The development and validation of a homologous tilapia vitellogenin enzyme linked immunosorbent assay (t-VTG-ELISA) as biomarker of estrogenic exposure

by

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Summary

Water is essential to all life but many freshwater resources are polluted through human activities. Humans and wildlife are exposed to a wide range of contaminants through their water, many of which pose a risk to health. Some of the contaminants released into the environment have been reported to have the capability to disrupt the endocrine functions in humans and wildlife and they can mimic or antagonise the action of estrogenic. These endocrine disrupting chemicals (EDCs) interact with physiological systems and cause alterations in development, growth and reproduction in wildlife and humans.

To achieve some measure of assessing the potential harm that the contaminants pose, we need to know the environmental concentration of the chemical concerned and to monitor their effect on the organisms. The water supply sector need to include EDCs in standard systems of routine water source monitoring which include indicator bacteria and nutrient species but before the system can be incorporated, methods to measure the occurrence of EDCs in aquatic environment need to be developed and validated and a reliable guidelines data need to be in place.

The aim of this study was to develop an enzyme-linked immunosorbent assay (ELISA) to quantify vitellogenin (VTG) in *Oreochromis mossambicus* (Mozambique tilapia) VTG has been used successfully as a biomarker for estrogenic contamination in different studies. For this study, VTG was isolated and purified from plasma of 17β-estradiol exposed tilapia by gel filtration chromatography. The purity of the VTG isolate was confirmed by polyacrylamide gel electrophoreses (SDS-PAGE). Polyclonal antibodies against t-VTG were raised in rabbits and the specificity of the anti-t-VTG was confirmed by western blot. Using purified t-VTG as a standard and anti-t-VTG antibody, a homologous competitive ELISA was developed and validated. The standard curves of the ELISA, which were generated on different days, were

identical which indicate that the assay is reliable, reproducible and repeatable. The intra-assay and inter-assay coefficient variation was 2.41 (n = 4) and 8.71 (n = 10) respectively. The serial dilution of plasma VTG from exposed tilapia showed a good parallelism with the standard t-VTG within the working range of the assay. The serial dilution of the reference fish did not cover the whole range of the t-VTG standard curve. By using the standard curve and the dilution of the exposed plasma, we were able to demonstrate that the ELISA was able to quantify VTG.

With good laboratory practise, this ELISA can be use to quantify VTG in chemically exposed fish. It will also be ideal to continue analyzing the antibody to determine the appropriate dilutions necessary to ensure that the assay work its optimal capabilities.

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1.1 Background

Water is our most abundant resource and covers about 71 % of the earth's surface (Manahan, 2000). It occurs in all spheres of the environment, in the oceans as a vast reservoir of saltwater, on land as fresh surface water in lakes and rivers, underground as groundwater, in the atmosphere as water vapour, in the polar icecaps as solid ice. Water bodies such as rivers and dams are used for diverse purposes such as domestic and industrial supply, crop irrigation, transport, recreation, sport, commercial fisheries, energy generation, land drainage, flood protection and waste transport and disposal (Abel, 2002).

The most important fact is that water is essential to all life - from the tiniest microbes to humans. Every living cell is composed primarily of water. Water dissolves salt and transports nutrients to and within living cells without dissolving and destroying the organic molecules of the living cell. It also transports waste elements away from the cell. The metabolic needs of every living being are often affected by the distribution and availability of water (Experimental Lakes Area, 2006).

Many freshwater resources are polluted by human activities (Mason, 1996). Humans and wildlife are exposed to a wide range of contaminants in water, many of which pose a risk to health. This is the result of an increasing global population in combination with the human desire for a higher material standard of living, causing worldwide pollution on a massive scale (Manahan, 2000; Mason, 1996). Pollution alters the natural and benevolent nature of water, be it the chemical composition, temperature, or microbial composition - to such an extent that harm may occur to resident organisms or to land animals and humans (Heath, 1995).

Rivers are the water resource that is most susceptible to contamination. Discharges from mining and associated activities contribute with pollution by heavy metals such as zinc, lead and copper (Abel, 2002). In agricultural areas, surface water runoff and groundwater infiltration into lakes and rivers introduce plant nutrients from fertilisers and pesticides in large quantities to water bodies. Large urban settlements contribute many forms of liquid wastes especially from domestic effluents and other diffuse sources (Ehiri & Birley, 2002; Kahara, 2002). One of the characteristics of these types of pollution is environmental toxicity (Crosby, 1998; Shaw & Chadwick, 1998).

Toxicology is the science of poisons; it deals with the adverse effects of chemical agents on biological systems (Crosby, 1998). Environmental toxicity / pollution affect the well-being of millions of species ranging from single-celled organisms to complex animals such as the blue whale and humans (Shaw & Chadwick, 1998). Some of the toxic chemicals released into the environment, are reported to have the capability to alter the embryonic and early postnatal development of animals and interfere with the development of reproductive, endocrine, immune and nervous systems of living organisms (Grünfeld & Bonefeld-Jorgensen, 2004; Gray *et al.*, 2002; Mendes, 2002; Mason, 1996). In particular some of these disrupt the normal endocrine (specialised cells and ductless glands) functions in humans and wildlife (Kime, 1999) - these contaminants are collectively termed endocrine disrupting chemicals (EDCs). Several species of metals as well as pesticides have been classified as EDCs (Fatoki & Awofolu, 2003; Children's Health Environmental Coalition, 2002; Kime, 2001).

In many parts of the world it is a considerable and increasing process to protect water of good quality (Water quality management in SA, 2006; Mason, 1996). In recent years humans have become more aware of their environment, probably realising that if we continue depositing our

waste products at the current rate in the environment, our world as we know it will have a finite future (Shaw & Chadwick, 1998). It is for this fundamental philosophical reason that we are becoming interested in the effects of the chemicals that we use (Shaw & Chadwick, 1998).

One hopes that having some (however small) knowledge and understanding of the effects of EDCs upon the inhabitants of an ecosystem might perhaps prevent their deleterious effect in the future (Shaw & Chadwick, 1998). However, assessing the effects of such waste-related chemicals upon the many different animals and plants is an almost insurmountable task. It would not be possible to examine the effects of each pollutant chemical on each or all of the individual species. To achieve some measure of assessing the potential harm that environmental pollutants pose, we need to know the environmental concentration of the chemicals concerned (Shaw & Chadwick, 1998). This is not readily achievable within the context of environmental monitoring and a need exists to assess, in a collective way, the effects of pollutants on organisms (Abel, 2002), especially as more and more chemicals are being introduced.

For this reason it is important that the water supply sector include methods of testing for EDCs in standard systems of routine water sources monitoring. The indicator bacteria such as total coliforms as well as nutrient species such as nitrate and phosphate are being used as part of the standard systems water monitoring as indicators of pollution (Ehiri & Birley, 2002), but at present, specific guidelines (based on VTG synthesis, competitive receptor binding and yeast assay) to test for EDCs in water are still at the development phase and government sponsored committees have been formed in the USA, the European Union and Japan to evaluate the effects of EDC (Soto *et al.,* 2006; Vanparys *et al.,* 2006; Gray *et al.,* 2002). To develop such guidelines, reliable data are needed on the occurrence, magnitude and frequency of contaminants that will safeguard the health of humans and wildlife against EDC activities (International Council for

Science, 2006; Burger, 2005). There are very little data in South Africa on EDCs contamination (Burger, 2005). This need can be met through the development and validation of bioassays that can quantify EDC activity in water resources (Leusch *et al.,* 2006; Vanparys *et al.,* 2006; Fossi *et al.,* 2002).

Such bioassays should at least be able to identify estrogenic agonist / antagonist chemicals and be able to assess interactions among the EDCs to which humans and wildlife are exposed to (Soto *et al.,* 2006). The development of such bioassays can be achieved by employing a reliable biomarker (Safe, 2005). Vitellogenin (VTG) in male fish was used successfully as a sensitive and reliable biomarker for estrogenic contamination (Ho *et al*., 2006; Mosconi *et al.,* 2006; Lutz *et al.*, 2005). Fish are well suited as biomonitor of environmental pollutants and can provide suitable data on the occurrence of a pollutant of the aquatic environment and their potential effects (Kime, 2001).

1.2 Research problem

Methods to measure the occurrence of endocrine disrupting compounds in aquatic environments are limited in South Africa.

1.3 Rationale

The use of *Oreochromis mossambicus* (Mozambique tilapia) as a sentinel species and Vitellogenin (VTG) as a biomarker will provide a useful biomarker of EDC exposure.

1.4 Aim of study

To use the Tilapia's VTG to develop a test system (ELISA method) to test for possible estrogenic exposure in *O. mossambicus.*

1.5 Objectives

The aim of the study was achieved through these objectives:

- Stimulate VTG in both male and female tilapias by exposing the fish to synthetic 17β-estradiol.
- Quantify VTG through VTG standard preparation, Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis and Western-blot analysis
- Isolate and purify VTG using gel filtration chromatography and using the purified VTG for the production of polyclonal antibodies in rabbits.
- Develop an enzyme linked immunosorbent assay (ELISA) method using polyclonal antibody to quantify VTG induction in adult male *O. mossambicus*.
- Validate the test system by doing intra- and inter-specific assays.

2.1 The Endocrine System

A disrupted endocrine system has serious implications for an animal or human, it damages the health of the animal/human and the pathological conditions associated with such a disruption are disorders in the reproduction, osteoporosis and cancer in the breast, testis and prostate (Grünfeld & Bonefeld-Jorgensen, 2004; Schultis & Metzger, 2004; Ohno *et al.,* 2003; Mendes, 2002). The endocrine system in animals and humans (Fig. 2.1) is a complex one consisting of specialised cells and ductless glands that secrete chemical messengers called hormones directly into the circulatory system to be distributed to selected cells or organs (Fig. 2.2) (Farabee, 2001; Safe *et al.,* 2000; O'Riordan *et al.,* 1988; Lewis, 1973).

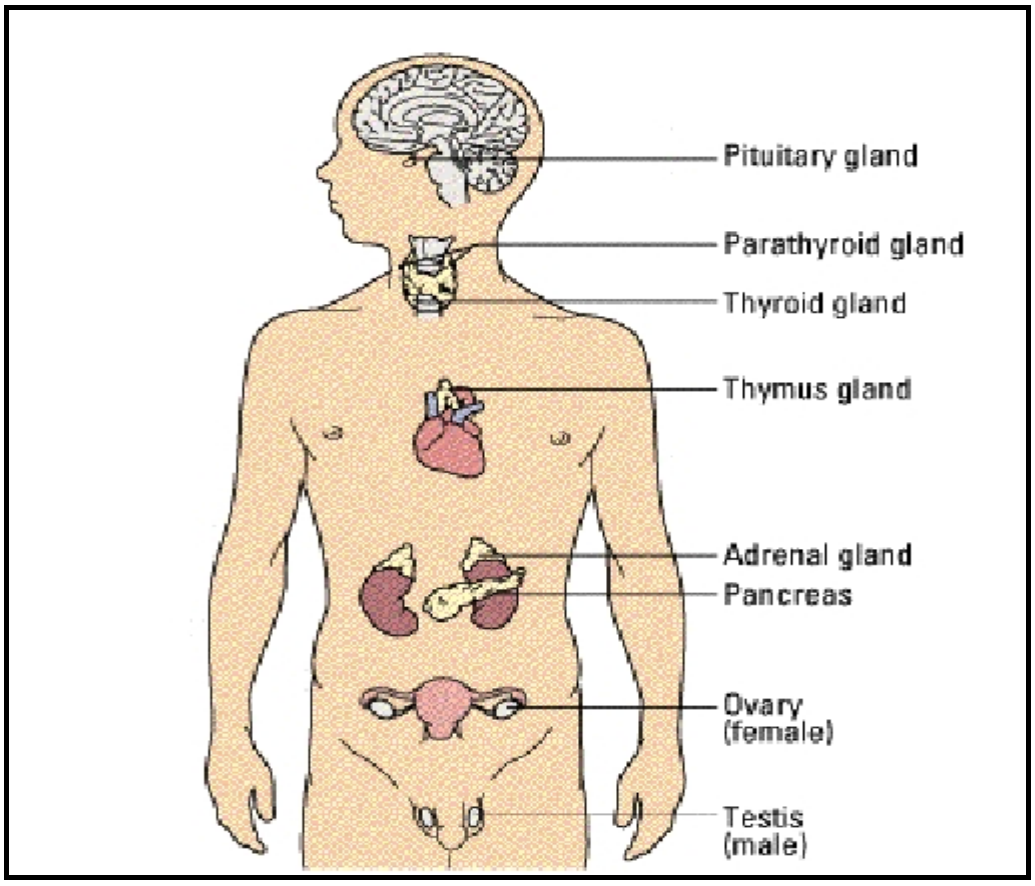

Figure 2.1: The Endocrine System (Tarrant *et al.,* 2005).

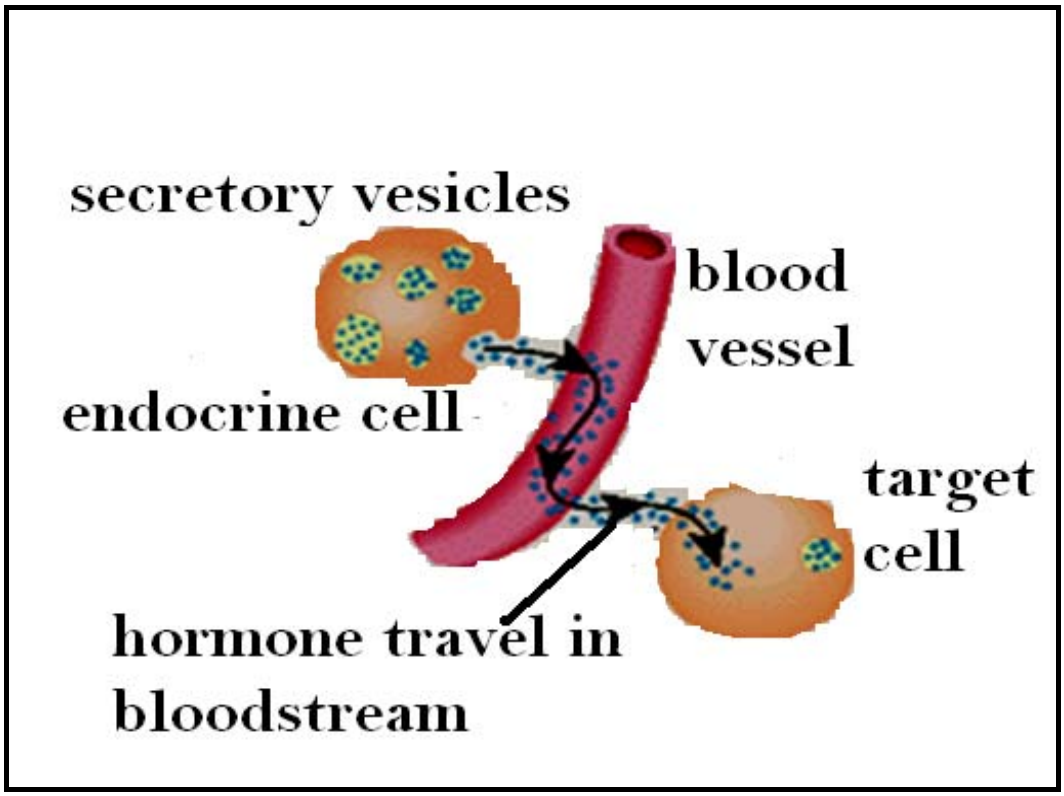

Figure 2.2: Hormones released into the bloodstream from endocrine gland cells (Schafersman, 2003).

This system regulates and coordinates vital activities of the body, including regulation and coordination of activities such as body-fluids homeostasis, management of stress, and perhaps most importantly, reproduction and fertility aspects (Kime, 2001; Lister & Kraak, 2001; Palmer *et al.,* 1998). Endocrine glands include the pituitary (anterior and posterior lobe), the thyroid, adrenals (cortex and medulla) and the gonads (ovaries and testes). The pituitary secretes growth hormone and oxytoxin which stimulate contractions of the uterus during labour and also causes the release of milk from a nursing mother. The thyroid secretes thyroxine which stimulates and maintains metabolic activities (heart beat, blood pressure) and calcitonin which inhibit the release of calcium from bones and regulates the levels of calcium in the blood. The adrenal cortex secrets three main groups of hormones i.e. glucocorticoids which affect carbohydrate, protein and fat metabolism it also helps people cope with stress, mineralocorticoids regulates the

re-absorption of sodium and the excretion of potassium by the kidneys and this affects water and salt balance in the body and sex hormones, these steroid hormones are essential for normal body function., while the adrenal medulla secretes adrenalin and non-adrenalin hormones which stimulates the heart muscle, they increase rate and strength of heartbeat. The gonads produce steroid sex hormones which regulate body changes that begin with puberty, and include the estrogens and progesterone from the ovaries and androgens from the testes (Saxena, 2002).

Hormones are grouped in three classes based on their structure, including the steroids (estradiol and testosterone), peptides and proteins (thyroid-stimulating hormone) as well as amines (thyroxine). Steroid hormones are lipids derived from cholesterol, and consist of testosterone, which is a male sex hormone and estradiol (estrogen), which is similar in structure to testosterone but is responsible for many female sex characteristics (Meintjies *et al.,* 2000). Steroid hormones are secreted by the gonads, adrenal cortex and placenta. The peptides are short chains of amino acids and are secreted by the pituitary, parathyroid, heart, stomach, liver and kidneys. Amines are derived from the amino acid tyrosine and are secreted from the thyroid and the adrenal medulla (O'Riordan *et al.,* 1988).

This study focused much on steroid hormones, especially the female hormone estrogen. The biochemical synthesis of steroid hormones is similar in the ovary, testes and the adrenal gland. The synthesis and metabolism of the estrogens is outlined in Figure 2.3.

Estradiol, the major estrogen produced by the ovaries, is synthesised by a group of enzymes known as the aromatase complex. The process involves three steps: 1) hydroxylation of the methyl group; 2) oxidation of this group and 3) hydroxylation at the 3α position (Greenspan & Forsham, 1986). Estradiol influences the development and maintenance of female sex

characteristics, and the maturation and function of the sex organs (Hylland & Haux, 1997; Toppari *et al.,* 1996), while androgens serve a similar purpose in males.

