POTAMONAUTES SPP. AS A BIOINDICATOR FOR OESTROGENIC ENDOCRINE DISRUPTING CHEMICALS

by

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Supervisor: Professor EJ Pool

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"*Alle Dinge sind Gift und nichts ist ohne Gift,*

allein die Dosis macht es, dass ein Ding kein Gift ist."

All things are poison and nothing is without poison,

only the dosage makes a thing not poison.

– Paracelsus (1493-1541)

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DECLARATION

I, **Dewald Schoeman**, declare that the thesis entitled '***Potamonautes* spp. as a bioindicator for oestrogenic endocrine disrupting chemicals**' is my work and has not been submitted for any degree or examination at any other university and that all sources of my information have been quoted as indicated in the text and/or list of reference.

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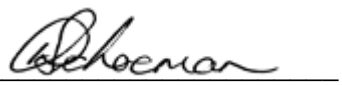

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I dedicate my thesis:

In memoriam

Greg Palm

(1952 – 2017)

Husband, Father, Mentor, Friend, Brother

"See you, my boy!"

I will see you again, uncle Greg (John 11:24, 25)

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LIST OF ABBREVIATIONS

%	percent
±	plus-minus
°C	degrees Celsius
α	alpha
β	beta
β-galactosidase	beta-galactosidase
µg	microgram(s)
µg/kg	micrograms per kilogram
µg/l	micrograms per litre
µg/ml	micrograms per millilitre
µg/m^3	micrograms per cubic metre
µl	microlitre(s)
µm	micrometre(s)
AG	androgenic gland
AGH	androgenic gland hormone
AHR	aryl hydrocarbon receptor
ALP	alkali-labile phosphate
ANOVA	analysis of variance
BALB/c	Bagg albino mouse, genotype c/c
BCF	bioconcentration factor
BPA	bisphenol A
Cd	cadmium
CFA	complete Freund's adjuvant
Con-A	concanavalin A
DEHP	di-(2-ethylhexyl) phthalate

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DES	diethylstilboestrol
dH_2O	deionised water
DMSO	dimethyl sulfoxide
dPBS	Dulbecco's phosphate buffered saline
E_1	oestrone
EAE	experimental autoimmune encephalomyelitis
EDCs	endocrine disrupting chemicals
ELA	experimental lakes area
ELISA	enzyme-linked immunosorbent assay
ER(s)	oestrogen receptor(s)
EREs	oestrogen responsive elements
E-SCREEN	oestrogen proliferative screening assay
FH	female haemolymph
FHP	female hepatopancreas
FICZ	6-formylindolo[3,2-b]carbazole
FOVA	female ovary
FSH	female-specific haemocyanin
GC-MS	gas chromatography coupled mass spectrometry
GIH	gonad-inhibiting hormone
GSH	gonad-stimulating hormone
GSI	gonadosomatic index
HAT	hypoxanthine-aminopterin-thymidine
hER	human oestrogen receptor
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
HI-FBS	heat-inactivated foetal bovine serum

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HL	haemolymph
HP	hepatopancreas
HPG	hypothalamic-pituitary-gonadal axis
HPSI	hepatosomatic index
HRP	horseradish peroxidase
HSA	human serum albumin
Hsp70	heat shock protein 70 kDa
HT	hypoxanthine-thymidine
IFA	incomplete Freund's adjuvant
IgG	immunoglobulin G
IU/ml	international units per millilitre
kDa	kilodalton
km	kilometre(s)
lacZ	gene coding for β-galactosidase
LC-MS	liquid chromatography coupled mass spectrometry
LPV 1, 2	lipovitellin 1 and 2
m^2	square metre
M	molar
mA	milliampere
mAb(s)	monoclonal antibody/antibodies
MCF-7	breast cancer cell line (Michigan Cancer Foundation)
MF	methyl farnesoate
mg	milligram(s)
mg/ml	milligram(s) per millilitre
MH	male haemolymph

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min(s)	minute(s)
ml	millilitre(s)
mM	millimolar
mRNA	messenger ribonucleic acid
MW	molecular weight marker
MWCO	molecular weight cut-off value
ng/l	nanograms per litre
ng/m^3	nanograms per cubic metre
ng/ml	nanograms per millilitre
NaOH	sodium hydroxide
nm	nanometre(s)
NP	nonylphenol
OCs	organochlorine pollutants
OD	optical density/densities
OECD	organisation for economic co-operation and development
OVA	unpurified ovarian homogenate
P.	*Potamonautes*
P. perlatus	*Potamonautes perlatus*
P. sidneyi	*Potamonautes sidneyi*
P. warreni	*Potamonautes warreni*
pAb(s)	polyclonal antibody/antibodies
PAGE	polyacrylamide gel electrophoresis
Pb	lead
PBDE(s)	polybrominated diphenyl ether(s)
PBS	phosphate buffered saline

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PC	phosphatidylcholine
PCB(s)	polychlorinated biphenyl(s)
PCP	pentachlorophenol
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PF(s)	purification factor(s)
PGM(s)	platinum group metals
PMSF	phenylmethylsulfonyl fluoride
pre-VTG	precursor form of vitellogenin
PV	phosvitin
pVn	purified vitellin
rcf	relative centrifugal force
RME	receptor-mediated endocytosis
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription polymerase chain reaction
SAS	saturated ammonium sulphate solution
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
secs	seconds
SEM	standard error of the mean
spp.	species
SOD	superoxide dismutase
T_4	thyroxine/3,5,3',5'-tetraiodothyronine
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin

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Th_{17}	interleukin-17-producing inflammatory T-cells/lymphocytes
TMB	tetramethylbenzidine
T_{regs}	regulatory T-cells/lymphocytes
TSH	thyroid stimulating hormone
TTR	transthyretin
V	volt
v/v	volume to volume ratio
VIH	vitellogenesis inhibiting hormone
Vn	vitellin
vs	versus
VSH	vitellogenesis stimulating hormone
VTG	vitellogenin
Vtg2	gene coding for vitellogenin-protein 2
w/v	weight to volume ratio
WWTP(s)	wastewater treatment plant(s)
XO-SG	X-organ sinus gland complex
YES	yeast oestrogen screening assay
Zn	zinc

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SUMMARY OF THESIS

Environmental pollutants, such as endocrine disrupting chemicals (EDCs), are a health concern as they can adversely affect animal health by interfering with the normal function of hormones. Oestrogenic EDCs can cause adverse developmental and reproductive effects by mimicking or inhibiting endogenous oestrogens. However, these effects are difficult to detect as they often only manifest long after the initial exposure. Vitellogenin (VTG) is the precursor to the major yolk protein vitellin (Vn) and is produced by egg laying females in response to oestrogens. The VTG gene is also present in males, but silent. Thus, the presence of VTG and Vn in animals, as well as the synthesis thereof in response to oestrogens, can serve a dual purpose in biomonitoring experiments. These proteins can be monitored over a period of time to establish the reproductive cycle of an organism and can also serve as a biomarker for oestrogenic pollutants.

Potamonautes (*P*) *perlatus* is a crustacean native to South Africa (SA) and belongs to one of the most widely distributed genera of freshwater crabs in Africa. In SA, data on endocrine disruption in aquatic invertebrates, particularly crustaceans, is sparse. To that end, this study aimed to accomplish three objectives: 1) to generate data for South African aquatic invertebrates through the production of monoclonal antibodies (mAbs) against Vn from *P. perlatus* females; 2) to use the mAbs to establish a western blot and ELISA for the quantification of VTG and Vn; 3) to determine the reproductive cycle of *P. perlatus* females by measuring the seasonal synthesis of VTG.

In this study, Vn was purified from the ovaries of *P. perlatus* females by ammonium sulphate precipitation. The purified product was characterised by SDS-PAGE, native PAGE, Oil Red O lipid stain, and concanavalin-A (Con-A) lectin blot. Analysis of SDS-PAGE revealed that compared to the unpurified ovarian homogenate, three major subunits each of approximately 75 kDa, 78 kDa, and 98 kDa remained after purification. Native PAGE analysis showed that the purified product had a native molecular weight of approximately 266 kDa, similar to that of the female-specific protein, VTG, found in the haemolymph of *P. perlatus* females. Furthermore, Oil Red O lipid staining and Con-A lectin blotting revealed that the purified product was also lipidated and glycosylated. These results confirmed the successful purification of Vn. The purified Vn (pVn) was subsequently used for the production of mAbs against Vn.

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Immunisation of BALB/c mice with pVn was effective as evident by the consecutive increase in anti-pVn polyclonal antibodies. From fusion, hybridoma colonies were plated into a total of 1 422 wells and yielded a total of 97 positive hybridoma clones through four repetitions of screening and cloning. From these, clone 2H4A3B6H3 was selected based on its high affinity and specificity for pVn and used for further characterisation. The mAb produced by clone 2H4A3B6H3 could detect pVn in a range of 150-5 000 ng/ml. In a competition ELISA between plate-bound pVn and soluble VTG, the anti-pVn mAb could detect VTG in the female haemolymph of *P. perlatus*, whereas no VTG was detected in the male haemolymph. Western blotting confirmed the specificity of the anti-pVn mAb for a single band in the haemolymph as well as the ovaries of a *P. perlatus* female. However, no bands were detected in the male haemolymph used in the western blot. Furthermore, Oil Red O lipid stain and Con-A lectin blotting confirmed that the bands detected in the western blot corresponded to VTG and Vn, respectively. Thus, the mAb from clone 2H4A3B6H3 was able to detect both VTG in the female haemolymph and Vn in the ovaries. This anti-pVn mAb was subsequently used to determine the reproductive cycle of *P. perlatus* females.

To determine the reproductive cycle of *P. perlatus* females, 24 female crabs were sampled from the Jonkershoek river in Stellenbosch. Qualitative measurements of the organs involved in the reproductive process of *P. perlatus* females were recorded. The anti-pVn mAb was used to quantify the biochemical marker VTG by western blotting and used in conjunction with the qualitative measurements to determine the reproductive cycle of *P. perlatus* females. The VTG content in the HP exhibited marked fluctuations, suggesting the involvement of the HP in vitellogenesis as is found in other crustaceans belonging to the suborder of Pleocyemata. During spring, there was a marked decrease in the VTG and Vn content of the haemolymph and ovaries, respectively, indicative of oviposition. The HP exhibited a concomitant increase in VTG content, corroborating the culmination of the female reproductive cycle in spring and demonstrates the synthesis of VTG in preparation for the commencement of the next cycle. This data also suggests that *P. perlatus* females oviposit only once a year in a warmer period and after the rainy season. Furthermore, with the exception of summer, reproductively active females were found throughout the year, suggesting that *P. perlatus* females in this region exhibit a continuous reproductive cycle.

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This study has produced and characterised mAbs against Vn that was purified from the ovaries of *P. perlatus* females. These anti-pVn mAbs can potentially be used in a rapid detection assay for the quantification of Vn as a biomarker for oestrogenic EDCs. The reproductive activity of *P. perlatus* females demonstrated that VTG and Vn are detectable throughout most of the year. Therefore, *P. perlatus* females can potentially be employed as bioindicators for studies aimed at investigating oestrogenic environmental pollutants with the potential to inhibit vitellogenesis. Together, these two components can be used to detect oestrogenic pollutants that interfere with vitellogenesis in *P. perlatus* females.


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CHAPTER 1: PROBLEM STATEMENT AND AIM OF THE STUDY

Endocrine disrupting chemicals (EDCs) are a group of environmental pollutants that are introduced to the environment through agricultural, industrial, and other anthropogenic activities (Aspelin, 2003; Briggs, 2003; Dong *et al.*, 2012). These chemicals are a concern as they can persist and accumulate in the environment (Markman *et al.*, 2007; Chen *et al.*, 2011). They can also adversely affect the endocrine system of animals and humans (Bergman *et al.*, 2013). The endocrine system is responsible for the coordination of essential biological processes such as the development, growth, and reproduction of organisms. Thus, by interfering with these processes, EDCs can cause severe adverse effects. The most well-known case of EDCs in humans is that of diethylstilboestrol (DES) – a potent synthetic oestrogen used by mothers between the 1940s and 1970s to prevent miscarriages and preterm births. It was only discovered after 1970 that exposure to DES during pregnancy caused severe reproductive abnormalities in both DES-exposed sons and daughters (Herbst *et al.*, 1971; Henderson *et al.*, 1976; Rubin, 2007; Palmer *et al.*, 2009; Hoover *et al.*, 2011; Virtanen & Adamsson, 2012). Reports have also implicated EDCs in the disruption of the endocrine system of wildlife. Fish found in rivers contaminated with oestrogenic chemicals have been reported to become "feminised" and developed "ovotestes" – a gonadal abnormality whereby ovarian tissue is found within the testicular tissue (Jobling *et al.*, 2005; Tyler & Jobling, 2008). A study spanning over 7 years attributed the collapse of the fathead minnow, *Pimephales promelas*, population to the chronic exposure to the synthetic oestrogen 17α-ethynyloestradiol (Kidd *et al.*, 2007).

These studies demonstrate that the consequences of EDCs only manifest some time after exposure and are, therefore, difficult to detect early on and link to EDCs. To this end, the organisation for economic co-operation and development (OECD) has proposed a list of standardised test methods that can be employed to evaluate the potential of chemicals for endocrine disruption (Hass *et al.*, 2005). *In vitro* assays serve as preliminary standardised tests with the aim of providing data on the selected endocrine-mediated mode(s) of action used by EDCs. Although these assays provide a reasonable foundation for the endocrine disrupting potential of chemicals, they do not accurately demonstrate the ability of EDCs to cause adverse effects in an intact organism. Endocrine disrupting chemicals in exposed organisms generate metabolites that, in some cases, only become active after metabolism, or are sometimes more active than their parent compounds (Jobling & Sumpter, 1993; Cheek *et al.*, 1999; Hamers *et*

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al., 2008). This underpins the need for the succeeding step in the OECD conceptual framework which requires the use of *in vivo* animal models.

Organisms that reflect the state of their habitat or environment are known as biological indicators, or bioindicators (McGeoch, 1998). Ideally, they are ubiquitous, easy to handle, have short generation times, display a reasonable sensitivity toward environmental stressors, such as pollutants, and play an important role in the food chain (Zhou *et al.*, 2008). Benthic macroinvertebrates are a diverse class of aquatic invertebrates that inhabit the lowest level of water bodies and fulfil many of these criteria. Additionally, these organisms exhibit mostly sedentary habits and seldom move away in response to pollution (Voshell Jr *et al.*, 1997). For these reasons, benthic macroinvertebrates have been employed for qualitative interpretations in environmental assessments. A number of studies have employed crustaceans, particularly crabs, for such assessments and used them as bioindicators for the accumulation of EDCs (Voorspoels *et al.*, 2004; Micheletti *et al.*, 2007). However, these international studies were able to do so as there was existing data on the crabs that were used as models. Data on South African invertebrates is sparse and even more so with regards to their use as models for endocrine disruption.

Potamonautes perlatus is a freshwater crab indigenous to South Africa and belongs to the most widely distributed genus of freshwater crabs in Africa (Cumberlidge, 1999). It is found predominantly in the Western Cape but also appears North and East of Clanwilliam and has been reported to occur at relatively high densities along the Buffalo River (Hill & O'Keeffe, 1992; Daniels, 2003). Some studies have employed *Potamonautes* species (spp.) as bioindicators but have only focussed on their ability to accumulate heavy metals (Sanders *et al.*, 1999; Snyman *et al.*, 2002; Reinecke *et al.*, 2003; van Stormbroek, 2007; Somerset *et al.*, 2015). No research has been done on the use of *Potamonautes* spp. as bioindicators for EDCs. However, the presence or absence of bioindicators used solely as a measure of environmental pollution is not sufficient. Such qualitative assessments can be supported by using quantitative measurements such as biomarkers. Biomarkers are measurable changes that typically occur at the biochemical or cellular level in organisms in response to stressors (Hamza-Chaffai, 2014).

Vitellogenin (VTG), the precursor to the major yolk protein vitellin (Vn), is produced in response to endogenous oestrogens and can be found in all oviparous organisms (Matozzo *et al.*, 2008; Jia *et al.*, 2013). In crustaceans, the hepatopancreas (HP) is generally considered to be the primary site of VTG synthesis (Subramoniam, 2011). From the HP, VTG is secreted into the haemolymph and whereby it is transported to the ovaries. Vitellogenin is then taken

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up by the ovaries and broken down into various proteins, of which Vn is a major component. Vitellin then serves as a source of nutrients for developing embryo (García *et al.*, 2008). Vitellogenin and Vn are immunologically indistinguishable proteins that, in crustaceans, are lipidated, glycosylated, and conjugated to carotenoids (Okuno *et al.*, 2002). The function of VTG and Vn, as well as the synthesis thereof in response to oestrogens, can serve a dual purpose. These proteins can be monitored over a period of time to establish the reproductive cycle of an organism and can also serve as a biomarker for oestrogenic pollutants. The aim of the current study is to investigate *P. perlatus* VTG as a potential biomarker. To that end, this study aims to accomplish three objectives: 1) produce monoclonal antibodies (mAbs) against Vn from *P. perlatus* females; 2) use the mAbs to establish a western blot and ELISA for the quantification of VTG and Vn; 3) determine the reproductive cycle of *P. perlatus* females by measuring the seasonal synthesis of VTG.

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1.1 REFERENCES

1. Aspelin, A. L. (2003). Pesticide usage in the United States: Trends during the 20th century. *CIPM Technical Bulletin,* 105, 1-213.

2. Bergman, Å., Heindel, J. J., Jobling, S., Kidd, K. A. & Zoeller, R. T. (2013). *State of the science of endocrine disrupting chemicals 2012: An assessment of the state of the science of endocrine disruptors prepared by a group of experts for the United Nations Environment Programme and World Health Organization,* Geneva, Switzerland: World Health Organization, 1-260.

3. Briggs, D. (2003). Environmental pollution and the global burden of disease. *British Medical Bulletin,* 68, 1-24.

4. Cheek, A. O., Kow, K., Chen, J. & McLachlan, J. A. (1999). Potential mechanisms of thyroid disruption in humans: Interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. *Environmental Health Perspectives,* 107, 273–278.

5. Chen, F., Ying, G. G., Kong, L. X., Wang, L., Zhao, J. L., Zhou, L. J. & Zhang, L. J. (2011). Distribution and accumulation of endocrine-disrupting chemicals and pharmaceuticals in wastewater irrigated soils in Hebei, China. *Environmental Pollution,* 159, 1490-1498.

6. Cumberlidge, N. (1999). *The freshwater crabs of West Africa: Family Potamonautidae,* Paris, France: IRD Editions, 77-79, 128 & 334.

7. Daniels, S. R. (2003). Examining the genetic structure among populations of the common Cape river crab *Potamonautes perlatus* from river systems in South Africa reveals hydrographic boundaries. *Journal of Crustacean Biology,* 23, 936-950.

8. Dong, C. D., Chen, C. F. & Chen, C. W. (2012). Determination of polycyclic aromatic hydrocarbons in industrial harbor sediments by GC-MS. *International Journal of Environmental Research and Public Health,* 9, 2175-2188.

