THE DEVELOPMENT OF AN INVERTEBRATE BIOMARKER ASSAY FOR ENVIRONMENTAL MONITORING

Report to the
WATER RESEARCH COMMISSION

by

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WRC Report No. KV 351/15
ISBN 978-1-4312-0745-9

February 2016

Obtainable from

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The publication of this report emanates from a project entitled *The development of an invertebrate biomarker assay for environmental monitoring* (WRC Project No K8/1056).

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EXECUTIVE SUMMARY

Potamonautes is a crustacean genus that is widely distributed over Sub-Saharan Africa. In South Africa, two species from this genus namely *Potamonautes warren* and *Potamonautes perlatus* have previously been used as bio-indicators for monitoring environmental water. Studies using *Potamonautes perlatus* focused mainly on its use as bio-indicator for monitoring adverse effects due to heavy metals in the Eerste River, Stellenbosch, South Africa. The current study investigated vitellogenin (VTG) in this species. Conflicting evidence in the literature are currently available on the use of crustacean VTG as biomarker for estrogenic contaminants in the environment. VTG is a yolk protein precursor and serves to supply nutrients to the developing embryo. The synthesis of VTG is controlled by sex hormones. Males do not normally synthesise this protein, but in oviparous vertebrates, males synthesise VTG upon exposure to environmental estrogenic substances. Conflicting reports on the ability of male crustaceans to synthesise VTG are available in the literature. The aim of this study is to develop monoclonal antibodies against VTG that can be used in an ELISA to elucidate if male *Potamonautes perlatus* synthesise VTG upon exposure to estrogenic chemicals. The specific aims of this study were to:

1. Isolate VTG from female crabs.
2. Produce and characterise monoclonal antibodies against the isolated VTG.
3. Develop sensitive immunoassays such as ELISAs for VTG using the newly produced monoclonal antibodies.
4. Employ the newly developed ELISAs in laboratory studies to determine if male *Potamonautes perlatus* exposed to estrogen synthesise VTG.

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LIST OF ABBREVIATION

Con A	–	Concanavalin A
DMSO	–	Dimethyl Sulphoxide
DNA	–	Deoxyribose Nucleic Acid
DPBS	–	Dulbecco's Phosphate Buffer Saline
EDCs	–	Endocrine Disrupting Chemicals
ELISA	–	Enzyme-Linked Immunosorbent Assay
FBS	–	Foetal Bovine Serum
FCA	–	Freund's Complete Adjuvant
FIA	–	Freund's Incomplete Adjuvant
HAT	–	Hypoxanthine Aminopterin Thymidine
HRP	–	Horseradish Peroxidase
HSA	–	Human Serum Albumin
HT	–	Hypoxanthine Thymidine
IM	–	Immunization
IgG	–	Immunoglobulin G
IgM	–	Immunoglobulin M
OD	–	Optical Density
PBS	–	Phosphate Buffered Saline
PEG	–	Polyethylglycerol
PI	–	Protease Inhibitors
pVTG	–	Purified Vitellogenin
RPMI	–	Roswell Park Memorial Institute
SDS-PAGE	–	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TMB	–	Tetramethylbenzidine
VN	–	Vitellin
VTG	–	Vitellogenin

1. INTRODUCTION

Due to the exponentially expanding global population (United Nations, Department of Economic and Social Affairs, Population Division, 2013), fresh drinkable water has become a scarce commodity. The rising global pollution puts major strains on the existing limited freshwater supply. Industrial waste disposal, mining chemicals and agricultural pesticides are all common pollutants of freshwater and threaten global freshwater reserves (Schwarzenbach, et al., 2010). Pollutants used in agriculture are commonly found in the freshwater supply. These pollutants affect the aquatic as well as terrestrial environment of that surrounding water source.

Various physiological disturbances due to pollution have also been observed in humans, including, diarrhoea, bronchitis, asthma, skin rashes and blurred vision as well as lowered immunity and, as a consequence, increased susceptibility to disease (Baker, et al., 1988). One of the more noteworthy observed disorders due to pollution is endocrine disruption in mammalian and non-mammalian species. This disruption results in growth abnormalities and other physiological disorders e.g. hypothyroidism (World Health Organisation, 2012). Phthalate esters, an endocrine disruptor, has been identified as causing physiological abnormalities such as cryptorchidism (Mylchreest, et al., 1999), reduced daily sperm production (Andrade, et al., 2006) and reduced testicular testosterone levels (Borch, et al., 2004). Phytoestrogens causes impaired erectile function (Pan, et al., 2008) and can induce permanent oestrous (Lewis, et al., 2002). Dicarboximide fungicide exposure in humans can result in decreased testis weight and sperm head abnormalities (Elzeinova, et al., 2009) and increase in apoptotic germ cells in the testis (Cowin, et al., 2010). Herbicides are known to result in testicular and epididymal malformations (Wolf, et al., 1999), and chlorinated pesticides known to cause hypospadias (Wolf, et al., 1999). Humans and farm livestock are at risk of ingesting polluted water or consuming fish and other food sources from the affected water source (Schwarzenbach, et al., 2010). The extents to which these pollutants are present in the environment need to be identified and monitored.

1.1 The Use of Bio-indicators for Monitoring Aquatic Systems

A bio-indicator is characterised as a biological process, species or community that can be used to assess the quality of the environment and its fluctuations over time (Holt & Miller, 2011). These fluctuations are often attributed to anthropogenic disturbances (e.g. pollution, deforestation) or natural stressors (e.g. drought, flooding and earthquakes). Bio-indicator species effectively indicate the condition of the environment due to their moderate tolerance of environmental variability. The function of a bio-indicator is to monitor the environment, ecological processes or to monitor biodiversity. For a bio-indicator to be useful in environmental bio-monitoring, tools to monitor the changes in the level of the specific biomarkers are essential.

Aquatic invertebrates have been identified and utilized as suitable bio-indicators for the monitoring of environmental pollution (Hodgkinson & Jackson, 2005). Aquatic macroinvertebrates possess many of the attributes required for bio-indicator. Taxonomic and functional diversity can capture a myriad of responses to different stressors and disturbances, including presence of fine sediment, metals, nutrients and hydrologic alterations. These organisms have been used to assess biological, ecological and biodiversity indicators (Miller, et al., 2007).

The freshwater crustacean, *Daphnia*, is known as an excellent model organism for evaluating multiple stressors within an aquatic environment. It is a widespread keystone species in most freshwater ecosystems, where it is routinely exposed to a multitude of anthropogenic (e.g. excavation) and natural stressors (e.g. earthquakes) (Althuler, et al., 2011). Its fully sequenced genome, well-documented life history and ecology, and a variety of responses to anthropogenic stressors provide a vast platform for environmental monitoring. *Daphnia* has been used as a model organism in studies in the fields of evolution, ecology, toxicology and genomics (Colbourne, et al., 2011) linking genetic expression to adverse outcomes at the individual and population level (Snell et al., 2003; Snape et al., 2004; Fedorenkova et al., 2010), responses to communities and ecosystems (Whitham et al 2006).

The use of *Daphnia* species illustrates the benefits of selecting an aquatic bio-indicator, specifically crustacean macroinvertebrates. A crustacean species common to the freshwater reserves in Africa is *Potamonautes*. Its ubiquitous nature is a key factor to its selection as a bio-indicator organism. The crustacean, found in most water bodies in Sub-Saharan Africa, can be utilized in monitoring environmental changes.

1.2 *Potamonautes perlatus* as an Aquatic Bio-indicator

The freshwater river crab, *Potamonautes perlatus* (*P. perlatus*), has been described as an ideal bio-indicator for monitoring environmental changes (Schuwerack, et al., 2001). *P. perlatus* is a decapod of the phylum *Crustacea* and belongs to one of the most commonly found aquatic infra-order and superfamily *Brachyura Potamoidea,* in Africa (De Grave, et al., 2009). Due to the vast distribution of the genus, *Potamonautes*, across most of the African continent (Cumberlidge, 1999), and especially Sub-Saharan Africa (Cumberlidge & Daniels, 2007), the freshwater crab can act as a bio-indicator to identify the level of endocrine disrupting chemicals (EDCs) within fresh water sources. *P. perlatus* has previously been used to detect heavy metal pollutant concentrations in water but the physiological changes within the crab were not monitored (Sanders, et al., 1999).