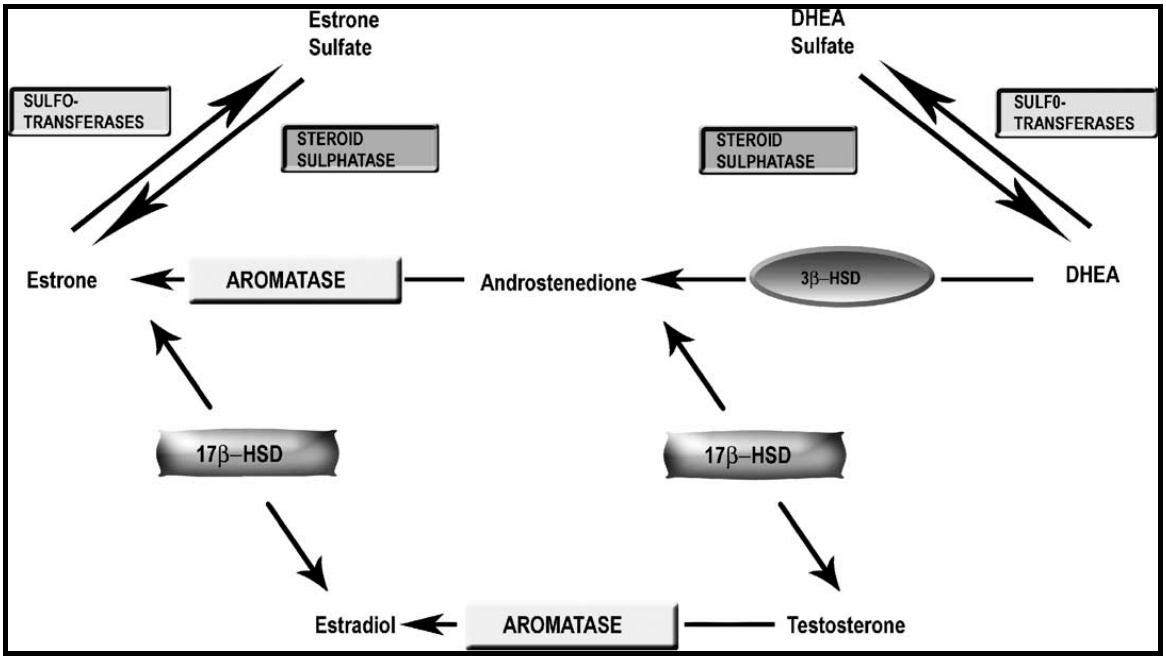

Figure 2.3: Biosynthesis and metabolism of estrogen (Fischer, 2004).

According to Tyler *et al.* (1998) estrogens are secreted primarily by follicle cells surrounding the oocytes of the ovary. These are steroidal molecules with a structure based on a phenanthrene ring. The main naturally occurring estrogens in all classes of vertebrates are estradiol-17β, estrone and estriol. Steroid hormones have a lipophilic nature and exert their actions by either diffusing through cell membranes, or binding to steroid hormones receptors in the cell nucleus within the cytoplasm (Palmer *et al.,* 1998), or by interacting with receptor protein via the plasma membrane, either through receptors or membrane-bound kinases (Toppari *et al.,* 1996). The protein sequences of the estrogens receptors (ERs), and the genes encoding them, are similar across the vertebrates' classes. ERs are found in many tissues including the brain, pituitary, gonads and accessory sex organ. Estrogens play pivotal roles during sexual development in all vertebrates; they are most clearly responsible for the development of feminine secondary sexual characteristics, in controlling female reproductive cycles and infertility (Nimrod & Benson,

1998; Palmer *et al.,* 1998; Toppari *et al.,* 1996). It is a key hormone in the initiation (puberty) and the end (menopause) of reproductive life in women (McLachlan, 2006).

Another role of estrogens in oviparous (egg-laying) vertebrates is to stimulate the liver to produce vitellogenin (VTG) (Fig. 2.4) (Wang *et al.,* 2005; Tatarazako *et al.,* 2004; Nishi *et al.,* 2002; Hashimoto *et al.,* 2000; Tyler *et al.,* 1998). VTG is the precursor protein of the major constituents in yolk, and the vitellin envelope proteins that form the eggshell. The protein is carried to the ovaries via blood and is incorporated into growing oocytes (Hennies *et al.,* 2003).

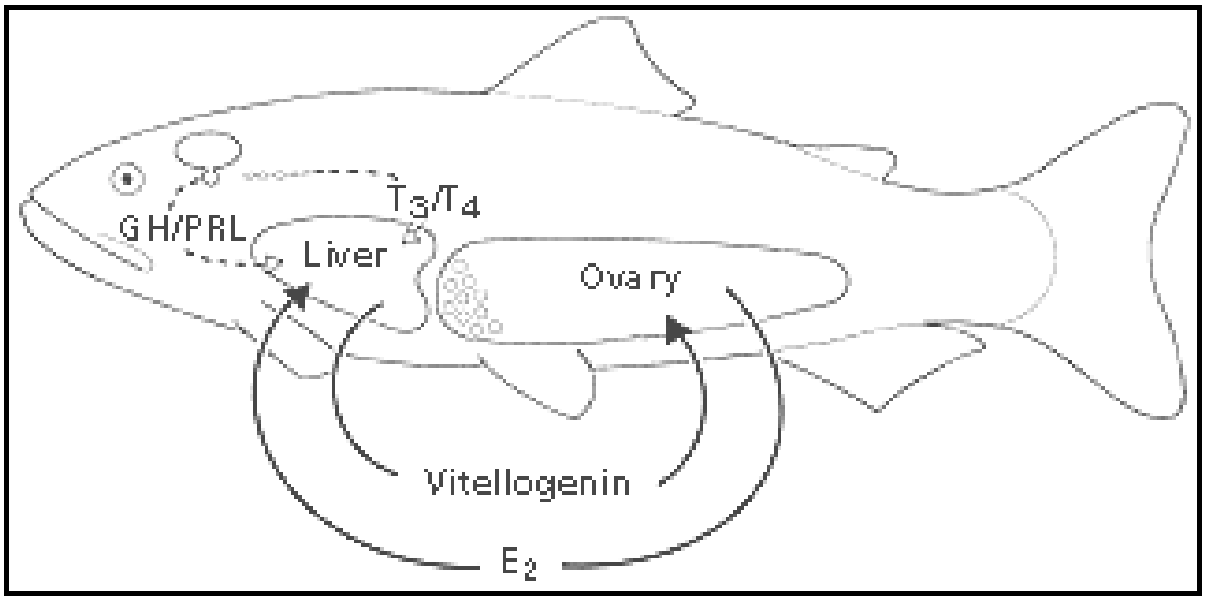

Figure 2.4: Hormonal control of vitellogenin synthesis. 17ß-estradiol (E_2) from cells of ovarian follicles is the principal hormone that stimulates vitellogenin synthesis in hepatocytes (liver cells) (Sumpter & Jobling, 1995).

Thus in oviparous animals, estrogens are vital for oocytes growth, egg formation, and provision of yolk for the developing embryo (Fenske *et al.,* 2001; Kime, 2001; Palmer *et al.,* 1998). Both the volume of natural estrogen and the timing of their release into the blood are carefully controlled by the endocrine system. Inappropriate exposure to this hormone or synthetic forms

thereof, at the wrong time in the life cycle, and/or at uncharacteristic concentrations, may cause adverse effects (Saxena, 2002; Tyler *et al.,* 1998) such as gonadal intersex observed in fish (Kavanagh *et al.*, 2004), early maturation in female fish (Matthiessen, 2003), and demasculinization of male fish (Orlando *et al.*, 2004).

2.2 Endocrine disrupting chemicals (EDCs)

It has been established that a wide variety of anthropogenic chemicals are being introduced into the environment that are potentially capable of modulating and/or disrupting the endocrine system of animals and humans (Oh *et al.,* 2006; Gray *et al.,* 2002; Tyler *et al.,* 2002; Tyler *et al.,* 1998). These chemicals are termed endocrine disrupting chemicals (EDCs). There are various definitions for EDCs (Bettaso *et al.,* 2002; Brion *et al*., 2002; Mendes, 2002; Kime, 2001; Lister & van der Kraak, 2001; Lopez de Alda & Barcelo, 2001; Kavlock, 1999; Menditto & Turrio-Baldassari, 1999; Parks *et al.,* 1999; Lomax *et al.,* 1998). The one used most by researchers worldwide is:

"Exogenous agents that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that is responsible for the maintenance of homeostasis, reproduction, development and/or behaviours" (United States Environmental Protection Agency (USEPA), 1997).

EDCs pose an increasing threat to the health of wildlife and humans and natural ecosystem (Ohno *et al.,* 2003; Mendes, 2002). They interfere with the endocrine function in a number of ways by:

- Binding and activating the estrogen receptor, therefore mimicking the effects of endogenous hormone;

- Binding but not activating the estrogen receptor, therefore antagonizing the effects of normal endogenous hormone;
- Altering the pattern of synthesis and metabolism of natural hormones;
- Modifying the metabolism of natural hormones (Soto *et al.*, 2006; Mendes, 2002; Pait & Nelson, 2002).

In the simplest scenario, the environmental contaminant binds directly to a hormone receptor stimulating or blocking the expression of hormone-induced genes (Guillette & Craine, 1996). EDCs constitute, therefore, a class of substances defined not by chemical nature but by biological effect. This implies that a wide variety of pollutants such as pesticides, polycyclic aromatic hydrocarbons (PAHs), phthalate plasticizers can be collectively referred to as EDCs (Lopez de Alda & Barcelo, 2001). These chemicals bio-accumulate in the fat and tissue of the organism due to their lipophilic nature and persistence in the environment (Palmer & Palmer, 1995). Research conducted on endocrine disruption has shown that some of the chemicals such as alkylphenols, which have long been claimed to be inert, are capable of causing widespread disruption of the endocrine system of both laboratory animals and wildlife (nearly all classes of vertebrates) (Lutz & Kloas, 1999).

Natural and synthetic hormones, natural compounds and several hundreds of chemicals have been cited as potential EDCs based on their estrogenic-like effects, including industrial contaminants, insecticides, and herbicides (Grünfeld & Bonefeld-Jorgensen, 2004; Nimrod & Benson, 1998) as illustrated in table 2.1. For this study an important class of EDCs are those acting as estrogenics (xeno-estrogens). They mimic the effects of estrogens because they have bioactivity similar to endogenous estrogens (Nimrod & Benson, 1998). This means they have the capability to bind to the estrogen receptors as agonist and to initiate responses similar to

those of the endogenous steroid hormone (Brion *et al.,* 2002; Nimrod & Benson, 1998; Palmer *et al.,* 1998) resulting with a disrupted endocrine system. EDCs are compounds that 1) are not easily broken down; 2) are long-lasting, remaining intact in the environment and in living organisms for many years; and 3) can accumulate in the natural world and within the fat and tissue of animals and humans (CBR-Tulane & Xavier University, 2004).

2.3 Effects attributable to EDCs

During the last decade, a variety of effects have been observed in the environment that has been attributed to EDCs (Bowman *et al.,* 2002). A limited number are summarised in table 2.2. Effects may be cumulative, with many effects not yet observed in the environment, making EDCs even more hazardous. It is possible that these effects will only be appearing in subsequent generations and with irreversible effects (Kime, 2001).

Scientific studies have reported that EDCs might be linked to reproductive, developmental, and behavioural effects in humans and wildlife (Skakkebæk *et al*., 2001; Colborn *et al*., 2000; Bowerman *et al*., 1998; Guillette *et al*., 1994). The decline in quality and quantity of human sperm coinciding with the introduction of chemicals in the environment was observed and reported (Soto *et al.,* 2006; Yoshida *et al.,* 2001; Lutz & Kloas. 1999; Toppari *et al.,* 1996). Reports confirmed an increased incidence of male genital tract defects (cryptorchidism, hyperspadism and testicular cancer) (Soto *et al.,* 2006; Fischer, 2004; Yoshida *et al.,* 2001; Korner *et al.,* 1998). In females an increase in breast cancer has been associated with EDCs (Safe, 2005; Lutz & Kloas, 1999; Toppari *et al.,* 1996).

Table 2.1: A few types of EDCs with estrogenic effect and their uses (adapted from Colborn *et al.*, 2000).

EDCs:	**Hormone affected/ Mechanism:**	**Class/ Uses:**
Synthetic and natural hormones o 17β-estradiol o Ethinylestradiol	• Reproductive system	• Reproductive hormone • Main ingredient of the oral contraceptive pill
Natural compounds • Phytoestrogens • Mycoestrogens	• Anti-estrogen	• Produced by plants • Produced by fungi
Anthropogenic chemicals 1. Persistent Organohalogens • Dioxin and furans • PCBs*	• Anti-estrogen	• Not manufactured deliberately • Produced during incineration, paper manufacture, coal fired utilities • Used as heat transfer fluids and in electrical installation • As additives in pesticides, paints, paper
2. Pesticides • DDT** • Endosulfan • Dieldrin • Methoxychlor 3. Herbicides	Competes with estrogen to bind to the estrogen receptor	• Insecticides used for malaria control in Africa • Herbicide used in blackcurrants and strawberries farming

• Alachlor		• Anti-fouling compound
• Atrazin	• Decrease thyroid hormone inhibits ligand binding to androgen and estrogen receptors	• Used in the production of epoxy and polycarbonate plastic resins
4. Fungicides	• Reproductive	
• Tributyltin	• Binds to estrogen receptor	
5. Bisphenol A	• Inhibit binding to estrogen receptor	• Used in the manufacture of paints, batteries and electronic devices
6. Phthalates	• reproductive	•
	• estrogenic	•
7. Metals	• Glucocorticoid	• Battery production
• Lead	• Reproductive/ thyroid	
• Cadmium	• estrogen receptor agonists	
• Arsenic		• It conducts electricity and is also used in most paints and thermometers
• Mercury		
8 Alkylphenols		• Used to make APE surfactant (detergent)
Nonylphenol	Reproductive/thyroid/development	
Octylphenols		

***PCBs-Polychlorinatedbiphenyl; **DDT-, Dichlorodiphenyltrichloroethane**

It is also reported that daughters of mothers who took the synthetic estrogen DES (diethylstilbestrol) during pregnancy to prevent miscarriage have higher rates of reproductive problems, reproductive cancer and malformed reproductive organs (Schrager & Potter, 2004; Nollar *et al.,* 1990) while theirs sons have increased incidence of genital abnormalities including sperm and semen abnormalities and a possible increased risk of prostate and testicular cancer as compared to men without such exposures (Schrager & Potter, 2004). In a study involving children of women who consumed PCB-contaminated fish caught in Lake Michigan, showed that these children had lower birth weights, smaller head circumferences, and lower gestational ages (USEPA, 1997). In Japan, infants born to mothers who consumed rice oil contaminated with PCBs had dark pigmentation of the skin, low birth weight, early eruption of the teeth and swollen gums and eyelids (Rice, 1995).

The effects of EDCs has also been observed and documented in wildlife (Table 2.2). In mammals, the reproduction impairment in the male Florida panther has been suggested to result from exposure to xeno-estrogens (Anthropogenic chemicals with estrogenic activity). These animals inhabited an area that was heavily contaminated with PCBs, and males had low sperm counts, low ejaculation volume and a high incidence of sperm malformation (Murray *et al.,* 2001; Tyler *et al.,* 1998; Facemire *et al.,* 1995). In Canada, masculinisation of female black bears and grizzly bears has been suggested to have risen from excess exposure to EDCs (Tyler *et al.,* 1998). Pseudo-hermaphroditic offspring were produced by polar bears inhabiting a contaminated site in the Arctic (McLachlan *et al.,* 2006).

From the 1950's, declines in bird populations have been reported from North America and Europe. This phenomenon was associated with high body burdens of organochlorine pesticides (Dawson, 2000). Eggshell thinning in many species resulting from 1,1,1-trichloro-2,2-bis(4-

chlorophenyl)ethane (DDT) exposure have been reported (Baker, 2001; Kime, 2001; Fry, 1995; Cooke, 1973; Hickey & Anderson, 1968). Evidence from studies on fish-eating birds suggested that contaminants affected their development and reproductive capacity (Bosveld & Van der Berg, 2002). Other studies reported on reproductive impairment in birds as characterised by high embryonic and chick mortality, oedema, and retardation of growth (Gilbertson *et al.,* 1991).

In reptiles a decrease in the alligator population and developmental abnormalities of gonad morphology was apparently a result of a pesticide spill in Lake Apopka in the 1980's (Crain & Guillette, 1998). Furthermore, male juvenile alligators from this lake had poorly organised testes with unique aberrant structures within the seminiferous tubules and a greatly reduced plasma testosterone concentration but elevated plasma estradiol-17β concentration (Crain & Guillette, 1998; Guillette *et al.,* 1994). Not only males were affected, but females (ovaries) from Lake Apopka synthesised significantly higher concentrations of estradiol-17β than ovaries from control females (Guillette *et al.,* 1994). Natural and synthetic estrogens have been shown to induce ovarian development at male producing temperatures in the American alligators, however estrogen induced ovaries were smaller as compared to the temperature induced ovaries (Milnes *et al.*, 2006).

Table 2.2: Selected examples of wildlife reproductive and developmental abnormalities attributed to EDCs.