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http://etd.uwc.ac.za/

9. García, F., Cunningham, M., Garda, H. & Heras, H. (2008). Embryo lipoproteins and yolk lipovitellin consumption during embryogenesis in *Macrobrachium borellii* (Crustacea: Palaemonidae). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 151, 317-322.

10. Hamers, T., Kamstra, J. H., Sonneveld, E., Murk, A. J., Visser, T. J., Van Velzen, M. J., Brouwer, A. & Bergman, Å. (2008). Biotransformation of brominated flame retardants into potentially endocrine-disrupting metabolites, with special attention to 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47). *Molecular Nutrition & Food Research,* 52, 284-298.

11. Hamza-Chaffai, A. (2014). Usefulness of bioindicators and biomarkers in pollution biomonitoring. *International Journal of Biotechnology for Wellness Industries,* 3, 19-26.

12. Hass, U., Ministerråd, N. & Råd, N. (2005). *OECD conceptual framework for testing and assessment of endocrine disrupters as a basis for regulation of substances with endocrine disrupting properties,* Copenhagen, Denmark: Nordic Council of Ministers, 25.

13. Henderson, B. E., Benton, B., Cosgrove, M., Baptista, J., Aldrich, J., Townsend, D., Hart, W. & Mack, T. M. (1976). Urogenital tract abnormalities in sons of women treated with diethylstilbestrol. *Pediatrics,* 58, 505-507.

14. Herbst, A. L., Ulfelder, H. & Poskanzer, D. C. (1971). Adenocarcinoma of the vagina: Association of maternal stilbestrol therapy with tumor appearance in young women. *New England Journal of Medicine,* 284, 878-881.

15. Hill, M. & O'Keeffe, J. (1992). Some aspects of the ecology of the freshwater crab (*Potamonautes perlatus,* Milne Edwards) in the upper reaches of the Buffalo River, Eastern Cape Province, South Africa. *Southern African Journal of Aquatic Science,* 18, 42-50.

16. Hoover, R. N., Hyer, M., Pfeiffer, R. M., Adam, E., Bond, B., Cheville, A. L., Colton, T., Hartge, P., Hatch, E. E. & Herbst, A. L. (2011). Adverse health outcomes in women exposed *in utero* to diethylstilbestrol. *New England Journal of Medicine,* 365, 1304-1314.

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17. Jia, X., Chen, Y., Zou, Z., Lin, P., Wang, Y. & Zhang, Z. (2013). Characterization and expression profile of vitellogenin gene from *Scylla paramamosain*. *Gene*, 520, 119-130.

18. Jobling, S. & Sumpter, J. (1993). Detergent components in sewage effluent are weakly oestrogenic to fish: An *in vitro* study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic Toxicology*, 27, 361-372.

19. Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., Tyler, C. R., van Aerle, R., Santos, E. & Brighty, G. (2005). Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations. *Environmental Health Perspectives*, 114, 32-39.

20. Kidd, K. A., Blanchfield, P. J., Mills, K. H., Palace, V. P., Evans, R. E., Lazorchak, J. M. & Flick, R. W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences*, 104, 8897-8901.

21. Markman, S., Guschina, I. A., Barnsley, S., Buchanan, K. L., Pascoe, D. & Müller, C. T. (2007). Endocrine disrupting chemicals accumulate in earthworms exposed to sewage effluent. *Chemosphere*, 70, 119-125.

22. Matozzo, V., Gagné, F., Marin, M. G., Ricciardi, F. & Blaise, C. (2008). Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review. *Environment International*, 34, 531-545.

23. McGeoch, M. A. (1998). The selection, testing and application of terrestrial insects as bioindicators. *Biological Reviews of the Cambridge Philosophical Society*, 73, 181-201.

24. Micheletti, C., Critto, A. & Marcomini, A. (2007). Assessment of ecological risk from bioaccumulation of PCDD/Fs and dioxin-like PCBs in a coastal lagoon. *Environment International*, 33, 45-55.

25. Okuno, A., Yang, W. J., Jayasankar, V., Saido-Sakanaka, H., Huong, D. T. T., Jasmani, S., Atmomarsono, M., Subramoniam, T., Tsutsui, N. & Ohira, T. (2002). Deduced primary structure of vitellogenin in the giant freshwater prawn, *Macrobrachium rosenbergii*, and yolk processing during ovarian maturation. *Journal of Experimental Zoology*, 292, 417-429.

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http://etd.uwc.ac.za/

26. Palmer, J. R., Herbst, A. L., Noller, K. L., Boggs, D. A., Troisi, R., Titus-Ernstoff, L., Hatch, E. E., Wise, L. A., Strohsnitter, W. C. & Hoover, R. N. (2009). Urogenital abnormalities in men exposed to diethylstilbestrol *in utero*: A cohort study. *Environmental Health,* 8, 1-6.

27. Reinecke, A., Snyman, R. & Nel, J. (2003). Uptake and distribution of lead (Pb) and cadmium (Cd) in the freshwater crab, *Potamonautes perlatus* (Crustacea) in the Eerste River, South Africa. *Water, Air, and Soil Pollution,* 145, 395-408.

28. Rubin, M. M. (2007). Antenatal exposure to DES: Lessons learned … future concerns. *Obstetrical & Gynecological Survey,* 62, 548-555.

29. Sanders, M., Du Preez, H. & Van Vuren, J. (1999). Monitoring cadmium and zinc contamination in freshwater systems with the use of the freshwater river crab, *Potamonautes warreni*. *Water SA Pretoria,* 25, 91-98.

30. Snyman, R., Reinecke, A. & Nel, J. (2002). Uptake and distribution of copper in the freshwater crab *Potamonautes perlatus* (Crustacea) in the Eerste River, South Africa. *African Zoology,* 37, 81-89.

31. Somerset, V., Van der Horst, C., Silwana, B., Walters, C. & Iwuoha, E. (2015). Biomonitoring and evaluation of metal concentrations in sediment and crab samples from the North-West Province of South Africa. *Water, Air, & Soil Pollution,* 226, 1-25.

32. Subramoniam, T. (2011). Mechanisms and control of vitellogenesis in crustaceans. *Fisheries Science,* 77, 1-21.

33. Tyler, C. R. & Jobling, S. (2008). Roach, sex, and gender-bending chemicals: The feminization of wild fish in English rivers. *Bioscience,* 58, 1051-1059.

34. van Stormbroek, T. (2007). *Bioaccumulation of metals in freshwater crabs (Potamonautes perlatus) of the Lourens River, Western Cape, South Africa.* M.Tech. thesis, Cape Peninsula University of Technology.

35. Virtanen, H. E. & Adamsson, A. (2012). Cryptorchidism and endocrine disrupting chemicals. *Molecular and Cellular Endocrinology,* 355, 208-220.

http://etd.uwc.ac.za/

36. Voorspoels, S., Covaci, A., Maervoet, J., De Meester, I. & Schepens, P. (2004). Levels and profiles of PCBs and OCPs in marine benthic species from the Belgian North Sea and the Western Scheldt Estuary. *Marine Pollution Bulletin*, 49, 393-404.

37. Voshell Jr, J. R., Smith, E. P., Evans, S. K. & Hudy, M. (1997). Effective and scientifically sound bioassessment: Opinions and corroboration from academe. *Human and Ecological Risk Assessment: An International Journal*, 3, 941-954.

38. Zhou, Q., Zhang, J., Fu, J., Shi, J. & Jiang, G. (2008). Biomonitoring: An appealing tool for assessment of metal pollution in the aquatic ecosystem. *Analytica Chimica Acta*, 606, 135-150.

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CHAPTER 2: LITERATURE REVIEW

2.1 Environmental pollutants: endocrine disrupting chemicals (EDCs)

There is a worldwide concern over the number of pollutants found in the environment. This increase in environmental pollutants can be attributed to an increase in agricultural, industrial, domestic, and other anthropogenic activities (Aspelin, 2003; Briggs, 2003; Dong *et al.*, 2012). Ultimately, these pollutants spill or are discharged into the environment and due to their chemical nature, many can persist in the environment and accumulate in organisms (Markman *et al.*, 2007; Ricciardi *et al.*, 2008; Chen *et al.*, 2011). Endocrine disrupting chemicals (EDCs) can be described as one group of these pollutants; xenobiotic compounds that interfere with the endogenous hormones of an organism. Endocrine disrupting chemicals can interfere on a variety of levels, such as impeding on the synthesis, secretion, transport, binding, action, or elimination of an organism's endogenous hormones (Segner *et al.*, 2003). Consequently, homoeostasis is disturbed and the organism's behaviour, development, and reproductive capability can also be adversely affected (Vos *et al.*, 2000). Table 2.1 lists some examples of EDCs, their target hormones, and their sources.

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Table 2.1. Examples, sources, and targets of some EDCs

EDC	Examples	Source(s)	Target Hormones
Phthalates	Butyl benzyl phthalate	Plasticizers [a]	Thyroid [b]
Pharmaceuticals	Diethylstilboestrol (DES)	Contraceptives Menopausal therapy	Oestrogens [c]
Parabens	Methyl-, Ethylparaben	Cosmetics Personal Care Products	Oestrogens [d]
Polychlorinated biphenyls (PCBs)	2,5-dichloro-4-hydroxybiphenyl	Coolants Electrical Equipment	Thyroid [e]
Bisphenols	Bisphenol A (BPA)	Rubber Flame Retardants	Oestrogens [f]
Insecticides	Chlorpyrifos [g]	Agriculture	Androgens [g]

Adapted from Esplugas *et al.*, (2007); [a]Henley & Korach, (2006); [b]Breous *et al.*, (2005); [c]Li *et al.*, (2014b); [d]Watanabe *et al.*, (2013); [e]Cheek *et al.*, (1999); [f]Diamanti-Kandarakis *et al.*, (2009); [g]Viswanath *et al.*, (2010).

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To date, EDCs have been reported to interfere with the activity of oestrogen, androgen, and thyroid hormones. Polychlorinated biphenyls (PCBs) and phthalates have been reported to interfere with thyroid homeostasis by affecting thyroid hormone synthesis and competing for binding to thyroid receptors and transport proteins (Cheek *et al.*, 1999; Breous *et al.*, 2005). The majority of studies on androgenic EDCs have identified many as being anti-androgenic but some EDCs have been shown to act as androgen agonists (Jolly *et al.*, 2006; Viswanath *et al.*, 2010; Liu *et al.*, 2012). Although EDCs have been shown to disrupt thyroid and androgenic hormones, oestrogenic EDCs have received the most attention (Chang *et al.*, 2009).

Oestrogenic EDCs are structurally similar to the endogenous oestrogen, 17 beta (β)-oestradiol, and are capable of mimicking or blocking this oestrogenic effect (Jobling & Sumpter, 1993; Orton *et al.*, 2009). A number of chemicals have been found to affect the oestrogen pathway, including plasticisers, pesticides, and industrial chemicals (Nims *et al.*, 1998; Monteiro *et al.*, 2000; Lovekamp & Davis, 2001; Lovekamp-Swan *et al.*, 2003; Porcher *et al.*, 2009; Craig *et al.*, 2010; Quignot *et al.*, 2010; Craig *et al.*, 2011; Peretz *et al.*, 2011). Oestrogenic EDCs also exhibit poor water solubility and are often associated with solids (Campbell *et al.*, 2006; Vega-Morales *et al.*, 2013).

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2.2 Exposure routes of EDCs and environmental levels

There are an estimated 800 chemicals with known or suspected EDC potential and humans can be exposed to these EDCs in a number of ways, including through food products, from the environment and workplace, and from pharmaceuticals (Matthiessen *et al.*, 1999; Bergman *et al.*, 2013). In certain countries, synthetic oestrogens are administered to livestock as growth-promoting agents to increase product output (Daxenberger *et al.*, 2001). Consequently, these products may be contaminated with synthetic oestrogens and, upon ingestion, it can impact the health of the consumers (Chighizola & Meroni, 2012). Dust and air have also received attention as possible sources of exposure to EDCs. A comprehensive study revealed that 48% of Swiss public buildings contained PCBs with some cases having an indoor air concentration of 1 $\mu g/m^3$ (Kohler *et al.*, 2005). The investigation into the occurrence of EDCs in dust and air of 120 homes revealed that phthalates and alkylphenols were among the most abundant EDCs and concentrations ranged between 50-1 500 ng/m^3 (Rudel *et al.*, 2003). The introduction of dioxins into the environment from waste incineration has raised much awareness from both

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scientists and the public. Fumes from the combustion of commercial and industrial waste release certain EDCs such as dioxins into the environment (Shibamoto *et al.*, 2007).

Workers in certain industries are more at risk for EDC exposure than others. The handling and spraying of pesticides place farmers at risk of exposure to EDCs in the form of pesticides. Some reports have found associations between pesticide exposure and hormone profiles of farmers (Meeker *et al.*, 2009a; Lacasaña *et al.*, 2010; Aguilar-Garduño *et al.*, 2013). At an electronic waste dismantling area in Guangdong, China, it was found that the levels of polybrominated diphenyl ether (PBDE) flame retardants were 11-20 times higher in the dismantling workers than in the non-exposed reference group (Qu *et al.*, 2007). The study did not, however, investigate any effects of the PBDE accumulation on hormone levels. Another study investigated the accumulation of the phthalate di-(2-ethylhexyl) phthalate (DEHP) in workers from a DEHP-manufacturing plant (Liss *et al.*, 1985). By comparing the DEHP levels before and after their shift, the study reported a significant increase ($P = 0.015$) in the highly exposed workers. Although these studies only reported on the associations between EDCs and altered hormone levels, it has been suggested that these associations could be used as potential intermediary indicators to link environmental exposure levels with the clinical manifestations of diseases caused by EDCs (Meeker *et al.*, 2009b).

Effluents from wastewater treatment plants (WWTPs) are considered to be one of the major EDC exposure routes (Fernandez *et al.*, 2007; Ying *et al.*, 2008). Wastewater from industrial, municipal, or agricultural sources is treated at WWTPs to remove pollutants and often the effluents are returned to the environment (Paxéus, 1996; Clara *et al.*, 2005). However, not all of the pollutants are removed and some get reintroduced into the environment through the resultant effluents (Monteiro & Boxall, 2010). The total concentration of oestrogens in the effluents from five Australian sewage treatment plants was reported to range between 2 446 ng/l and 6 579 ng/l (Ying *et al.*, 2009). In Germany, Bolz *et al.*, (2001) reported levels of BPA at 272 ng/l and <0.5-15 µg/kg in rivers and sediment, respectively. Fernandez *et al.*, (2007) investigated the concentrations of oestrogenic compounds in four Canadian municipal WWTPs and reported that 17α-ethynyloestradiol occurred most frequently and at concentrations ranging from ≤5-178 ng/l. Water composites from sewage treatment plant effluents in Spain were reported to contain concentrations of nonylphenol (NP) and its metabolites at 15-35 µg/l and at 10-820 µg/kg in the river sediments (Petrovic *et al.*, 2002). During the wastewater treatment process, various metabolites are formed due to degradation of parent compounds and the

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disruptive effects of EDCs are most often attributed to these metabolites (Giger *et al.*, 1984; Jobling & Sumpter, 1993; Ahel *et al.*, 1994; Cheek *et al.*, 1999; Hamers *et al.*, 2008).

Certain pharmaceuticals, such as those designed as synthetic hormones aimed at improving pregnancy outcomes, also exhibit endocrine disrupting activity (Bergman *et al.*, 2013). During the mid-1900s, a potent synthetic oestrogen, diethylstilboestrol (DES), was used to prevent preterm births and miscarriages, but in later years it was discovered that maternal usage of DES caused teratogenic effects. The *in utero* DES-exposed sons and daughters were reported to have many reproductive abnormalities (Henley & Korach, 2006). Moreover, similar to DES, some EDCs are able to cross the placenta and affect the developing foetus if the mother is exposed during pregnancy. Meerts *et al.*, (2002) investigated the accumulation and endocrine disrupting effects of a PCB metabolite on the offspring of exposed pregnant rats. They found that the metabolite accumulated in the foetal liver and was also bound to the thyroid hormone transport protein, transthyretin (TTR). Furthermore, there was a significant decrease in the foetal levels of thyroxine (T_4) and a significant increase in thyroid-stimulating hormone (TSH). This demonstrates that EDCs can affect the immediately exposed individuals, and can also negatively impact the health and development of their offspring. This is especially evident during foetal development.

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2.3 Impact of EDC exposure

2.3.1 Human impact

Hormones are very important during the developmental period of an organism. Therefore, exposure to EDCs during this particularly sensitive period can result in endocrine-dependent developmental defects (Fenton *et al.*, 2002; Ohsako *et al.*, 2002; Fisher, 2004). Studies have implicated the *in utero* exposure to phthalates in malformations of the male reproductive tract. Owing to its antiandrogenic properties, it has been demonstrated that phthalates can inhibit enzymes involved in testosterone production. This, in turn, can disrupt androgen-dependent developmental processes and give rise to abnormalities like cryptorchidism and hypospadias (Parks *et al.*, 2000; Foster, 2006; Lague & Tremblay, 2008).

Furthermore, there is also evidence showing that EDCs can disrupt the immune system, which can ultimately compromise the individual's ability to combat infections or predispose them to inflammatory conditions. Recently, the aryl hydrocarbon receptor (AHR), which functions in the metabolism of dioxins, has been shown to be involved in the regulation of T cell

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differentiation. Mice exposed to the dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), an AHR ligand, were found to have an expanded population of the regulatory T cells (T_{regs}) and also experienced alleviated symptoms from experimental autoimmune encephalomyelitis (EAE). Conversely, treatment with another AHR ligand, 6-formylindolo[3,2-b]carbazole (FICZ), skewed the T cell population towards the interleukin-17-producing inflammatory T cells (Th_{17}) (Quintana *et al.*, 2008; Zhang *et al.*, 2010). Thus, exposure to certain dioxins can suppress the immune system by increasing T_{reg} population or promote an aberrant Th_{17}-mediated inflammatory condition. Some EDCs can also potentiate allergic responses, and modulate inflammatory products secreted by macrophages (Lee *et al.*, 2003; Hong *et al.*, 2004). From these studies, it is evident that EDCs pose a potential hazard to humans, but before EDCs even reach humans, they are introduced into the environment where they can also exert adverse effects.

2.3.2 Environmental impact

Not all EDCs are effectively removed from WWTPs and in this way some EDCs are often introduced into the aquatic environment through wastewater effluent. Although the majority of EDCs are removed during the wastewater treatment process, the effluent still contains residual levels which are sufficient to induce endocrine disruptive responses (Rodgers-Gray *et al.*, 2000; Tilton *et al.*, 2002; Huggett *et al.*, 2003; Aerni *et al.*, 2004). Accordingly, the introduction of EDCs into the aquatic environment from wastewater effluents put aquatic animals most at risk for exposure (Rodgers-Gray *et al.*, 2000). This has made the Baltic Sea a particular area of interest due to its susceptibility to pollution from industrial and municipal waste by the surrounding countries (Rheinheimer, 1998). This has made the levels of EDCs in marine mammals from the Baltic Sea the focus of some studies.