To identify estrogenic EDCs (eEDCs), a biomarker specific to eEDC effects in *P. perlatus* is necessary. In isolating a biomarker for estrogenic endocrine disruption, gender-specific proteins and hormones must be assessed to monitor potential changes. Comparative studies between male and female crabs are required to illustrate the fluctuations in proteins and hormone production. Vitellogenin (VTG), a precursor to egg yolk protein, has previously been identified as an ideal biomarker (Denslow, et al., 1999) for eEDCs and is found in crustacean species, primarily in females (Meusy & Payen, 1988). Trace quantities have been detected within male crustaceans.

1.3 Vitellogenin

Vitellogenin (VTG) is synthesized by oviparous vertebrates and invertebrates and is known as a female-specific protein (Piulach, et al., 2003); however, evidence has shown it is present in trace quantities in male species exposed to estrogen or estrogen-like compounds (Denslow, et al., 1999). VTG is the major precursor to the egg yolk protein, vitellin (VN), and is used as a nutrient source for the developing embryo (Baker, 1988). In its native form, VTG is an oligomeric phospho-lipo-glycoprotein with a size range of between 300-600 kDa (Wallace, 1985) or 200-700 kDa (Valle, 1993). The subunits span a size ranging from 160-200 kDa (Wallace, 1985) depending on the species and is immunologically and biochemically indistinguishable from its breakdown protein, vitellin (Derelle et al., 1986; Pateraki & Stratakis, 1997). In the crustacean, *Potamon potamios*, both VTG and VN were purified and characterised as a large lipo-glyco-carotenoprotein. The identified molecular weights of the native proteins were approximately 562 kDa for VTG and 501 kDa for VN, according to the pore limit electrophoresis performed (Pateraki & Stratakis, 1997). In a later study, it was

determined that the subunits of vitellin within *Potamon potamios* ranged between 85-115 kDa (Pateraki & Stratakis, 2000).

VTG mostly consists between two to three subunits, although in the Chinese mitten crab, *Eriocheir sinensis*, and the crucifix crab, *Charybdis feriatus*, two subunits were found (proteinaceous/carbohydrate/lipid base). The subunits in the Chinese mitten crab were 97 kDa and 74 kDa (Chen, et al., 2004), and in the crucifix crab 105 kDa and 76 kDa. However, in *Potamon potamios* and *Callinetus sapdius* three subunits were found (*P. potamios* – 115, 105, 85 kDa (Pateraki & Stratakis, 2000); *C. sapdius* – 119.5, 87.9, 78.5 kDa (Zmora, et al., 2007)).

The synthesis of VTG occurs in the liver of vertebrates (Byrne, et al., 1989), fat bodies of insects (Sappington & Raikhel, 1998) and in the intestines of nematodes (Nakamura, et al., 1999). In all organisms, the VTG is transported to the ovaries via the circulatory system. It was identified that the site of VTG synthesis is the hepatopancreas in various crustacean species such as *Uca pugilator* (Quackenbush & Keeley, 1988), *Penaeus vannamei* (Quackenbush, 1989), *Penaeus monodon* (Tseng, et al., 2001) and *Macrobrachium rosenbergii* (Chen, et al., 1999). Synthesis has, however, been detected in the ovaries of certain crustacean species e.g. *Penaeus semisulcatus* (Browdy, et al., 1990) and *Callinectes sapdius* (Lee & Watson, 1995). VTG has been shown to be synthesised in the hepatopancreas and the ovaries of *Echioneir sinensis* (Li, et al., 2006), *Callinectes sapdius* (Zmora, et al., 2007) and *Penaeus monodon* (Tiu, et al., 2006).

VTG is present in the hepatopancreas, which is the site of synthesis in most crustacean species. The protein is secreted into the haemolymph where it is transported to the ovaries for receptor-mediated endocytosis and, certain invertebrates; VTG is also produced in the ovaries. These three sites within *P. perlatus* are ideal areas from which to isolate VTG. The ovary would be the most ideal site of isolation due to the fact that high VTG concentrations relative to other proteins in this organ. The hepatopancreas and haemolymph contain lower levels of VTG relative to the total protein. VTG is broken into VN within the ovary, and the two proteins are immunologically indistinguishable, therefore either protein can be utilized in antibody production.

VTG is transported via the circulation to the oocytes and selectively internalised by receptor-mediated endocytosis (Opresko & Wiley, 1987). The constituents of vertebrate VTG are lipovitellin I, II and phosvitin. These products are cleaved from VTG within the oocytes after endocytosis (Wahli, 1988). Phosvitin is a domain that contains a high concentration of serine residues (Wang, et al., 2000). This is not present within invertebrates. A polyserine region

does exist though it is not homologous to phosvitin (Denslow, et al., 1999). Synthesis of VTG occurs along ribosomes associated with rough endoplasmic reticulum, where it is transferred to the Golgi apparatus and packaged into secretory granules (Giorgi, et al., 2005). In invertebrates, post-translational modifications occur through proteolytic cleavage by dibasic endoproteases and are divided into large (140-190 kDa) and small (40-60 kDa) subunits (Tufail, et al., 2005). These subunits are secreted into the haemolymph as oligomeric proteins (400-600 kDa), commonly existing as dimers. These modifications are necessary to facilitate transport of carbohydrates, lipids, sulphates and other nutrients to the ovaries (Giorgi, et al., 1999).

It has been identified that expression of the VTG gene occurs in the hepatopancreas and the ovaries of certain crustacean species, indicating that synthesis occurs in both organs. The common factor among the various studies in identifying the site of synthesis of VTG in crustaceans is that it occurs predominantly (Zmora et al., 2007; Jia et al., 2013) or solely (Girish, et al., 2014) in the hepatopancreas.

In the ovaries, VTG is utilized for the production of vitellin, the major yolk protein in all oviparous animals. Within the ovary, vitellin is broken down to provide amino acids, lipids, carbohydrates, minerals and carotenoids required for embryogenesis (Byrne et al., 1989; Khalaila et al., 2004). Site of vitellogenesis is dependent on the specific species; in the liver of vertebrates (Byrne, et al., 1989), fat bodies of insects (Sappington & Raikhel, 1998) intestines of nematodes (Nakamura, et al., 1999), and hepatopancreas of crustaceans (Girish, et al., 2014). Internalization into the ovaries occurs via receptor-mediated endocytosis, confirmed by the VTG receptors detected on oocytes (Subramoniam, 2011).

Ecdysteroids are involved in controlling reproduction cycles and vitellogenin concentration is often synchronous with these cycles (Huberman, 2000), therefore the production of vitellogenin is indirectly regulated by ecdysteroids. Ecdysteroids are sterol hormones synthesized by arthropods for the purpose of moulting. Moulting encompasses the replacement of the cuticle that surrounds an animal's body (Fingerman, 1997). Within crustaceans, ecdysteroids is synthesised by a pair of glands known as Y organs. The effects of these steroids are regulated by methyl farnesoate, which is a lipid-based hormone (Lafont & Mathieu, 2007). Vitellogenesis is hormone-regulated in the reproductive cycle of female crustaceans. This leads to uptake of VTG and production of the yolk protein, vitellin, in the ovary (Valle, 1993).

1.4 Monoclonal Antibody Production

Monoclonal antibody production remains to be one of the most popular immunological applications, with its uses found in many diagnostic as well as therapeutic fields. This application utilises hybridoma technology, of significant importance for the production of monoclonal antibodies. Monoclonal antibodies are currently being employed for several therapeutic applications, including the treatment of carcinomas, autoimmune disorders, allergy and transplantation (Costa, et al., 2010). Another application, one applicable to this study, is bio-monitoring; the development of antibodies against a biomarker used to identify changes within the environment (Kamata, et al., 2005; Marx, et al., 2001; Parks, et al., 1999).