Species	Site	Observation	Contaminant
Invertebrate			
Mollusca (Oehlmann *et al.,* 2000)	Laboratory experiments	Formation of additional female organs, an enlargement of the accessory pallial sex glands	Bisphenol A and Octylphenol
Gastropods (Safe *et al.,* 2000)	Marine	Pseudohermaphroditism, imposex, intersexes, sterility, population declines	Tributyl tin
Fish			
Catfish (Barnhoorn *et al.,* 2004)	Gauteng	Intersex	Nonylphenol
English sole (Lomax *et al.,* 1998)	N/A	Reproductive dysfunction	Xeno estrogens
Caged male carp (Folmar *et al.,* 2000, Van der Kraak *et al.,* 2001)	United Kingdom and U.S.	Elevated level of VTG and Hermaphrodites	Sewage effluent
Rainbow trout (Folmar *et al.,* 2000; Van der Kraak *et al.,* 2001)	United Kingdom and UK.	Elevated level of VTG and Hermaphrodites	Sewage effluent
Roach (Sumpter & Jobling, 1995)		Hermaphrodites	Sewage treatment Works (STW)
Trout (Sumpter & Jobling, 1993)	River	Hermaphrodites, vitellogenin	STW
Eel (Sumpter & Jobling, 1995)	England and Wales	Hermaphrodites, vitellogenin	STW
Salmonid (Van der Kraak *et al.,* 2001)	France Lake trout	Blue sac disease, early life stage mortality	TCDD
White croakers (LeBlanc & Bain,	L.A. harbour	Papillomas	STW

1997)			
Eelpout	Swedish Baltic Coast	Male biased sex ratios	Effluent from pulp mill
Mosquito fish (Toft *et al.,* 2003)	Lake	Disturbed sexual characteristics, lower sperm counts, reduced gonopodial lengths, and a lower testosterone peak	EDCs Sewage effluent
Trout, roach (Safe *et al.,* 2000)	English river	Hermaphrodites, vitellogenin in males, altered testis development	Sewage effluent
Reptiles			
Alligator (Sumpter, 1995)	Lake Apopka, FL	Decreased viability, abnormal gonadal development	DDE
Birds			
Wild birds (Fry, 1995)	Global	Egg shell thinning, mortality, developmental abnormalities	DDT
Mammals			
Mink (Safe *et al.,* 2000)	Great Lakes	Population decline, developmental toxicity, hormonal alterations	PCBs and dioxins
Amphibians			
Xenopus laevis (Kloas *et al.,* 1999, Palmer *et al.,* 1998)	N/A	Higher number of female phenotype and VTG	Endosulfan, chlordane, dieldrin, and toxaphene, Bisphenol-A
Rodents			
Rats (Safe *et al.,* 2000)	N/A	Reproductive toxicity	Bisphenol-A

Environmental pollutants have contributed to the world-wide decline in many amphibian populations (Urbatzka, 2006; Lutz *et al.*, 2005). Skewed sex ratios in favour of the female phenotypes have been observed in the African clawed frogs (*Xenopus laevis*) exposed during metamorphosis to environmentally relevant doses of ammonium perchlorate, estrogens, nonylphenol and bisphenol A (Milnes *et al.,* 2006). However, a limited number of studies are still available on the effects of EDCs on reproductions of amphibians in the wild (Lutz *et al.,* 2005; Carey & Bryant, 1995). Estrogenic effects have been found in the African clawed frog after exposure to a variety of pesticides (Palmer *et al.,* 1998; Palmer & Palmer, 1995). Hayes & partners (2002) reported that exposures of frogs to the herbicide Atrazine inhibits testosterone and induces estrogen secretion resulting in 100% females, while Milnes & partners (2006) reported hermaphroditism when *Xenopus laevis* were exposed to Atrazine. DDT has also been implicated when northern cricket frogs has died in a stream adjacent to a cotton field (Fergusen & Gilbert, 1967).

The first evidence of endocrine disruption in molluscs came in 1970, when Blaber reported a penis like structure in a female dogwelk. A few cases in what was now called imposex were reported and the organisms were exposed to higher tributyl tin (TBT) levels (Oehlmann *et al.,* 2000; Smith, 1981). In marine gastropods, females developed male characteristics, including a penis (Bryan *et al.,* 1986).

Wildlife studies showed elevated plasma levels of VTG, a lower sperm count and intersex phenomena in male fish from contaminated lake and rivers (Urbatzka *et al.,* 2006). Male crucian carp inhabiting the Youngsan River exhibited abnormalities with regard to sex ratio, intersex and blood VTG induction (Ho *et al.,* 2006). In Cootes paradise region of Hamilton Harbour, Canada gonadal intersex have been observed in male white perch, the area was affected by domestic and

industrial effluent (Kavanagh *et al.,* 2004). Female sex characteristics have been observed in male fish inhabiting water near municipal sewage outlets in England, while the fish that lived farther downstream had less severe symptoms (Van Aerle *et al.,* 2001; Jobling *et al.,* 1998; 1996). In Florida, United State of America, masculinisation and behavioural changes have been observed in female mosquito fish (*Gambusia affinis)* living downstream from pulp and paper mills - these effects are a result of exposure to the large amounts of phytosterols (Parks *et al.,* 2001). Other effects that were observed in fish downstream from bleached Kraft mill effluent discharges in the USA included reduced plasma sex steroid concentration, decreased gonad size and absence of secondary sexual characteristics (Sepulveda *et al.,* 2003). Fathead minnows exposed to feedlot effluent had significant alterations in their reproductive biology; demasculinization of male fish (lower testicular testosterone synthesis and smaller testis size) and defeminization of females (decreased estrogen-androgen ratio) were observed and documented (Orlando *et al.,* 2004). Exposure to exogenous estrogen has produced reproductive and developmental toxicity in fish (Zaroogian *et al.,* 2000).

EDCs not only affect the species that are being exposed but secondary exposure also occurs via the food chain, such as in the case of fish-eating birds, domestic animals and humans (Bosveld & Van den Berg, 2002; Bøgh *et al.,* 2001). Concerns about environmental estrogens are therefore even more widespread because of their potential to cause deleterious physiological effects in humans and wildlife resulting in poor developmental/reproductive performances (Hashimoto *et al.,* 2000).

2.3.1 *In-vivo* laboratory studies

Numerous studies have been conducted where fish species have been exposed to selected EDCs under controlled conditions. Werner *et al.* (2003) exposed lake trout (*Salvelinus namaycush*) to

1mg/mL 17α-ethinylestradiol to examine the relationship between VTG induction and metallothionein (MT) protein expression. They showed that the MT concentration in livers declined while that of the VTG increased. VTG was induced in carp (*Cyprinus carpio*) using 1 mg/kg estradiol and a radio-immunoassay developed to measure VTG in a variety of cyprinid fish including bream, roach, minnow as species native to rivers, and goldfish, zebrafish, fathead minnow as non-natives species used in laboratory-based eco-toxicology work – with the carp-VTG assay being very reproducible (Tyler *et al.,* 1996). VTG was also induced, isolated and used to develop an ELISA in the zebrafish (*Danio rerio*) using 10 ng/L 17α-ethynylestradiol (Fenske *et al.,* 2001) and 1 µg/l estradiol (Holbech *et al.,* 2001) as well as 100 µg/l estradiol (Brion *et al.,* 2002). To develop a sensitive and quantitative ELISA for the detection of VTG in the Fathead Minnow, these minnows (*Pimelas promelas*) were exposed to 10 ng/L 17α-ethynylestradiol (Mylchreest *et al.,* 2003) to compare ELISAs for detecting VTG and ~ 25 µg estradiol/g body weights (Parks *et al.,* 1999).

Male Summer Flounder (*Paralichthys dentatus)* were exposed to various concentration (0.1 mg/kg body weight (bw), 1.0 mg/kg bw, and 10.0 mg/bw) of estradiol to measure VTG and to conduct pathological evaluation of the kidney, livers and testes (Folmar *et al.,* 2001). Since VTG was induced, the liver was softer than normal, and it presented with hyalinuria, while the testes showed inhibited testicular growth as compared to the control. Mills *et al.,* (2001) also exposed Flounder to 0.2, 2 and 20mg/kg 17β-estradiol, 30, 60 and 120 mg/kg of DDT and DDE and 2, 20, and 200mg/kg octylphenol to assess the effects of these chemicals on hepatosomatic and gonadosomatic indices, plasma steroid hormone levels, vitellogenin production and gonadal development. Medaka (*Oryzias latipes*) was exposed to 10 and 100 ng/l 17α-ethynylestradiol (Scholz *et al.,* 2004) to measure the protein expression and VTG gene expression was strongly induced by 100 ng/l ethynylestradiol while at 10 ng/L only a slight VTG band could be

amplified, with Kordes *et al.* (2002) also inducing VTG in the medaka with 17α-ethynylestradiol and developing a sensitive sandwich ELISA with the use of monoclonal antibodies.

Nonylphenol (NP) effects were investigated on Grey mullet (*Liza aurata*) by exposing the fish to 0.25 and 250 mg/kg body weight NP, and it exerted estrogenic effects only at the highest dose injected (Cionna *et al.,* 2006). Fertilised eggs of the Rainbow trout (*Onchorhynchus mykiss*) were continuously exposed to real NP concentrations of 1.05 and 10.17 µg/L NP during their embryonic, larval and juvenile developmental period to investigate the estrogenic action of NP (Ackermann *et al.,* 2002). The expression of VTG and zona radiata proteins was reported in NP exposed Atlantic salmon (*Salmo salar*), the fish were exposed to waterborne NP at 5, 15 and 50 g/L, both plasma and surface mucus showed similar and parallel NP-induced expression patterns (Meucci & Arukwe, 2005).

More species were exposed under controlled conditions to selected EDCs. Amphibians like the African clawed frog (*Xenopus laevis)* (Mitsui *et al.,* 2003; Van Wyk *et al.,* 2003; Palmer *et al.,* 1998) as well as juvenile green turtles (*Chelonia mydas)* (Herbst *et al.,* 2003) were exposed to unknown concentrations of estradiol to stimulate VTG. While Urbatzka *et al.* (2006) investigated the effects of EDCs on the hypothalamus-pituitary-gonad axis, regulating reproduction in *Xenopus laevis* by determining their potential impact on gene expression of gonadotropin releasing hormone, luteinizing hormone *b*-subunit and follicle-stimulating hormone b-subunit in brain and pituitary. Mice treated with the estrogenic chemical diethylstilbestrol (DES) showed abnormal morphology of the ovarian follicle and oocytes (Guillette & Craine, 1996).

Oreochromis mossambicus (Mozambique tilapia) were exposed to doses of estradiol of 1 mg/100 g body weight of estradiol (Kishida & Specker, 1993) and 5 µg/g body weight (Takemura & Kim, 2001) for the induction of two forms of VTG and to develop a competitive ELISA and sandwich ELISA respectively. In another study Tilapia was exposed to estradiol and 5α-dihydrotestosterone (DHT) to examine the effects of these chemicals on the production of VTG, insulin-like growth factor-I (IGF-I) and IGF-binding proteins (Riley *et al.,* 2004).

O. mossambicus has been used successfully as a sentinel to examine the relationship between vitellogenesis and estradiol (Takemura & Kim, 2001; Kishida & Specker, 1993). For *O. mossambicus*, unlike in small fish like the medaka and zebrafish, it is easier to purify VTG from blood, so the fish is not sacrificed unless one has to do histopathology testing. Since this is a study in the South African context, it was deemed appropriate to evaluate/test Southern African fish species, making the native *O. mossambicus* an obvious choice (Nussey, 1994).

2.4 EDCs exposure in South Africa

During the last decade, much effort has been made globally including South Africa to identify anthropogenic substances in the environment that may interfere with the hormonal system of humans or animals (Burger, 2005; Baker, 2001). Research on the effects of EDCs on several species in South Africa is ongoing. EDCs are present in South African waters (Barnhoorn *et al.,* 2004; Fatoki & Awofuki, 2003), with several sources showing estrogenic activity (Timmerman, 2003; Aneck-Hahn, 2002; De Jager *et al.,* 2002). This indicated significant environmental pollution by possible EDCs. Recently the first evidence of intersex in the Sharptooth Catfish (*Clarias gariepinus*) inhabiting South African waters has been reported (Barnhoorn *et al.,* 2004). These fish were caught in a water source receiving effluent from industrial sites, agricultural

activities, informal settlements and municipal treatment plants. The water had high levels of the known EDC *p*-NP.

Another concern in South Africa is the continued use of chemicals listed as potential EDCs in most developed and developing countries, including DDT (Van Wyk, 2002). DDT is an organochlorine pesticides and a hydrophobic molecule that acts by interfering with the ion transport system in the neuronal cell membrane (Shaw & Chadwick, 1998). This interference inhibits neurotransmission and eventually has detrimental effects on animals if exposed to a sufficiently high dose. This molecule is extremely stable and is therefore persistent in water, soil, and animal and plant tissue. The endocrine disrupting effects that have been ascribed to DDT include thinning of eggshells, damage to male reproductive ability and behavioural changes in wildlife (Anon, 1999), and stimulation of VTG synthesis in male Mosquito fish and Rainbow trout (Tyler *et al.,* 1998).

It is not only the case with DDT. Lead (Pb) has been shown among other metals to have estrogen-mimicking and other endocrine-disrupting effects (Children's Health Environmental Coalition (CHEC), 2002). Yet South Africa has no laws restricting the use of Pb in toys and paint. This lack of regulation led to thousands of painted wooden toys being sold with Pb levels up to 1,300 times higher than international limits (Sunday Times, 2005). Recent studies in other countries show evidence that continuous exposure to low-level Pb concentrations can cause reproductive problems. In humans, maternal blood-Pb levels are associated with increased incidence of preterm delivery and decreased gestational age, and low birth weight (Rice, 1995). Reductions in foetal growth put the foetus at increased risk of morbidity and mortality. An increased blood-lead level has been found to be associated with spontaneous abortion (Lanphear, 2004).

A study conducted in the Western Cape, Republic of South Africa, has shown that endosulfan and other pesticides used on farms spilled into nearby rivers after the first rain (Dalvie *et al.,* 2003; Gronen *et al.,* 1999). As a consequence of these, surface waters were contaminated (Schulz *et al.,* 2001). South Africa has no endosulfan regulatory standard and research abroad conducted indicated that elevated levels could negatively affect the central nervous system. Hyperactivity, nausea, dizziness, headache, or convulsions have also been observed in adults exposed to high doses (Environmental Justice Foundation (EJF), 2003). Severe poisoning may result in death. Studies of the effects of endosulfan on animals suggest that long-term exposure to endosulfan can also damage the kidneys, testes, and liver and may possibly affect the body's ability to fight infection (EJF, 2003). However, it is not known if these effects also occur in humans. Endosulfan accumulates in the environment and it is not readily detoxified by soil microorganisms (Schulz *et al.,* 2001).

According to Meintjies *et al.* (2000) estrogen and estrogen mimicking substances were found in the South African water environment at different locations (including Kruger National Park, KwaZulu-Natal, Vaal River, Bethlehem, Transvaal, Pretoria and Johannesburg), and the substances identified were endosulfan, atrazine, DDT, PCBs, NP and Bisphenol A. Most of the studies were undertaken as early as 1974. During that time there was no realisation that the substances were part of the estrogen-mimicking group.

2.5 Biomarkers of endocrine disruption

There is a need to develop and validate reliable, sensitive and cost-effective methods to quantify endocrine disrupting effect/ activity of chemicals which humans and wildlife are exposed to (Leusch *et al.,* 2006; Soto *et al.,* 2006). Increasing research efforts have been devoted to the

development and validation of such assays (Vanparys *et al.,* 2006). But in order for such assays to be developed, a suitable biomarker needs to be identified (Soto *et al.,* 2006).

VTG synthesis is the most reliable and easily detected biomarker of EDC exposure both *in-vivo* and *in-vitro* (Lutz *et al.,* 2005). *In-vivo* experiments concentrate on determining which chemicals are estrogenic and at what levels and on examining the effects of EDCs on the species themselves, these also provide evidence for cause-effect relationship but they are generally impractical for routine screening of environmental samples (Tarrant *et al.,* 2005) and they are more costly and time consuming (Schultis & Metzger, 2004). *In-vitro* test measure the potential estrogen activity in a variety of environmental matrices (Tarrant *et al.,* 2005), and they offer important benefits for these tests are rapid, inexpensive and reproducible, making them very attractive as large scale screening tools (Leuch *et al.,* 2006; Vanparys *et al.,* 2006; Tarrant *et al.,* 2005).

The *in-vivo* studies have focused mainly upon intersex, gonad morphology, liver size and VTG synthesis in male and juvenile fish (Tarrant *et al.,* 2005). These have been identified as the most reliable and easily detected biomarkers of endocrine disruption in fish. But VTG is the outstanding one as fish are not sacrificed. *In-vitro* bioassay such as human cell line, competitive binding, VTG and yeast assay have been developed and employed with documented results (Soto *et al.,* 2006; Vanparys *et al.,* 2006). In *in-vitro* studies, VTG is being synthesised in the primary-cultured hepatocytes and is directed at wildlife monitoring (Tarrant *et al.,* 2005; Monteverdi & Giulio, 1999). The endpoint of both the *in-vivo* and *in-vitro* assays in response to estrogenic effect is the amount of VTG produced (Schmid *et al.,* 2002) which can be determined directly by antibody based methods, enzyme linked immunosorbent assay (ELISA), radioimmunoassay, and SDS polyacrylamide gel electrophoresis (SDS-PAGE), radial

immunodiffusion and indirectly by protein-bound phosphorus or calcium (Tarrant *et al.,* 2005; Cheek *et al.,* 2004; Johnsen *et al.,* 1999).

Some of the other techniques used to measure the estrogenic activity of the xeno-estrogens are based on the ability of estrogens to induce proliferation of their target organs and they are E-SCREEN assays (Silva *et al.,* 2006; Oh *et al.,* 2000), flow cytometric cell cycle analysis (Vanparys *et al.,* 2006) and Uterotrophic assays (Kang *et al.,* 2000; Odum *et al.,* 1999; Ashby *et al.,* 1997). There are also competitive receptor binding assay to test the affinity of known estrogenic chemicals for the receptor binding site (Leuch *et al.,* 2006; Olsen *et al.,* 2005), uterine epithelial cell height, uterine peroxidase activity and reduction in ovary and follicle size have been used as biomarkers of exposure to xeno-estrogens but their use as routine assay are limited (Menditto & Turrio-Baldassari, 1999).

As mentioned above, VTG is the well established biomarker for estrogenic activity and it can be used both *in-vivo* and *in-vitro.* When used *in-vitro* it has shown to predict an *in-vivo* response to similar treatment (Jessen *et al.,* 2003; Mitsui *et al.,* 2003; Takemura & Kim, 2001). Among the methods which can be used to determine VTG, the ELISA approach is the most rapid and reliable method. It provides ease of performance, high sensitivity and a very specific non radioactive method for measuring VTG in large numbers of samples simultaneously (Tarrant *et al.,* 2005; Cheek *et al.,* 2004; Parks *et al.,* 1999). In this study, *O. mossambicus* was used as a sentinel organism for *in-vivo* VTG induction and the development of a sensitive, competitive ELISA based on polyclonal antibodies specific for *O. mossambicus.* VTG has been induced *in-vivo* and used successfully to develop and validate ELISA in various fish species as mentioned in 2.3.1.