Sørmo *et al.*, (2003) and Sørmo *et al.*, (2005) investigated organochlorine pollutants (OCs) in the grey seal, *Halichoerus grypus*. The former study reported higher levels of OCs in seals from the Baltic Sea compared to their Atlantic counterparts, while the latter study implicated these OCs in the decrease of thyroid hormone levels in the seals. Another study reported "surprisingly high" levels of PCBs in free-ranging Pacific killer whales (*Orcinus orca)* compared to what was reported in the literature for other marine mammals (Ross *et al.*, 2000). There have also been reports of the *in utero* transfer of EDCs from mother to offspring in some animals, similar to what occurs in humans (Ross *et al.*, 2000; Sørmo *et al.*, 2005).

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Other aquatic animals such as frogs and fish have also been studied for the impact of environmental pollution from agricultural run-off and municipal wastewater. Hayes *et al.*, (2006) investigated the impact of a pesticide mixture on the larval growth and development, sex differentiation, and immune function of Northern leopard frogs, *Rana pipiens*, as well as the levels of plasma corticosterone in the exposed African clawed frogs, *Xenopus laevis*. The authors found that while exposure to the single pesticides demonstrated adverse effects, these effects were more pronounced upon exposure to pesticide mixtures. They also suggested that the negative impacts on sex differentiation and growth, and the immunosuppressive effects caused by EDCs could be responsible for the decline in amphibian populations. Another study reported a decline in the fathead minnow, *Pimephales promelas*, population in the Experimental Lakes Area (ELA), Canada after a 7-year chronic exposure to (5-6 ng/l) to 17α-ethynyloestradiol. The study reported feminisation of males as evident by induction of the female-specific protein vitellogenin (VTG), both at the transcript and protein level, as well as intersex males and altered oogenesis in the females (Kidd *et al.*, 2007). This study also demonstrated the danger of EDC accumulation over a long period of time – that prolonged exposure to EDCs impairs the reproductive activity of a population, which can ultimately result in the collapse thereof.

The occurrence of intersex individuals has been implicated in the decline of fish populations exposed to oestrogenic EDCs that arise from sewage effluents (Jobling *et al.*, 2002). Intersex fish are typically "feminised", i.e. they have reproductive abnormalities such as feminised reproductive tracts, the presence of ovarian tissue within the testicular tissue, known as an "ovotestes", and could also include abnormal levels of sex steroid hormones (Jobling *et al.*, 2005). Some studies have attributed the incidence of intersex fish to the discharge of oestrogen-containing effluents from WWTPs into the environment (Jobling *et al.*, 2005; Tyler & Jobling, 2008). Collectively, these studies strongly suggest that EDCs can have serious environmental implications. Yet, some scepticism remains as to the potency of EDCs and their affinity for endogenous receptors and thereby question the risk that EDCs pose.

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2.3.3 Endocrine disrupting chemicals: a cause for concern?

Generally, EDCs exhibit a decreased potency or binding affinity for specific hormone receptors, which often underestimates the risk they pose in endocrine disruption (Arnold *et al.*, 1996; Folmar *et al.*, 2002; Nakada *et al.*, 2004). However, there are certain factors that should

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be considered when implicating EDCs in endocrine disruption. This includes the bioavailability of EDCs at the target cell which would, consequently, affect the responsiveness of the target cell to a threshold concentration of EDCs. The resistance of EDCs to metabolism promotes persistence in the environment as well as accumulation in organisms (Arnold *et al.*, 1996). This accumulation would directly influence the concentration of EDCs at the target and consequently, determine the responsiveness of the cell. Thus, despite there being different classes of EDCs, each with different target hormones, many of these compounds have common properties. These include: being present in the environment at high concentrations; having a lipophilic nature and tendency to persist which, in turn, causes them to bioaccumulate in exposed organisms; and constantly being introduced into the environment from WWTPs (Tyler *et al.*, 1998).

Once an "effective" concentration in the plasma or cytosol is reached, the EDCs can outcompete endogenous hormones for binding to hormone receptors and consequently induce the expression of hormone-responsive elements (Arnold *et al.*, 1996). This, in turn, is likely to cause an aberrant expression of hormone-responsive elements that can ultimately manifest as disease (Bhan *et al.*, 2014). Therefore, only minor effects might be observed in acute exposures where there is little accumulation and a low concentration of EDCs at the target cell. Conversely, continuous exposure to EDCs would most likely lead to bioaccumulation and bring about a more pronounced effect, more accurately reflecting the risks that EDCs can pose (Arnold *et al.*, 1996; Rodgers-Gray *et al.*, 2000; Hayes *et al.*, 2006; Kidd *et al.*, 2007; Xuereb *et al.*, 2011; Park *et al.*, 2014).

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Ecotoxicological assessments have generally been centred around acute toxicity testing with little focus on the toxicity due to chronic exposures, or the potential of certain chemicals to accumulate (Christen *et al.*, 2010). Thus, EDCs might not appear to be a health hazard over short periods of time but given the low-level and continuous exposure to these pollutants, along with their persistent and accumulative potential, exposure over long periods of time could cause EDCs to outcompete endogenous hormones (Brooks *et al.*, 2006). This could, ultimately, lead to the disruption of endocrine-mediated processes. To that end, Meeker *et al.*, (2009b) similarly remarked that the significant changes in hormone levels attributed to the environmental and occupational exposure to EDCs in the majority of studies can be regarded as subclinical. Yet, the extent to which these hormone changes might be associated with an increased risk of adverse health effects in the long term is still unknown.

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Additionally, results obtained from the studies conducted by Hayes *et al.*, (2006) and Kidd *et al.*, (2007) further suggest that EDCs are indeed a probable cause for concern. Endocrine disrupting chemicals are poorly soluble in water, but are highly fat-soluble and consequently stored in the fats of exposed organisms (Bergman *et al.*, 2013). Moreover, it is this lipophilic nature of EDCs, that causes them to be taken up easily but also impedes elimination from the body (Bergman *et al.*, 2013). For this reason, these compounds are of particular cause for concern as it can lead to the bioaccumulation of EDCs in organisms at the lower trophic levels of a food chain. As predators from higher trophic levels feed on the organisms of the lower trophic levels, the accumulated EDCs can progress upwards in the food chain and ultimately reach the higher trophic levels through biomagnification (Sørmo *et al.*, 2005; Burger *et al.*, 2007; Bergman *et al.*, 2013). Although empirical evidence on the effects of EDC biomagnification at the population-level is sparse, adverse effects such as population extinction or the collapse of ecosystems may yet be observed as a consequence of biomagnification (Hayes *et al.*, 2006; Kidd *et al.*, 2007). This lack of evidence to support a cause and effect relationship between the exposure to EDCs and adverse biological effects has raised some scepticism as to whether EDCs are actually a cause for concern.

However, the number of EDC exposure routes and their potential impact on human health and the environment underpins the importance of detecting EDCs in the environment. Ecological evaluation involves assessing an ecosystem at its various levels so as to determine the most effective way to restore the contaminated environment to a healthy, functional ecosystem (Burger, 2008). Such an evaluation includes quantifying the contaminants found in the organisms of that ecosystem, determining the reproductive success of a specific species, as well as ascertaining the effects of the contaminants and at which levels they occur (Burger, 2008). Thus, the detection of EDCs would constitute the first step in the ecological evaluation and subsequent restoration of a contaminated environment. Ecological evaluation can encourage the proper management of pollution and ultimately aid in the remediation of EDC-polluted ecosystems and possibly limit human exposure as well. Prompt and effective detection involves the use of assays to determine the levels of EDCs in the environment and the demonstration that the levels are sufficient to induce adverse effects.

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2.4 Assays for oestrogenic EDCs

2.4.1 Organisation for economic co-operation and development (OECD) conceptual framework for testing and assessment of endocrine disruptors

A number of assays have been developed to screen and identify potential EDCs with the major focus being on identifying chemicals with possible oestrogenic activity. Thus, the organisation for economic co-operation and development (OECD) has developed a conceptual framework which can be used to test and assess potential EDCs. This framework is comprised of five levels which provide OECD test guidelines and standardised test methods that can be used to evaluate chemicals for endocrine disruption (Hass *et al.*, 2005). Level one is based on existing data and non-test information, such as the physical and chemical properties of the compound(s) under investigation. Level two is based on *in vitro* assays and serves to provide data on selected endocrine mechanisms or pathways. This data can be obtained by means of receptor binding affinity assays, transcriptional assays, or MCF-7 cell proliferation assays. Level three makes use of *in vivo* assays to provide data about selected endocrine mechanisms or pathways. There are a number of assays that can be used to obtain this kind of data but requires the use of intact *in vivo* animal models. Level four and five are both aimed at obtaining data on the adverse effects of EDCs by monitoring endocrine relevant endpoints. The assays employed at these levels span over longer time periods so as to provide more comprehensive data on the adverse effects (Hass *et al.*, 2005). The type of assay used would depend on the intention of the study, while a combination of *in vitro* and *in vivo* assays would provide a solid foundation for an effective ecological evaluation with the aim of remediation.

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2.4.2 Chemical assays

Chemical assays are those that can be used at the OECD level one and are typically employed in the quantification EDC levels in environmental samples, such as sediment or water, obtained from environments suspected to be polluted (Paxéus, 1996; Huang & Sedlak, 2001; Drewes *et al.*, 2005). However, this type of assay provides no information on the effect(s) of EDCs. It merely aims to identify potential EDCs based on certain features and provides no indication as to whether a chemical does, in fact, possess endocrine disrupting activity.

The most common techniques used to quantify environmental samples are gas/liquid chromatography coupled mass spectrometry (G/LC-MS) and enzyme-linked immunosorbent assays (ELISAs). Both of these techniques display a reasonable sensitivity that favour their use

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in the quantification of EDCs, where G/LC-MS offers a higher sensitivity than ELISA (Drewes *et al.*, 2005). However, the concentrations of EDCs in the samples are often below the detection limits of both of these assays (Huang & Sedlak, 2001; Laganà *et al.*, 2004; Drewes *et al.*, 2005; Zhao *et al.*, 2009). It has also been remarked that natural organic matter can sometimes interfere with the analysis of both GC-MS and ELISA (Huang & Sedlak, 2001). Despite their drawbacks, these techniques can still be used to quantify EDC levels from environmental samples effectively. However, the quantification of environmental EDC levels alone might only be sufficient to declare a location as potentially hazardous. To confirm that the area poses adverse risks, biological assays or bioassays are needed.

2.4.3 Bioassays

In comparison to chemical assays, bioassays are those typically used at OECD level two and can elucidate the possible mechanism(s) of EDC action in organisms. Many bioassays are aimed at determining the mode(s) of action of EDCs and based on *in vitro* systems such as competitive ligand binding assays, recombinant oestrogen receptor assays, transcriptional assays, and cell proliferation assays.

The binding of oestrogenic EDCs to oestrogen receptors (ERs) has been the focus of some structural studies. The consensus is that the structural similarities between endogenous oestrogens and oestrogenic EDCs allow the latter to bind to ERs and induce or inhibit expression of oestrogen responsive genes (Liu *et al.*, 2012; Li *et al.*, 2013). Studies have demonstrated that the presence of a ring structure or a phenolic ring in the compound generally allows for binding to the ER (Tabira *et al.*, 1999; Blair *et al.*, 2000; Bergman *et al.*, 2013).

The yeast oestrogen screening (YES) assay makes use of a transformed yeast strain with a human oestrogen receptor (hER), oestrogen responsive elements (EREs), and *lacZ*. The purpose of the YES assay is to establish whether oestrogenic chemicals are able to bind to the ER and subsequently activate EREs. Potential oestrogenic chemicals are incubated with the transformed yeast and the binding of oestrogens to the hER triggers activation of the EREs and the subsequent expression of *lacZ*. The level of oestrogenicity is expressed as β-galactosidase activity (Arnold *et al.*, 1996; Folmar *et al.*, 2002).

The oestrogen proliferative screening (E-SCREEN) assay measures the oestrogenicity of a chemical by quantifying the proliferation of human MCF-7 breast cancer cells as an endpoint. The assay compares the number of cells between negative controls, not exposed to oestrogen,

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positive controls, exposed to 17β-oestradiol, and those exposed to the potentially oestrogenic chemicals under investigation (Soto *et al.*, 1995; Folmar *et al.*, 2002).

Although these types of assays generally provide a good indication as to the endocrine disrupting potential of compounds *in vitro*, they may not exhibit the same effects *in vivo* (Schlumpf *et al.*, 2001; Folmar *et al.*, 2002; Sugiyama *et al.*, 2005). This stands to reason why *in vitro* assays are followed by *in vivo* assays in the OECD framework for EDCs. Also, as mentioned previously, the metabolites of many EDCs possess more endocrine disruptive activity than their parent compounds. This warrants the need for the use of intact *in vivo* models which can provide a more accurate insight into the potential toxicity of pollutants and confirm the endocrine disruptive potential of suspected EDCs.

Biological indicators/bioindicators are organisms, or a group of organisms, that serve as representatives and reflect the state of their environment. They are responsive to a variety of changes that impact on their environment and often also exposed to the same, or similar combinations of environmental pollutants that humans are exposed to (McGeoch, 1998; Altshuler *et al.*, 2011; Bergman *et al.*, 2013). Furthermore, organisms that are continuously exposed in the environment can be studied to determine the effects of life-long exposure on one generation as well as future generations. From an environmental perspective, this will also provide valuable information in the evaluation of population-level effects (Clubbs & Brooks, 2007). These characteristics validate the use of bioindicators in the investigation of potentially toxic environmental pollutants like EDCs.

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2.5 Aquatic invertebrates as animal models for environmental toxicology

2.5.1 Aquatic invertebrates

Invertebrates make up approximately 95% of the earth's animals (Matthiessen *et al.*, 1999; Lewbart, 2011). Aquatic invertebrates are vital components within their respective ecosystems, whereby they provide nutrients to higher order organisms and functioning in decomposition (Matthiessen *et al.*, 1999; Bouchard, 2004). They are ubiquitous and diverse organisms, which make them good models for the assessment of environmental pollutants as they can be found in most ecosystems (Holt & Miller, 2011). The general sensitivity that aquatic invertebrates display toward pollutants, some being more tolerant than others, also makes them suitable potential models for toxicological studies (Bouchard, 2004; Pérez & Beiras, 2010). Therefore, the absence of a certain species within an aquatic ecosystem may suggest that the environment

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is polluted. Furthermore, the importance of invertebrates in ecosystems necessitates the need for protection against EDC-mediated toxicity and neglecting to intervene may prove to have far-reaching, or even devastating ecological consequences (deFur, 2003).

There are several reasons why aquatic invertebrates are suitable bioindicators for environmental pollution, including having short lifespans and generation times. Organisms with short life spans allow for studies to be conducted in a shorter amount of time. Short generation times allow studies to determine the effect of toxicants over multiple generations. Organisms that have both a short lifespan and generation time would make good models for determining whether pollutants have teratogenic effects.

2.5.2 Daphnia as a bioindicator for endocrine disruption

Due to its many advantages, the crustacean *Daphnia* has been used extensively as a bioindicator in environmental toxicology. *Daphnia* are small and have a short generation time which makes them more economical and easier to maintain in the laboratory (Hodkinson & Jackson, 2005; Barnett, 2012). Furthermore, having sequenced the entire genome of *Daphnia* adds to its value as a model organism in that it allows for the identification of genes responsive to given environmental conditions (Hodkinson & Jackson, 2005). The value of *Daphnia* as a model for endocrine disruption has been demonstrated by some studies. Parks & LeBlanc, (1996) found that exposure of daphnids to the biocide pentachlorophenol (PCP) adversely affected the fecundity of the organisms by reducing the elimination of testosterone metabolites. Also, Haeba *et al.*, (2008) found that the organochlorine dicofol skewed the sex ratio in exposed daphnids in favour of male neonates. The authors hypothesised that it could have been as a result of the inhibition of a steroidogenic enzyme that catalyses the conversion of androgens to oestrogens, thus acting as an anti-oestrogen.

Daphnia has also been employed in the use of transgenerational studies to determine the effects of EDCs on offspring and subsequent generations. Brennan *et al.*, (2006) found that first and second generational daphnids had an increased mortality and reduced fecundity after exposure to environmental oestrogens. Another study, spanning over 5 generations of daphnids, demonstrated that BPA decreases the number of offspring in each generation in a dose-dependent manner. The authors concluded that this might be attributed to a magnification of BPA from one generation to the next (Mansilha *et al.*, 2013). Other studies reported similar results for the offspring of pollutant exposed aquatic invertebrates (Lee *et al.*, 2008; Guo *et al.*,

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2012). This demonstrates that certain aquatic invertebrates have the potential to be employed as bioindicators for EDCs. However, aquatic macroinvertebrates that are found at the benthic level of the environment can also be used as bioindicators for environmental pollutants such as heavy metals and EDCs.

2.5.3 Benthic macroinvertebrates as bioindicators for endocrine disruption

Benthic macroinvertebrates, such as crabs and other crustaceans, are aquatic invertebrates that inhabit the bottom of water bodies and are large enough to be visible with the naked eye (Walag & Canencia, 2016). This group is regarded as the assemblage of choice when it comes to using living organisms to assess the condition of an environment, particularly streams (Voshell Jr *et al.*, 1997; Moeykens, 2002). The reason being that they possess the required characteristics that allow them to be classified as bioindicators. However, this group of organisms exhibits additional traits that add to their value as models for environmental pollution. This includes having sufficiently long life cycles that will allow time for exposure to pollution to occur, while at the same time the recovery rate from the exposure is not too short for the impact to go unnoticed (Voshell Jr *et al.*, 1997). To sample these organisms also does not require great effort or any technical expertise (Voshell Jr *et al.*, 1997).

The chemical nature of certain EDCs further contributes to the utility of benthic macroinvertebrates as bioindicators since these compounds tend to be associated with sediment rather than remain suspended in water (Takahashi *et al.*, 2003; Fu & Wu, 2005; Micheletti *et al.*, 2007). This puts benthic macroinvertebrates, particularly crabs, in a favourable position as they inhabit a number of aquatic habitats, such as muddy lagoons (Park & Kwak, 2013). Accordingly, the combination of their sedentary habits, restricted migratory activities, and proximity to the benthos increases the likelihood of benthic macroinvertebrates coming into contact with pollutants (Voshell Jr *et al.*, 1997; Roose *et al.*, 1998; Voorspoels *et al.*, 2004; Fu & Wu, 2005; Micheletti *et al.*, 2007). From this, it is clear that benthic macroinvertebrates occupy a unique position in the ecosystem that makes them susceptible to pollutants and, therefore, quite suitable for the role of bioindicators.