Hybridomas technology incorporates the production of antibodies against a desired antigen. Antibody production relies on the intricate and complex immune system of mammals to generate these antibodies once the mammal has been immunized with the antigen. In this study, a BALB/c mouse will be used as the antibody generating mammal and will be immunized with purified VTG, harvested from the ovaries of female freshwater crabs. Once immunized antigen presenting cells collect the antigen and reveal the antigen to B-lymphocytes which produce antibodies against the presented antigen. It is essential that the protein used in immunization possesses antigenic properties or that it is presented to the immune system using an adjuvant. Antigenicity is the ability of an antigen to elicit an immune response. An adjuvant is a vector in which an antigen is transported and presented to the immune system in the event that the antigen cannot sufficiently elicit an immune response. The initial immunization results in secretion of Immunoglobulin M (IgM). Subsequent immunizations result in the decrease in IgM secretions and increase the secretion of Immunoglobulin G (IgG) for a more rapid response to the same antigen; these are produced by mature lymphocytes.

Splenocytes within the mouse produce these antibodies against different epitopes on the antigen. Harvesting of the spleen to immortalize and culture the splenocytes is essential in isolating antibodies against various epitopes. Fusion of the splenocytes with Sp2/0-Ag14 murine myeloma cells forms what is known as hybridomas. Hybridomas are immortalized splenocytes that continually produce antibodies. As the cells fuse at random, it is not initially possible to determine which cells produce the ideal antibodies. The initial stages of hybridoma cell culturing gives rise to polyclonal antibody producing cells. Through multiple screening and cloning processes cell are isolated and cell lines are cultured eventually resulting in monoclonal antibody producing cells. These cells produce antibodies that bind

specifically to one epitope on the antigen. Each cell line may produce antibodies that bind to a specific epitope that is different to the antibodies of other cell lines.

Isolation of hybridomas that produce an epitope-specific antibody requires confirmation through immunochemical assays. Enzyme-linked Immunosorbent Assays (ELISAs) are able to identify if an antibody binds to an antigen. Although this technique is highly sensitive and, thus, able to detect low quantities of antibodies produced, it is not specific enough to confirm antibody-antigen specificity. Gel electrophoresis is a technique developed to separate proteins and DNA molecules based on molecular size. This isolates the antigen by is molecular weight, separating it from other proteins, and possibly other undesired antigenic molecules. Combined with Western blotting, a visual confirmation can be performed identifying whether or not the antibody produced is against the correct antigen.

1.5 Significance of the study

The soaring demands for freshwater stresses the need for the preservation of this valuable resource. It is imperative that measures be put in place to ensure that the drinkable water is free of harmful chemical by-products resulting from pollutants. Currently endocrine disruptors pose a threat to our freshwater system as they readily contaminate the water supplies, with no routine monitoring system currently in place. To date, literature does not reveal toxicity levels for these disruptors in the human or animal population.

The freshwater river crab genus, *Potamonautes*, is widely distributed in various regions of the African continent. As such, it can be used as a bio-indicator to monitor endocrine disruption. The objective of this study was to develop an ELISA based assay using monoclonal antibodies to monitor *Potamonautes* VTG as biomarker for EDCs in water resources.

The specific aims of this study were to:

1. Isolate VTG from female crabs.
2. Produce and characterise monoclonal antibodies against the isolated VTG.
3. Develop sensitive immunoassays such as ELISAs for VTG using the newly produced monoclonal antibodies.
4. Employ the newly developed ELISAs in field studies to determine if male *Potamonautes perlatus* exposed to estrogen synthesise VTG.

2. METHODS

2.1 Animal collection and exposure

Male *Potamonautes perlatus* were collected in the Eerste River at a site in Jonkershoek, Stellenbosch, South Africa. Upon arrival at the laboratory, animals were placed in containers (2 males per container) and allowed to acclimatize for one week. The laboratory receives normal daylight and animals were kept at 21ºC. The water used for replacement was normal tap water that was left in an open container for 24 hours before use to allow hypochlorite residues in the water to vapourize. Animals were fed rat food pellets (1 pellet per 4 crabs every second day). Cages were cleaned the day after feeding. Crab hemolymph was collected in citrate containing vacuum tubes. The tubes were centrifuged and the supernatants were stored in aliquots at -80ºC.

2.2 Vitellogenin (VTG) Isolation and Purification

Ovaries from female *Potamonautes perlatus* crabs were collected and the appropriate volume of protease inhibitors (1:20) (Sigma Aldrich) was added to make up to a protein concentration of 100mg/ml. The ovaries were homogenised by sonication (Tissuemiser, Fisher Scientific), centrifuged at 4000rpm for 20 minutes, and stored as 50µl aliquots at -80°C. VTG was isolated from ovary homogenates by ammonium sulphate precipitation. Saturated ammonium sulphate was added to the ovary homogenate to produce a 50% saturated ammonium sulphate solution (SAS) and incubated for 1 hour on ice. Thereafter the suspension was centrifuged at 16 000rcf at 4°C for 25 minutes. The supernatant was collected and saturated ammonium sulphate was added again to produce a 60% SAS. Like before, the suspension was incubated for 1 hour and centrifuged at 16 000rcf at 4°C for 25 minutes. After centrifugation the supernatant was discarded and the pellet resuspended in PBS to be dialysed.

Dialysis was performed using a membrane with a molecular weight cut-off value of 10 kDa (Thermo Scientific). The purified VTG (pVTG) was dialysed twice for 1 hour against 1000 x volume of PBS. After dialyses the pVTG was stored in 50µl aliquots at -80°C.

2.3 Protein Determination

A protein determination was performed on the pVTG using the Bradford method. HSA (1mg/ml) was used as standard. Doubling dilutions of both HSA and samples were prepared in PBS containing protease inhibitors (1:20) as diluent. Bradford's reagent (Biorad) was added to the diluted samples, after which the plate was read at 620nm with a spectrophotometer. A standard curve was prepared using the HSA samples and the concentration of the purified pVTG was read off this curve.

2.4 SDS PAGE

The purity of the isolated VTG was checked using SDS PAGE. The reagents used for preparation of the SDS PAGE was bought as a kit (Fluka SDS Gel Preparation Kit, Sigma Aldrich) and a 7.5% separating gel and 3% stacking gel was prepared according to the kit instructions. The gel was run in a Tris-Glycine running buffer (25mM Tris, 192mM Glycine, 0.1% SDS) (Sigma Aldrich). After running the gel it was fixed using Laemmli's fixative (20% methanol, 10% acetic acid) for 15 minutes then rinsed for 15 minutes with deionised water (dH_2O). Proteins were visualised by EZBlue staining (Sigma) for 45 minutes.

2.5 Native Polyacrylamide Gel Electrophoresis (PAGE)

For the purpose of visualising lipoproteins a Native PAGE was performed. A 7.5% separating gel was prepared with separating gel buffer (1.5M Tris, pH 8.8) and 3% stacking gel was prepared with stacking gel buffer (0.5M Tris, pH 6.8). All samples and molecular weight markers (Prism Ultra Protein Ladder, Abcam) were mixed with sample buffer (0.125M Tris, 20% Glycerol, 0.004% Bromophenol Blue, pH 6.8) and a protein content equivalent to 30µg was added to each well. The gel was run in a Tris-Glycine (25mM Tris, 192mM Glycine, pH 6.8) running buffer at 250mV and 10mA for 90 minutes at room temperature. The gel was then fixed using Laemmli's fixative (20% methanol, 10% acetic acid) for 15 minutes then rinsed for 15 minutes with deionised water (dH_2O). Proteins were visualised by EZBlue staining (Sigma) for 45 minutes.

