,000, exposed females showed absorbance values of 1.6, whereas unexposed males showed very low background readings of 0.1. Blood from the exposed females was stored at -80°C as an in-house standard for VTG.

VTG Competition ELISA

A typical tilapia VTG competition ELISA standard curve is displayed in Figure 4. The standard curve was linearized by plotting percentage of maximum values on a logarithmic Y-axis. The sensitivity of the ELISA (the amount of VTG which gave 90% of binding) was 80 ng/mL VTG with a practical operating range betweem 80 ng/mL and 5.4 ug/mL VTG. The standard curve displayed in Figure 4 is the result of six standard curves over a period of six months and the ELISA precision

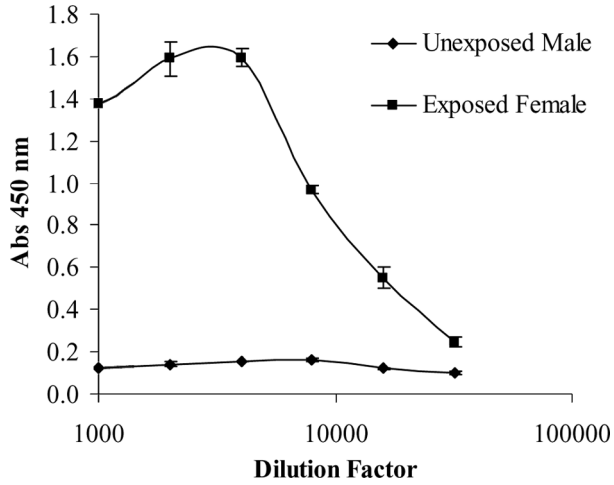

Figure 3. Screen for VTG induction in sexually mature female tilapia blood exposed to DES as well as untreated males. Female, sexually mature female tilapia fish were exposed for 7 days to 60 ng/l DES. Male tilapias were kept under the same conditions without DES treatment. Blood were screened for VTG using the optimal ELISA assay concentrations.

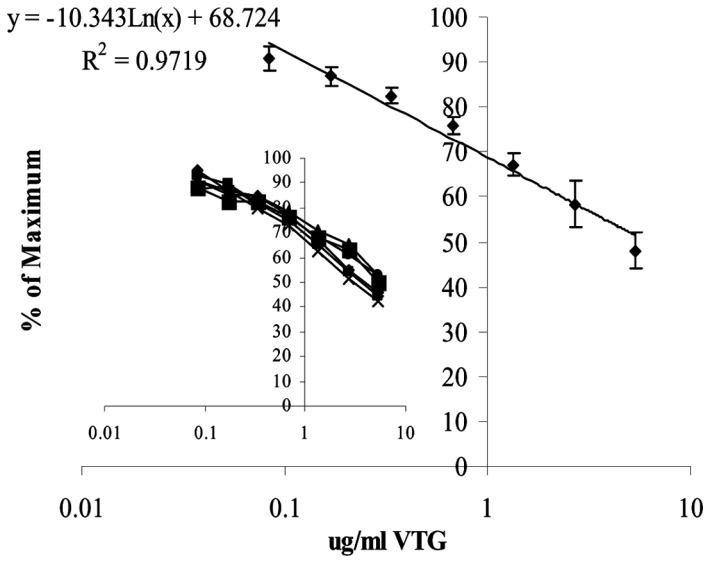

Figure 4. A typical tilapia VTG competition ELISA standard curve. In-house VTG standard were diluted from 84.1 ng/ml to 5.4 μg/ml VTG. The competition ELISA procedure was followed as explained in the methods and materials section.

(intra- and inter-assay variation) was calculated from this (Table 1). Intra-assay variation was less than 3.4%, whereas inter-assay variation was 8.4% at 50% binding and less then 2.9% at 90% and 80%.