Ideally, a bioindicator should be a relatively simple organism with certain phylogenetically conserved traits. These traits may be genetic and/or cellular that would make it possible for the observed experimental effects to be extrapolated to higher, more complex organisms (Stokes, 2004). One such trait is that of the basic structure and function of the hypothalamic-pituitary-

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gonadal (HPG) axis, which is conserved in all vertebrates (Ankley *et al.*, 2009; Bergman *et al.*, 2013). In the last two decades, steroid receptors associated with the HPG, i.e. those specific for oestrogens, androgens, and progesterone, have been found in benthic macroinvertebrates, such as crabs and other crustaceans (Paolucci *et al.*, 2002; Ye *et al.*, 2008; Ye *et al.*, 2010a; Yang *et al.*, 2012; Wu *et al.*, 2014). The corresponding vertebrate-type steroid hormones have also been reported in crabs and other crustaceans (Warrier *et al.*, 2001; Okumura & Sakiyama, 2004; Gunamalai *et al.*, 2006; Ye *et al.*, 2010b). Accordingly, some studies, albeit few, have attempted to employ benthic macroinvertebrates in the accumulation of EDCs, with a particular focus on crustaceans (Micheletti *et al.*, 2007; Ricciardi *et al.*, 2010; Wen & Pan, 2016). The combination of steroid receptors associated with the HPG axis and their corresponding vertebrate-type steroids strongly suggests that benthic macroinvertebrates can be used as a foundation from which cross-species extrapolation can be done.

2.6 *Potamonautes perlatus* (*P. perlatus*)

The genus *Potamonautes* is found throughout sub-Saharan Africa, with the exception of the Saharan desert and the Maghreb, and is the most widely distributed genus of freshwater crabs in Africa (Cumberlidge, 1999) (figure 2.1). In Southern Africa, the most abundant species of freshwater crabs are *Potamonautes* (*P.*) *sidneyi*, *P. perlatus*, and *P. warreni* (de Kock, 2001). *Potamonautes perlatus*, also known as the Cape River crab, is predominantly found in the Western Cape, but also occurs North and East from Clanwilliam; an area stretching approximately 900 km (Daniels, 2003). Along the Buffalo River, densities of *P. perlatus* have been reported to range between 1.72 and 5.25 crabs/m^2, demonstrating the abundance of *P. perlatus* in the freshwater systems of Southern Africa (Hill & O'Keeffe, 1992).

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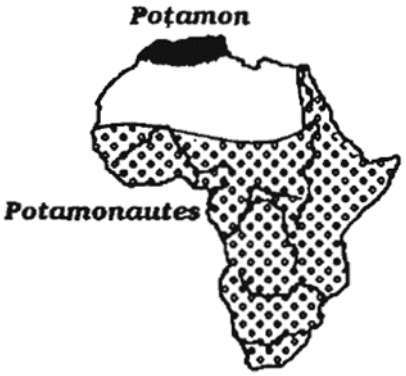

Figure 2.1. Distribution (shaded area) of *Potamonautes* across the African continent (adapted from Cumberlidge, (1999)).

Studies have investigated the efficiency of *Potamonautes* species (spp.) as bioindicators, but have only demonstrated its ability to accumulate heavy metals. Sanders *et al.*, (1999) measured the levels of cadmium (Cd) and zinc (Zn) in water and sediment samples from an impacted environment as well as a non-impacted environment. They compared these levels to those found in the freshwater crab, *Potamonautes warreni*. The results showed that *P. warreni* accumulated Zn at levels higher than those found in the impacted environment, whereas the Cd level in *P. warreni* was found to be equal to that of the impacted and the non-impacted environment. Somerset *et al.*, (2015) conducted a similar study using *P. warreni* but investigated the mining areas in the North-West Province, South Africa. The results indicated that *P. warreni* was able to accumulate lead (Pb), Cd, and three of the platinum group metals (PGMs) from the environment.

Reinecke *et al.*, (2003) employed another species, *P. perlatus*, as a bioindicator and used the bioconcentration factor (BCF) to express its ability to accumulate the heavy metals Pb and Cd. The authors reported relatively high BCF values for both Pb and Cd, indicative of metal accumulation from the environment. The whole body concentrations of the crabs for Cd was also significantly ($P < 0.05$) higher than that of the water or the sediment. Other studies have conducted similar investigations using *P. perlatus* (Snyman *et al.*, 2002; van Stormbroek, 2007). However, to date, no studies have investigated the ability of *P. perlatus*, or any other *Potamonautes* spp., to accumulate EDCs or the effects thereof.

Sex-specific differences in the accumulation of and response to environmental pollutants is a factor that has been reported but has not received much attention. This is an important factor to consider in the evaluation of potential environmental pollutants as the genetic,

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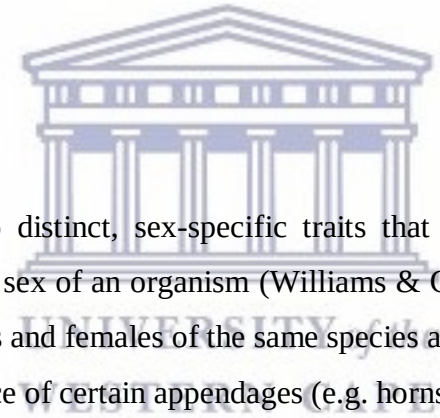
morphological, physiological, and behavioural makeup differs between the sexes. These differences, in turn, would influence parameters such as the uptake, fate, distribution, effects, and elimination of pollutants on organisms (Burger, 2007). Some studies have reported sex-specific differences in the accumulation of and biochemical response to both heavy metals and EDCs. The majority of these studies reported that the level of environmental pollutants was higher in males than in females (Ishizuka *et al.*, 1996; Sastre *et al.*, 1999; Na & Park, 2012; Ghaeni *et al.*, 2015). Ishizuka *et al.*, (1996) and Burger, (2007) have attributed this observation to the ability of females to eliminate pollutants from their body by transferring the contaminants to their eggs. This would explain why the majority of studies have reported significantly higher levels of pollutants in males than in females and demonstrates that these sex-specific responses offer an advantage when it comes to assessing the impact of environmental pollutants (Pereira *et al.*, 2009). However, utilisation of this advantage is dependent on the distinguishability between male and female of an organism; a trait only apparent in sexually dimorphic organisms.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2.7 Sexual dimorphism

Sexual dimorphism refers to distinct, sex-specific traits that allow for the differentiation between the male and female sex of an organism (Williams & Carroll, 2009). It is defined as the differences between males and females of the same species and includes variations in size, colour, the presence or absence of certain appendages (e.g. horns or feathers), or a variation in patterns (Ralls & Mesnick, 2002; Todd & Davis, 2007; Hierlihy *et al.*, 2013). In contrast to sexually dimorphic species, monomorphic species appear identical in that no clear feature(s) differentiates one sex from the other (Ralls & Mesnick, 2002).

Crabs are easily distinguished based on abdominal patterns, the female crabs having broader abdomens than the male crabs. The male crab has a narrower abdomen and has either a T-shaped or triangular pattern (Haley, 1969; Cumberlidge, 1999). In females, the broad shape might allow for the retention of the eggs during embryogenesis (Castilho-Westphal *et al.*, 2013). In contrast to this, the narrower abdomen of males may serve to accommodate the localisation of the male reproductive system (Cumberlidge, 1999).

In addition to macroscopic or physical sexual dimorphism, organisms also demonstrate sex-specific differences on a biochemical level, known as biochemical sexual dimorphism. This was evident in the observation of a female-specific haemocyanin (FSH) found only in adult

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female crabs of the mud crab, *Scylla olivacea*. Characterisation revealed that it was indeed a haemocyanin and was found only in the haemolymph of adult female crabs, and not in the haemolymph of juveniles or that of adult males (Chen *et al.*, 2007).

Another well-known example of biochemical sexual dimorphism is the female-specific protein, VTG. The VTG gene is present in both males and females, but expressed only in females and is silent in males (Palmer & Palmer, 1995; Yehezkel *et al.*, 2000; Matozzo *et al.*, 2008; Xuereb *et al.*, 2011). Where maternal viviparous organisms provide nutrients to their offspring via the placenta or an analogous organ, yolk proteins serve as a source of nutrients to the offspring of oviparous organisms (Chen *et al.*, 2004; Ghekiere *et al.*, 2005; Lodé, 2012). Vitellogenin is the precursor to vitellin (Vn), one of the major constituents of yolk proteins (Gerber-Huber *et al.*, 1987; Jia *et al.*, 2013).

2.8 Vitellogenin (VTG): the female-specific protein

Vitellogenin is a lipoglycoprotein that is synthesised in the hepatic tissue in response to endogenous oestrogens (Babin *et al.*, 2007; Matozzo *et al.*, 2008). After synthesis, VTG is processed and post-translationally modified through phosphorylation, lipidation, glycosylation, and conjugation of carotenoids respectively (Pateraki & Stratakis, 1997; Okuno *et al.*, 2002). The mature form of VTG is then released as a dimer into the circulatory system and transported to, and sequestered by the ovaries through receptor-mediated endocytosis (RME) (Yehezkel *et al.*, 2000; Warrier & Subramoniam, 2002; Krishnan *et al.*, 2008; Roth & Khalaila, 2012). In the ovaries, VTG is cleaved into its individual subunits lipovitellin 1 (LPV1), lipovitellin 2 (LPV2), and phosvitin (PV) – the major yolk protein constituents, or Vn (Meusy, 1980). Respectively, each of the VTG derivatives contributes nutritionally to the development of the growing embryo (García *et al.*, 2008). The lipovitellins act as a source of amino acids and lipids required by the developing embryo for protein synthesis and energy utilisation (Valle, 1993; Yaron & Sivan, 2006; García *et al.*, 2008). Phosvitin is a highly phosphorylated protein that provides phosphorous and acts as a carrier of divalent cations such as calcium (Samaraweera Mudiyanselage, 2012).

Glycosylation is an essential post-translational modification, functioning in the folding and secretion of VTG (Wojchowski *et al.*, 1986; Helenius, 1994; Wilder *et al.*, 2010). Invertebrate VTG predominantly contains carbohydrate residues mannose and N-acetylglucosamine, but typically lack sialic acid residues (Valle, 1993; Khalaila *et al.*, 2004; Roth *et al.*, 2010). Lipids

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are non-covalently conjugated to VTG, consisting of both nonpolar lipids and phospholipids (Raag *et al.*, 1988; Thompson & Banaszak, 2002). Phospholipids constitute the majority of lipids, predominantly phosphatidylcholine (PC) and phosphatidylethanolamine (PE). To a lesser extent, crustacean VTG contains neutral lipids such as glycerides and sterols (Adiyodi & Adiyodi, 1970; Pateraki & Stratakis, 1997).

A distinguishing feature of crustacean VTG is the presence of carotenoids such as astaxanthin, lutein, and β-carotene; consequently, crustacean VTG is defined as a lipoglycocarotenoprotein (Pateraki & Stratakis, 1997). The predominant carotenoid, astaxanthin, is said to function in pigmentation, but also provide a protection against reactive oxygen species (ROS) (Jittivadhna *et al.*, 2010; Maoka *et al.*, 2012). Furthermore, crustacean VTG lacks a PV domain – a feature characteristic of vertebrate and some insect VTG (van het Schip *et al.*, 1987; Kang *et al.*, 2008; Tufail *et al.*, 2010).

The role of VTG as a female-specific protein puts it in a unique position to be utilised as a marker for oestrogen-mimicking EDCs in male crabs. Only a few studies have attempted to use male crabs as bioindicators in conjunction with VTG as a biomarker for oestrogen-mimicking EDCs (Ricciardi *et al.*, 2010).

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2.9 REFERENCES

1. Adiyodi, K. & Adiyodi, R. G. (1970). Endocrine control of reproduction in decapod Crustacea. *Biological Reviews,* 45, 121-164.

2. Aerni, H. R., Kobler, B., Rutishauser, B. V., Wettstein, F. E., Fischer, R., Giger, W., Hungerbühler, A., Marazuela, M. D., Peter, A. & Schönenberger, R. (2004). Combined biological and chemical assessment of estrogenic activities in wastewater treatment plant effluents. *Analytical and Bioanalytical Chemistry,* 378, 688-696.

3. Aguilar-Garduño, C., Lacasaña, M., Blanco-Muñoz, J., Rodríguez-Barranco, M., Hernández, A., Bassol, S., González-Alzaga, B. & Cebrian, M. (2013). Changes in male hormone profile after occupational organophosphate exposure. A longitudinal study. *Toxicology,* 307, 55-65.

4. Ahel, M., Giger, W. & Koch, M. (1994). Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment - I. Occurrence and transformation in sewage treatment. *Water Research,* 28, 1131-1142.

5. Altshuler, I., Demiri, B., Xu, S., Constantin, A., Yan, N. D. & Cristescu, M. E. (2011). An integrated multi-disciplinary approach for studying multiple stressors in freshwater ecosystems: *Daphnia* as a model organism. *Integrative and Comparative Biology,* 51, 623-633.

6. Ankley, G. T., Bencic, D. C., Breen, M. S., Collette, T. W., Conolly, R. B., Denslow, N. D., Edwards, S. W., Ekman, D. R., Garcia-Reyero, N. & Jensen, K. M. (2009). Endocrine disrupting chemicals in fish: Developing exposure indicators and predictive models of effects based on mechanism of action. *Aquatic Toxicology,* 92, 168-178.

7. Arnold, S. F., Robinson, M. K., Notides, A. C., Guillette Jr, L. J. & McLachlan, J. A. (1996). A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens. *Environmental Health Perspectives,* 104, 544-548.

8. Aspelin, A. L. (2003). Pesticide usage in the United States: Trends during the 20th century. *CIPM Technical Bulletin,* 105, 1-213.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

9. Babin, P. J., Carnevali, O., Lubzens, E. & Schneider, W. J. (2007). Molecular aspects of oocyte vitellogenesis in fish. Babin, P. J., Cerdà, J. & Lubzens, E. (eds.), *In: The fish oocyte: From basic studies to biotechnological applications.* Berlin, Germany: Springer Netherlands, 44.

10. Barnett, R. E. (2012). *A study assessing the comparative molecular toxicities of heavy metals to multiple strains of Daphnia magna.* M.Res. thesis, University of Birmingham.

11. Bergman, Å., Heindel, J. J., Jobling, S., Kidd, K. A. & Zoeller, R. T. (2013). *State of the science of endocrine disrupting chemicals 2012: An assessment of the state of the science of endocrine disruptors prepared by a group of experts for the United Nations Environment Programme and World Health Organization,* Geneva, Switzerland: World Health Organization, 1-260.

12. Bhan, A., Hussain, I., Ansari, K. I., Bobzean, S. A., Perrotti, L. I. & Mandal, S. S. (2014). Bisphenol-A and diethylstilbestrol exposure induces the expression of breast cancer associated long noncoding RNA HOTAIR *in vitro* and *in vivo*. *The Journal of Steroid Biochemistry and Molecular Biology,* 141, 160-170.

13. Blair, R. M., Fang, H., Branham, W. S., Hass, B. S., Dial, S. L., Moland, C. L., Tong, W., Shi, L., Perkins, R. & Sheehan, D. M. (2000). The estrogen receptor relative binding affinities of 188 natural and xenochemicals: Structural diversity of ligands. *Toxicological Sciences,* 54, 138-153.

14. Bolz, U., Hagenmaier, H. & Körner, W. (2001). Phenolic xenoestrogens in surface water, sediments, and sewage sludge from Baden-Württemberg, South-West Germany. *Environmental Pollution,* 115, 291-301.

15. Bouchard, R. W. (2004). *Guide to aquatic invertebrates of the Upper Midwest: Identification manual for students, citizen monitors, and aquatic resource professionals,* University of Minnesota, USA: Water Resources Research Center, 11.

16. Brennan, S. J., Brougham, C. A., Roche, J. J. & Fogarty, A. M. (2006). Multi-generational effects of four selected environmental oestrogens on *Daphnia magna. Chemosphere,* 64, 49-55.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

17. Breous, E., Wenzel, A. & Loos, U. (2005). The promoter of the human sodium/iodide symporter responds to certain phthalate plasticisers. *Molecular and Cellular Endocrinology*, 244, 75-78.

18. Briggs, D. (2003). Environmental pollution and the global burden of disease. *British Medical Bulletin,* 68, 1-24.

19. Brooks, B. W., Riley, T. M. & Taylor, R. D. (2006). Water quality of effluent-dominated ecosystems: Ecotoxicological, hydrological, and management considerations. *Hydrobiologia,* 556, 365-379.

20. Burger, J. (2007). A framework and methods for incorporating gender-related issues in wildlife risk assessment: Gender-related differences in metal levels and other contaminants as a case study. *Environmental Research,* 104, 153-162.

21. Burger, J. (2008). Environmental management: Integrating ecological evaluation, remediation, restoration, natural resource damage assessment and long-term stewardship on contaminated lands. *Science of the Total Environment,* 400, 6-19.

22. Burger, J., Fossi, C., McClellan-Green, P. & Orlando, E. F. (2007). Methodologies, bioindicators, and biomarkers for assessing gender-related differences in wildlife exposed to environmental chemicals. *Environmental Research,* 104, 135-152.

23. Campbell, C. G., Borglin, S. E., Green, F. B., Grayson, A., Wozei, E. & Stringfellow, W. T. (2006). Biologically directed environmental monitoring, fate, and transport of estrogenic endocrine disrupting compounds in water: A review. *Chemosphere,* 65, 1265-1280.

24. Castilho-Westphal, G. G., Ostrensky, A., Pie, M. R. & Boeger, W. A. (2013). Morphology of the female reproductive system and reproductive cycle of the mangrove land crab *Ucides cordatus* (L.) in the Baía de Antonina, Paraná, Brazil. *Acta Zoologica,* 94, 86-93.

25. Chang, H. S., Choo, K. H., Lee, B. & Choi, S. J. (2009). The methods of identification, analysis, and removal of endocrine disrupting compounds (EDCs) in water. *Journal of Hazardous Materials,* 172, 1-12.

UNIVERSITY of the
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http://etd.uwc.ac.za/

26. Cheek, A. O., Kow, K., Chen, J. & McLachlan, J. A. (1999). Potential mechanisms of thyroid disruption in humans: Interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. *Environmental Health Perspectives*, 107, 273–278.

27. Chen, F., Ying, G. G., Kong, L. X., Wang, L., Zhao, J. L., Zhou, L. J. & Zhang, L. J. (2011). Distribution and accumulation of endocrine-disrupting chemicals and pharmaceuticals in wastewater irrigated soils in Hebei, China. *Environmental Pollution*, 159, 1490-1498.

28. Chen, H. Y., Ho, S. H., Chen, T. I., Soong, K., Chen, I. M. & Cheng, J. H. (2007). Identification of a female-specific hemocyanin in the mud crab, *Scylla olivacea* (Crustacea: Portunidae). *Zoological Studies*, 46, 194-202.

29. Chen, L., Jiang, H., Zhou, Z., Li, K., Li, K., Deng, G. Y. & Liu, Z. (2004). Purification of vitellin from the ovary of Chinese mitten-handed crab (*Eriocheir sinensis*) and development of an antivitellin ELISA. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 138, 305-311.

30. Chighizola, C. & Meroni, P. L. (2012). The role of environmental estrogens and autoimmunity. *Autoimmunity Reviews*, 11, A493-A501.

31. Christen, V., Hickmann, S., Rechenberg, B. & Fent, K. (2010). Highly active human pharmaceuticals in aquatic systems: A concept for their identification based on their mode of action. *Aquatic Toxicology*, 96, 167-181.