2.6 Western Blot

Immediately after SDS-PAGE proteins were electrophoretically transferred to nitrocellulose membrane (Santa Cruz Biotechnology) in transfer buffer (25mM Tris, 192mM Glycine, 10% Methanol) with a TE70X semi-dry transfer unit (Hoefer). Membranes were incubated with 2% human serum albumin (WP Blood Transfusion Service, South Africa) for 2 hours to blocking non-specific binding. Thereafter membranes were incubated with anti-VTG antiserrum (1: 2000) overnight at 4°C. The following day membranes were washed three times for 15 minutes each with wash buffer (0.1% Tween-20 in PBS). Membranes were then incubated with rabbit-anti-mouse IgG HRP-conjugate (1:5 000) (Southern Biotech), each for 1 hour. Thereafter membranes were washed four times for 15 minutes each with wash buffer. The membrane was then stained with tetramethylbenzidine (TMB) (Whitehead Scientific) peroxidise substrate to visualise positive bands.

2.7 Production of Vitellogenin Antiserum

Male BALB/c mice were purchased from the University of Cape Town Animal Unit. Animals were acclimatized in the animal holding facility for 1 week prior to being used for production of anti-purified VTG (pVTG) antibodies. The initial immunization comprised of pVTG (50 µg) in 100µl PBS, emulsified with 100µl Complete Freund's Adjuvant per mouse (Sigma Aldrich). Subsequent immunizations were administered with the same amount of antigen suspended in Incomplete Freund's Adjuvant (Sigma Aldrich). Prior to each immunization tail-blood was collected in PBS (20 µl blood mixed with 180 µl PBS), centrifuged at 12.1rcf for 5 minutes. The plasma fraction was stored in aliquots at -20°C until needed.

2.8 ELISA for anti-VTG

A 96-well microtitre plate (Nunc, Germany) was coated with 1µg/ml pVTG and incubated overnight at 4°C. Plates were blocked with 2% HSA (Western Cape Regional Blood Bank) in PBS (blocking buffer) at 200µl per well, and incubated for 1 hour at room temperature. Thereafter the plate was washed 5 times with 0.1% Tween-20 in PBS (wash buffer). Plasma samples (pre- and post-immunization with pVTG) or hybridoma culture supernatants were diluted 1 in 1000 with wash buffer containing 0.2 % HSA (antibody diluent). The diluted plasma was added at 50µl/well. The plate was incubated for 1 hour at room temperature, after which the plate was washed 5 times with wash buffer. Rabbit-anti-mouse IgG HRP-conjugate (diluted 1 in 5000 with antibody diluent) (Southern Biotech) was then added at 50µl per well. The plate was again incubated for 1hour at room temperature and washed 7 times with wash buffer. Chromogenic substrate, TMB (Whitehead Scientific), was added at 100µl per well and incubated for 15 minutes away from direct light. The enzyme reaction was stopped with 50 µl per well stop solution (0.5M Sulphuric Acid). The optical density (OD) was measured at 450nm.

2.9 Fusion

After sacrificing and exsanguination, the spleen of the BALB/c mouse was placed in cell culture wash buffer in order to prevent dehydration. The spleen was then homogenised by compressing the organ through a mesh into wash buffer. The homogenized tissue was pipetted into a 10 ml tube (SPL Life Sciences) and allowed to settle. The supernatant was aspirated into a 50 ml tube (SPL Life Sciences) and centrifuged using C-28A centrifuge (BOECO, Germany) at 1500 rpm for 10 minutes and thereafter at 3000 rpm for 10 minutes, due to ineffective initial centrifugation. The supernatant was decanted and the pellet was

resuspended in 2 ml wash buffer. A cell count was performed on the resultant splenocytes and added to Sp2/0-Ag14 cells at a ratio of 3:1.

The cell suspension was then centrifuged at 3000 rpm for 10 minutes and the supernatant was discarded. The residual wash buffer was removed with a 70% ethanol swab. Polyethylene glycol was then added to the cell pellet at a rate of 100 µl/20 seconds to a final volume of 1 ml. The cells were mixed gently during every interval. The cells were incubated for 1 minute at 37°C. Serum-free media (1% Antibiotic/Antimycotic, 1% Glutamax, 0.5% Gentamycin, RPMI-1640) was added to the cell mixture at the previous rate to a volume of 2 ml. The addition of media was repeated until final volume of 5 ml was reached and the mixture was filled to 50 ml with serum-free media. The solution was centrifuged at 1000 rpm for 10 minutes.

The supernatant was decanted and the cells were resuspended in Full Media (0.01% Mecaptoethanol, 1% Antibiotic/Antimycotic, 1% Glutamax, 0.25% Sodium Pyruvate, 0.5% Gentamycin, 10% Heat-Inactivated Hyclone Foetal Bovine Serum (FBS (Thermo Scientific)), Ex Cell Media) supplemented with 1% Hypoxanthine Aminopterin Thymidine (HAT). The fused cells (from here on referred to as hybridomas) were pipetted at 100 µl/well into five 96-well tissue culture plates (Thermo Fisher Scientific) and incubated at 37°C, 5% CO_2 for 48 hours. An additional 100 µl/well of Full Media supplemented with HAT was added after the initial incubation period. Medium was changed after 5 days to Full Media containing 1 % Hypoxanthine Thymidine (HT). Hybridoma culture supernatants were screened after 7-10 days for antibodies.

2.10 Cloning of hybridomas

Hybridomas screening positive for anti-VTG were selected for cloning. Cells were collected and centrifuged. The cell pellet was resuspended in a small volume of Full medium. After counting the cells using a hemocytometer, cells were diluted to 10 cells/ml using Full medium containing 10 % hybridoma enhancing supplement. Cells were seeded into 96 well culture dishes at 100µl per well and incubated at 37°C, 5% CO_2 for 5 days after which the medium was supplemented with full medium at 100µl per well. The cell culture supernatants were screened after 10 days for antibody production. The well with the highest positive culture was recloned as above.

2.11 Antibody optimization

The hybridomas clones secreting monoclonal antibodies against VTG were cultured in small tissue culture flasks to produce supernatant containing anti-VTG antibodies. A stock of supernatant was collected from each clone. A 96-well microtitre plate (Nunc, Germany) was

coated with 1µg/ml pVTG and incubated overnight at 4°C. Plates were blocked with 2% HSA (Western Cape Regional Blood Bank) in PBS (blocking buffer) at 200µl per well, and incubated for 1 hour at room temperature. Thereafter the plate was washed 5 times with 0.1% Tween-20 in PBS (wash buffer). A dilution curve of monoclonal antibody containing cell culture supernatant diluted in wash buffer containing 0.2 % HSA (antibody diluent) was added to wells at 50 µl/well. The plate was incubated for 1 hour at room temperature, after which the plate was washed 5 times with wash buffer. Rabbit-anti-mouse IgG HRP-conjugate (diluted 1 in 5000 with antibody diluent) (Southern Biotech) was then added at 50µl per well. The plate was again incubated for 1hour at room temperature and washed 7 times with wash buffer. Chromogenic substrate, TMB (Whitehead Scientific), was added at 100 µl per well and incubated for 15 minutes away from direct light. The enzyme reaction was stopped with 50µl per well stop solution (0.5M Sulphuric Acid). The optical density (OD) was measured at 450nm. From the antibody dilution curves optimum monoclonal antibody dilutions were obtained for setting up competition ELISAs for each clone.

2.12 Competition ELISA

Using the monoclonal antibody concentrations determined in the previous experiment, a competition ELISA was performed with the monoclonal antibodies using male haemolymph, female haemolymph and pVTG as secondary antigens. This ELISA was performed using plates coated with pVTG as mentioned previously. The secondary antigens were pipetted at a 1/2 dilution curve (25 µl per well) before the monoclonal antibody was added (25 µl per well). The remaining steps were performed according to previously completed ELISAs.

2.13 Animal exposure

Male *Potamonautes perlatus* were collected in the Eerste River at a site in Jonkershoek, Stellenbosch, South Africa. After acclimatization (**Section 2.1**), animals were separated into 3 groups of 6 each. Group 1 served as a no treatment control, Group 2 received DMSO (100 µl/l water), while Group 3 received the 100 µl of the synthetic estrogen diethylstilbestrol (DES) made up as a stock solution at 100 µg/ml in DMSO i.e. the final concentration of DES in the exposure water was 10 µg/l. Water was replaced every second day with water containing the appropriate additives for each group. After a 7 day exposure period, crab hemolymph was collected in citrate containing vacuum tubes. The tubes were centrifuged and the supernatants were stored in aliquots at -80ºC. The hemolymph samples were assayed for total protein and VTG.