Consequently, this study contributed to meeting an urgent need for assays to assess the quantity and effects of environmental estrogens in South Africa. Assays so developed should be independent of physiological fluctuation like age, sex and seasonal changes (Siroka & Drastichova, 2004). One such assay is the development of a tilapia VTG ELISA (t-VTG-ELISA).

3.1 Introduction

A bio-indicator, or biomarker, is a measurable response of a living organism to environmental changes (Siroka & Drastichova, 2004; Joubert, 2000). This response can be as a result of exposure to contaminants and may predict any future harm or may itself be a harmful effect (Joubert, 2000). A bio-indicator response will thus indicate the presence of a contaminant in the environment and the possible threat of the contaminant to the organism. Furthermore it could be an indication that the contaminant has reached the affected tissue or organ in sufficient amounts to cause the observed response (Melancon, 1995).

Vitellogenin (VTG), an egg yolk precursor protein, has been used successfully as a sensitive biomarker for studies of artificial estrogenic compounds in aquatic environments (Scholz *et al*., 2004; Cheek *et al.*, 2001; Hashimoto *et al.*, 2000; Palmer *et al.*, 1998). It is synthesised in the liver of oviparous vertebrates under the control of 17β-estradiol (E_2) by an estrogen receptor-dependent mechanism (Berg *et al.,* 2004; Werner *et al.*, 2003; Takemura & Kim, 2001; Folmar *et al.,* 2000; Nimrod & Benson, 1998,) as shown in Fig. 3.1, where an estrogen (steroid hormone) is taken up by the liver cells (e.g. hepatocytes) and then crosses the nuclear membrane, the estrogen receptor (ER) is maintained in an inactive form, following the binding of estrogen to the receptor, the ER changes its form to an active one, the ER will then seek out specific DNA segments which will result in the production of the target protein (VTG) (Wang *et al.,* 2005). VTG is then transported to the ovary through the bloodstream and incorporated into the oocytes (Hennies *et al.,* 2003; Hashimoto *et al.*, 2000; Lomax *et al.*, 1998). Once in the oocytes, VTG is processed to yolk proteins, including lipovitelline and phosvitin which accumulate in yolk granules (Cheek *et al.,* 2004; Fenske *et al.*, 2001; Palmer *et al.,* 1998), to form the principal nutrient reserve of the developing embryo (Werner *et al.*, 2003; Monteverdi & Giulio, 1999).

The accumulation of yolk protein occurs mainly during later stages of oocyte development and accounts for the rapid growth of the oocyte.

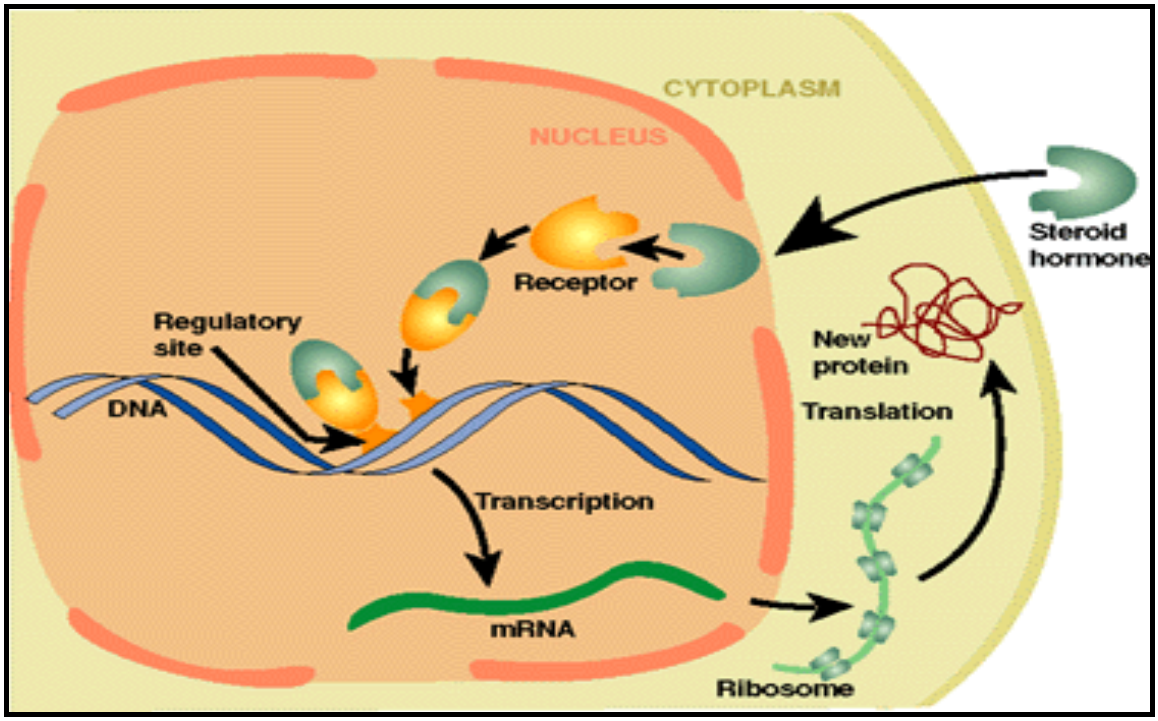

Figure 3.1: Hormone entering a cell, binding a receptor, binding DNA, and initiating a response (Center for Bioenvironmental Research at Tulane & Xavier University, 2004).

VTG synthesis is normally limited to the reproductive female of oviparous vertebrates (Jung *et al.,* 2005; Brion *et al.,* 2002; Cheek *et al.,* 2001) but male and juvenile female organisms possess the *Vtg gene*, which remains inactive under normal conditions when little E_2 is produced but can be induced by exposure to (xeno-) estrogens (Werner *et al.*, 2003; Brion *et al.,* 2002; Cheek *et al.*, 2001; Hashimoto *et al.*, 2000). VTG synthesis appears to be estrogen-specific; therefore the induction of VTG in male or immature fish serves as a sensitive and reliable biomarker for exposure to estrogen agonists in oviparous vertebrates (Cionna *et al.,* 2006; Mosconi *et al.,* 2006; Kordes *et al.,* 2002; Lomax *et al.*, 1998).

Both *in vitro* and *in vivo* assays to measure VTG induction have been developed to assess exposure to environmental estrogen in a number of vertebrates (Lutz *et al.,* 2005; Scholz *et al.*, 2004; Nilsen *et al.*, 2003). As a result of the specific association between VTG synthesis and estrogen stimulation and the low background production of this protein in all but mature females, VTG is a highly specific biomarker for estrogen exposure in male fish. The protein is usually easy to measure and forms the basis of a bioassay for estrogenicity (Lomax *et al.*, 1998; Shaw & Chadwick, 1998; Heppell *et al.*, 1995). In female teleosts, blood levels of the egg yolk precursor VTG can be used as a definitive marker for the onset and progress of maturation. In males, VTG accumulates in the plasma due to the absence of ovary and receptive (maturing) oocytes (Schmid *et al.*, 2002; Gimeno *et al.*, 1998), thus it facilitates sampling (Hylland & Haux, 1997). The accumulation of VTG in male fish can result in the loss of calcium from scales, kidney dysfunction leading to death, abnormalities in reproductive health and inhibition of testicular growth (Solė *et al.,* 2000).

VTG is an ancient protein belonging to a multigene family that includes a variety of lipoproteins found in various vertebrate and invertebrate species (Heppell *et al.*, 1995). It is a high molecular weight protein (Marx *et al.*, 2001) and is strongly antigenic (Heppell *et al.*, 1999; Hylland & Haux, 1997) therefore it has been exploited to develop antibodies and immunoassays.

During this study, the liver of *O. mossambicus* was exposed via an injection to E_2, to produce VTG that was used to develop a species-specific ELISA to enhance analyses during field studies and *in vivo* studies using EDC in the South African context. Vitellogenesis due to estradiol treatment of *O. mossambicus* has been investigated before (Takemura & Kim, 2001; Kishida & Specker, 1993) and two VTG molecules were identified and an *O. mossambicus* VTG ELISA (t-VTG-ELISA) system was established. One homologous competitive ELISA against the VTG of

the *O. mossambicus* was reported (Kishida & Specker, 1993). A Tilapia VTG ELISA has been developed in South Africa. However, the antibodies used in this ELISA were not species-specific (Van Wyk, 2002) which implies that this VTG ELISA can only be used to as a screening assay to indicate the presence of plasma VTG in male or immature fish. The anti-VTG antibody ELISA development approach followed for this study was highly specific for O. *mossambicus* – the argument being that a sensitive and specific homologous competitive ELISA should be available for use during research in South Africa. A specific t-VTG ELISA is more sensitive and accurate to detect VTG levels at a very low level, while the non-specific t-VTG ELISA might be relevant as a large scale screening tools.

3.2 *Oreochromis mossambicus* (Mozambique tilapia) as bioassay organism

Sentinel organisms are defined by Stahl (1997) as:

"Any non-human organism that can react to an environmental contaminant before the contaminant impacts humans".

This offers the possibility of expanding human understanding and responses to environmental health concerns (Van der Schalie *et al.*, 1999). Sentinel organisms provide an early warning system of potential risks before disease develops in humans and animals.

Fish as a sentinel organism provide suitable data on pollution of the aquatic environment since it is their natural habitat (Cheek *et al.,* 2001; Kime, 2001) and water sources are the ultimate sink for most chemicals, whether natural or anthropogenic (Van der Oost *et al.*, 2003; Sumpter, 1998). Studies have been conducted where estrogenic properties of chemicals were investigated using vitellogenin expression in male fish as a biomarker, and include the following fish species zebrafish (*Danio rerio*) (Brion *et al.*, 2002; Fenske *et al.*, 2001; Holbech *et al.*, 2001), sheephead

minnow (*Cyprinodon variegatus*) (Folmar *et al.*, 2000) and English sole (*Pleuronectes vetulus*) (Lomax *et al.,* 1998).

For the purpose of this study, *O. mossambicus* (Fig. 3.2) was chosen as test organism. *O. mossambicus* is indigenous to Southern Africa and has its evolutionary origin in the Zambezi River Basin and has spread southwards through most of the warmer regions of Southern Africa (Skelton, 1993). This fish species is common in the Kruger National Park and is widely distributed in all the perennial rivers and most seasonal rivers throughout the Park (Nussey, 1994). Tilapia is a warm-water fish that prefers temperatures in the range of 24-32°C. Growth rates decline rapidly at temperatures below 20°C with little or no growth registered at temperatures below 15 °C. At these temperatures these fish are also more susceptible to diseases and mortality is often experienced at temperatures below 11°C. Females reach sexual maturity at an earlier age than males. The growth rate in males is 40% faster than that of females, making them the preferred sex for production purposes.

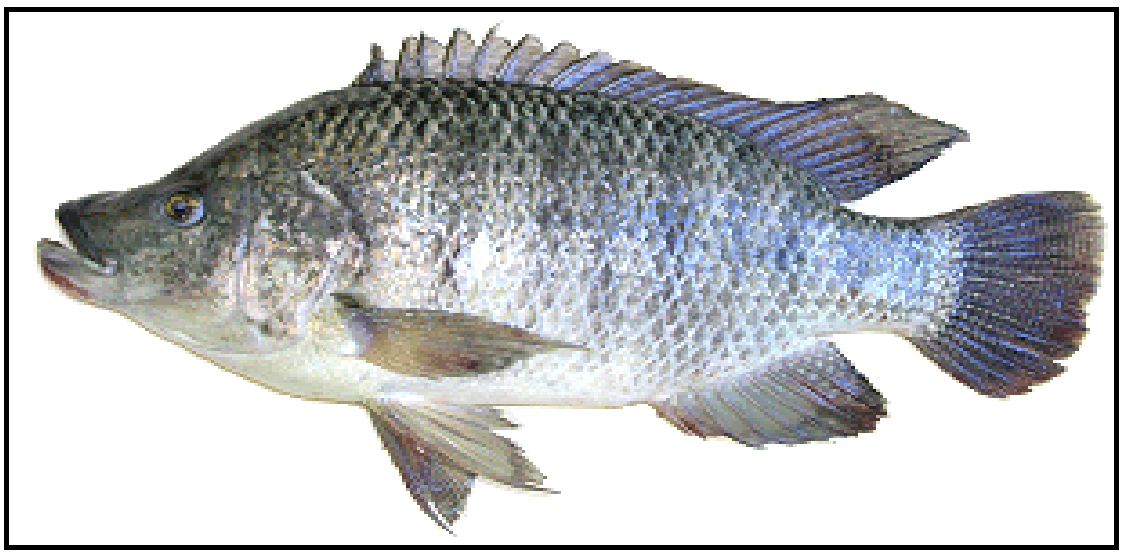

Figure 3.2 ***Oreochromis mossambicus*** **(The Mozambique tilapia) (Aquastel, 2005).**

Oreochromis mossambicus were chosen as a test organism because they:

- Occupy a position within a food chain leading to humans;
- Are widely available and abundant;
- Are amenable to laboratory testing;
- Are genetically stable, thus uniform populations can be tested;
- Are available throughout the year;
- Are more resistant to viral, bacterial and parasitic diseases than other species;
- Are tolerant to high salinity, high water temperatures, low dissolved oxygen and high ;
- Are larger than smaller fishes like zebrafish, making it is easier to obtain large plasma samples (Department of Primary Industries and Fisheries (DPI), 2004; Brion *et al.*, 2002; Popma & Masser, 1999; Barnhoorn, 1996).

Although the use of fish VTG as indicator of EDC occurrence in water has been studied intensively internationally, the development of specific VTG ELISAs is still ongoing in South Africa (Van Wyk, 2002). Validated homologous VTG-ELISAs derived South African freshwater fish species such as the *O. mossambicus* are a necessity for use as biomarkers in local bio-monitoring programmes.

3.3 Enzyme-linked immunosorbent assay (ELISA) for measuring the effects of EDC

The ELISA is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample (Wikipedia, 2006). It utilizes two antibodies, one of which is specific to the antigen and the other of which is coupled to an enzyme. Horseradish peroxidase is an enzyme that is often employed (Cruse & Lewis, 1995). This second antibody gives the assay its "enzyme-linked" name, and will cause a chromogenic substrate to produce a colorimetric signal.

Advantages of enzyme immunoassay:

- Very high sensitivity, detectability, and specificity are possible;
- Equipment and reagents required are relatively cheap;
- Assays may be very rapid and simple;
- Reproducibility is high and evaluation is objective;
- Feasible under field conditions;
- No radiation hazards;
- Once the ELISA has been developed and validated, it requires only the minimally invasive technique of collecting a blood sample (Crowther, 2001; Tijssen *et al.,* 1985).

Although the development of a t-VTG-ELISA offers the good advantages, there are limitations to using such assays:

- Limited detection of VTG protein;
- Assay duration;
- Similarities in responsiveness;
- It is specific to a certain species (Tarrant *et al.,* 2005; Mason, 1996).

For the purpose of this study a homologous competitive ELISA was developed. Competitive ELISA simply means that two reactants are competing to bind to a single receptor. The assay was based on a paper by Fenske *et al.* (2001), which describes a competition for the zebrafish (zf)-VTG antibody between zf-VTG coated on the wells of a microtiter plate and free VTG molecules in sample solutions.

Researchers have proposed the use of functional assays for the screening of potential estrogenic compounds in water sources (Palmer *et al.*, 1998). Such assays would use biomarkers of

estrogenicity, which produce specific and predictable responses to an estrogenic stimulus (Palmer *et al.*, 1998). The assay should demonstrate not merely the presence of a compound in the tissue or body fluids, but should also measure a biological effect. An ideal test for estrogen-related substances should meet several criteria mentioned below:

- Have widespread applicability across many animal groups;
- Require a small and easily obtainable sample without causing undue harm or distress to the animals;
- Measure a physiological response to xenobiotics compounds;
- Measure a response through known biochemical pathways;
- Be responsive to a large class of estrogenic chemicals;
- Be quantifiable (Palmer & Palmer, 1995).

The study approach for VTG production and developing the ELISA to measure it, meets these criteria because the mechanisms will model estrogen action. Besides meeting the point mentioned above by Palmer & Palmer (1995), our VTG test also meets two fundamental requirements for it to be immunogenic as described by Tijssen *et al.* (1985); 1) it should be foreign to the organism to activate the defence mechanism and, 2) it must also be of suitable complexity to react with the different components of the immune system necessary to induce the immune response.

The tilapia–vitellogenin enzyme linked immunosorbent (t-VTG-ELISA) assay developed during this project, is intended to shift the monitoring of the "health'' of the aquatic environment, from water quality and counting dead fish to the response of the living organism to environmental changes/ pollution.

4.1 Introduction

As mentioned in Section 3.2, fish as sentinel organisms were used successfully to monitor their biochemical, physiological and toxicological responses to foreign exogenous compounds (xeno-estrogens) as a prelude to their use in humans, (Palace *et al.,* 2002; Cheek *et al.,* 2001; Kime, 1999; Wilson & Goulding, 1986). Blood was used to assess vitellogenin (VTG) levels produced by the laboratory fish after stimulation with estrogen. The particles, different in density, shape and size, were separated from the blood by centrifugation separation techniques. An ultraviolet (UV) spectrophotometer was used to determine the absorbance of the compound that is proportional to the concentration of the compound.

The molecular weights of VTG were verified by polyacrylamide gel-electrophoresis, a technique that separates molecules in complex mixtures according to size and charge (Elliot & Elliot, 2005; Stryer, 1995). Chromatographic techniques are convenient for separating and purifying of one or more biological compounds from a mixture of compounds. In this project gel-filtration chromatography was employed. For the development of the ELISA, antibodies were used. The ones used in this study were raised or induced by injection of a suspension of the appropriate antigen (VTG) into a rabbit.