32. Clara, M., Strenn, B., Gans, O., Martinez, E., Kreuzinger, N. & Kroiss, H. (2005). Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. *Water Research*, 39, 4797-4807.

33. Clubbs, R. L. & Brooks, B. W. (2007). *Daphnia magna* responses to a vertebrate estrogen receptor agonist and an antagonist: A multigenerational study. *Ecotoxicology and Environmental Safety*, 67, 385-398.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

34. Craig, Z. R., Leslie, T. C., Hatfield, K. P., Gupta, R. K. & Flaws, J. A. (2010). Mono-hydroxy methoxychlor alters levels of key sex steroids and steroidogenic enzymes in cultured mouse antral follicles. *Toxicology and Applied Pharmacology,* 249, 107-113.

35. Craig, Z. R., Wang, W. & Flaws, J. A. (2011). Endocrine-disrupting chemicals in ovarian function: Effects on steroidogenesis, metabolism and nuclear receptor signaling. *Reproduction,* 142, 633-646.

36. Cumberlidge, N. (1999). *The freshwater crabs of West Africa: Family Potamonautidae,* Paris, France: IRD Editions, 77-79, 128 & 334.

37. Daniels, S. R. (2003). Examining the genetic structure among populations of the common Cape river crab *Potamonautes perlatus* from river systems in South Africa reveals hydrographic boundaries. *Journal of Crustacean Biology,* 23, 936-950.

38. Daxenberger, A., Ibarreta, D. & Meyer, H. H. (2001). Possible health impact of animal oestrogens in food. *Human Reproduction Update,* 7, 340-355.

39. de Kock, E. (2001). *Aspects of the general biology and the bioaccumulation of metals in the freshwater crab, Potamonautes warreni.* M.Sc. thesis, Rand Afrikaans University.

40. deFur, P. L. (2003). Use and role of invertebrate models in endocrine disruptor research and testing. *ILAR Journal/National Research Council,* 45, 484-493.

41. Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., Zoeller, R. T. & Gore, A. C. (2009). Endocrine-disrupting chemicals: An Endocrine Society scientific statement. *Endocrine Reviews,* 30, 293-342.

42. Dong, C. D., Chen, C. F. & Chen, C. W. (2012). Determination of polycyclic aromatic hydrocarbons in industrial harbor sediments by GC-MS. *International Journal of Environmental Research and Public Health,* 9, 2175-2188.

43. Drewes, J. E., Hemming, J., Ladenburger, S. J., Schauer, J. & Sonzogni, W. (2005). An assessment of endocrine disrupting activity changes during wastewater treatment through the use of bioassays and chemical measurements. *Water Environment Research,* 77, 12-23.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

44. Esplugas, S., Bila, D. M., Krause, L. G. T. & Dezotti, M. (2007). Ozonation and advanced oxidation technologies to remove endocrine disrupting chemicals (EDCs) and pharmaceuticals and personal care products (PPCPs) in water effluents. *Journal of Hazardous Materials,* 149, 631-642.

45. Fenton, S. E., Hamm, J. T., Birnbaum, L. S. & Youngblood, G. L. (2002). Persistent abnormalities in the rat mammary gland following gestational and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Toxicological Sciences,* 67, 63-74.

46. Fernandez, M. P., Ikonomou, M. G. & Buchanan, I. (2007). An assessment of estrogenic organic contaminants in Canadian wastewaters. *Science of the Total Environment,* 373, 250-269.

47. Fisher, J. S. (2004). Environmental anti-androgens and male reproductive health: Focus on phthalates and testicular dysgenesis syndrome. *Reproduction,* 127, 305-315.

48. Folmar, L. C., Hemmer, M. J., Denslow, N. D., Kroll, K., Chen, J., Cheek, A., Richman, H., Meredith, H. & Grau, E. G. (2002). A comparison of the estrogenic potencies of estradiol, ethynylestradiol, diethylstilbestrol, nonylphenol and methoxychlor *in vivo* and *in vitro*. *Aquatic Toxicology,* 60, 101-110.

49. Foster, P. (2006). Disruption of reproductive development in male rat offspring following *in utero* exposure to phthalate esters. *International Journal of Andrology,* 29, 140-147.

50. Fu, C. T. & Wu, S. C. (2005). Bioaccumulation of polychlorinated biphenyls in mullet fish in a former ship dismantling harbour, a contaminated estuary, and nearby coastal fish farms. *Marine Pollution Bulletin,* 51, 932-939.

51. García, F., Cunningham, M., Garda, H. & Heras, H. (2008). Embryo lipoproteins and yolk lipovitellin consumption during embryogenesis in *Macrobrachium borellii* (Crustacea: Palaemonidae). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 151, 317-322.

52. Gerber-Huber, S., Nardelli, D., Haefliger, J. A., Cooper, D. N., Givel, F., Germond, J. E., Engel, J., Green, N. M. & Wahli, W. (1987). Precursor-product relationship between vitellogenin and the yolk proteins as derived from the complete sequence of a *Xenopus* vitellogenin gene. *Nucleic Acids Research,* 15, 4737-4760.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

53. Ghaeni, M., Pour, N. A. & Hosseini, M. (2015). Bioaccumulation of polychlorinated biphenyl (PCB), polycyclic aromatic hydrocarbon (PAH), mercury, methyl mercury, and arsenic in blue crab *Portunus segnis* from Persian Gulf. *Environmental Monitoring and Assessment,* 187, 1-9.

54. Ghekiere, A., Fenske, M., Verslycke, T., Tyler, C. & Janssen, C. (2005). Development of a quantitative enzyme-linked immunosorbent assay for vitellin in the mysid *Neomysis integer* (Crustacea: Mysidacea). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 142, 43-49.

55. Giger, W., Brunner, P. H. & Schaffner, C. (1984). 4-Nonylphenol in sewage sludge: Accumulation of toxic metabolites from nonionic surfactants. *Science,* 225, 623-625.

56. Gunamalai, V., Kirubagaran, R. & Subramoniam, T. (2006). Vertebrate steroids and the control of female reproduction in two decapod crustaceans, *Emerita asiatica* and *Macrobrachium rosenbergii*. *Current Science,* 90, 119-123.

57. Guo, F., Wang, L. & Wang, W. X. (2012). Acute and chronic toxicity of polychlorinated biphenyl 126 to *Tigriopus japonicus*: Effects on survival, growth, reproduction, and intrinsic rate of population growth. *Environmental Toxicology and Chemistry,* 31, 639-645.

58. Haeba, M. H., Hilscherová, K., Mazurová, E. & Bláha, L. (2008). Selected endocrine disrupting compounds (vinclozolin, flutamide, ketoconazole and dicofol): Effects on survival, occurrence of males, growth, molting and reproduction of *Daphnia magna*. *Environmental Science and Pollution Research,* 15, 222-227.

59. Haley, S. (1969). Relative growth and sexual maturity of the Texas ghost crab, *Ocypode quadrata* (Fabr.) (Brachyura, Ocypodidae). *Crustaceana,* 17, 285-297.

60. Hamers, T., Kamstra, J. H., Sonneveld, E., Murk, A. J., Visser, T. J., Van Velzen, M. J., Brouwer, A. & Bergman, Å. (2008). Biotransformation of brominated flame retardants into potentially endocrine-disrupting metabolites, with special attention to 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47). *Molecular Nutrition & Food Research,* 52, 284-298.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

61. Hass, U., Ministerråd, N. & Råd, N. (2005). *OECD conceptual framework for testing and assessment of endocrine disrupters as a basis for regulation of substances with endocrine disrupting properties*, Copenhagen, Denmark: Nordic Council of Ministers, 25.

62. Hayes, T. B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., Lee, M., Mai, V. P., Marjuoa, Y. & Parker, J. (2006). Pesticide mixtures, endocrine disruption, and amphibian declines: Are we underestimating the impact? *Environmental Health Perspectives*, 114, 40-50.

63. Helenius, A. (1994). How N-linked oligosaccharides affect glycoprotein folding in the endoplasmic reticulum. *Molecular Biology of the Cell*, 5, 253–265.

64. Henley, D. V. & Korach, K. S. (2006). Endocrine-disrupting chemicals use distinct mechanisms of action to modulate endocrine system function. *Endocrinology*, 147, s25-s32.

65. Hierlihy, C. A., Garcia-Collazo, R., Chavez Tapia, C. B. & Mallory, F. F. (2013). Sexual dimorphism in the lizard *Sceloporus siniferus*: Support for the intraspecific niche divergence and sexual selection hypotheses. *Salamandra*, 49, 1-6.

66. Hill, M. & O'Keeffe, J. (1992). Some aspects of the ecology of the freshwater crab (*Potamonautes perlatus*, Milne Edwards) in the upper reaches of the Buffalo River, Eastern Cape Province, South Africa. *Southern African Journal of Aquatic Science*, 18, 42-50.

67. Hodkinson, I. D. & Jackson, J. K. (2005). Terrestrial and aquatic invertebrates as bioindicators for environmental monitoring, with particular reference to mountain ecosystems. *Environmental Management*, 35, 649-666.

68. Holt, E. A. & Miller, S. W. (2011). Bioindicators: Using organisms to measure environmental impacts. *Nature Education Knowledge* [Online], 3, 10. Available at: http://www.nature.com/scitable/knowledge/library/bio-indicators-using-organisms-to-measure-environmentalimpacts-16821310, [Accessed: 17/12/2016].

http://etd.uwc.ac.za/

69. Hong, C. C., Shimomura-Shimizu, M., Muroi, M. & Tanamoto, K. (2004). Effect of endocrine disrupting chemicals on lipopolysaccharide-induced tumor necrosis factor-alpha and nitric oxide production by mouse macrophages. *Biological and Pharmaceutical Bulletin*, 27, 1136-1139.

70. Huang, C. H. & Sedlak, D. L. (2001). Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem mass spectrometry. *Environmental Toxicology and Chemistry*, 20, 133-139.

71. Huggett, D. B., Foran, C. M., Brooks, B. W., Weston, J., Peterson, B., Marsh, K. E., La Point, T. W. & Schlenk, D. (2003). Comparison of *in vitro* and *in vivo* bioassays for estrogenicity in effluent from North American municipal wastewater facilities. *Toxicological Sciences*, 72, 77-83.

72. Ishizuka, M., Hoshi, H., Minamoto, N., Masuda, M., Kazusaka, A. & Fujita, S. (1996). Alterations of cytochrome P450-dependent monooxygenase activities in *Eriocheir japonicus* in response to water pollution. *Environmental Health Perspectives*, 104, 774-778.

73. Jia, X., Chen, Y., Zou, Z., Lin, P., Wang, Y. & Zhang, Z. (2013). Characterization and expression profile of vitellogenin gene from *Scylla paramamosain*. *Gene*, 520, 119-130.

74. Jittivadhna, K., Flegel, T. W., Ruenwongsa, P. & Panijpan, B. (2010). Biochemistry in decapod crustaceans. *Bioscience Explained* [Online], 5, 2. Available at: http://www.bioscience-explained.org/ENvol5_2/pdf/decaeng.pdf, [Accessed: 18/03/2015].

75. Jobling, S., Coey, S., Whitmore, J., Kime, D., Van Look, K., McAllister, B., Beresford, N., Henshaw, A., Brighty, G. & Tyler, C. (2002). Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biology of Reproduction*, 67, 515-524.

76. Jobling, S. & Sumpter, J. (1993). Detergent components in sewage effluent are weakly oestrogenic to fish: An *in vitro* study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic Toxicology*, 27, 361-372.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

77. Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., Tyler, C. R., van Aerle, R., Santos, E. & Brighty, G. (2005). Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations. *Environmental Health Perspectives,* 114, 32-39.

78. Jolly, C., Katsiadaki, I., Le Belle, N., Mayer, I. & Dufour, S. (2006). Development of a stickleback kidney cell culture assay for the screening of androgenic and anti-androgenic endocrine disrupters. *Aquatic Toxicology*, 79, 158-166.

79. Kang, B., Nanri, T., Lee, J., Saito, H., Han, C., Hatakeyama, M. & Saigusa, M. (2008). Vitellogenesis in both sexes of gonochoristic mud shrimp, *Upogebia major* (Crustacea): Analyses of vitellogenin gene expression and vitellogenin processing. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 149, 589-598.

80. Khalaila, I., Peter-Katalinic, J., Tsang, C., Radcliffe, C. M., Aflalo, E. D., Harvey, D. J., Dwek, R. A., Rudd, P. M. & Sagi, A. (2004). Structural characterization of the N-glycan moiety and site of glycosylation in vitellogenin from the decapod crustacean *Cherax quadricarinatus*. *Glycobiology*, 14, 767-774.

81. Kidd, K. A., Blanchfield, P. J., Mills, K. H., Palace, V. P., Evans, R. E., Lazorchak, J. M. & Flick, R. W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences,* 104, 8897-8901.

82. Kohler, M., Tremp, J., Zennegg, M., Seiler, C., Minder-Kohler, S., Beck, M., Lienemann, P., Wegmann, L. & Schmid, P. (2005). Joint sealants: An overlooked diffuse source of polychlorinated biphenyls in buildings. *Environmental Science & Technology*, 39, 1967-1973.

83. Krishnan, M., Muthumeenakshi, P., Bharathiraja, C., Warrier, S. & Subramoniam, T. (2008). A comparative study on vitellogenin receptor of a lepidopteran insect (*Spodoptera litura*) and a decapod crustacean (*Scylla serrata*): Phylogenetic implication and co-evolution with vitellogenins. *Journal of Endocrinology and Reproduction,* 12, 13-23.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

84. Lacasaña, M., López-Flores, I., Rodríguez-Barranco, M., Aguilar-Garduño, C., Blanco-Muñoz, J., Pérez-Méndez, O., Gamboa, R., Bassol, S. & Cebrian, M. E. (2010). Association between organophosphate pesticides exposure and thyroid hormones in floriculture workers. *Toxicology and Applied Pharmacology,* 243, 19-26.

85. Laganà, A., Bacaloni, A., De Leva, I., Faberi, A., Fago, G. & Marino, A. (2004). Analytical methodologies for determining the occurrence of endocrine disrupting chemicals in sewage treatment plants and natural waters. *Analytica Chimica Acta,* 501, 79-88.

86. Lague, E. & Tremblay, J. J. (2008). Antagonistic effects of testosterone and the endocrine disruptor mono-(2-ethylhexyl) phthalate on *INSL3* transcription in Leydig cells. *Endocrinology*, 149, 4688-4694.

87. Lee, K. W., Raisuddin, S., Hwang, D. S., Park, H. G., Dahms, H. U., Ahn, I. Y. & Lee, J. S. (2008). Two-generation toxicity study on the copepod model species *Tigriopus japonicus*. *Chemosphere*, 72, 1359-1365.

88. Lee, M. H., Chung, S. W., Kang, B. Y., Park, J., Lee, C. H., Hwang, S. Y. & Kim, T. S. (2003). Enhanced interleukin-4 production in $CD4^+$ T cells and elevated immunoglobulin E levels in antigen-primed mice by bisphenol A and nonylphenol, endocrine disruptors: Involvement of nuclear factor-AT and Ca^{2+}. *Immunology,* 109, 76-86.

UNIVERSITY of the
WESTERN CAPE

89. Lewbart, G. A. (2011). *Invertebrate medicine,* 2nd Ed, John Wiley & Sons, 3.

90. Li, Y., Hamilton, K. J., Lai, A. Y., Burns, K. A., Li, L., Wade, P. A. & Korach, K. S. (2014b). Diethylstilbestrol (DES)-stimulated hormonal toxicity is mediated by ERα alteration of target gene methylation patterns and epigenetic modifiers (*DNMT3A*, *MBD2*, and *HDAC2*) in the mouse seminal vesicle. *Environmental Health Perspectives*, 122, 262-268.

91. Li, Y., Luh, C. J., Burns, K. A., Arao, Y., Jiang, Z., Teng, C. T., Tice, R. R. & Korach, K. S. (2013). Endocrine-disrupting chemicals (EDCs): *In vitro* mechanism of estrogenic activation and differential effects on ER target genes. *Environmental Health Perspectives*, 121, 459-466.

http://etd.uwc.ac.za/

92. Liss, G. M., Albro, P. W., Hartle, R. W. & Stringer, W. T. (1985). Urine phthalate determinations as an index of occupational exposure to phthalic anhydride and di (2-ethylhexyl) phthalate. *Scandinavian Journal of Work, Environment & Health,* 11, 381-387.

93. Liu, X., Ji, K. & Choi, K. (2012). Endocrine disruption potentials of organophosphate flame retardants and related mechanisms in H295R and MVLN cell lines and in zebrafish. *Aquatic Toxicology,* 114, 173-181.

94. Lodé, T. (2012). Oviparity or viviparity? That is the question... . *Reproductive Biology,* 12, 259-264.

95. Lovekamp-Swan, T., Jetten, A. M. & Davis, B. J. (2003). Dual activation of PPARα and PPARγ by mono-(2-ethylhexyl) phthalate in rat ovarian granulosa cells. *Molecular and Cellular Endocrinology,* 201, 133-141.

96. Lovekamp, T. & Davis, B. (2001). Mono-(2-ethylhexyl) phthalate suppresses aromatase transcript levels and estradiol production in cultured rat granulosa cells. *Toxicology and Applied Pharmacology,* 172, 217-224.

97. Mansilha, C., Silva, P., Rocha, S., Gameiro, P., Domingues, V., Pinho, C. & Ferreira, I. M. (2013). Bisphenol A migration from plastic materials: Direct insight of ecotoxicity in *Daphnia magna*. *Environmental Science and Pollution Research,* 20, 6007-6018.

98. Maoka, T., Tokuda, H., Suzuki, N., Kato, H. & Etoh, H. (2012). Anti-oxidative, anti-tumor-promoting, and anti-carcinogenesis activities of nitroastaxanthin and nitrolutein, the reaction products of astaxanthin and lutein with peroxynitrite. *Marine Drugs,* 10, 1391-1399.

99. Markman, S., Guschina, I. A., Barnsley, S., Buchanan, K. L., Pascoe, D. & Müller, C. T. (2007). Endocrine disrupting chemicals accumulate in earthworms exposed to sewage effluent. *Chemosphere,* 70, 119-125.

100. Matozzo, V., Gagné, F., Marin, M. G., Ricciardi, F. & Blaise, C. (2008). Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review. *Environment International,* 34, 531-545.

http://etd.uwc.ac.za/

101. Matthiessen, P., Reynoldson, T., Billinghurst, Z., Brassard, D., Cameron, P., Chandler, G., Davies, I., Horiguchi, T., Mount, D., Oehlmann, J., Pottinger, T., Sibley, P., Thompson, H. & Vethaak, A. (1999). Field assessment for endocrine disruption in invertebrates. deFur, P. L., Crane, M., Ingersoll, C. & Tattersfield, L. (eds.), *In: Endocrine disruption in invertebrates: Endocrinology, testing, and assessment.* Brussels, Belgium: SETAC Press, 199 & 201.

102. McGeoch, M. A. (1998). The selection, testing and application of terrestrial insects as bioindicators. *Biological Reviews of the Cambridge Philosophical Society*, 73, 181-201.