3. RESULTS AND DISCUSSION

3.1 The isolation of VTG from ovaries.

Vitellogenin were isolated from the ovarian tissue of crabs and the hemolymph was analysed for purity by comparing the protein banding pattern of the purified VTG to those of male hemolymph, female hemolymph, crude ovary homogenate using SDS PAGE (Figure 1). From the gel it can be seen that the ovary homogenate contained many protein bands. The isolated VTG displayed 3 bands at 100 kDa, 85 kDa and 80 kDa. The molecular weights of these bands correspond to vitellogenin peptide (vitellin) molecular weights found for other crustacean species.

Hemolymph samples displayed two major bands in the 75 kDa region. These bands correspond to the molecular weights of hemocyanin, the major oxygen carrier in the crab circulatory system. A minor band at molecular weight 210 kDa can be seen in the female hemolymph. This band is not visible in male hemolymph and corresponds to the molecular weight of native VTG.

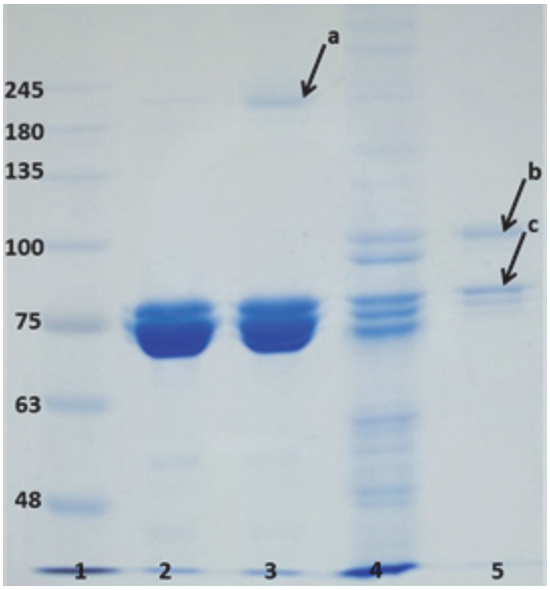

Figure 1: Potamonautes perlatus protein samples were separated using 7 % acrylamide SDS PAGE. All samples applied were at 15 ug/lane and samples were molecular weight marker (lane 1), male hemolymph (lane 2), female hemolymph (lane 3), ovary homogenate (lane 4) and purified ovary VTG (5). The molecular weights of the markers are given on the left (kDa). Arrows a, b, and c indicate bands that correspond to the molecular weight of VTG and vitellin.

3.2 Anti-VTG antibody production

Antiserum collected from mice pre- and post-immunizations were screened for antibodies against pVTG using a direct ELISA as described in methods. Pre-immunization plasma did not contain any antibodies against VTG. High antibody titres were obtained after the third immunization. The antiserum was screened for its ability to differentiate between male (low VTG) and female hemolymph (high VTG). Figure 2 shows that the ELISA absorbances at a 1/1280 dilution of antiserum were very high for female hemolymph and pVTG (OD450 > 2) while the absorbance for male hemolymph was much lower (OD450 = 0.9). The data shows that the antiserum can be used to differentiate between male and female hemolymph using ELISA.

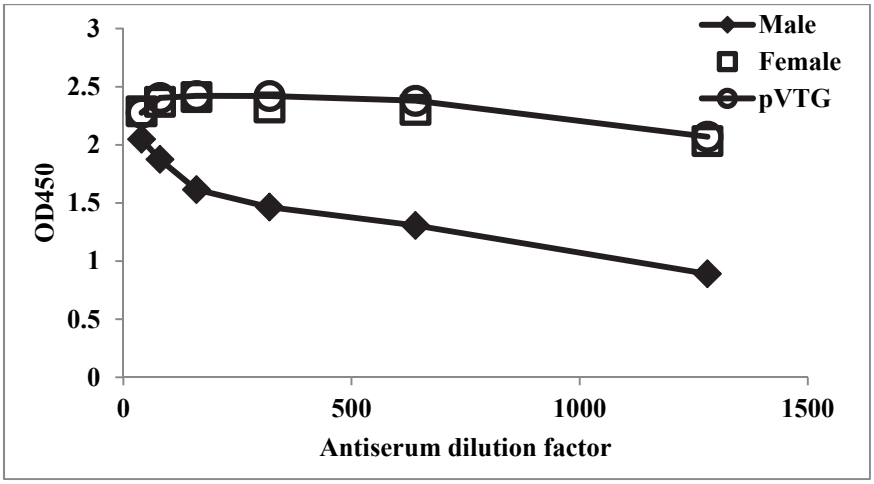

Figure 2: The detection of male and female hemolymph and pVTG by a direct ELISA using post-immunization 3 antiserum as described in the methods section.

Figure 3 shows that Western blotting analysis of hemolymph samples, using post-immunization 3 antiserum, specifically detected a minor band at 245 kDa and a major band at 210 kDa in female hemolymph. Minor bands at 170 kDa and 75 kDa were detected in both male and female hemolymph. Western blotting analysis of pVTG post-immunization 3 plasma shows that the antibodies generated detect a major band at 100 kDa and minor band at 75 kDa in purified VTG samples. The band detected at 75 kDa is probably due to cross-reactivity of the antiserum with hemocyanin. From the Western Blotting data it can be seen that the antiserum generated detects the bands previously found for VTG from other crustacean species. The results obtained from this study showed polyclonal antibodies were successfully raised against purified *Potamonautes perlatus* VTG, and that the antibody can be used in both ELISA and Western Blotting systems for VTG analysis.

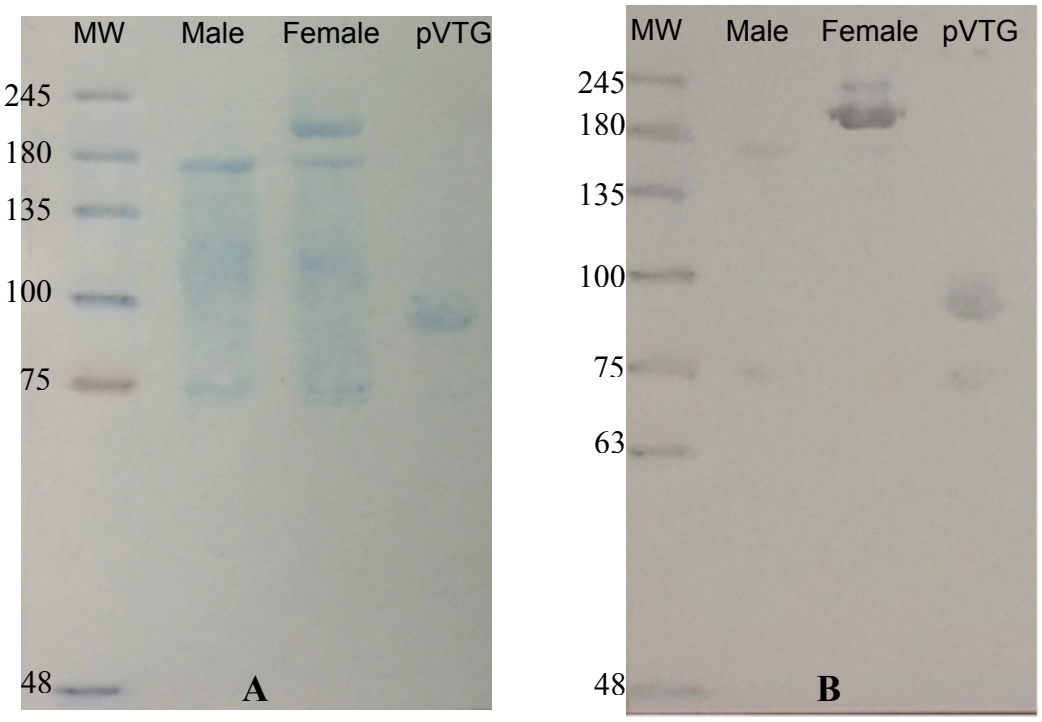

Figure 3: Potamonautes perlatus protein samples were separated using 7 % acrylamide SDS PAGE and then transferred to nitrocellulose. All samples applied were at 15 ug/lane and samples were molecular weight marker (lane 1), male hemolymph (lane 2), female hemolymph (lane 3) and purified ovary VTG (4). The molecular weights of the markers are given on the left (kDa). Protein bands on the nitrocellulose was detected with amido black stain (A) or by post-immunization serum diluted to 1 in 1000 as described in methods (B).