4.2 *Oreochromis mossambicus* (The Mozambique tilapia)

Female and male *O. mossambicus* were obtained from the general stock of the University of Zululand, Kwazulu-Natal, were maintained in the aquarium of the Department of Zoology, University of Johannesburg (UJ). The fish were kept in tanks through which tap water was circulated. The water quality of tap water is represented in table 4.1. The fish were held on a 12:12 hour day: night photoperiod and fed on commercial pelleted food according to methods used by Barnhoorn (1996).

Table 4.1: Summary of selected water quality variables values of tap water including target water quality ranges (TWQR) for aquatic ecosystem and aquaculture from Grant (2004).

Variable	Unit	Tap water	TWQR aquatic ecosystem	TWQR Agriculture
pH		7.6		
Conductivity	µs	220		
Total Dissolved solids	ppm	110		
Alkalinity $CaCO_3$	mg/l	60.00	NA	20-100
Aluminium (Al)	mg/l	0.03	0005	0.03
Boron (B)	mg/l	0.15	NA	NA
Cadmium (Cd)	mg/l	<0.003	0.15µg/l	0.02µg/l
Calcium (Ca)	mg/l	17.00	NA	NA
Chloride (Cl)	mg/l	21.00	NA	0-600
Chromium (Cr)	mg/l	0.09	0.012	0.002
Iron (Fe)	mg/l	<0.01	NA	0.01
Magnesium (Mg)	mg/l	5.20	NA	NA
Nitrate (N)	mg/l	2.60	NA	0-0.05
Potassium (K)	mg/l	4.10	NA	NA
Silicon (Si)	mg/l	4.70	NA	NA
Sodium (Na)	mg/l	13.00	NA	NA
Zinc (Zn)	mg/l	<0.01	0.002	0.03

All the standard operating procedures used during this study are available from the appendix on page 87.

4.3 Experimental Design

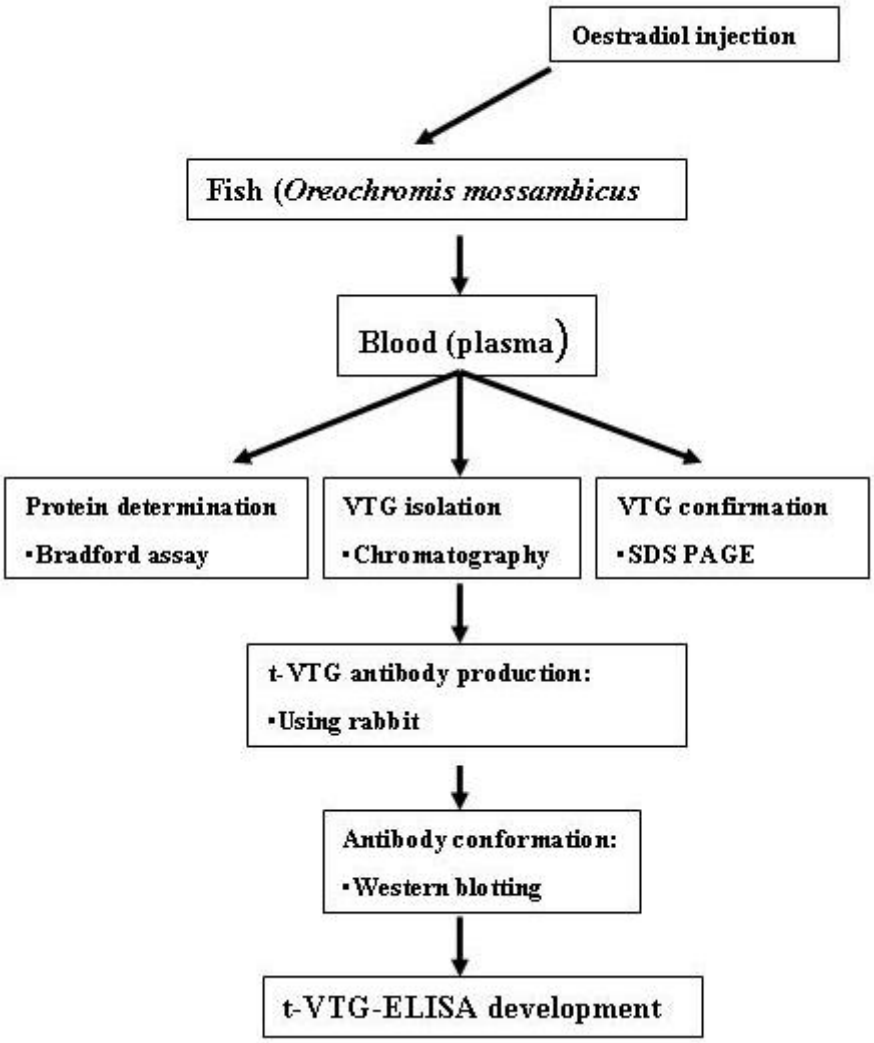

Figure 4.1: Flow diagram of the experimental design.

The aim of this study was to use the Tilapia's VTG to develop a test system (ELISA method) to test for possible estrogenic exposure in *O. mossambicus* (fig. 4.1). This was achieved by stimulating VTG in the *O. mossambicus* by injecting the fish with 17β-estradiol. The VTG was purified and verified by gel filtration chromatography and SDS-PAGE respectively, and the VTG protein concentration was determined by Bradford protein determination. The isolated VTG solution was concentrated by Amicon Ultra-4 centrifugal filter devices (Millipore), these devices are used for concentrating and purifying biological samples. This was done to increase

the concentration of the VTG. The concentrated VTG was used to raise polyclonal antibodies in two rabbits. The antibody production was undertaken by Harlan Sera lab (UK). The specificity of the antibodies raised against the t-VTG was examined by Western-blotting of plasma proteins separated by SDS-PAGE. To fulfil the aim of the study, the ELISA was developed based on the protocol by Kishida & Specker (1993) with optimized assay conditions.

4.3.1 Production-stimulation as well as harvesting of tilapia – Vitellogenin (t-VTG) from males and females

4.3.1.1 VTG used to raise antibody

VTG was induced in both male and female *O. mossambicus* by injecting the fish intra-peritoneal with 10µg/g body weight 17β-Estradiol (Sigma product no. E-8875) as it has shown to be effective when Tyler *et al.* (1990) performed it. 17β-estradiol was prepared for a 10µg/g body weight by dissolving it in 10 ml 70 % ethanol and after it has dissolved; 10 ml cottonseed oil was added according to methods used by Lomax *et al.* (1998). The solution was heated and mixed to allow the solvent to evaporate. One female and one male were used as reference fish, as to compare the effect of estradiol on the fish with reference to VTG stimulation. Before the injection, blood was first drawn from the fish caudal aorta using ice-cold heparinized 1-mL syringes (Nussey, 1994) to verify the presence or the absence of VTG prior to stimulation. The fish received two injections, one on day 0 and the second one on day 7. The fish were taken out of water using a small net, then laid down on a table and the eyes covered with a wet towel to keep it calm and in position during injection as well as during drawing blood as performed by Nussey (1994) and shown in Fig. 4.2**.** Aprotinin (a proteolytic enzyme inhibitor) was added to the Eppendorf tubes containing blood at 20µl/mL (Tyler *et al.,* 1990). The centrifuge was set at 3,000g and 4ºC for 15 minutes, the blood separated and the plasma then withdrawn. The plasma

was filtered using Whatman 02µm pore-size filters to get rid of the cells. The processed samples were stored in Eppendorf tubes at -20ºC until VTG isolation and analysis.

Figure 4.2: Blood drawn from *O. mossambicus.*

4.3.1.2 VTG used as a standard in ELISA development

For the production of standard VTG stock which was used as a standard in ELISA development a different protocol was used whereby the fish received three injections, one every other day, and were bled two days after the last injection according to methods used by Kishida & Specker (1993).

4.3.2 Purification of t-VTG

Gel-filtration chromatography was performed in a 4°C cold room and trypsin inhibitor aprotinin was added to the samples for reduced proteolysis. Before application of samples to the column, the column was equilibrated with a 150 mM Potassium phosphate buffer, pH 7.6, at a flow rate of 0.5 ml/minute. The isolation procedure was similar to the protocol of Santos *et al.* (2001). The proteins were fractionated using a Sephacryl S-300 column (Pharmacia) linked with a peristaltic pump (Amersham) and a fraction collector (Amersham). The gel filtration chromatography procedure was previously effective for purification of catfish VTG (Santos *et*

al., 2001). Fractions of 3 ml/tubes were collected throughout. A total of 30, 40 and 37 tubes/fraction were collected. The absorbance (optical density) was measured at 280nm after collection. Fractions with the highest absorbance were then set aside as fractions containing VTG and to confirm the purity of the t-VTG, the isolate were subjected to protein determination and gel-electrophoresis.

4.3.3 Protein determination

The VTG protein concentration was determined using the Bradford assay (Bradford, 1970). Bovine serum albumin (BSA) was used as the protein standard. Five standards were prepared each with a different concentration. The standards and VTG absorbance were read using a spectrophotometer (Hitachi 150-20) at 595 nm. A standard curve was prepared using absorbance over concentration of the standards. The concentration of VTG was read off in µg/µL from the standard curve.

4.3.4 Sodium Dodecyl Sulfate-PolyAcrylamide Gel-Electrophoresis (SDS-PAGE)

This was done to confirm the purity of the t-VTG and to determine the molecular mass of VTG. The serum samples were electrophoresed under denaturing conditions in polyacrylamide gels SDS-PAGE. BioRad precast 4-19% gradient gels and 10% running gel were used. Before loading the samples on the gels, the protein-containing samples (4% dilution) were mixed with Laemmli sample buffer and denatured by heating the solution for 5 minutes at 100ºC. The samples were allowed to cool down before they were loaded on the gels. A high molecular Precision Plus Protein Standard (BioRad) was used as a marker to verify the molecular weights of the separated proteins. The electrophoretic separation of VTG was carried out for 35 minutes at 200 V for pre-cast gels and for 3 hours at 10mA/gel for 10 % running gel. The gels were

stained with Coomassie brilliant blue solution. To view the bands on the gel, the gel was destained after 24 hours with destaining solution.

4.3.5 Concentrating the purified t-VTG

This was done to increase the concentration of the purified t-VTG. Amicon Ultra-4 centrifugal filter devices (Millipore) were used. The purified fractions with higher absorbance at 280nm were pooled, added to the Amicon Ultra filter unit and centrifuged at 4,000 x g for 20 minutes. The concentrated solutes were recovered, aliquoted and stored at -80°C until antibody production.

4.3.6 t-VTG antibody Production

This was done to raise antibodies specific for t-VTG. Harlan Sera-Lab Limited, Leicestershire, UK, conducted the immunization program. Polyclonal anti-tilapia (t)-vitellogenin (VTG) antibodies (PAS 9633 and 9634) against purified t-VTG in rabbits was raised. In this standardised 77-day protocol, the rabbits were injected with t-VTG in adjuvant and then boosted on five subsequent occasions with t-VTG adjuvant on days 14, 28, 42, 56 and 70. Test bleeds took place on days 0, 35, 49 and 63 and tested using an ELISA. The rabbits were terminated for final bleeding on day 77 after the first antigen injection. The antiserum from each rabbit were collected, aliquot and stored at –80°C.

4.3.7 Western Blotting

To examine the specificity of the raised against t-VTG, a western blot was performed. This was done by separating plasma samples from estrogen induced female and male *O. mossambicus* and the non-induced male and female tilapia on SDS-PAGE. The gel was equilibrated in cold Towbin transfer buffer for 15 minutes, this allowed for the removal of electrophoresis buffer.

The nitro-cellulose membrane was soaked into distilled water for 1 minute after which it was also equilibrated in Towbin buffer. The blotting paper and the sponge were also equilibrated in Towbin buffer and the air bubbles trapped in both the sponge and the blotting paper were rolled out. After the blotting cassette was arranged, the cassette was placed into the transfer tank system (Mini Protean, Biorad) and the proteins were transferred for 20 hours at 36 volts. The transfer was done in a cold room at 4˚C.

After the transfer, the membrane was allowed to air-dry for 10 minutes, while the gel was placed in gel staining solution overnight. The membrane was placed in Ponceau S for 15 minutes to stain transferred proteins. The membrane was de-stained with distilled water until the background became white and the transferred protein appeared as red bands.

After the protein transfer, the membrane was soaked in 20 ml fresh blocking solution A and placed on a Mini Orbital Shaker (Stuart Scientific) set at 50 rpm. After 3 hours, the membrane was placed into 15 ml of fresh blocking solution A containing 5µl of primary antibody (Harlan: Anti-Tilapia-VTG, PAS 9634) and incubated for 20 hours on an orbital shaker. After incubation, the membrane was washed three times in 250 ml phosphate buffered saline for 10 minutes each and once in 200 ml Tris buffered saline for 10 minutes. The membrane was then placed in 15 ml blocking solution B (phosphate/azide free) containing secondary antibody (Sigma: anti-rabbit IgG, A-6154) and incubated for 3 hours at room temperature. The membrane was washed four times in 200 ml TBS for 10 minutes each time before ECL western blot detection and X-ray. ECL reagent (Amersham Pharmacia Biotech) were poured on the membrane and allowed to stand for 1 minute without agitation. The excess reagent was drained and the membrane was wrapped with a cling-wrap. Under safe light condition, the wrapped membrane was placed with

the protein side up in the x-ray cassette together with 1 x-ray film. The x-ray cassette was closed for 10 seconds and after the 10 seconds the cassette was opened and the x-ray film removed and placed into the developer solution for 1 minute 30 seconds, and then transferred to the distilled water for 30 seconds, followed by the fixer solution for 5 minutes. After that, the film was rinsed in water and allowed to dry.

4.3.8 ELISA development and general techniques

A homologous competitive ELISA for quantitative measurement of VTG was developed according to the method described by Kishida & Specker (1993), with some modification. Purified VTG from the gel-filtration chromatography was used as a standard.

4.3.8.1 Coating the plates

The wells of 96-well microtiter plates (NUNC) were coated with 100 µl/well of 500 ng/ml of VTG, diluted in coating buffer (bicarbonate buffer, pH 9.6) for standards, samples and specific binding. For determination of non-specific binding, five wells were coated with coating buffer only. The plates were sealed and incubated overnight (16-18 hours) in a refrigerator at 4˚C.

4.3.8.2 Pre-incubation of samples/ standards

In a non-coated plate (AEC Amersham, SA), the standards (t-VTG) was diluted in PBS-T-blocking buffer to a concentration of 2,000 ng t-VTG/ml. From this stock solution, serial dilution (1,000 ng/ml, 500 ng/ml, 250 ng/ml etc) was prepared in PBS-T-blocking buffer. In parallel, samples with an unknown VTG content were also diluted in PBS-T-blocking buffer. For non-specific as well as specific binding, 100 µl/well of only blocking buffer was mixed with 100 µL/well of antibody solution. The suspensions were mixed and the plates were also sealed and incubated overnight in the refrigerator at 4ºC.

4.3.8.3 Antibody incubation

On the second day, the coated plates were washed manually three times with washing buffer (PBS-T buffer). The plates were blocked with 150 µl/well of blocking buffer for 1 hour at room temperature. After the blocking step, the plates were washed again three times and the antigen-antibody mixture (200 µl/well) was transferred into the coated plate and the plates were sealed and incubated overnight in the refrigerator at 4ºC.

On the third day, the plates were washed three times with washing buffer and horse radish peroxidase (HRP) conjugated-goat anti-rabbit IgG (1:2,000 dilution factor) (Sigma) was added to the well and incubated for 6-8 hours at room temperatures.

4.3.8.4 Revelation

The plates were washed again for three times and then 125 µl/well of the enzyme substrate solution added and incubated in the dark at room temperature for 30 minutes. The reaction was stopped by the addition of 30 µl 3M sulphuric acid. The intensity of colour development was then measured at 490 nm in a universal microplate reader (ELx 800).

4.3.8.5 Validation of the t-VTG ELISA

The objective in validation is to be able to define an assay in terms of statistically quantifiable parameters with measured confidence (Crowther, 2001). The robustness of the t-VTG-ELISA was accomplished by calculating the inter- and intra-plate variation using the same samples within one assay and between different assays. The assay was run in different plates and on different days. The standard deviation values of the sample were divided by the mean value. The sensitivity and specificity of the assay was assessed by comparing the slopes of ten different standard curves. Plasma from reference and estrogen exposed tilapia were also serially diluted in

PBS-T-blocking buffer to assess the parallelism between the binding curves of standards and the samples.

5.1 Stimulation of VTG in male and female fish and protein determination

The total plasma protein concentration as determined by the Bradford method (Section 4.3.3) is presented in tables 5.1, 5.2 and 5.3. The protein concentration of estrogen-stimulated females increased as compared to the reference. The plasma protein concentration for the control female *O. mossambicus* was 14.33 µg/µl for Day 14, increasing to 19.95 µg/µl on day 28, while the concentration for the two estrogen-stimulated females (at 10 µg/g body weight) was 20.60 µg/µl and 23.80 µg/µl respectively for Day 14 and 23.16 µg/µl and 32.03 µg/µl for Day 28 (Table 5.1). The concentration for the estrogen stimulated exposed female and male (at 1µg/g) was 26 µg/µl and 36 µg/µl for day 0 respectively and 230 µg/µl and 258 µg/µl for day 14 (Table 5.2).

Table 5.1: The total protein concentrations (µg/µl) of the estrogen-stimulated and control *O. mossambicus*.

Experimental fish	Day 0	Day 14	Day 21	Day 28
Control male	19.11	N/A	27.92	27.16
Control female	24.49	14.33	14.72	19.95
Female 2	18.21	20.6	32.95	23.66
Female 3	16.35	23.8	26.21	32.03

The difference between stimulated and reference *O. mossambicus* was examined for the production of VTG using SDS-PAGE. As illustrated in Figs. 5.1 and 5.2, the plasma of the estrogen-stimulated females showed a 210- and 140-kDa protein as identified by Takemura & Kim (2001) on day 14, 21 and 28. The plasma of the reference female did not show any VTG synthesis on the gel while the male control, even though its protein concentration was very high as compared to the reference female on day 28 (27.16µg/µl), no VTG band could be identified.

Table 5.2: Total protein concentration (µg/µl) of estrogen stimulated *O. mossambicus*.

Experimental fish	Day 0	Day 14	Day 21
Exposed female	26.0	230	94
Exposed male	36.0	258	116

Table 5.3: Total protein concentration (mg/ml) of estrogen stimulated *O. mossambicus* (for a standard VTG stock).