103. Meeker, J. D., Barr, D. B. & Hauser, R. (2009a). Pyrethroid insecticide metabolites are associated with serum hormone levels in adult men. *Reproductive Toxicology*, 27, 155-160.

104. Meeker, J. D., Sathyanarayana, S. & Swan, S. H. (2009b). Phthalates and other additives in plastics: Human exposure and associated health outcomes. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 364, 2097-2113.

105. Meerts, I. A., Assink, Y., Cenijn, P. H., Van den Berg, J. H., Weijers, B. M., Bergman, Å., Koeman, J. H. & Brouwer, A. (2002). Placental transfer of a hydroxylated polychlorinated biphenyl and effects on fetal and maternal thyroid hormone homeostasis in the rat. *Toxicological Sciences,* 68, 361-371.

106. Meusy, J. (1980). Vitellogenin, the extraovarian precursor of the protein yolk in Crustacea: A review. *Reproduction Nutrition Développement,* 20, 1-21.

107. Micheletti, C., Critto, A. & Marcomini, A. (2007). Assessment of ecological risk from bioaccumulation of PCDD/Fs and dioxin-like PCBs in a coastal lagoon. *Environment International,* 33, 45-55.

108. Moeykens, M. D. (2002). *Studies of benthic macroinvertebrate use for biomonitoring of mid-Atlantic highland streams.* Ph.D. thesis, Virginia Polytechnic Institute and State University.

109. Monteiro, P. R. R., Reis-Henriques, M. A. & Coimbra, J. (2000). Polycyclic aromatic hydrocarbons inhibit *in vitro* ovarian steroidogenesis in the flounder (*Platichthys flesus* L.). *Aquatic Toxicology,* 48, 549-559.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

110. Monteiro, S. C. & Boxall, A. B. (2010). Occurrence and fate of human pharmaceuticals in the environment. Whitacre, D. M. (ed.), *In: Reviews of Environmental Contamination and Toxicology*. New York, USA: Springer New York, 71.

111. Na, C. K. & Park, H. J. (2012). Distribution of heavy metals in tidal flat sediments and their bioaccumulation in the crab *Macrophthalmus japonicas* in the coastal areas of Korea. *Geosciences Journal*, 16, 153-164.

112. Nakada, N., Nyunoya, H., Nakamura, M., Hara, A., Iguchi, T. & Takada, H. (2004). Identification of estrogenic compounds in wastewater effluent. *Environmental Toxicology and Chemistry*, 23, 2807-2815.

113. Nims, R. W., Lubet, R. A., Fox, S. D., Jones, C. R., Thomas, P. E., Reddy, A. B. & Kocarek, T. A. (1998). Comparative pharmacodynamics of CYP2B induction by DDT, DDE, and DDD in male rat liver and cultured rat hepatocytes. *Journal of Toxicology and Environmental Health Part A*, 53, 455-477.

114. Ohsako, S., Miyabara, Y., Sakaue, M., Ishimura, R., Kakeyama, M., Izumi, H., Yonemoto, J. & Tohyama, C. (2002). Developmental stage-specific effects of perinatal 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure on reproductive organs of male rat offspring. *Toxicological Sciences*, 66, 283-292.

115. Okumura, T. & Sakiyama, K. (2004). Hemolymph levels of vertebrate-type steroid hormones in female kuruma prawn *Marsupenaeus japonicus* (Crustacea: Decapoda: Penaeidae) during natural reproductive cycle and induced ovarian development by eyestalk ablation. *Fisheries Science*, 70, 372-380.

116. Okuno, A., Yang, W. J., Jayasankar, V., Saido-Sakanaka, H., Huong, D. T. T., Jasmani, S., Atmomarsono, M., Subramoniam, T., Tsutsui, N. & Ohira, T. (2002). Deduced primary structure of vitellogenin in the giant freshwater prawn, *Macrobrachium rosenbergii*, and yolk processing during ovarian maturation. *Journal of Experimental Zoology*, 292, 417-429.

117. Orton, F., Lutz, I., Kloas, W. & Routledge, E. J. (2009). Endocrine disrupting effects of herbicides and pentachlorophenol: *In vitro* and *in vivo* evidence. *Environmental Science & Technology*, 43, 2144-2150.

UNIVERSITY of the
WESTERN CAPE
http://etd.uwc.ac.za/

118. Palmer, B. D. & Palmer, S. K. (1995). Vitellogenin induction by xenobiotic estrogens in the red-eared turtle and African clawed frog. *Environmental Health Perspectives,* 103, 19-25.

119. Paolucci, M., Cristo, C. D. & Cosmo, A. D. (2002). Immunological evidence for progesterone and estradiol receptors in the freshwater crayfish *Austropotamobius pallipes*. *Molecular Reproduction and Development,* 63, 55-62.

120. Park, K. & Kwak, I. S. (2013). Expression of stress response HSP70 gene in Asian paddle crabs, *Charybdis japonica*, exposure to endocrine disrupting chemicals, bisphenol A (BPA) and 4-nonylphenol (NP). *Ocean Science Journal,* 48, 207-214.

121. Park, K., Kwak, T. S. & Kwak, I. S. (2014). Vitellogenin gene characterization and expression of Asian paddle crabs (*Charybdis japonica*) following endocrine disrupting chemicals. *Ocean Science Journal,* 49, 127-135.

122. Parks, L. G. & LeBlanc, G. A. (1996). Reductions in steroid hormone biotransformation/elimination as a biomarker of pentachlorophenol chronic toxicity. *Aquatic Toxicology,* 34, 291-303.

123. Parks, L. G., Ostby, J. S., Lambright, C. R., Abbott, B. D., Klinefelter, G. R., Barlow, N. J. & Gray, L. E. (2000). The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicological Sciences,* 58, 339-349.

124. Pateraki, L. E. & Stratakis, E. (1997). Characterization of vitellogenin and vitellin from land crab *Potamon potamios*: Identification of a precursor polypeptide in the molecule. *Journal of Experimental Zoology,* 279, 597-608.

125. Paxéus, N. (1996). Organic pollutants in the effluents of large wastewater treatment plants in Sweden. *Water Research,* 30, 1115-1122.

126. Pereira, P., de Pablo, H., Subida, M. D., Vale, C. & Pacheco, M. (2009). Biochemical responses of the shore crab (*Carcinus maenas*) in a eutrophic and metal-contaminated coastal system (Óbidos lagoon, Portugal). *Ecotoxicology and Environmental Safety,* 72, 1471-1480.

http://etd.uwc.ac.za/

127. Peretz, J., Gupta, R. K., Singh, J., Hernández-Ochoa, I. & Flaws, J. A. (2011). Bisphenol A impairs follicle growth, inhibits steroidogenesis, and downregulates rate-limiting enzymes in the estradiol biosynthesis pathway. *Toxicological Sciences,* 119, 209-217.

128. Pérez, S. & Beiras, R. (2010). The mysid *Siriella armata* as a model organism in marine ecotoxicology: Comparative acute toxicity sensitivity with *Daphnia magna*. *Ecotoxicology,* 19, 196-206.

129. Petrovic, M., Solé, M., López De Alda, M. J. & Barceló, D. (2002). Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: Integration of chemical analysis and biological effects on feral carp. *Environmental Toxicology and Chemistry,* 21, 2146-2156.

130. Porcher, J., Devillers, J. & Marchand-Geneste, N. (2009). Mechanisms of endocrine disruptions: A tentative overview. Devillers, J. (ed.), *In: Endocrine disruption modeling.* 1st ed. Boca Raton, USA: CRC Press, 27-30.

131. Qu, W., Bi, X., Sheng, G., Lu, S., Fu, J., Yuan, J. & Li, L. (2007). Exposure to polybrominated diphenyl ethers among workers at an electronic waste dismantling region in Guangdong, China. *Environment International,* 33, 1029-1034.

132. Quignot, N., Desmots, S., Barouki, R. & Lemazurier, E. (2010). Effects of endocrine disruptors on steroidogenic enzymes gene expression and on aromatase activity in two human cell lines. *Toxicology Letters,* 196, S318-S319.

133. Quintana, F. J., Basso, A. S., Iglesias, A. H., Korn, T., Farez, M. F., Bettelli, E., Caccamo, M., Oukka, M. & Weiner, H. L. (2008). Control of T_{reg} and T_{H17} cell differentiation by the aryl hydrocarbon receptor. *Nature,* 453, 65-71.

134. Raag, R., Appelt, K., Xuong, N. & Banaszak, L. (1988). Structure of the lamprey yolk lipid-protein complex lipovitellin-phosvitin at 2.8 Å resolution. *Journal of Molecular Biology,* 200, 553-569.

135. Ralls, K. & Mesnick, S. (2002). Sexual dimorphism. Perrin, W. F., Würsig, B. & Thewissen, J. G. M. (eds.), *In: Encyclopedia of Marine Mammals.* San Diego, USA: Academic Press, 1005.

http://etd.uwc.ac.za/

136. Reinecke, A., Snyman, R. & Nel, J. (2003). Uptake and distribution of lead (Pb) and cadmium (Cd) in the freshwater crab, *Potamonautes perlatus* (Crustacea) in the Eerste River, South Africa. *Water, Air, and Soil Pollution,* 145, 395-408.

137. Rheinheimer, G. (1998). Pollution in the Baltic sea. *Naturwissenschaften,* 85, 318-329.

138. Ricciardi, F., Matozzo, V., Binelli, A. & Marin, M. G. (2010). Biomarker responses and contamination levels in crabs (*Carcinus aestuarii*) from the Lagoon of Venice: An integrated approach in biomonitoring estuarine environments. *Water Research,* 44, 1725-1736.

139. Ricciardi, F., Matozzo, V. & Marin, M. G. (2008). Effects of 4-nonylphenol exposure in mussels (*Mytilus galloprovincialis*) and crabs (*Carcinus aestuarii*) with particular emphasis on vitellogenin induction. *Marine Pollution Bulletin,* 57, 365-372.

140. Rodgers-Gray, T. P., Jobling, S., Morris, S., Kelly, C., Kirby, S., Janbakhsh, A., Harries, J. E., Waldock, M. J., Sumpter, J. P. & Tyler, C. R. (2000). Long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. *Environmental Science & Technology,* 34, 1521-1528.

141. Roose, P., Cooreman, K. & Vyncke, W. (1998). PCBS in cod (*Gadus morhua*), flounder (*Platichthys flesus*), blue mussel (*Mytilus edulis*) and brown shrimp (*Crangon crangon*) from the Belgian continental shelf: Relation to biological parameters and trend analysis. *Chemosphere,* 37, 2199-2210.

142. Ross, P. S., Ellis, G., Ikonomou, M., Barrett-Lennard, L. & Addison, R. (2000). High PCB concentrations in free-ranging Pacific killer whales, *Orcinus orca*: Effects of age, sex and dietary preference. *Marine Pollution Bulletin,* 40, 504-515.

143. Roth, Z. & Khalaila, I. (2012). Identification and characterization of the vitellogenin receptor in *Macrobrachium rosenbergii* and its expression during vitellogenesis. *Molecular Reproduction and Development,* 79, 478-487.

144. Roth, Z., Parnes, S., Wiel, S., Sagi, A., Zmora, N., Chung, J. S. & Khalaila, I. (2010). N-glycan moieties of the crustacean egg yolk protein and their glycosylation sites. *Glycoconjugate Journal,* 27, 159-169.

http://etd.uwc.ac.za/

145. Rudel, R. A., Camann, D. E., Spengler, J. D., Korn, L. R. & Brody, J. G. (2003). Phthalates, alkylphenols, pesticides, polybrominated diphenyl ethers, and other endocrine-disrupting compounds in indoor air and dust. *Environmental Science & Technology*, 37, 4543-4553.

146. Samaraweera Mudiyanselage, C. H. (2012). *Production and characterization of phosphopeptides from egg yolk phosvitin.* Ph.D. thesis, Iowa State University.

147. Sanders, M., Du Preez, H. & Van Vuren, J. (1999). Monitoring cadmium and zinc contamination in freshwater systems with the use of the freshwater river crab, *Potamonautes warreni*. *Water SA Pretoria,* 25, 91-98.

148. Sastre, M., Reyes, P., Ramos, H., Romero, R. & Rivera, J. (1999). Heavy metal bioaccumulation in Puerto Rican blue crabs (*Callinectes* spp.). *Bulletin of Marine Science,* 64, 209-217.

149. Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B. & Lichtensteiger, W. (2001). *In vitro* and *in vivo* estrogenicity of UV screens. *Environmental Health Perspectives,* 109, 239-244.

150. Segner, H., Caroll, K., Fenske, M., Janssen, C., Maack, G., Pascoe, D., Schäfers, C., Vandenbergh, G., Watts, M. & Wenzel, A. (2003). Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: Report from the European IDEA project. *Ecotoxicology and Environmental Safety,* 54, 302-314.

151. Shibamoto, T., Yasuhara, A. & Katami, T. (2007). Dioxin formation from waste incineration. Ware, G. W., Whitacre, D. M. & Gunther, F. A. (eds.), *In: Reviews of Environmental Contamination and Toxicology.* New York, USA: Springer New York, 1-2.

152. Snyman, R., Reinecke, A. & Nel, J. (2002). Uptake and distribution of copper in the freshwater crab *Potamonautes perlatus* (Crustacea) in the Eerste River, South Africa. *African Zoology,* 37, 81-89.

153. Somerset, V., Van der Horst, C., Silwana, B., Walters, C. & Iwuoha, E. (2015). Biomonitoring and evaluation of metal concentrations in sediment and crab samples from the North-West Province of South Africa. *Water, Air, & Soil Pollution,* 226, 1-25.

http://etd.uwc.ac.za/

154. Sørmo, E. G., Jüssi, I., Jüssi, M., Braathen, M., Skaare, J. U. & Jenssen, B. M. (2005). Thyroid hormone status in gray seal (*Halichoerus grypus*) pups from the Baltic Sea and the Atlantic Ocean in relation to organochlorine pollutants. *Environmental Toxicology and Chemistry,* 24, 610-616.

155. Sørmo, E. G., Skaare, J. U., Jüssi, I., Jüssi, M. & Jenssen, B. M. (2003). Polychlorinated biphenyls and organochlorine pesticides in Baltic and Atlantic gray seal (*Halichoerus grypus*) pups. *Environmental Toxicology and Chemistry,* 22, 2789-2799.

156. Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N. & Serrano, F. O. (1995). The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. *Environmental Health Perspectives,* 103, 113-122.

157. Stokes, W. S. (2004). Selecting appropriate animal models and experimental designs for endocrine disruptor research and testing studies. *ILAR Journal,* 45, 387-393.

158. Sugiyama, S. i., Shimada, N., Miyoshi, H. & Yamauchi, K. (2005). Detection of thyroid system–disrupting chemicals using *in vitro* and *in vivo* screening assays in *Xenopus laevis*. *Toxicological Sciences,* 88, 367-374.

159. Tabira, Y., Nakai, M., Asai, D., Yakabe, Y., Tahara, Y., Shinmyozu, T., Noguchi, M., Takatsuki, M. & Shimohigashi, Y. (1999). Structural requirements of para-alkylphenols to bind to estrogen receptor. *European Journal of Biochemistry,* 262, 240-245.

160. Takahashi, A., Higashitani, T., Yakou, Y., Saitou, M., Tamamoto, H. & Tanaka, H. (2003). Evaluating bioaccumulation of suspected endocrine disruptors into periphytons and benthos in the Tama River. *Water Science and Technology,* 47, 71-76.

161. Thompson, J. R. & Banaszak, L. J. (2002). Lipid-protein interactions in lipovitellin. *Biochemistry,* 41, 9398-9409.

162. Tilton, F., Benson, W. H. & Schlenk, D. (2002). Evaluation of estrogenic activity from a municipal wastewater treatment plant with predominantly domestic input. *Aquatic Toxicology,* 61, 211-224.

163. Todd, B. D. & Davis, A. K. (2007). Sexual dichromatism in the marbled salamander, *Ambystoma opacum*. *Canadian Journal of Zoology,* 85, 1008-1013.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

164. Tufail, M., Naeemullah, M., Elmogy, M., Sharma, P., Takeda, M. & Nakamura, C. (2010). Molecular cloning, transcriptional regulation, and differential expression profiling of vitellogenin in two wing-morphs of the brown planthopper, *Nilaparvata lugens* Stål (Hemiptera: Delphacidae). *Insect Molecular Biology,* 19, 787-798.

165. Tyler, C., Jobling, S. & Sumpter, J. (1998). Endocrine disruption in wildlife: A critical review of the evidence. *Critical Reviews in Toxicology,* 28, 319-361.

166. Tyler, C. R. & Jobling, S. (2008). Roach, sex, and gender-bending chemicals: The feminization of wild fish in English rivers. *Bioscience,* 58, 1051-1059.

167. Valle, D. (1993). Vitellogenesis in insects and other groups: A review. *Memórias do Instituto Oswaldo Cruz,* 88, 1-26.

168. van het Schip, F. D., Samallo, J., Broos, J., Ophuis, J., Mojet, M., Gruber, M. & Geert, A. (1987). Nucleotide sequence of a chicken vitellogenin gene and derived amino acid sequence of the encoded yolk precursor protein. *Journal of Molecular Biology,* 196, 245-260.

169. van Stormbroek, T. (2007). *Bioaccumulation of metals in freshwater crabs (Potamonautes perlatus) of the Lourens River, Western Cape, South Africa.* M.Tech. thesis, Cape Peninsula University of Technology.

170. Vega-Morales, T., Sosa-Ferrera, Z. & Santana-Rodríguez, J. (2013). Evaluation of the presence of endocrine-disrupting compounds in dissolved and solid wastewater treatment plant samples of Gran Canaria Island (Spain). *BioMed Research International,* 2013, 1-15.

171. Viswanath, G., Chatterjee, S., Dabral, S., Nanguneri, S. R., Divya, G. & Roy, P. (2010). Anti-androgenic endocrine disrupting activities of chlorpyrifos and piperophos. *The Journal of Steroid Biochemistry and Molecular Biology,* 120, 22-29.

172. Voorspoels, S., Covaci, A., Maervoet, J., De Meester, I. & Schepens, P. (2004). Levels and profiles of PCBs and OCPs in marine benthic species from the Belgian North Sea and the Western Scheldt Estuary. *Marine Pollution Bulletin,* 49, 393-404.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

173. Vos, J. G., Dybing, E., Greim, H. A., Ladefoged, O., Lambré, C., Tarazona, J. V., Brandt, I. & Vethaak, A. D. (2000). Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. *CRC Critical Reviews in Toxicology,* 30, 71-133.

174. Voshell Jr, J. R., Smith, E. P., Evans, S. K. & Hudy, M. (1997). Effective and scientifically sound bioassessment: Opinions and corroboration from academe. *Human and Ecological Risk Assessment: An International Journal,* 3, 941-954.

175. Walag, A. & Canencia, M. (2016). Physico-chemical parameters and macrobenthic invertebrates of the intertidal zone of Gusa, Cagayan de Oro City, Philippines. *AES Bioflux,* 8, 71-82.