3.3 The production of monoclonal antibodies against VTG

After fusion cells were transferred to 5x96 well culture trays. The initial screening of the five plates resulted in the following wells being identified for further screening:

Table 1: Representation of the wells that were selected from each plate

Plate 1	E1	A7	E11	Plate 2	A3	H10	Plate 3	E1	A11
	G2	C8			H4			F3	H11
	C3	E8			G6			H5	A12
	H4	A9			B7			H8	B12
	E6	A11			E7			H10	D12
Plate 4	G1	E5	H11	Plate 5	G1	G6			
	E2	G5	E12		H1	C7			
	F2	C6			D3	E7			
	G2	D6			F3				
	F3	B10			E4				

The second screening performed, which included a competition ELISA, was to confirm the validity of the first screening and to isolate the most specific wells with the highest affinity. The seven wells with the highest affinity were selected.

Table 2: Selection of cells possessing highest affinity towards purified VTG

ORIGINAL WELL	Next Selection
PLATE 1 – E1	A1
PLATE 1 – G2	B1
PLATE 2 – H4	A3
PLATE 3 – E1	F3
PLATE 3 – H5	B4
PLATE 4 – G2	A6
PLATE 5 – E7	E8

The cells from each of these positive colonies were suspended in medium and plated into a 96 well tissue culture plate at one cell per well. A rescreening was performed on the new plates

to identify which wells were producing antibodies against pVTG. The well with the highest affinity from each plate was selected for cloning.

The Male vs. Female Indirect ELISA identified which samples could distinguish between male and female haemolymph.

Table 3: Next selection of cells possessing highest affinity including selection for male-female distinction

ORIGINAL WELL	Next Selection
PLATE 1 – E1 A1	B1
PLATE 2 – H4 A3	B4, B6, E4, E6
PLATE 3 – E1 F3	D9
PLATE 3 – H5 B4	E6, F4
PLATE 5 – E7 E8	B2

Various cell lines did not possess the adequate affinity for further screening and, therefore, were excluded from further culturing. The final screening yielded the following cell lines:

Table 4: Cell line selection of final screening

ORIGINAL WELL	Next Selection
PLATE 2 – H4 A3 B4	F7
PLATE 2 – H4 A3 B6	H3
PLATE 2 – H4 A3 E4	H11
PLATE 2 – H4 A3 E6	A6
PLATE 3 – E1 F3 D9	H3
PLATE 3 – H5 B4 F4	H5
PLATE 5 – E7 E8 B2	C8

The seven cell lines from Table 4 were isolated for culturing in a 15ml Tissue Culture flask. The supernatant from each cell line was collected and stored until a sufficient volume was obtained for further analysis.

Table 5: Selected cell lines and their respective codes.

CELL LINE	Code
2H4 A3 B4 F7	A
2H4 A3 B6 H3	B
2H4 A3 E4 H11	C
3E1 F3 D9 H3	D
3H5 B4 F4 H5	E
5E7 E8 B2 C8	F
2H4 A3 E6 A6	G

The positive clones isolated were then screened for ability to detect VTG using Western Blotting. The data obtained (Figures 4-6) showed that the three 2H4A3 clones, the 3H5B4F4H5 clone and the 5E7E8B2C8 detected both purified VTG and VTG from female hemolymph.

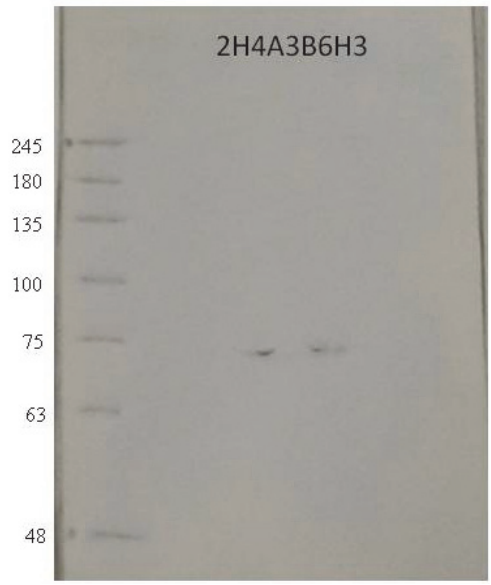

Figure 4: Western blot performed using the supernatant from the cell line 2H4AB6H3 confirming the production of antibodies against the protein VTG.

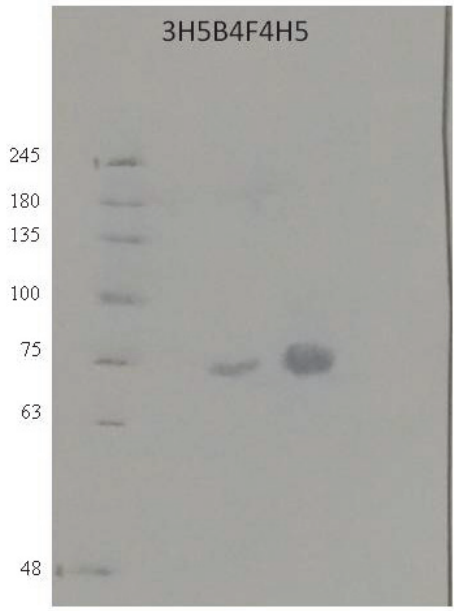

Figure 5: Western blot performed using the supernatant from the cell line 3H5BF4H5 confirming the production of antibodies against the protein VTG.

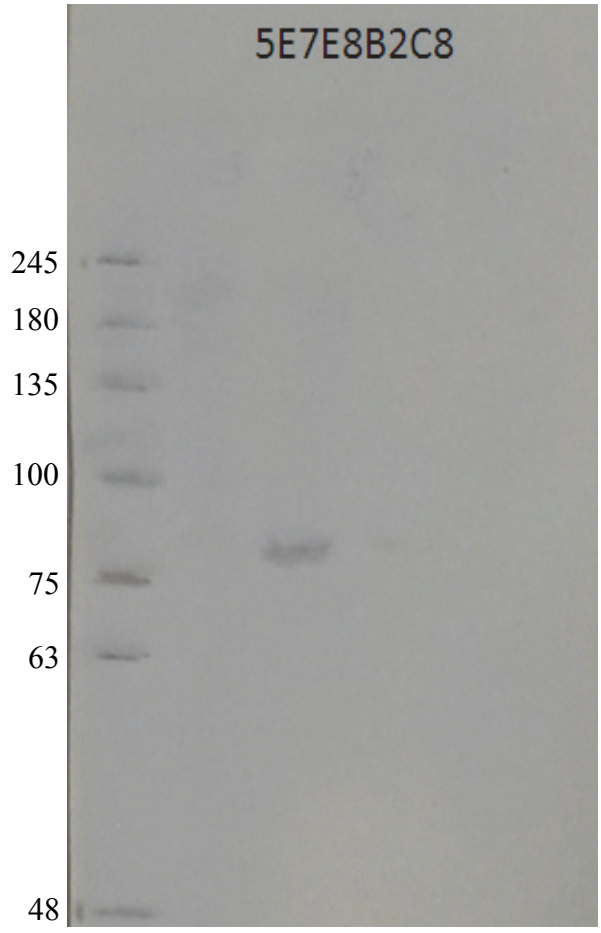

Figure 6: Western blot performed using the supernatant from the cell line 5E7E8B2C8 confirming the production of antibodies against the protein VTG.