Experimental fish	Day 0	Day 7
Exposed female	3.7	6.895
Exposed male	5.145	9.285

The SDS-PAGE revealed that the production of VTG is dependent on estrogen. On day 0, before the fishes were stimulated with estrogen, there was no presence of a 210 kDa or 140 kDa protein/band the size of VTG as shown in Figs. 5.1 and 5.2.

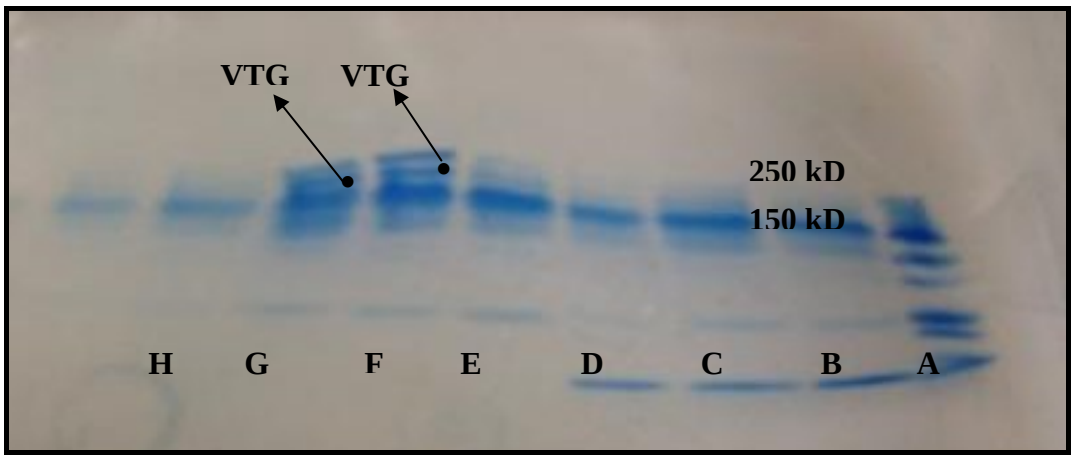

Figure 5.1: SDS-PAGE (10% running gel) of plasma from control and estrogen-treated fish. Lane A, molecular weight markers, Lane B, control male at day 0, Lane C, female 2 before estrogen treatment (day 0), Lane D, female 3 before estrogen treatment (day 0),

Lane E, control female at day 0, Lane F, estrogen treated female 2 at day 14, Lane G, estrogen treated female 3 at day 14 and Lane H, control female at day 14.

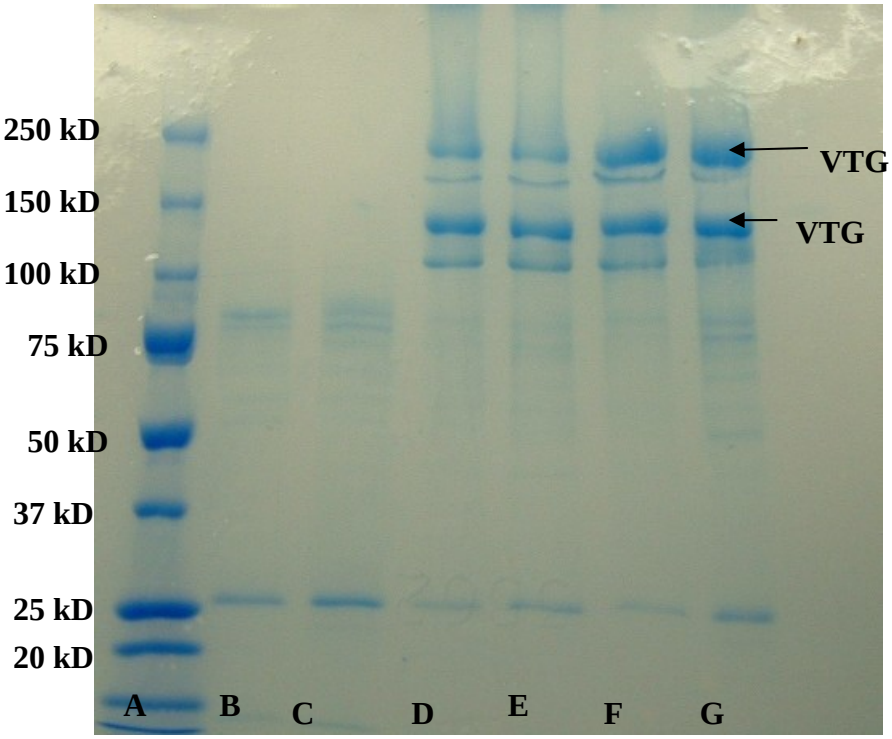

Figure 5.2: SDS-PAGE (pre-cast 4-9% gel) of plasma from estrogen treated fish. Lane A, molecular weight markers, Lane B, female before estrogen treatment (day 0), Lane C, male before estrogen treatment (day 0), Lane D, female after estrogen treatment (day 14), Lane E, male after estrogen treatment (day 14), Lane F, female after estrogen treatment (day 21), and Lane G, male after estrogen treatment (day 21).

5.2 Purification and verification of *O. mossambicus* VTG (t-VTG)

VTG was purified by gel filtration chromatography as described in chapter 4, using a Sephacryl S-300 column (Pharmacia) linked to a fraction collector (Amersham). Examples of the elution profiles after gel filtration chromatography from the estrogen-stimulated females are shown in Figs. 5.3 and 5.4.

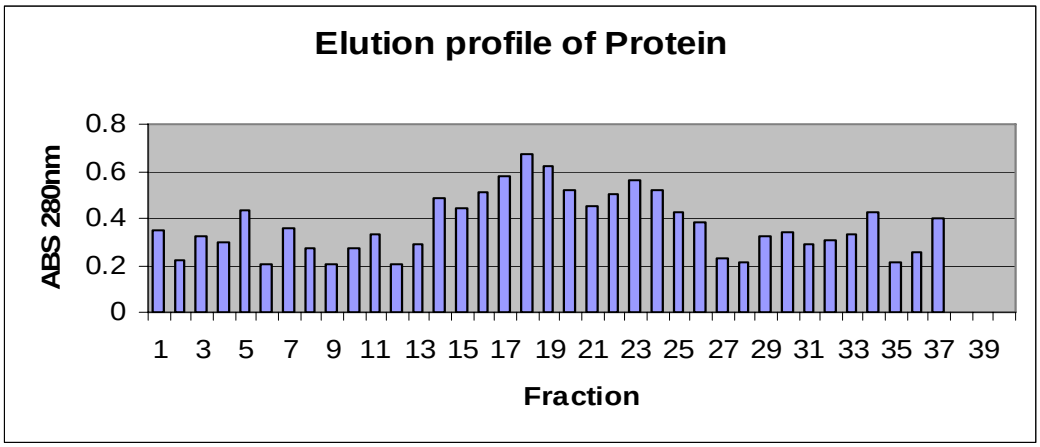

Figure 5.3: Elution profiles of proteins at a detection wavelength of 280 nm from female 2 (at 10 µg/g body weight).

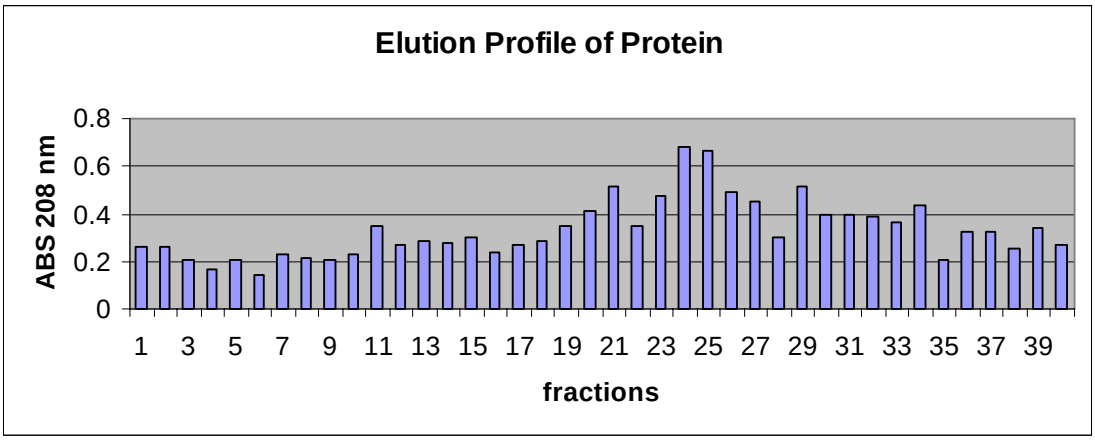

Figure 5.4: Elution profiles of proteins at a detection wavelength of 280 nm from female 3 (at 10 µg/g body weight).

Fractions with the highest peak were pooled and subjected to the Bradford method (Section 4.3.3) and SDS-PAGE (Section 4.3.4) to evaluate VTG purity. Apparent molecular weight of these peaks was estimated as 140 kDa (VTG 140) as shown in Fig. 5.5. It was concluded that the highest peak is where the VTG lies based on the molecular weight of the protein band.

To ensure a maximal purity of the isolated and purified protein (t-VTG), the t-VTG fraction was further purified using elution buffer and it was concentrated by means of Amicon ultra-4

centrifugal filter devices. The concentration of the purified t-VTG was 46.9 µg/µl for female 2 and 23.2 µg/µl for female 3.

5.3 Revelation of the VTG

SDS-PAGE revealed only the presence of VTG (Fig. 5.5) and a minor fragment whose molecular weight is closely related to that of VTG, indicating that the purification process was a success. The purified protein was used to raise polyclonal antibodies in rabbit.

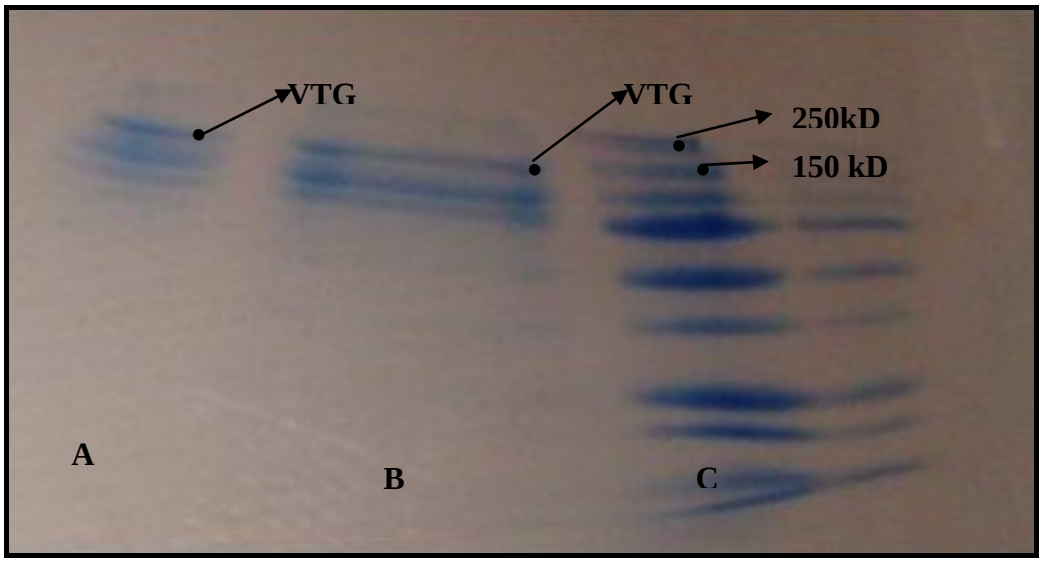

Figure 5.5: SDS-PAGE (10% running gel) of purified VTG. Lane A, VTG fractions from female 2 and Lane B, VTG fractions of female 3, and Lane C, molecular weight marker.

5.4 Antibody production

Harlan Sera lab (UK) used the purified t-VTG (140 kDa) from female 2 as an antigen to raise the polyclonal anti-tilapia (t)-vitellogenin (t-VTG) antibodies (PAS 9633 and 9634) against purified t-VTG in rabbits (Section 4.3.6). Immunisation of the two rabbit with VTG resulted with the production of a high titer of antibodies in one rabbit (PAS 9634) after terminal bleed after five booster injection. The production of the antibodies in the other rabbit (PAS 9633) was not as

high as the other one (PAS 9634) at the terminal bleed after five booster injection (Fig. 5.6). The antiserum (PAS 9634) was characterised by western blot analysis using purified VTG and plasma samples of control male and female and stimulated male and female.

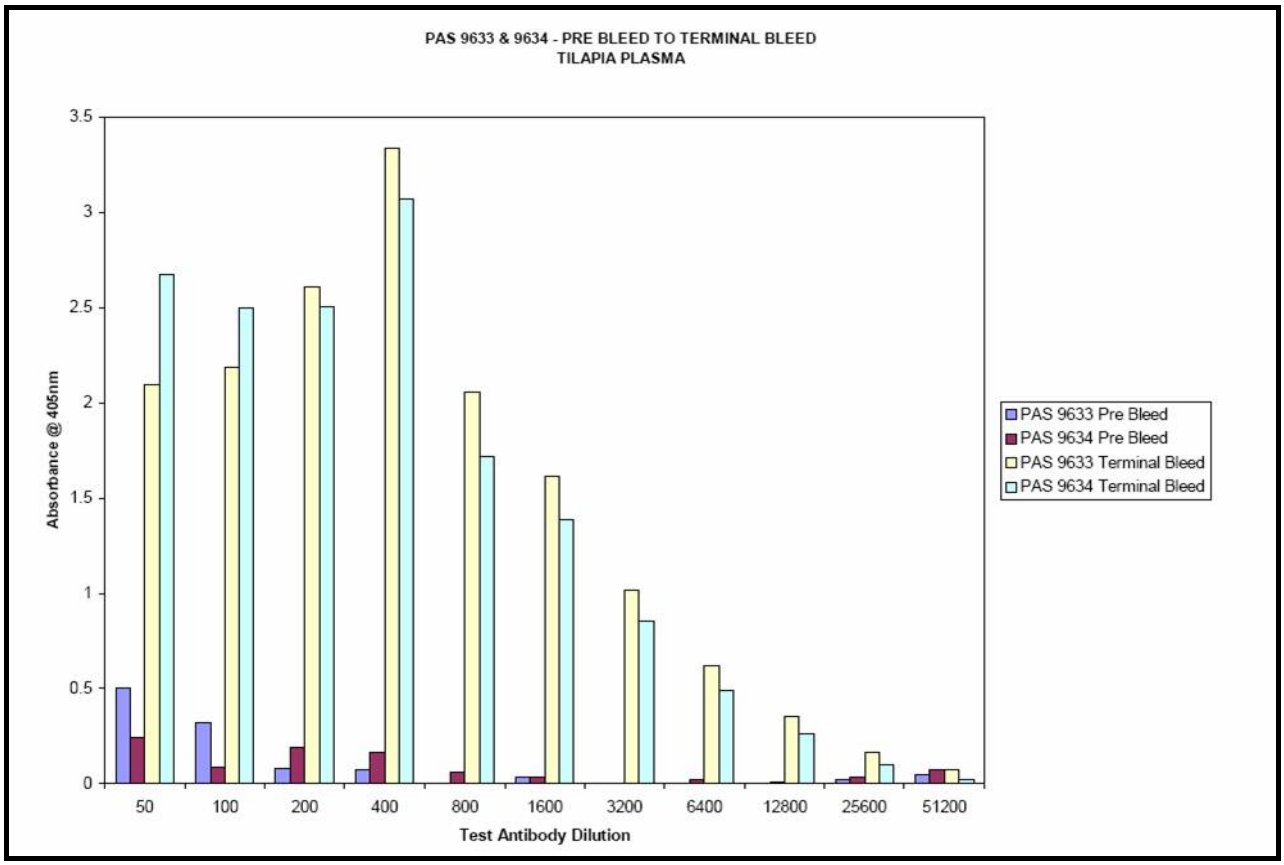

Figure 5.6: Presentation of the results of serial dilution test for both the antibody (PAS 9633 and PAS 9634) and the pre bleed. The absorbance (concentration) of the antibody is higher than the pre bleed that indicate that the antibody can be used in different dilutions for ELISA development.

5.5 ELISA development and validation

The anti-VTG was used to develop a competitive ELISA. During the ELISA development, optimal concentrations of the reagents were determined. Fig. 5.7 presents the optimal concentration of t-VTG and primary antibody (PAS 9634). The VTG coating was 500 ng/ml. The dilutions of the homologous primary antibody (PAS 9634) of between 1:4,000 and 1:1,000,

together with a secondary antibody (HRP conjugated-goat anti-rabbit IgG (Sigma) titre of 1:2,000, produced workable assay conditions.