Implementation of ELISA for *in vivo* Induced VTG

To validate the applicability of the competition ELISA for quantification of *in vivo* induced tilapia VTG, groups of 20 four-week-old tilapia fish were exposed to DES at various concentrations. Exposure to DES did not significantly influence the mortality of exposed juveniles, suggesting that neither the solvents nor the tested concentrations of DES had an acute toxicity effect under our test conditions. Figure 5 displays VTG concentrations of the whole body homogenates measured by the competition ELISA after 7 days of exposure. In the control group, the measured VTG concentration was 2.1 μg VTG/mg protein. Exposure of juveniles to 25 ng/L and 250 ng/L DES resulted in VTG concentrations of 3 μg/mL and 9 μg/mL, respectively. There was no significant difference in VTG concentration between the control and 25 ng/L DES groups. However, the 250 ng/L DES exposed tilapia have significantly higher VTG compared to the control ($p < 0.001$) and the 25 ng/L DES exposed group ($p < 0.001$).

Implementation of ELISA for *in vitro* Induced VTG

Liver slice cultures from sexually mature male tilapia was used to validate the competition ELISA for *in vitro* VTG synthesis (Figure 6). Liver slices were exposed to 0.5 μg/mL DES for a total of four days. VTG concentrations measured in the medium from the control samples did not change

Table 1. Characteristics of the tilapia VTG competition ELISA

	Binding (Percentage of maximum)		
	90%	80%	50%
CV[b] intra-assay (%)	3.4	1.2	1.1
CV interassay (%)	2.9	2.2	8.4

[a]For routine measurement 1:2000 VTG antigen and 1:1000 antibody dilution was selected.

[b]CV = coefficient of variation.

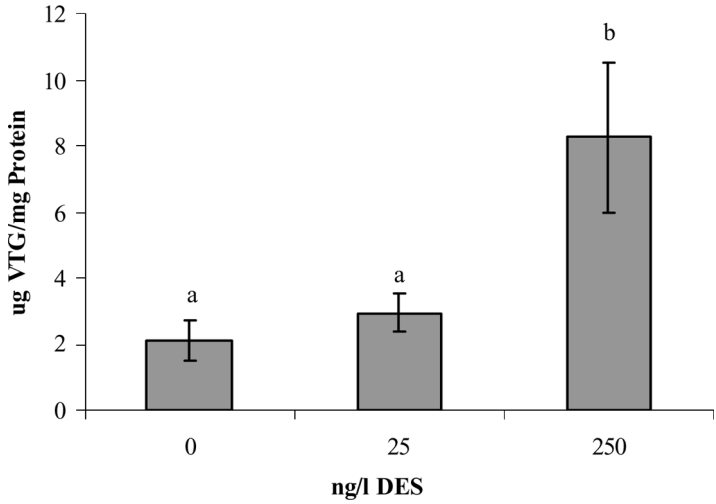

Figure 5. VTG concentration of four-week-old juvenile tilapia whole body homogenates. Fish were exposed to different concentrations of DES over a period of seven days. Vertical bars are the average concentration obtained for six individual fish, whereas error bars represent standard deviations. Groups marked with different letter are significantly different ($p < 0.001$).

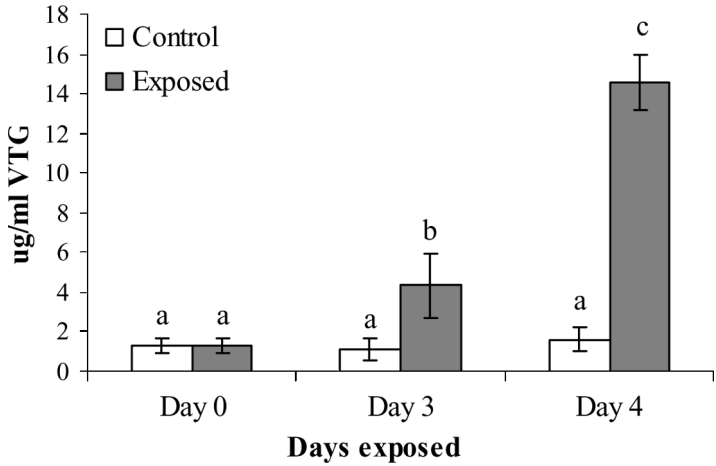

Figure 6. Vitellogenin synthesis by sliced liver cultures exposed to 0.5 µg/ml DES over a period of four days. Vertical bars represent the mean of four independent experiments, whereas errors bars represent standard deviations. Data bars marked with different letters differ significantly ($p < 0.001$).

over the culture period ($p > 0.01$). After three days exposure to 0.5 μg/mL DES, VTG concentrations increased significantly from 1.3 μg/mL (control) to 4.3 μg/mL VTG ($p < 0.001$). VTG concentrations increased from 4.3 μg/mL to 14.6 μg/mL VTG after four days in culture.