From the data presented above it can that several clones (4 isolated from the original 2H4 well, 3E1F3D9H3, 5E7E8B2C8, and 3H5B4F4H5) secreted antibodies that can be used to detect *P. Perlatus* VTG. The 2H4 clones, and 5E7E8B2C8 and 3H5B4F4H5 can also be used for VTG detection using Western Blots.

3.4 Setting up of a competitive ELISA for VTG using the newly produced monoclonal antibodies.

3.4.1 Optimization of Monoclonal Antibodies

An Indirect ELISA was performed with the antibody of each cell line to determine the optimal concentration (Figure 7 & 8). This ELISA was performed with every stock of antibody collected. The results are indicated in Table 6 for each clone screened. Clones "A", "B" and C required the lowest concentration for screening whereas Cell line "F" and "G" required the highest concentration. These findings were used for subsequent tests. Clones "A", "B" and C originated from the same parent hybrid and have very similar dilution curves, so only one of these were further characterized.

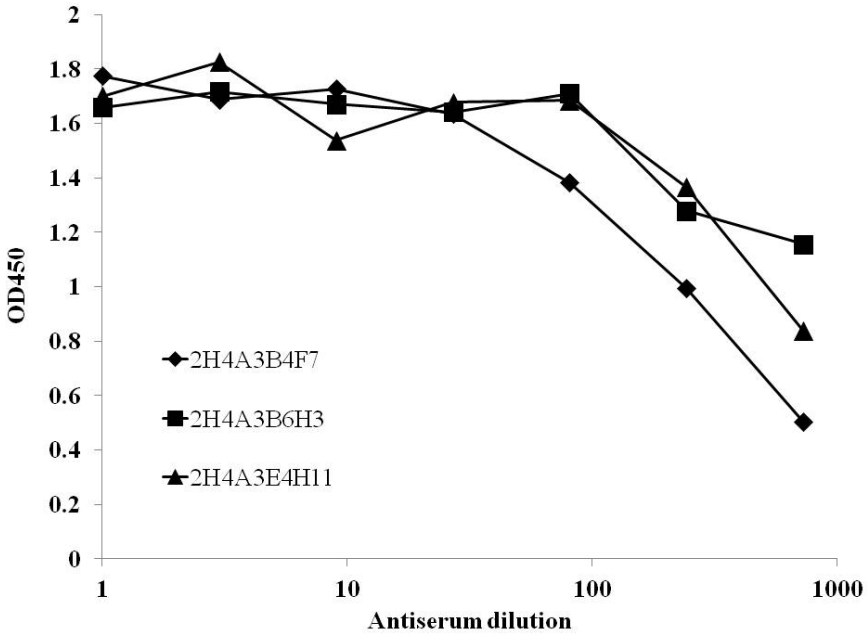

Figure 7: Antibody 3-fold dilution curve of clones 2H4A3B4F7 (A), 2H4A3B6H3 (B) and 2H4A3E4H11 (C) using an indirect ELISA as described in the methods. Plates were coated with 10 µg/ml pVTG in PBS at 50 µl/well.

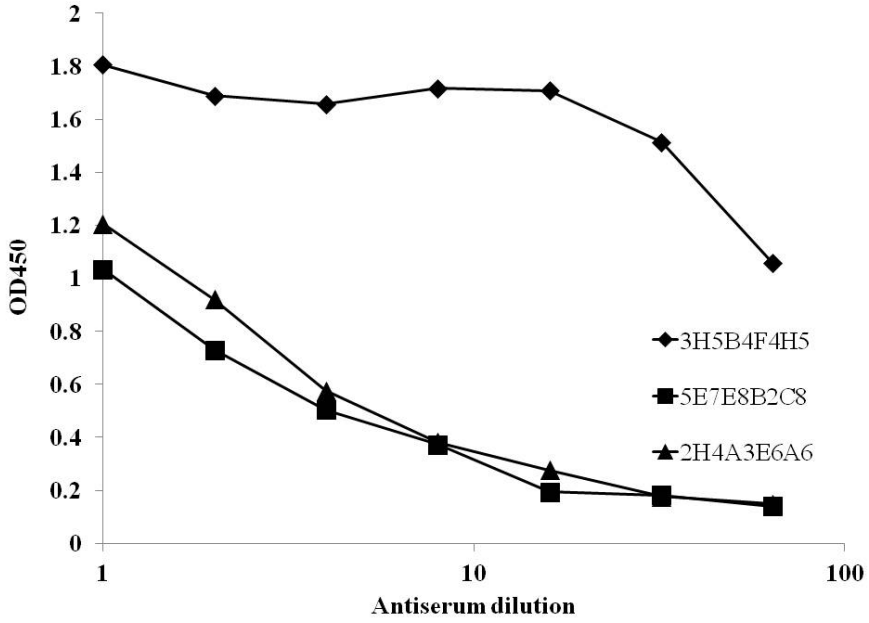

Figure 8: Antibody 2-fold dilution curve of cell lines 3H5B4F4H5 (E), 5E7E8B2C8 (F) and 2H4A3E6A6 (G) using an indirect ELISA as described in the methods. Plates were coated with 10 µg/ml of pVTG diluted with PBS at 50 µl/well.

Table 6: Concentration of Antibody to be used from each Cell Line

Cell Line	Clone origin	Optimum Concentration
A	2H4A3B4F7	1/250
B	2H4A3B6H3	1/250
C	2H4A3E4H11	1/250
E	3H5B4F4H5	1/80
F	5E7E8B2C8	Neat
G	2H4A3E6A6	Neat

3.4.2 Evaluation of Sensitivity and Specificity of Cell Lines

A Competition ELISA with a "free-antigen" dilution curve was performed to identify the sensitivity of the antibodies generated (Figure 9). Male haemolymph, female haemolymph and pVTG were used at the specified concentrations in the ELISA.

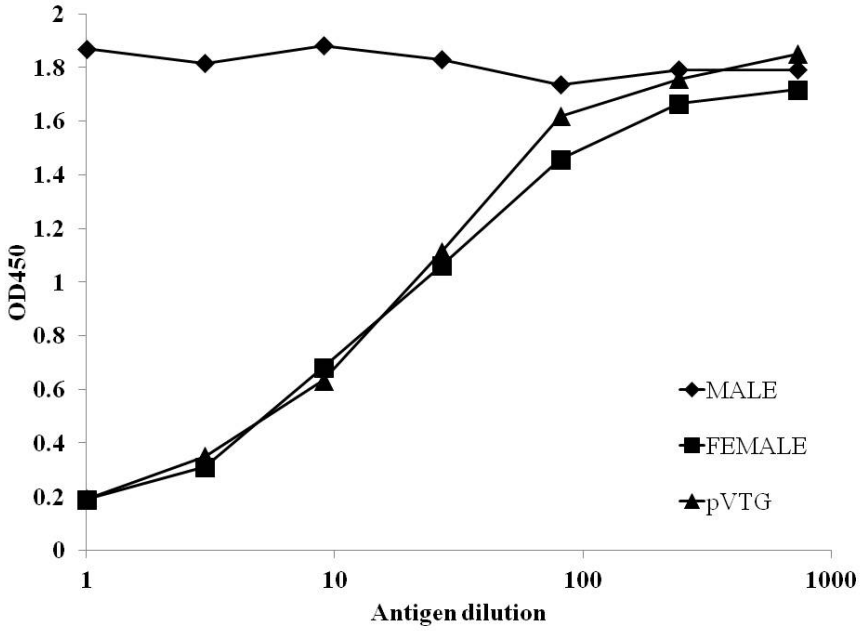

Figure 9: Antigen 3-fold dilution curve with antibody from cell line 2H4A3B6H3 using a competition ELISA as prescribed in the methods. Plates were coated with 10 µg/ml of pVTG diluted with PBS at 50 µl/well. The secondary antigen concentrations were set as follows: male haemolymph and female haemolymph added at 100 µg/ml diluted in PBS, pVTG added at 20 µg/ml diluted in PBS. Secondary antigens added at 50 µl/well.