176. Warrier, S. & Subramoniam, T. (2002). Receptor mediated yolk protein uptake in the crab *Scylla serrata*: Crustacean vitellogenin receptor recognizes related mammalian serum lipoproteins. *Molecular Reproduction and Development,* 61, 536-548.

177. Warrier, S. R., Tirumalai, R. & Subramoniam, T. (2001). Occurrence of vertebrate steroids, estradiol 17β and progesterone in the reproducing females of the mud crab *Scylla serrata*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 130, 283-294.

178. Watanabe, Y., Kojima, H., Takeuchi, S., Uramaru, N., Ohta, S. & Kitamura, S. (2013). Comparative study on transcriptional activity of 17 parabens mediated by estrogen receptor α and β and androgen receptor. *Food and Chemical Toxicology,* 57, 227-234.

179. Wen, J. & Pan, L. (2016). Short-term exposure to benzo [a] pyrene causes oxidative damage and affects haemolymph steroid levels in female crab *Portunus trituberculatus*. *Environmental Pollution,* 208, 486-494.

180. Wilder, M. N., Okumura, T. & Tsutsui, N. (2010). Reproductive mechanisms in Crustacea focusing on selected prawn species: Vitellogenin structure, processing and synthetic control. *Aqua-BioScience Monographs,* 3, 73-110.

181. Williams, T. M. & Carroll, S. B. (2009). Genetic and molecular insights into the development and evolution of sexual dimorphism. *Nature Reviews Genetics,* 10, 797-804.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

182. Wojchowski, D. M., Parsons, P., Nordin, J. H. & Kunkel, J. G. (1986). Processing of pro-vitellogenin in insect fat body: A role for high-mannose oligosaccharide. *Developmental Biology,* 116, 422-430.

183. Wu, X., Chen, H., Liu, Z. & Cheng, Y. (2014). Immunorecognition and distribution of progesterone receptors in the Chinese mitten crab *Eriocheir sinensis* during ovarian development. *Journal of Shellfish Research,* 33, 35-43.

184. Xuereb, B., Bezin, L., Chaumot, A., Budzinski, H., Augagneur, S., Tutundjian, R., Garric, J. & Geffard, O. (2011). Vitellogenin-like gene expression in freshwater amphipod *Gammarus fossarum* (Koch, 1835): Functional characterization in females and potential for use as an endocrine disruption biomarker in males. *Ecotoxicology,* 20, 1286-1299.

185. Yang, X., Zhao, L., Zhao, Z., Hu, B., Wang, C., Yang, Z. & Cheng, Y. (2012). Immunolocalization of estrogen receptor α in *Neomysis japonica* oocytes and follicle cells during ovarian development. *Tissue and Cell,* 44, 95-100.

186. Yaron, Z. & Sivan, B. (2006). Reproduction. Evans, D. H. & Claiborne, J. B. (eds.), *In: The physiology of fishes.* 3rd ed. Boca Raton, USA: CRC Press, 353.

187. Ye, H., Huang, H., Li, S. & Wang, G. (2008). Immunorecognition of estrogen and androgen receptors in the brain and thoracic ganglion mass of mud crab, *Scylla paramamosain*. *Progress in Natural Science,* 18, 691-695.

188. Ye, H., Huang, H., Song, P. & Wang, G. (2010a). The identification and distribution of progesterone receptors in the brain and thoracic ganglion in the mud crab *Scylla paramamosain* (Crustacea: Decapoda: Brachyura). *Invertebrate Neuroscience,* 10, 11-16.

189. Ye, H., Song, P., Ma, J., Huang, H. & Wang, G. (2010b). Changes in progesterone levels and distribution of progesterone receptor during vitellogenesis in the female mud crab (*Scylla paramamosain*). *Marine and Freshwater Behaviour and Physiology,* 43, 25-35.

http://etd.uwc.ac.za/

190. Yehezkel, G., Chayoth, R., Abdu, U., Khalaila, I. & Sagi, A. (2000). High-density lipoprotein associated with secondary vitellogenesis in the hemolymph of the crayfish *Cherax quadricarinatus*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 127, 411-421.

191. Ying, G. G., Kookana, R. S. & Kumar, A. (2008). Fate of estrogens and xenoestrogens in four sewage treatment plants with different technologies. *Environmental Toxicology and Chemistry*, 27, 87-94.

192. Ying, G. G., Kookana, R. S., Kumar, A. & Mortimer, M. (2009). Occurrence and implications of estrogens and xenoestrogens in sewage effluents and receiving waters from South East Queensland. *Science of the Total Environment,* 407, 5147-5155.

193. Zhang, L., Ma, J., Takeuchi, M., Usui, Y., Hattori, T., Okunuki, Y., Yamakawa, N., Kezuka, T., Kuroda, M. & Goto, H. (2010). Suppression of experimental autoimmune uveoretinitis by inducing differentiation of regulatory T cells via activation of aryl hydrocarbon receptor. *Investigative Ophthalmology & Visual Science*, 51, 2109-2117.

194. Zhao, J. L., Ying, G. G., Wang, L., Yang, J. F., Yang, X. B., Yang, L. H. & Li, X. (2009). Determination of phenolic endocrine disrupting chemicals and acidic pharmaceuticals in surface water of the Pearl Rivers in South China by gas chromatography–negative chemical ionization–mass spectrometry. *Science of the Total Environment,* 407, 962-974.

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CHAPTER 3: MONOCLONAL ANTIBODY PRODUCTION

3.1 INTRODUCTION

3.1.1 Monoclonal antibody (mAb) production

Monoclonal antibodies (mAbs) have a very restricted specificity for their cognate antigen as they are produced by identical cells or clones. Thus, the results obtained from using these homogeneous antibodies are highly reproducible. In contrast to this, polyclonal antibodies (pAbs) may exhibit a less restricted specificity and results are less consistently reproducible than with mAbs. This is because pAbs are produced by different clones of B-lymphocytes (Ansar & Ghosh, 2013). Also, pAbs are produced in limited supply which calls for new batches to be produced often, each batch exhibiting a different specificity than the last. In this sense, mAbs are superior in that they can be produced limitlessly without compromising the specificity of the antibody (Ansar & Ghosh, 2013).

The production of mAbs is aimed at obtaining a homogenous population of antibody-producing cells that produce antibodies to a single epitope on an antigen. This involves immunisation of an animal model with the antigen of interest; isolation of the B-lymphocytes from the immunised animal; fusion of the B-lymphocytes with myeloma cells; selection of only the successfully fused hybridomas, causing unfused cells to die off. Although a few methods for hybridoma selection exists, the culturing of hybridomas in the selective medium hypoxanthine-aminopterin-thymidine (HAT) is the most commonly used (Shay, 1987). The selective growth in HAT medium is attributed to the ability of the hybridomas to make use of the salvage pathway for nucleic acid synthesis as aminopterin blocks the *de novo* nucleic acid synthesis pathway. Utilisation of the salvage pathway requires the enzyme hypoxanthine-guanine-phosphoribosyl transferase (HGPRT), which allows the cell to use hypoxanthine and thymidine as alternative substrates for nucleic acid synthesis. B-lymphocytes possess the HGPRT enzyme, but have a finite lifespan, whereas HAT-sensitive myeloma cells have an infinite lifespan, but lack the HGPRT enzyme. Thus, only successfully fused hybridomas will survive in HAT medium in that they are able to propagate infinitely and also possess the HGPRT enzyme (Ansar & Ghosh, 2013).

Myeloma cells that are not HAT-sensitive can be sensitised by culturing them in the presence of 8-azaguanine prior to fusion with B-lymphocytes (NRC, 1999). The HAT medium is then replaced with hypoxanthine-thymidine (HT) medium, after which the supernatant of the

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hybridomas is screened for antibody production by enzyme-linked immunosorbent assay (ELISA). Only the hybridomas that produce highly specific mAbs are selected and grown in isolated cultures to obtain single clones, which could then be grown in bulk for the intended use (NRC, 1999).

3.1.2 Applications of mAbs

Monoclonal antibodies have been widely used in biomedical science and medicine, ranging from applications in therapeutics and diagnostics to the purification of proteins. To date, a large number of mAbs are used in the treatment of cancer and autoimmune diseases. The diagnostic application of mAbs has proven very valuable for the identification and quantification of biomarkers (Zola & Thomson, 2001). A biomarker is any measurable change in a biological system, ranging from subcellular to the level of ecosystems and occurs due to a change in the cellular environment (Hamza-Chaffai, 2014). The detection of cellular or biochemical biomarkers through the use of antibodies has been widely used in the diagnosis of cancer (Havrilesky *et al.*, 2008; Sung *et al.*, 2014).

This application has also been extended to monitoring the effects of environmental stressors, such as heavy metal pollutants. The chaperone protein, heat shock protein 70 (Hsp70), is commonly used as a biomarker for heavy metal exposure and can be detected by antibodies (Downs *et al.*, 2001; Kefaloyianni *et al.*, 2005; Metzger *et al.*, 2012). Heavy metals also induce the formation of reactive oxygen species (ROS) and consequently upregulate ROS-responsive enzymes such as superoxide dismutase (SOD) to neutralise these oxygen radicals (Ercal *et al.*, 2001; Farombi *et al.*, 2007). As a result, SOD is an established biomarker of heavy metal pollution and can be quantified by antibodies (Kumagai *et al.*, 1997).

3.1.3 Vitellogenin (VTG) as a biomarker for oestrogenic endocrine disrupting chemicals (EDCs)

Vitellogenin is an established biomarker for oestrogenic endocrine disrupting chemicals (EDCs) in certain vertebrate species, such as fish. Vitellogenin (VTG) is a female-specific protein found in all oviparous organisms and is produced in response to oestrogens. The VTG gene is present in male organisms but silent (Yehezkel *et al.*, 2000; Auttarat *et al.*, 2006; Xuereb *et al.*, 2011). Thus the presence of VTG, in the circulation of male oviparous species, provides a good indication as to the presence of oestrogenic environmental pollutants. The zebrafish,

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Danio rerio, and medaka, *Oryzias latipes*, are commonly used as models for research that involve EDC-induced VTG production (Segner *et al.*, 2003; Scholz *et al.*, 2004). Crustaceans are also oviparous organisms capable of producing VTG and have been employed as models for environmental pollutants. However, very few studies have investigated whether crustaceans can be used effectively to monitor the effects of oestrogenic EDCs through VTG induction.

To date, female crustaceans have been the main focus of studies to use VTG as a biomarker in crustaceans, while male crustaceans have received very little attention. Hannas *et al.*, (2011) exposed female daphnids (*Daphnia magna*) to 25 different chemicals and found that only some induced *Vtg2* messenger ribonucleic acid (mRNA). Using gravid female mysid shrimp, Ghekiere *et al.*, (2006) found that exposure to nonylphenol induces Vn production, while oestrone (E_1) decreased the level of Vn. Another study reported a similar result of VTG induction by EDCs (Park *et al.*, 2014). However, owing to the complexity of VTG regulation, it has been suggested that female crustaceans might not be the most suitable model for the investigation of VTG induction (Lye *et al.*, 2008; Xuereb *et al.*, 2011). Concerning models for oestrogen-mimicking EDCs, this gives male crustaceans an added precedence over female crustaceans as studies have reported higher levels of accumulated pollutants in males than in females. Albeit few, some studies have reported the induction of VTG-like proteins in male crabs (Locatello *et al.*, 2009; Ricciardi *et al.*, 2010).

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3.1.4 Vitellogenin bioassays

Bioassays for VTG are fairly well established. Vitellogenin induction is commonly detected by VTG-specific ELISAs, VTG mRNA, or the alkali-labile phosphate (ALP) method (Scholz *et al.*, 2004). The appropriate detection method depends on the intent of the study. The rapid induction of VTG mRNA favours reverse transcription polymerase chain reaction (RT-PCR) as a detection method for short-term exposure studies (Bowman *et al.*, 2000). However, the process involved in obtaining the mRNA makes it an impractical approach for continuous sampling from the same organism at different time intervals (Iguchi *et al.*, 2005). Therefore, if the study requires repeated sampling over a long period of exposure, an ELISA would be a better approach. The secretion of VTG into the circulation makes it obtainable in a non-destructive manner and possible to obtain samples repeatedly from the same organism (Iguchi *et al.*, 2005).

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Alternatively, VTG can also be measured indirectly by the ALP assay. This assay involves the measurement of protein-bound alkali-labile phosphates and can be expressed as a fraction of the total protein content in the sample (Gagné & Blaise, 2000; Hallgren, 2009). An advantage of this assay is that it can be used with various tissues as well as different species (Gagné & Blaise, 2000).

Currently, two methods for VTG induction have been established. The *in vivo* method requires that organisms be kept in aquaria and exposed to the substances under investigation, simulating how the organisms would encounter these substances in a natural setting (Christiansen *et al.*, 2000; Scholz *et al.*, 2004). Furthermore, the *in vitro* method involves the exposure of hepatic cell cultures to potentially oestrogenic EDCs under investigation (Smeets *et al.*, 1999; Scholz *et al.*, 2004).

3.1.5 Rationale and aim of the study

Reports of natural and synthetic compounds capable of interfering with the endogenous hormones in animals have been made as early as the 1930s, with additional evidence emerging in the 1940s (Schueler, 1946; Sluczewski & Roth, 1948). Thus, the ability of certain chemicals to cause endocrine disruption cannot be considered an "emerging issue" within the water industry (Snyder *et al.*, 2003). Endocrine disrupting chemicals are still a major concern, though, as many of these compounds are almost constantly introduced into the environment, persist, and accumulate in exposed organisms. A number of the chemicals that were originally designed for the purpose of disrupting normal endocrine function, as is the case with pesticides and oral contraceptives, also cause inadvertent adverse effects on nontarget organisms (Clotfelter *et al.*, 2004). These effects include changes in behaviour, skewing the sex ratios in the offspring of exposed organisms, reproductive abnormalities, and potential population declines (Kidd *et al.*, 2007; Martinović *et al.*, 2007; Haeba *et al.*, 2008). This underpins the need for bioindicators as *in vivo* models that can demonstrate the true environmental hazard of EDCs effectively.

Invertebrates possess a number of the required characteristics that make them suitable for the role of bioindicators. They are ubiquitous, inhabiting a large number and variety of ecosystems; diverse in taxa and demonstrate a reasonable sensitivity to pollutants; close enough to the benthic zone to enable exposure to pollutants (Micheletti *et al.*, 2007). Given the role of certain invertebrates, such as crustaceans, in ecosystems, they can also reflect the impact of EDCs on other species at higher trophic levels in the food chain (Stokes, 2004). Their value as

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bioindicators is further supported in that they have vertebrate-type steroids and the corresponding receptors associated with the HPG axis – a phylogenetically conserved trait from which extrapolations can be made to higher, more complex organisms (Stokes, 2004).

The OECD conceptual framework for EDC testing is a good guideline for the testing and assessment of potentially adverse effects caused by EDCs. To this end, an *in vivo* approach, supported by *in vitro* assays, would form a solid foundation to establish whether the chemical(s) under investigation is capable of endocrine disruption. The ubiquity of *Potamonautes* (*P.*) *perlatus* in Southern Africa, and by extension the distribution of the *Potamonautes* genus in Africa, makes this freshwater crab an apt model for environmental pollution. Research on the use of *P. perlatus* as a bioindicator is limited and has only focused on its potential to accumulate heavy metals. To the best of the author's knowledge, no research has been conducted on *P. perlatus* with respect to EDCs and, therefore, it will be used as a model in this study. However, standardised, *in vitro* laboratory assays are also necessary to assess endocrine-relevant endpoints and for this purpose, mAbs will be produced for the quantification of VTG in *P. perlatus*. Thus, the aim of this study is to produce and characterise mAbs against vitellin (Vn) purified from the ovaries of *P. perlatus* females. These mAbs will then be used in developing a potentially rapid detection assay for the quantification of Vn as a biomarker for oestrogenic environmental pollutants.

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3.2 MATERIALS AND METHODS

3.2.1 Vitellin (Vn) isolation and purification

Ovaries from female *Potamonautes perlatus* crabs were collected and weighed. A protease inhibitor cocktail containing phosphate buffered saline (PBS; Lonza, Belgium) with protease inhibitors (1:200, v/v) (Sigma-Aldrich, USA) was prepared and added to the ovaries to give 100 mg of ovarian tissue per ml of buffer. The ovaries were mechanically homogenised (Tissuemiser, Fisher Scientific), centrifuged at 2 469 relative centrifugal force (rcf) for 20 mins, and stored at -80°C. Vitellin was isolated from ovarian homogenates by ammonium sulphate precipitation as described by Chen *et al.*, (2004). Saturated ammonium sulphate was added to the ovarian homogenate to produce a 50% saturated ammonium sulphate solution (SAS, v/v) and incubated for 1 hour on ice. Thereafter, the suspension was centrifuged at 16 100 rcf at 4°C for 25 mins. The supernatant was collected and saturated ammonium sulphate was added again to produce a 60% SAS (v/v). The suspension was incubated for another hour and

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subsequently centrifuged at 16 100 rcf at 4°C for 25 mins. 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 (MWCO) value of 10 kilodalton (kDa) (Thermo Scientific, USA). The purified Vn (pVn) was dialysed twice for 1-hour intervals with PBS. After dialysis, the pVn was aliquoted and stored at -80°C. A 25 µl aliquot was taken after every major step to construct a purification table.

3.2.2 Protein determination: Bradford assay

Total protein concentrations were determined by the Bradford method in a 96-well plate (Greiner Bio-One, Germany) with human serum albumin (HSA; Western Cape Regional Blood Bank, RSA) (1 mg/ml, v/v) as a standard. In one column, a doubling dilution of the HSA was performed with the protease inhibitor cocktail. In the subsequent columns, doubling dilutions of samples were also performed with the protease inhibitor cocktail. Bradford's reagent diluted in deionised water (dH_2O) (1:5, v/v) (Bio-Rad, Germany) was then added to each well at 100 µl per well and the total protein concentrations were determined spectrophotometrically at 595 nm (FLUOStar Omega, BMG Labtech). A standard curve was constructed from the optical densities (OD) of the HSA standards and the total protein concentrations of the samples were determined from the standard curve.

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3.2.3 Characterisation of purified Vn (pVn)

3.2.3.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The success of the Vn purification was determined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE; SDS-PAGE). The polyacrylamide gel consisting of a 7.5% separating gel and 8% stacking gel was cast using the Fluka SDS Gel Preparation Kit (Sigma-Aldrich, USA). Samples used were male haemolymph, female haemolymph, an unpurified ovarian homogenate, and pVn. All samples and molecular weight markers (Prism Ultra Protein Ladder, Abcam, UK) were mixed with sample buffer (60 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and thermally denatured by boiling at 95°C for 5 mins. After thermal denaturation, the samples, male and female haemolymph (10 µg), unpurified ovary (10 µg) and pVn (1 µg), were loaded into the lanes of the gel at a protein content per lane as indicated. The gel was run in a Tris-Glycine

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running buffer (25 mM Tris, 192 mM Glycine, pH 8.6, 0.1% SDS) (Sigma-Aldrich, USA) at 250 V and 20 mA for 90 mins at room temperature. Thereafter, the gel was fixed for 15 mins using Laemmli's fixative (20% methanol, 10% acetic acid) and proteins were visualised after 45 mins of staining with EZBlue (Sigma-Aldrich, USA).