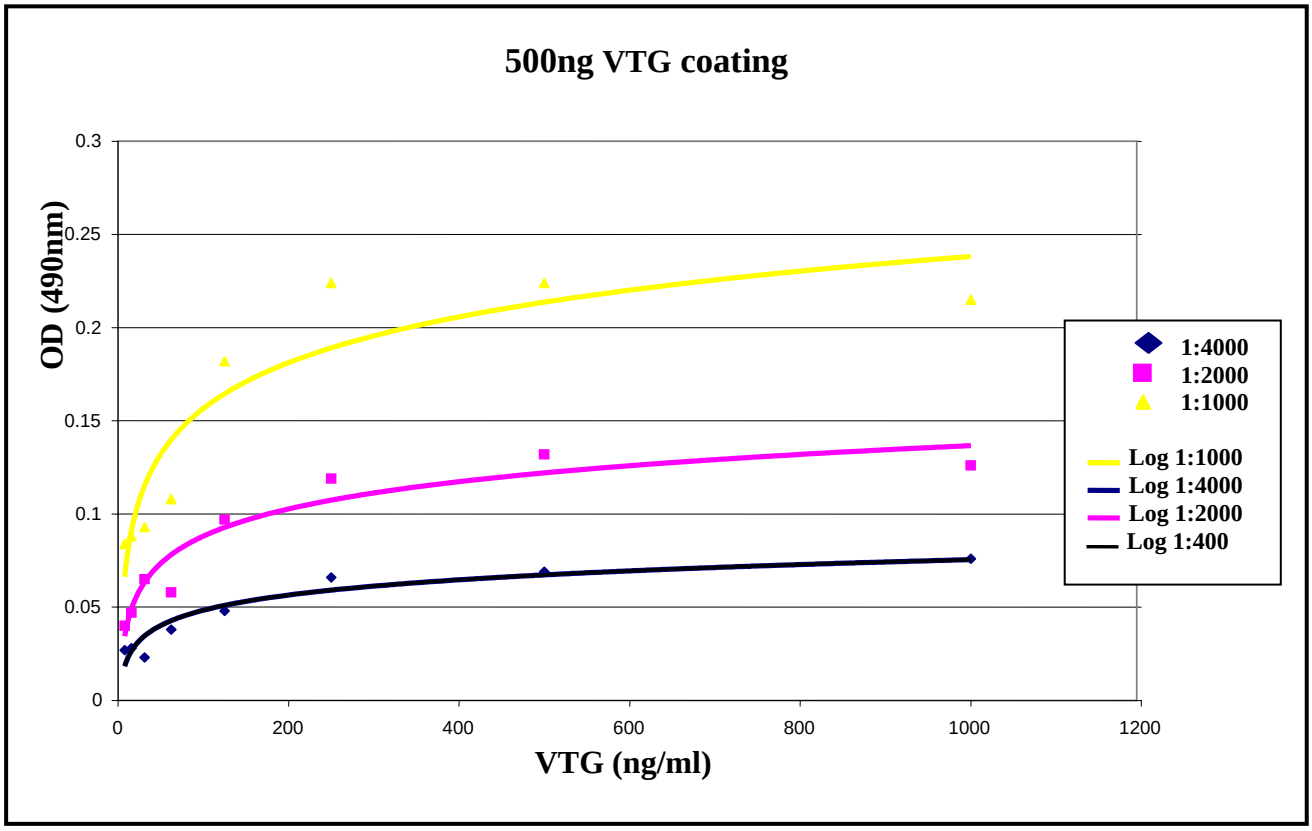

Figure 5.7: Determination of optimal concentration of t-VTG and primary antibody (PAS 9634) for the development of t-VTG-ELISA.

The effects of different VTG coating concentrations were also observed as shown in figs. 5.8 and 5.9. The maximal absorbance occurred at t-VTG coating concentration of between 200 and 300 ng/ml as shown in fig. 5.10. For the development and validation of the assay, therefore, a primary antibody dilution of 1:1000, second antibody dilution of 1:2000 and a VTG coating of 300 ng/ml t-VTG was used.

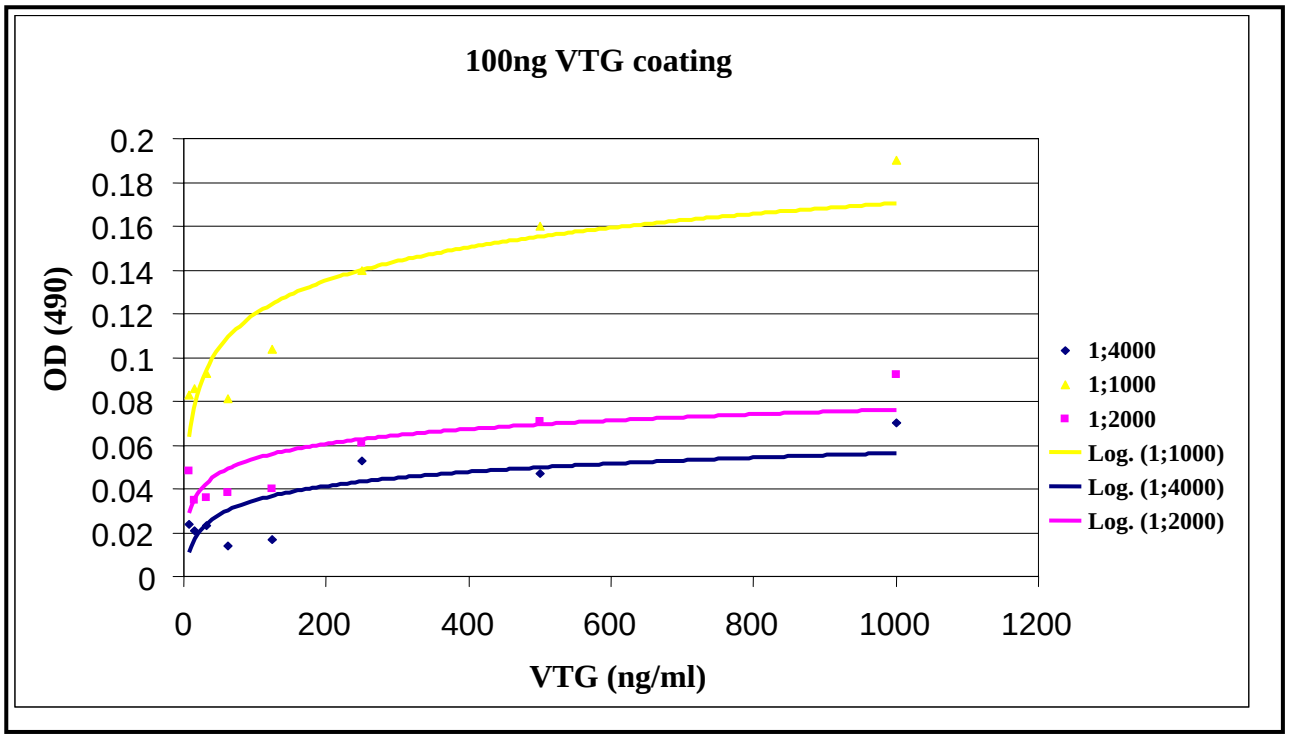

Figure 5.8: Determination of optimal concentration of t-VTG and primary antibody (PAS 9634) for the development of t-VTG-ELISA.

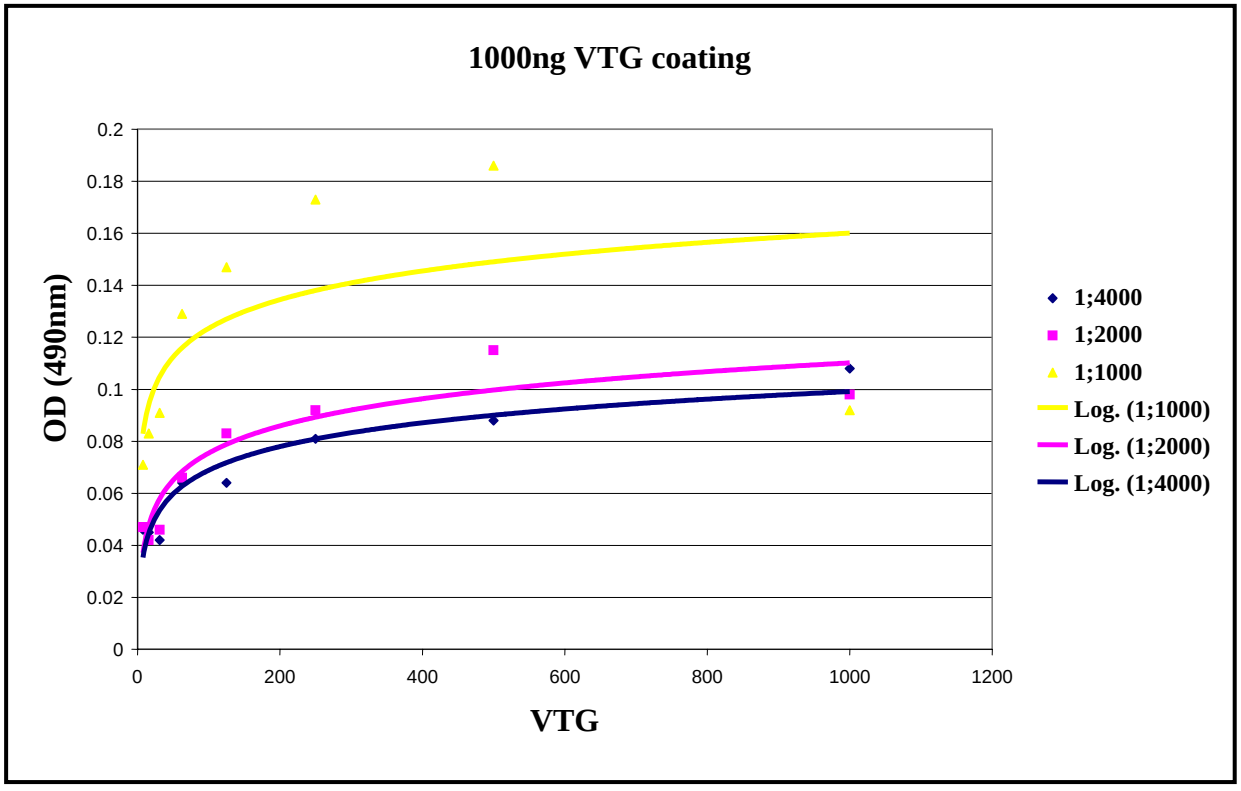

Figure 5.9: Determination of optimal concentration of t-VTG and primary antibody (PAS 9634) for the development of t-VTG-ELISA.

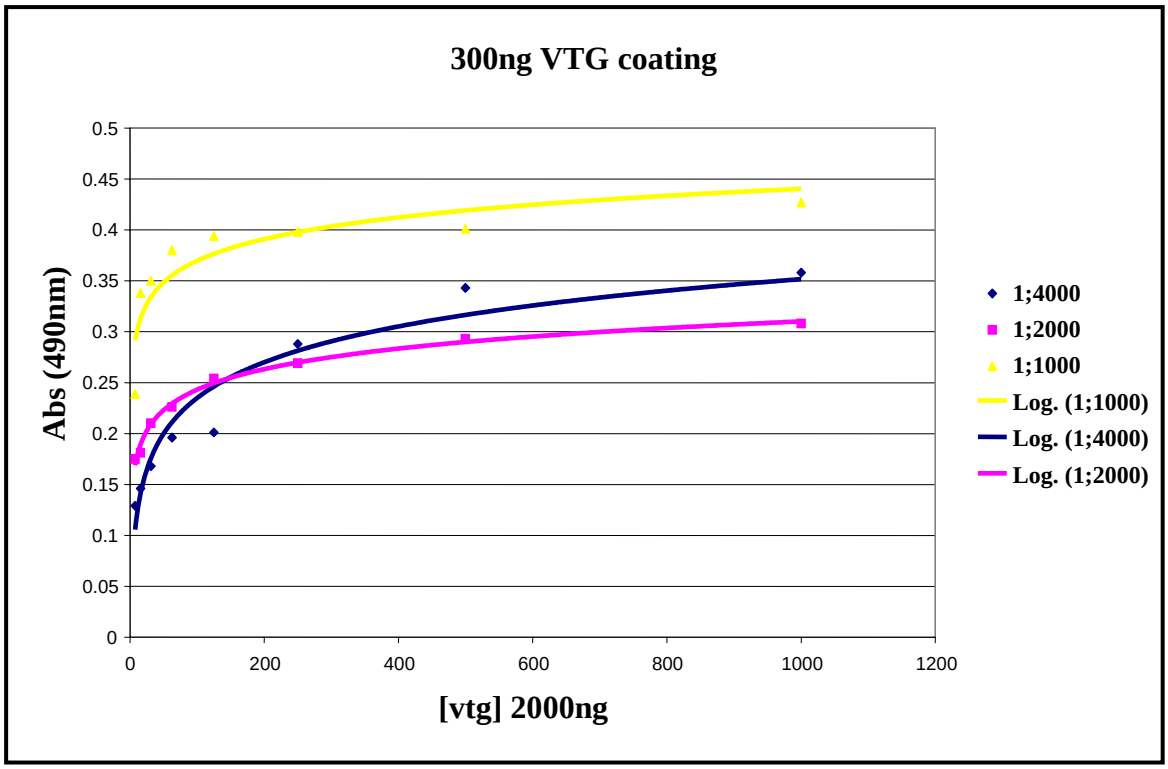

Figure 5.10: Determination of optimal concentration of t-VTG and primary antibody (PAS 9634) for the development of t-VTG-ELISA.

With the assay conditions described above, serial dilutions of purified t-VTG were used to prepare standard curves as shown in fig. 5.11. The five standard curves generated were nearly identical which indicate that the assay developed is repeatable and reproducible. The specificity of the assay was assessed by comparing the slopes of the standard curves. The slope values of the standard dilution curves were quite similar and ranged between -0.059 and -0.07. The intra-assay and inter-assay coefficient variation was 2.41 (n = 4) and 8.71 (n = 10) respectively. The serial dilution of exposed tilapia showed a good parallelism with the standard t-VTG within the working range of the assay as shown in fig. 5.12. The serial dilution of the reference fish did not cover the whole range of the t-VTG standard curve.

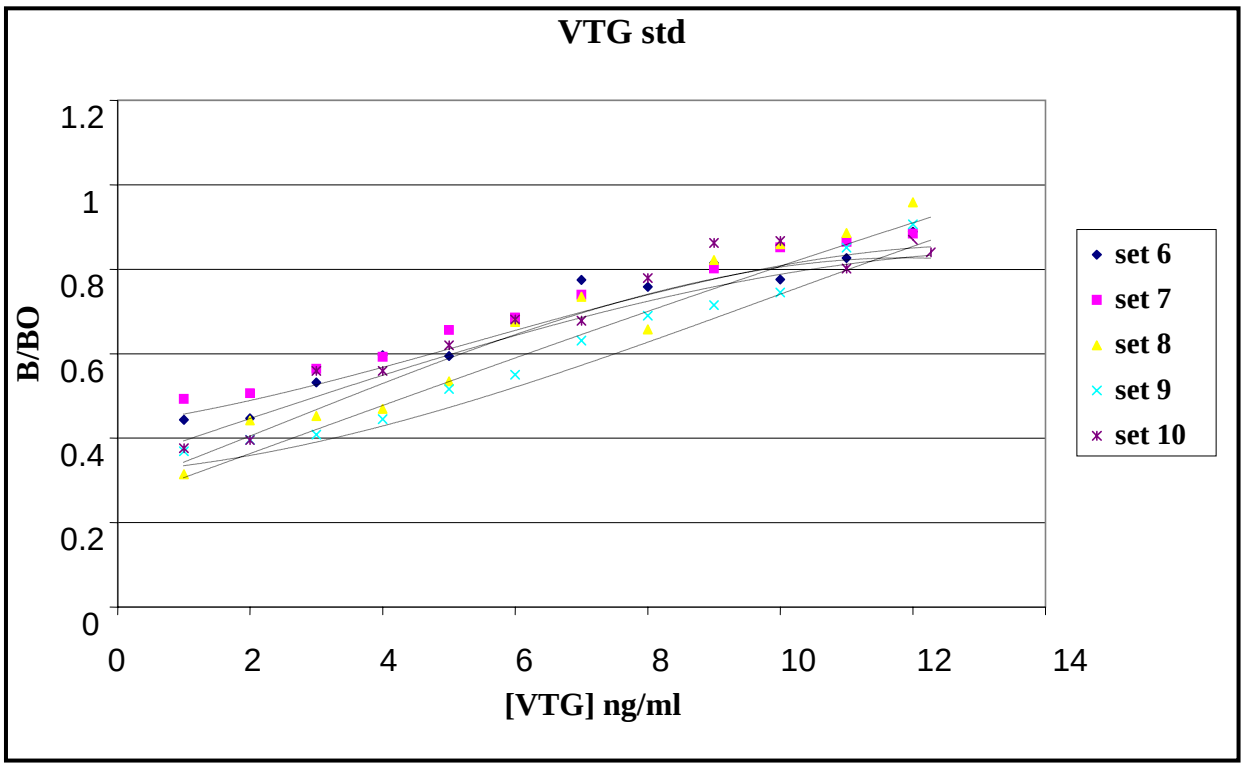

Figure 5.11: Binding curves obtained with serial dilutions of t-VTG standards (set 6 to 10) (B/Bo= binding displacement).

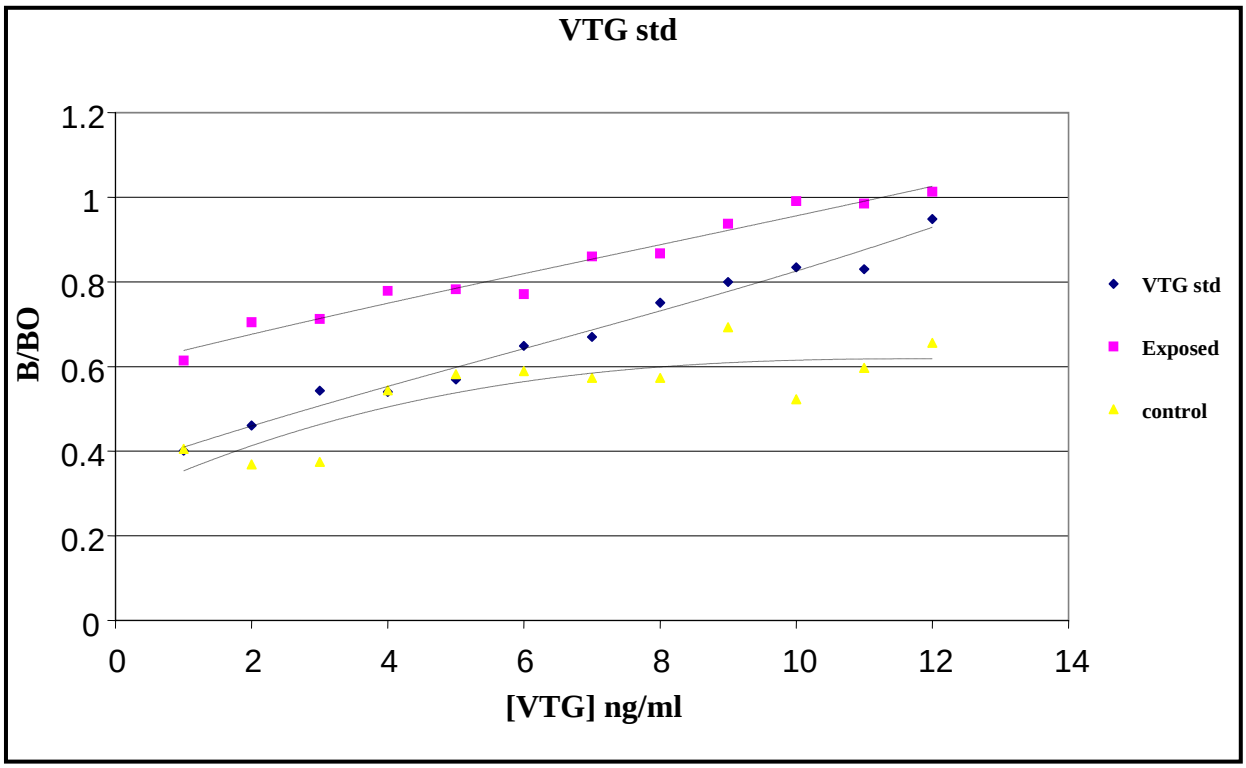

Figure 5.12: Binding curves of serial dilution of plasma samples from E_2 exposed male tilapia and a reference male (control) was compared to t-VTG standard.