The ELISA developed using cell line "B" (2H4A3B6H3) shows that the male haemolymph did not compete with the coated pVTG for the antibody, indicating that this sample does not contain any VTG. However, female haemolymph and pVTG competed effectively with the coated pVTG for antibody, resulting in ELISA absorbance readings. The lowering in ELISA absorbance was concentration dependent and the curves obtained for female haemolymph and pVTG shows good parallelism indicating that pVTG can be used in future experiments to obtain standard curves for determining VTG concentration in haemolymph.

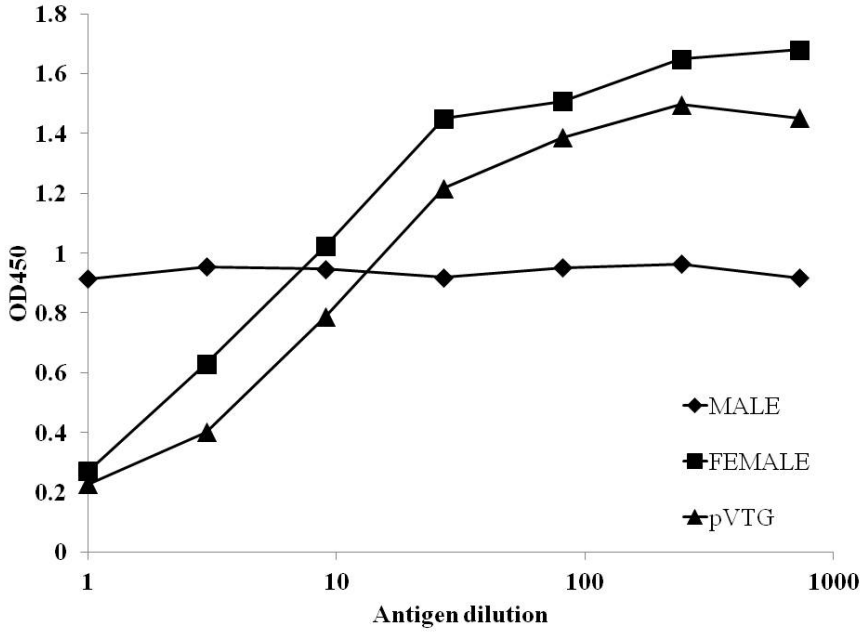

Figure 10: Antigen 3-fold dilution curve with antibody from cell line 3H5B4F4H5 using a competition ELISA as prescribed in the methods. Plates were coated with 10 µg/ml of pVTG diluted with PBS at 50 µl/well. The secondary antigen concentrations were set as follows: male haemolymph and female haemolymph added at 100 µg/ml diluted in PBS, pVTG added at 20 µg/ml diluted in PBS. Secondary antigens added at 50 µl/well.

Clone "E" (3H5B4F4H5) displayed a similar correlation in detecting pVTG and VTG in female haemolymph when compared to the previous cell line (2H4A3B6H3). The male haemolymph displayed a flat line, however at a slightly lower optical density than at the lowest pVTG concentration. This experiment was repeated several times, and repeated data was obtained. The reason for the lower absorbarbance of the male haemolymph is not known. Neither of clones "F" and "G" gave inhibition curves in the competition ELISA. This is probably due to the fact that these antibodies do not have a high enough affinity for the antigen. The results of this study shows that the monoclonal antibodies produced by clone 2H4A3B6H3 can be used in a quantitative competition ELISA for determining VTG concentrations in haemolymph. The data also shows that pVTG can be used as antigen to prepare standard curves for this ELISA.

3.5 The effects of laboratory exposure of crabs to DES on hemolymph total protein and VTG concentration.

The data presented in Table 7 shows that protein level is significantly increased by DES compared to the control. The vehicle used for DES administration to the water, namely DMSO, had no significant effect on protein levels in haemolymph compared to the control.

This data suggests that DES either upregulates protein synthesis or result in transfer of protein from other body compartments to the haemolymph.

Table 7: The effect of DES exposure of crabs on protein concentration of haemolymph. Protein concentration was determined using the Biorad method as described in methods. The * indicates p<0.01.

Group	Exposure	n	Mean	SEM
1	Control	6	738.952	169.303
2	DMSO	5	960.973	188.868
3	DES*	5	1773.239	211.041

The hemolymph samples were also analysed for VTG content using the competitive ELISA. Data showed that DES exposure did not have a significant effect on the VTG levels. Due to this a seasonal study was done to check if VTG levels have a strict seasonal pattern (Figure 11) indicating that assays should be done at a specific time. The data obtained showed that male hemolymph had very VTG throughout the year. The VTG concentration in female hemolymph samples from any single time point was very variable and the highest VTG concentration was seen in March 2015. However, there were no significant differences in the level of VTG at the different times of the year for the 2014-2015 periods.

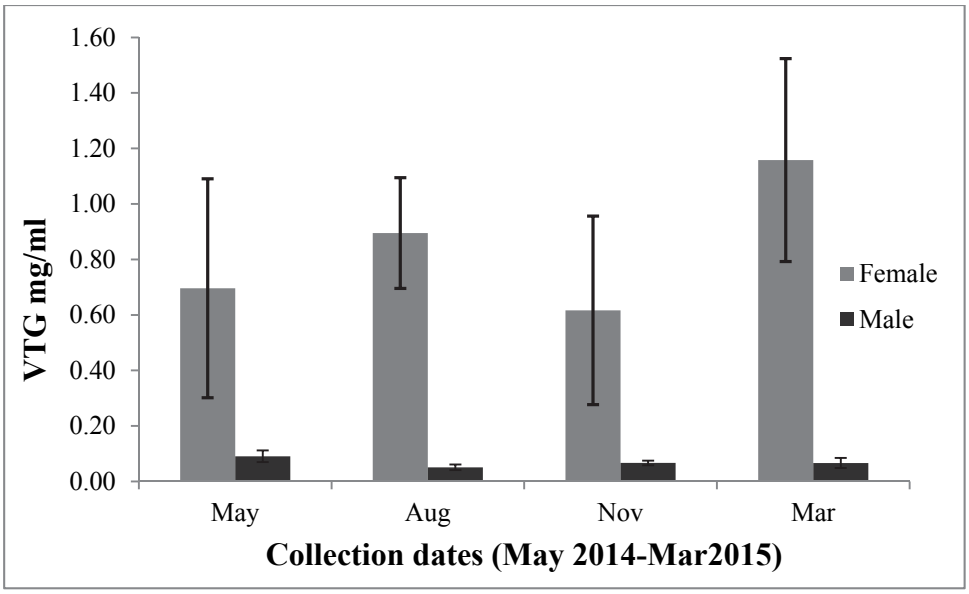

Figure 11: Hemolymph was collected from crabs collected on various dates during the 2014-2015 periods. The hemolymph samples were analysed for VTG using the competitive ELISA. Data recorded are the means and standard error of the mean for 6 animals per group.

4. CONCLUSIONS

All the original aims of the project were met. VTG from *Potamonautes perlatus* was successfully purified from ovarian tissue, the isolated VTG was then used to prepare both polyclonal and monoclonal antibodies specific for VTG. The antibodies produced were then used and implemented for various experiment to determine the VTG concentrations of samples. Studies using the antibodies showed that some of the monoclonal antibodies generated can be used in a competitive ELISA for *Potamonautes perlatus* VTG. The ELISA data showed that male hemolymph contains low VTG levels, while female hemolymph contains high VTG levels. Data also showed that the levels of male VTG are not modulated by the estrogenic substance DES. Seasonal VTG levels show that there were major differences in the level of VTG in female hemolymph at all the times throughout the year. This data also showed that there were no significant differences between the levels of VTG at any point throughout the year.

Future studies will use the newly developed *Potamonautes perlatus* VTG competitive ELISA on cultures of ovary and hepatopancreas to see if various chemical substances affect VTG synthesis in crabs.

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