DISCUSSION

In this study, commercially available anti-seabream VTG was used to set up, validate, and implement a competition ELISA which can be used for the quantification of *in* vitro and *in vivo* synthesized *Oreochromis mossambicus* VTG. Previous investigations have shown that anti-VTG is very species specific and, therefore, very often requires the production of species specific anti-VTG antiserum.[24] It is evident from the protein blot analysis (Fig. 1) that the anti-seabream VTG does recognize tilapia VTG. The antibody reacted with peptides in DES treated plasma, whereas no reaction was visible with untreated male plasma. These results are supported by the ELISA on serum from DES exposed females and unexposed males (Fig. 3). The antibody reacted with the DES exposed serum, whereas no crossreactivity is observed with the unexposed male fish. In a previous study conducted by Kishida and Specker,[27] vitellogenin was isolated by DEAE agarose ion-exchange chromatography from the plasma of the tilapia, *Oreochromis mossambicus*. The monomers had apparent molecular masses of 200 and 130 kDa. SDS-PAGE of the oocyte extract showed a major protein band at 106.6 kDa. The anti-seabream VTG used for this study similarly detected three bands at molecular weights 200 kDA, 140 kDa, and 130 kDA, respectively. The 140 kDa protein of tilapia may be a degradation product of a larger VTG molecule. Previous investigations have shown that VTG in many fish is rather unstable, and even the addition of protease inhibitors to a plasma sample cannot fully prevent its proteolysis.[25,28] A protein with a molecular weigh of approximately 110 kDa was detected in ovary extracts.

The validated ELISA is a competition-binding assay in which antibodies are preincubated together with the sample of analysis (or standards). The immobilized VTG on the plate and the soluble VTG in the sample or standard competes for antibody binding. The tilapia ELISA validated in the present study is sensitive and has a detection limit of 80 ng/mL VTG, which is comparable to radioimmunoassays for VTG.[28–30] The working range of the ELISA is very broad (80 ng/mL −5.4 μg/mL VTG), which makes it suitable for VTG quantification when studying very weak, as well as very potent estrogens. The ELISA is also very reliable and repeatable with intra- and inter-assay variation less than 3.4% at the detection limit (Table 1) and is comparable to VTG ELISAs for other teleosts.[31–33]

To test the applicability of the validated ELISA for *in vivo* analysis of VTG induction, four weak old tilapia juveniles were exposed to different concentrations of DES for seven days (Fig. 5). Due to the sensitivity of the validated ELISA, VTG concentration can be accurately determined in fish weighing as little as 10 mg. Exposure of juveniles to 250 ng/L DES induced significantly higher concentrations of VTG compared to control fish. A dose responsive effect to DES exposure can be observed where the juveniles induced higher VTG concentrations to higher DES levels. A similar induction of VTG has previously been shown for fathead minnow (*P. promelas*) exposed to various concentrations of estradiol-17β until 30 days post hatching.[31] Control juveniles showed VTG concentrations of 2.1 μg VTG/mg protein. This baseline level of VTG may be because of estrogenic substances in the food or small amounts of natural estrogens secreted by female fish in the breeding tanks. The ELISA was tested for the quantification of *in vitro* synthesized VTG using tilapia liver cultures as model culture system. VTG was detectable after exposing liver slices for three days to 0.5 μg/mL DES. After four days of DES exposure, an 11.2-fold increase in VTG (from 1.3 to 14.6 μg/mL VTG) was observed.

In conclusion, a tilapia competition VTG ELISA was validated using commercially available anti-seabream VTG as the detecting antibody to quantify tilapia VTG. This ELISA is very specific for VTG, has a wide detection range, is very sensitive, and has low inter- and intra-assay variations. We showed that the ELISA can be used for both *in vivo* and *in vitro* quantification of VTG. Our results show that the validated competition ELISA can be used as a useful tool to detect environmental estrogen contamination and endocrine disruption using tilapia VTG as a biomarker.

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