5.6 Discussion of results

The assay developed in this study is based on the fish *O. mossambicus* as the model organism. The choice of the *O. mossambicus* as a model is discussed on section 3.2. As mentioned in section 2.5 there are several assays that have been developed to study environmental estrogens like estrogen-dependent breast cancer cells (Soto *et al.*, 1992) and recombinant yeast screen assay (Aneck-Hahn *et al.*, 2005), but assays that are based on whole animal model systems are the most needed because there are many similarities in the organisation of endocrine communication across vertebrates classes and the favoured assays are based on the induction of VTG in non-mammalian vertebrate (Folmar *et al.,* 2000; Lister & Kraak, 2001; Palmer *et al.*, 1998). VTG is a well accepted biomarker for estrogenic chemicals (Kordes *et al.,* 2002) *In-vivo* assay such as the measurement of plasma VTG in fish is an important method and has been used successfully to screen existing or new chemicals for estrogenic potency (Shao *et al.,* 2005; Folmar *et al.,* 2002). ELISA methods have also been used successfully to quantify VTG in the plasma. They have shown to be sensitive, reliable and rapid.

The study has shown that estradiol can be used to induce the synthesis of VTG in male and juvenile female fish. During this study, VTG was stimulated in tilapia in order to assess the sensitivity of the *O. mossambicus* to exogenous estrogens, and to develop a homologous direct ELISA using the VTG. The stimulation was a success because the fish were taken care of, the fish were kept in tanks supplied with running tap water and with sodium chloride to reduce salt loss and prevent bacterial infection according to the methods used by Grant (2004). They were held on a 12:12 hour day: night photoperiod and fed on commercial pelleted food (Barnhoorn, 1996). The wellbeing of the fish was very important for this study because the VTG was purified and isolated from plasma which was drawn from the fish using injections.

The fish were stimulated with 17-β estradiol. This substance was used successfully in fishes and vertebrates in similar studies elsewhere in the world (Riley *et al.,* 2004; Takemura & Kim, 2004; Herbst *et al.*, 2003; Mitsui *et al.*, 2003; Brion *et al.*, 2002; Folmar *et al.*, 2000; Lomax *et al.*, 1998; Palmer *et al.*, 1998). All the experimental *O. mossambicus* that were stimulated with 17-β estradiol for this study produced two proteins that were absent from the reference fish (Section 4.3.1). The protocol used by Kishida & Specker (1993) to stimulate VTG in the fish proved to be more effective than the initial one by Tyler *et al.* (1990). The protein content in the blood of exposed female at day 7 was 6.895 mg/ml by Kishida and Specker (1993) protocol and 32.95 µg/µl on day 14 by Tyler *et al.* (1990) protocol.

For assay development the purification of the protein is the first essential step (Hennies *et al.,* 2003). Isolation and purification of VTG were performed in many studies using both anion-exchange liquid chromatography and gel filtration (Santos *et al*., 2001). However, for this study, the isolation and purification of these proteins were successfully carried out by only gel filtration chromatography. Furthermore, the fraction with the highest wavelength were further purified and concentrated by using Amicon Ultra-4 centrifugal filter devices (section 4.3.5), with the resultant proteins having molecular weights of 140 kD. This corresponds with the molecular weights of *O. mossambicus* VTG reported by Takemura & Kim (2004). The molecular weight of VTG varies across different organisms. VTG in most species has two different molecular weights (Brion *et al.*, 2002; Fenske *et al.*, 2001; Chang *et al.*, 1996). Some literature identifies the molecular size of *O. mossambicus* VTG as 200 and 130 kDa (Kishida & Specker, 1993). More importantly, the proteins identified in this study were considered to be VTG because they were detected only in the plasma of the 17β-estradiol treated females and males as demonstrated by SDS-PAGE (Figs. 5.1 and 5.2) and the plasma proteins levels in exposed fish increased as compared to non-induced fish (Table 5.1).

In fig. 5.6 after the purification process there is a minor fragment next to VTG. The fragment is believed to be degraded part of VTG. It is very important to do VTG analysis as soon as possible because it is unstable (Fenske *et al.*, 2001) under conditions such as repetitive freezing and –thawing resulting in VTG degradation even with the presence of protease inhibitor (Shao *et al.*, 2005; Roy *et al.*, 2004). Proteolytic degradation of plasma VTG has been reported in other species (Herbst *et al.*, 2003; Holbech *et al.*, 2001). But we tried to reduce the risk of degradation of VTG by adding protease inhibitor, aprotinin to the samples immediately after blood was collected and the purification procedure of VTG was carried out at low temperatures.

The purified VTG as the antigen source was used to raise polyclonal antibodies in two rabbits and were used to develop specific immunoassays. The homologous polyclonal antibodies were specific for t-VTG.

A homologous t-VTG-ELISA that uses a polyclonal antibody raised against purified *O. mossambicus* VTG was successfully developed and validated. The ELISA method developed was repeatable and it was sensitive enough to quantify VTG in the standards and the exposed fish. The intra-assay and inter-assay coefficient variation was 2.41 (n = 4) and 8.71 (n = 10) respectively. These variations are closely similar to values obtained for other fishes (Brion *et al.*, 2002; Johnsen *et al.*, 1999). The reproducibility of the assay was also confirmed by the parallelism observed between the standards and the plasma of E_2 exposed fish, indicating that anti-t-VTG was able to recognise the purified VTG and the plasma VTG in the similar manner. The assay could be used successfully to quantify VTG in fish exposed to EDCs.

6.1 Conclusion

The aim and objectives of the study as discussed in Chapter 1 were met. In particular:

1. VTG was stimulated both in male and female fish that were injected with 17β-estradiol. Injection is one of the commonly used methods in exposing larger model organism to estradiol or chemical of interest.
2. VTG production also demonstrated that *O. mossambicus* is sensitive to exogenous estrogen.
3. Polyclonal antibodies against t-VTG were raised.
4. The ELISA method was developed and validated. Through the ELISA method we have demonstrated that VTG induction can be quantified in adult male *O. mossambicus* exposed to graded concentrations of the natural steroid hormones.
5. The ELISA method with a little modification is ready to be used or added to a battery of tests to assess estrogenic activity in South African water sources.

The increasing global populations and the human desire for a higher material standard living will only result in worldwide pollution on a massive scale. Most of the pollutants might have endocrine disruption activities. The reliable guidelines data that will safeguard the health of human and wildlife against EDC activities need to be developed, validated and implemented. VTG induction in both male and juvenile fish has provided valuable background on the best protocol to use. This study has served as a means for meeting the needs for reliable biomarkers for the detection of estrogenic effects.

6.2 Recommendation

The method of stimulation used by Kishida & Specker (1993), resultant in a significant increase in the total plasma protein content as compared to the one by Tyler *et al.,* (1990). With the

method by Kishida & Specker (1993), the duration of the stimulation is shorter, blood can be taken for VTG analysis on day 7.

The ELISA method developed need to be modified and some of the factors to consider include good laboratory practice and use of a different blocking buffer, or maybe even a different ratio between coating concentration and anti-VTG concentrations. It will be ideal to continue analyzing the antibody to determine the appropriate dilutions necessary to ensure that the assay work its optimal capabilities.

If the antibody does not work at levels that are said to be optimal, and there is a great necessity to produce a new antibody from the purified t-VTG, the antibody should be produced immediately after purification process. In cases where the purified protein should be sent to another country for antibody production, it will be wise to start with the stimulation and purification once one have organized funding and the means of getting the protein to another country. VTG is an unstable protein and a degraded protein will not produce antibody of good quality.

Since it has been established that when male tilapia are exposed to estrogen produces VTG, it would be ideal to validate the t-VTG-ELISA on feral *O. mossambicus* and not on laboratory animals/ fish. The field studies results call for more attention and a drive to do attend to the problem from government agencies and media rather than laboratory work.

The study of EDCs is broad and there are lots of assays that have been developed to measure the biochemical effect after exposure like VTG, while others measure the proliferation of the target cell. What need to be done is for the water supply sector to incorporate the testing of EDCs in the standard systems of routine water sources monitoring

The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) (1998) have recommended that a two tier system, which encompasses a battery of in vitro and in vivo biological assays be used for screening and testing of EDCs. A screening tier (tier 1) would be used to identify substances with endocrine-disruption potential for further testing and a testing tier (tier 2) would identify adverse effects and establish dose-response relationship for hazard assessment.

In conclusion I would like to say this project has provided a tier 1 results and the t-VTG-ELISA will also be used in the future as a tier 1 screening. It would be wise to pursue projects that will be of tier 2 testing.

On the lighter side:

While we are still waiting for the water supply sector to incorporate the testing of EDCs in the standard water system, for the engineers to design equipment that will be able to remove all traces of EDCs from our water supply and the production of environmentally friendly chemicals to substitute EDCs, one can apply the principle of prevention is better than cure by:

- Educating yourselves and your family about EDCs.
- Buying organic food whenever possible.
- Avoiding the use of pesticides in your home, yard or on your pet.
- Avoiding fatty foods such as cheese and meat whenever possible.
- Keeping plastic teethers and toys away from young children.
- Supporting efforts to get strong government regulation of and increased research on EDCs (Marchese, 2006).

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Appendix

Standard operating procedures

Bradford (1970) Method for Protein Determination

Standard Curve:

Make up the following standards from the protein standard stock solution (BSA)

1mg/ml	40µl BSA + 360µl Sucrose buffer
0.5mg/ml	20µl BSA + 380µl Sucrose buffer
0.25mg/ml	10µl BSA + 390µl Sucrose Buffer
0.125mg/ml	5µl BSA + 395µl Sucrose Buffer
0.062mg/ml	2.5µl BSA + 397 Sucrose buffer

- Add 100µl of each standard to 5ml Bradford Reagent. Let it stand for 5 minutes.
- Read on the spectrophotometer at 595nm(Abs)
- Draw a curve: absorbance over concentration

Protein determination of samples:

- Add 100µl of sample to 5ml Bradford reagent and let it stand for 5 minutes (Note: sample may need to be diluted, make note of dilution used).
- Read at 595nm
- Read off concentration in µg/µl from the standard curve.

Gel construction

Clean the glass plates and spacers with 100 % ethanol. Construct vertical mini-gel system (Biorad) according to instruction manual.

For a 10 % Running gel:

- In a vacuum flask, add:

Monomer solution	6.7 ml
4 X Running gel buffer	5 ml
10 % SDS	0.2 ml
ddH_2O	8.0 ml

- De-gas the solution for 20 minutes, shaking lightly every five minutes
- After the solution has been degassed, add:

APS	100 µl
Temed	6.7 µl

- Gently swirl the contents without creating bubbles
- Pipette 5 ml of the gel solution between the plates
- Add 100 µl of water saturated butanol above the gel solution between the plates.
- Gel usually set in 1-2 hours

For a 4 % Stacking gel

- In a vacuum flask, add:

Monomer solution	0.88 ml
4 X Stacking gel buffer	1.66 ml
10 % SDS	66 µl
ddH_2O	4.06 ml

- Degas for 15 minutes, shaking lightly every five minutes
- After degassing the solution add:

APS	33.4 µl
Temed	3.3 µl

- Gently swirl the contents without creating bubbles
- Pour off the water saturated butanol from the running gel and add 150 µl of 4 X stacking gel buffer to the running gel.

- Tilt the gel unit from side to side to wash away any excess butanol that may have remained behind.
- Pour off the stacking gel buffer
- Clean the well combs with 100 % ethanol and add them to the gel unit, and fill the unit with 4 X stacking gel.
- Allow the gel to polymerise (should take two-three hours).

Sample preparation and gel running:

- After protein determination, calculate the volume of sample needed to get 15 µg of protein/ well.
- Take that volume and divide it by three to get the volume of 3 X Laemmli sample loading buffer needed to be added to each sample.
- Heat the samples with corresponding volume of sample loading buffer to 100ºC for five minutes.
- Remove and let cool.
- Add the sample to the gel.
- Run the gel at a constant current of 10 mA/ gel
- Run the gel until the colour begins to come out the bottom of the gel (should take about 3 hours).

Bradford Reagent

100 ml 85% Orthophosphoric acid

100 mg Coomassie Brilliant Blue G250

50 ml 95-97% Ethanol

Dissolve Coomassie in ethanol. Add phosphoric acid. Solution should be warm to the touch. Make up to 1 l with distilled water. Filter twice to remove precipitate. Caution: Solution foams when filtered. Work carefully.

Solution can be stored in the fridge for up to 1 month, although filtering is necessary to remove possible precipitate. Solution must be at room temperature before use.

Sucrose buffer

8.5 g Sucrose (0.25 M)

0.6 g Tris-base (50 mM)

80 ml distilled water

Adjust to pH 7.4 with 10 M HCl

Make up to 100 ml with distilled water

(Solution must be kept cold).

Protein Standard Stock Solution

100 mg BSA

Add 10 ml Sucrose buffer

Monomer Solution

(30.8%T, 2.7%C_{bis})

60 g Acrylamide

1.6 g Bisacrylamide

ddH_2O to 200 ml

(Caution: Acrylamide is neurotoxic and should be handled with care and store up to 3 months in the dark at 4ºC) .

4x Running Gel Buffer (Store up to 3 months in the dark at 4ºC)

(1.5M Tris-HCl, pH 8.8)

36.3 g Tris

Add 150 ml ddH_2O

Adjust to pH 8.8 with HCL (± 6 ml)

10% SDS

20 g SDS made up to 200ml

(Store for up to 6 months at room temperature. Caution: this chemical cause's nose bleeds and corrodes contact lenses if the incorrect laboratory procedures are not followed).

10% APS (For best results make a new one everyday).

0.5 g Ammonium Persulphate made up to 5ml with distilled water

3x Laemmli Sample Loading Buffer

3.75 ml 1.0M Tris Base (pH 6.8)

0.2 g SDS (2%)

1.5 ml β-Mercaptoethanol

3 ml Glycerol

0.001 g Bromophenol blue (dip tip of dissecting needle into Bromophenol Blue and stir into solution, add last)

Make up to 10 ml with distilled water.

Tank Buffer

(0.025M Tris, 0.192 M Glycine, 0.1 % SDS, pH 8.3)

6.06 g Tris

28.8 g Glycine

2 g SDS

dH_2O to 2 l

(Not necessary to check pH. Store in the fridge for up to 2 months max).

Standard Staining Solutions for Gels

(0.025% Coomassie Brilliant Blue, 40% Methanol, 7% Acetic acid)

0.5 g Coomassie Brilliant Blue

800 ml Methanol

Stir until dissolved

140 ml Acetic acid

Make up to 2 l with distilled water

(Store on shelf for up to 6 months).

Destaining Solution 1

(40% Methanol, 7% Acetic acid)

400 ml Methanol

70 ml Acetic acid

Make up to 1 l with distilled water

(Store on shelf for up to 6 months).

VTG elution buffers

Buffer B] 300 mMol (K_2HPO_4) = 0.3 Mol

174.2 X 0.3 = 52.26 g/ l or 104.52 g/2 l

75 mMol (KH_2PO_4) = 0.075 Mol

136.1 X 0.075 = 10.21 g/ l or 20.42 g/2 l

Buffer C] From buffer B or final buffer take 1l and fill up to 2 l again to get 150 mMol buffer as buffer C

Towbin Transfer buffer

6.06 g Tris base

28.82 g Glycine

Make to 1.6 l with distilled water (pH should be 8.2-8.4)

400 ml methanol

Adjust to final volume of 2 l with distilled water.

Phosphate buffer Saline (PBS)

23.0g Disodium hydrogen orthophosphate

5.92 g Sodium dihydrogen orthophosphate

11.68 g Sodium Chloride

Make up to 2 l with distilled water.

Tris buffer saline (TBS)

17.6 g Sodium Chloride

12 g Tris base

Make up to 1.6 with distilled water (adjust pH to 7.5 with 10N HCl)

Make up to 2 l with distilled water.

Ponceau S

1.0 g Ponceau S

50 ml acetic acid

Fill to 1 l with distilled water.

Blocking solution A

5 g non-fat milk powder

0.02 g Sodium azide

Make up to 100 ml with TBS

Phosphate/ azide-free Blocking solution B

5 g non-fat milk powder

10 µl Anti-foam A

Make up to 100 ml with TBS

Coating buffer (pH 9.6)

4.24 g Na_2CO_3

5.04 g $NaHCO_3$

Make up to 1 l with distilled water.

Phosphate buffered saline (PBS 10X stock) (pH 7.4)

16.7 g Na_2HPO_4

3.6 g NaH_2PO_4

85 g NaCl

Make up to 1 l with distilled water.

Washing buffer (PBS-T) (pH 7.4)

Take 200 ml from PBS 10X stock

Make up to 2 l with distilled water

Adjust pH

Add 1 ml Tween 20 and mix until Tween has dissolved.

Blocking buffer

200 ml of PBS-T

Add 2 g of BSA

Mix until BSA has dissolved.

Substrate buffer

Solution one: 14.2 g dibasic sodium phosphate and make it to 500 ml with distilled water.

Solution two: 10.5 g Citric acid and make it to 500 ml with distilled water

Phosphate citric acid buffer: 25 ml of solution one plus 25 ml of solution two. Then add 50 ml distilled water and adjust pH to 5 with solution one or two.

Substrate buffer: 20 ml Phosphate citric acid buffer.

2 tablets 0.5 mg/ ml OPD

10 µl 0.5 µl/ ml H_2O_2 (30 %)