3.2.3.2 Native PAGE

Native PAGE was performed to determine the native molecular weight of Vn. Native PAGE is performed under non-denaturing and non-reducing conditions, i.e. without denaturation and a reducing agent. This retains the structure and innate charge, as well as post-translational modifications such as glycosylation and lipidation. Samples were run on a 7.5% separating gel (1.5 M (w/v) Tris, pH 8.8) and 8% stacking gel (0.5 M (w/v) Tris, pH 6.8) cast with the Fluka SDS Gel Preparation Kit. Before loading the gel with samples, it was run for 1 hour in a Tris-Glycine running buffer (25 mM Tris, 192 mM Glycine, pH 8.3) without molecular weight markers or samples. All samples and molecular weight markers were mixed with sample buffer (0.125 M Tris, 20% glycerol, 0.004% bromophenol blue, pH 6.8) prior to loading the gel. For male and female haemolymph samples, a protein content equivalent to 2.5 µg was added per well and 0.5 µg per well for pVn. The gel was run at 250 V and 10 mA for 95 mins at room temperature. Afterwards, the gel was fixed and proteins were visualised as described in section 3.2.3.1.

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3.2.3.3 Lipid staining: Oil Red O

Vitellogenin and Vn are both lipoproteins, with lipids non-covalently bound to the protein portion (Thompson & Banaszak, 2002). Thus, in order to stain the lipid moiety, samples had to be run under non-reducing and non-denaturing conditions. The Oil Red O lipid stain was used to visualise the lipid moieties and was prepared according to the manufacturer's instructions (Sigma-Aldrich, USA). Briefly, male and female haemolymph samples (30 µg) and pVn (3 µg) were run on a native PAGE, as described in section 3.2.3.2, and subsequently transferred to a 0.45 µm supported nitrocellulose membrane (Santa Cruz Biotechnology, USA) in transfer buffer (25 mM Tris, 192 mM Glycine, pH 8.3, 10% methanol). The transfer was performed at room temperature for 125 mins at 30 V and 250 mA with a semi-dry transfer unit (TE70X Hoefer) and the membrane was briefly rinsed with dH_2O after the transfer. Oil Red O (Sigma-Aldrich, USA) was prepared according to the manufacturer's instructions. A 0.2%

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(w/v) Oil Red O stock solution was prepared in methanol. Thereafter, the staining solution was prepared by adding 10 ml of 1 N NaOH to the stock solution. The membrane was subsequently stained with the staining solution for 95 mins at room temperature. Excess staining solution was removed by incubating the membrane with destaining solution (3:1, glycerol: methanol, v/v). Red bands indicated a positive stain for lipids.

3.2.3.4 Concanavalin A (Con-A) lectin blot

Concanavalin A (Con-A) is a mannose-specific lectin that can be used to target glycosylated proteins, such as Vn (Okuno *et al.*, 2000). Thus, a lectin blot was performed with Con-A to detect Vn by its carbohydrate residues. Male and female haemolymph samples (2.5 µg) and pVn (0.5 µg) were run on a native PAGE, as described in section 3.2.3.2, and subsequently transferred onto a nitrocellulose membrane as described in section 3.2.3.3. After transfer, the membrane was stained with amido black (0.1% (w/v) amido black in 50% (v/v) methanol, 10% (v/v) acetic acid) for 5 mins to confirm a successful transfer and destained with amido black destain solution (20% (v/v) methanol, 7.5% (v/v) acetic acid). Subsequently, the membrane was incubated for 2 hours at room temperature with PBS containing 2% (v/v) HSA to block non-specific binding. Thereafter, the membrane was incubated overnight at 4°C with biotinylated Con-A (1:30 000, v/v) (Sigma-Aldrich, USA) prepared in diluent (0.1% (v/v) HSA in wash buffer). The following day, the membrane was washed 3 times with wash buffer (0.1% (v/v) Tween-20 (Merck, Germany) in PBS) for intervals of 15 mins each. The membrane was subsequently incubated for 1 hour with horseradish peroxidase (HRP)-conjugated StreptAvidin (1:5 000, v/v) (Southern Biotech, USA) prepared in diluent. Thereafter, the membrane was washed 4 times for intervals of 15 mins each and tetramethylbenzidine (TMB; KPL, USA) was used to visualise positive bands, indicative of glycosylated proteins.

3.2.3.5 Densitometry

ImageJ (version 1.50i) was used to semi-quantify the intensity of the major subunits of Vn by means of densitometry. Densitometry was done before and after the purification of Vn so as to determine the efficacy of the purification. Each measurement was repeated five times to allow for the calculation of statistical differences.

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3.2.4 Immunisation

Ethical clearance was obtained from the University of the Western Cape Ethics Committee to use BALB/c mice for immunisation purposes (Ref. number – ScRIRC06/06/11) and for the use of *P. perlatus* as an animal model (Ref. number – ScRIRC12/07/03). Two male BALB/c mice (University of Cape Town Animal Unit, RSA) were used for immunisation. Food and water were given *ad libitum* and the mice were kept under a 12-hour day/night cycle. The living conditions were ventilated and temperature controlled.

For immunisation purposes, pVn was prepared in PBS to a final concentration of 3 µg/200 µl. Of the pVn solution, 100 µl was used to perform the initial immunisation, while the other 100 µl were used for the booster immunisations. For the initial immunisation, the pVn solution was mixed (1:1, v/v) with complete Freund's adjuvant (CFA; Sigma-Aldrich, USA). Each mouse was then immunised intraperitoneally with 100 µl of this antigen preparation. This was followed by 2 booster immunisations which were of equal volume and antigen content but were suspended in incomplete Freund's adjuvant (IFA; Sigma-Aldrich, USA). Immunisations were administered 7 days apart. Prior to each immunisation, tail blood was collected in PBS (1:10, v/v). The final tail blood was collected 7 days after the last booster immunisation. The blood was centrifuged at 12 100 rcf for 5 mins and the supernatant was aliquoted and stored at -20°C to be used for ELISAs.

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3.2.5 Anti-pVn polyclonal serum

3.2.5.1 Success of pVn immunisation: enzyme-linked immunosorbent assay (ELISA)

The sera collected from the tail bleeds of immunised mice were used to perform an indirect ELISA to determine the success of the immunisation with pVn. Coating of 96-well microtiter plates (Nunc, Denmark) was done with pVn (10 µg/ml) in PBS at 50 µl per well and incubated overnight at 4°C. The following day, the ELISA was carried out at room temperature. Non-specific sites were blocked with 200 µl per well of 2% (w/v) HSA prepared in PBS and incubated for 1 hour, after which the plate was washed 5 times with wash buffer. The serum samples, pre-immunisation serum (1:500, v/v) and post-immunisation serum 1-3 (1:500, v/v), were prepared in diluent. A doubling dilution of each serum sample was performed in diluent to a final volume of 50 µl per well. The plate was then incubated for another hour and washed 5 times thereafter. A rabbit-anti-mouse immunoglobulin G (IgG) HRP-conjugate (1:5 000, v/v) was prepared in diluent and used as secondary antibody for the serum samples, at 50 µl per

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well. The plate was incubated for 1 hour with the rabbit-anti-mouse IgG secondary antibody. The plate was then washed 7 times and subsequently incubated for 15 mins with 100 µl of TMB per well for the colour development of positive reactions. The reaction was stopped after 15 mins with stop solution (0.5 M (v/v) sulphuric acid) and the OD was determined at 450 nm (MultiSkan EX, Thermo Electron).

3.2.5.2 Concanavalin A ligand binding assay

A Con-A ligand binding assay was used to validate the success of the plate coating with pVn. The Con-A ligand binding assay was performed as described in section 3.2.5.1 with the exception of biotinylated Con-A (1:30 000) prepared in diluent which was used for primary ligand binding. Also, HRP-conjugated StreptAvidin (1:5 000) was prepared in diluent and used in place of the secondary antibody and incubated for only 30 mins instead of 1 hour.

3.2.6 Cell culture and media

Sp2/0-Ag14 murine myelomas (Sigma-Aldrich, USA) were used as fusion partners for the splenic B-lymphocytes of pVn-immunised mice. The myelomas were cultured in full medium, i.e. Ex-Cell 610-HSF hybridoma medium (SAFC Biosciences, USA) supplemented with 2 mM glutamax (Gibco, USA), 1 X antibiotic/antimycotic (10 000 IU/ml penicillin, 10 mg/ml streptomycin, 25 µg/ml amphotericin B) (PAA, UK), 50 µg/ml gentamicin (Sigma-Aldrich, USA), 100 mM sodium pyruvate (Sigma-Aldrich, USA), and 10% heat-inactivated foetal bovine serum (HI-FBS; HyClone, GE Healthcare Life Sciences, USA). All cells were incubated at 37°C, 5% carbon dioxide, and 95% humidity.

Wash buffer and serum-free medium were prepared as media to maintain cell viability during fusion. Wash buffer comprised of Dulbecco's phosphate buffered saline (dPBS) (Whitehead Scientific, RSA) supplemented with 2 mM glutamax and 1 X antibiotic/antimycotic. Serum-free medium consisted of Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich, USA) supplemented with 2 mM glutamax, 1 X antibiotic/antimycotic, and 50 µg/ml gentamicin.

After fusion, the hybridomas were cultured in full medium supplemented with hypoxanthine-aminopterin-thymidine (HAT) (Sigma-Aldrich, USA) and 0.001% (v/v) mercaptoethanol (Sigma-Aldrich, USA). After 8 days, the HAT medium was replaced with full medium

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supplemented with hypoxanthine-thymidine (HT) (Sigma-Aldrich, USA) and 0.001% mercaptoethanol.

Freezing of cells was performed on ice and with cold freeze medium. Freeze medium consisted of Ex-Cell 610-HSF hybridoma medium supplemented with 2 mM glutamax, 1 X antibiotic/antimycotic, 10% (v/v) HI-FBS, and 10% (v/v) dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA).

3.2.7 Fusion

The immunised mice were sacrificed by cervical dislocation, after which the spleen was harvested and the splenic B-lymphocytes were used for fusion with the myelomas. The spleen was macerated in wash buffer and centrifuged at 347 rcf for 10 mins. The cell pellet was resuspended in wash buffer and a cell count was performed of both the splenic B-lymphocytes and the myelomas. The myelomas and splenic B-lymphocytes were added together in a 1:3 ratio, respectively, and made up to a final volume of 50 ml in serum-free medium. The cells were then centrifuged at 157 rcf for 10 mins. Thereafter, the supernatant was discarded and the cell pellet was resuspended over 1 min in 1 ml of polyethylene glycol (PEG; Sigma-Aldrich, USA). The cells and PEG were incubated at 37°C for 1 min to allow fusion to occur. Serum-free medium was added in a dropwise manner: first, 2 ml over 2 mins, then 2 ml over 1 min, and 2 ml over 30 secs. Thereafter, the suspension was made up to 50 ml of serum-free medium and centrifuged at 154 rcf for 10 mins. The pellet was resuspended in full medium supplemented with HAT and plated into five 96-well microtiter plates (Nunc, Germany) at 100 µl per well. After 48 hours, another 100 µl of full medium containing HAT was added to the wells. The medium was replaced with fresh full medium containing HAT 5 days and again 8 days after fusion.

Once hybridoma colonies were macroscopic, they were big enough to be screened for antibody production by ELISA. The first screening was done by indirect ELISA. All subsequent screening assays were done as competition and indirect ELISAs to obtain hybridoma colonies that produce mAbs with a high affinity and specificity.

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3.2.8 Screening of hybridoma cells for mAb production: ELISA

3.2.8.1 Indirect ELISA

Hybridoma cultures were screened for mAb production by indirect ELISA to determine which cultures produced the most specific mAbs for pVn. The ELISA was carried out as described in section 3.2.5.1 with the exception of hybridoma culture supernatant being used instead of the serum obtained from tail bleeds. The serum samples obtained from the mice tail bleed were prepared in diluent (1:1 000, v/v); pre-immunisation serum was used as a negative control and post-immunisation serum 3, was used as a positive control in all the ELISAs.

3.2.8.2 Competition ELISA

Hybridoma cultures were also screened to determine which cultures produced mAbs with the highest affinity for pVn. The ELISA was carried out as described in section 3.2.5.1 with the exception of adding 50 µl of pVn (20 µg/ml), prepared in diluent, to each well along with 50 µl of the hybridoma culture supernatant.

3.2.9 Cloning

After screening, the hybridoma cultures with the highest affinity and specificity for pVn were selected for cloning. The objective of cloning was to isolate the hybridoma colonies that demonstrated high affinity and specificity and culture them as single colonies per well in a 96-well microtiter plate. The hybridoma clones were named after the wells that they were cultured from.

Hybridomas screening positive for pVn were resuspended in their culture medium and a cell count was performed using a haemocytometer. A pre-dilution (1:100, v/v) was done in full medium supplemented with HT and subsequently diluted again (v/v) to obtain 5 cells per 100 µl of medium. These cells were then seeded into 48-well microtiter plates (Nunc, Germany) at 100 µl per well. After 96 hours, another 100 µl of HT supplemented full medium was added to each well. Thereafter, 50 µl of medium was added every 72 hours until colonies were once again macroscopic and ready for screening. This screening and cloning process was repeated 4 times until 7 hybridoma clones that demonstrated the highest specificity and affinity for pVn were obtained.

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3.2.10 Freezing of hybridoma clones

Once the hybridoma clones that produced anti-pVn mAbs with the highest specificity and affinity were obtained, they were frozen in freeze medium that was prepared as described in section 3.2.6. Cells were centrifuged at 347 rcf for 5 mins and the pellet resuspended in cold freeze medium. The cell suspension was aliquoted as 1 ml per cryovial (Nunc, Germany) and stored at -80°C. A single cryovial was thawed and seeded one week after freezing to determine the success of freezing.

3.2.11 Concentration and partial purification of anti-pVn mAb

The anti-pVn mAbs were concentrated and partially purified from hybridoma culture supernatant with centrifugal filter units (MWCO: 10 kDa; Merck, Germany). The hybridoma clone that exhibited the highest specificity and affinity for pVn and was cultured in large culture flasks (SPL Life Sciences, Korea). Culturing was done in full medium as described for the Sp2/0-Ag14 murine myelomas in section 3.2.6 but without FBS.

Once confluent, the supernatant was collected from each flask and centrifuged at 2 469 rcf for 5 mins to remove big particles of debris. Thereafter, the supernatant was transferred to centrifugal filter units and centrifuged at 2 510 rcf at 4°C in 30 min increments until the supernatant was concentrated to approximately 1 ml. Saturated ammonium sulphate was then added to produce a 50% (v/v) SAS, which was incubated overnight at 4°C. The following day, the 50% SAS was centrifuged at 16 100 rcf at 4°C for 25 mins. The supernatant was removed and the pellet was resuspended in PBS to be dialysed. Dialysis was performed as described in section 3.2.1. A 25 µl aliquot was taken after every major step to construct a purification table.

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3.2.12 Characterisation of anti-pVn mAbs for Vn

The sensitivity of the mAbs for Vn, as well as whether the mAbs could detect VTG in the female haemolymph, was determined by a competition ELISA. Confirmation of the ELISA results and further characterisation of the anti-pVn mAbs was done by western blotting.

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3.2.12.1 Sensitivity of anti-pVn mAbs for Vn and VTG: competition ELISA

A competition ELISA was performed to determine the sensitivity of the mAbs for Vn and to see if the mAbs could detect VTG in the female haemolymph. The 96-well microtiter plates were coated with pVn as the primary antigen and competing antigens were added along with the anti-pVn mAbs to allow for competition of the mAbs between the soluble and plate-bound antigens.

The competition ELISA was carried out as described in section 3.2.8.2, except that competing antigens were added to the plate at 50 µl per well. Competing antigens were pVn (20 µg/ml, v/v), or male or female haemolymph (100 µg/ml, v/v), prepared in diluent. After the addition of the competing antigens, a three-fold dilution of each antigen was performed in diluent at a final volume of 50 µl per well. The female haemolymph used was previously confirmed to have VTG.

3.2.12.2 Western blot

To determine if the anti-pVn mAbs could detect the native form of Vn, a western blot was performed with the anti-pVn mAbs and compared to a western blot with the pVn-antiserum, which was used as an internal control.

The western blot was carried out as described in section 3.2.3.4, except that membranes were incubated overnight at 4°C with pVn-antiserum (1:8 000, v/v) or anti-pVn mAbs (0.1 µg/ml), respectively, prepared in diluent. Positive bands visualised by TMB colourimetric substrate indicated binding of the pVn-antiserum or the anti-pVn mAbs to the native structure of those proteins.

3.3 RESULTS & DISCUSSION

3.3.1 Isolation and purification of Vn

Vitellin is commonly purified by ultracentrifugation, column chromatography, ammonium sulphate precipitation, or a combination of these methods (Tsukimura *et al.*, 2000; Vazquez-Boucard *et al.*, 2002; Serrano-Pinto *et al.*, 2003; Chen *et al.*, 2004; Zmora *et al.*, 2007; Ding *et al.*, 2010; Revathi *et al.*, 2012). However, it has been remarked that the number and size of Vn subunits might differ depending on the purification method used (Kawazoe *et al.*, 2000; Tsukimura, 2001; García-Orozco *et al.*, 2002). In this study, Vn was purified from the ovaries

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of vitellogenic *P. perlatus* females by means of ammonium sulphate precipitation. Ammonium sulphate precipitation is a purification technique that makes use of the differences in the solubility of proteins in a heterogeneous solution. Therefore, different proteins will precipitate out of solution at different percentages of a SAS. In this way, the protein of interest can be isolated with a reasonable purity. A purification table demonstrates how effectively target proteins were purified from a crude protein solution. In this study, Vn was isolated from a crude ovarian homogenate.

The crude ovarian homogenate was a concentrated protein solution of a small volume (table 3.1). Despite having an increased volume after the purification, the protein concentration of the purified product was still reasonably high (table 3.1). This was also evident in the need to load a higher protein content of the crude ovarian homogenate (i.e. 10 µg) onto the SDS-PAGE, compared to the lower protein content that was required for pVn (i.e. 1 µg). After running the SDS-PAGE, the OD of the major pVn subunits before and after purification was determined by means of densitometry. The OD of the different subunits, together with the protein content loaded onto the SDS-PAGE, could then be used to calculate the purification factor (PF). The OD of each major subunit was divided by the protein content that was loaded onto the SDS-PAGE. This is represented for each major subunit as the OD/µg protein. This value was subsequently expressed as a factor of the crude ovarian homogenate. In this way, the PF demonstrates how effectively each major pVn subunit was purified by comparing it to the crude ovarian homogenate.

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A PF value lower than 1 demonstrates that some of the desired protein was lost during the purification process and that the purification was, therefore, not so effective. If very little or none of the desired protein was lost during the purification process, the PF value would be higher than 1, demonstrating an effective purification. From the PFs, it is evident that each of the major pVn subunits were purified effectively as each subunit had a PF higher than its corresponding subunit from the crude ovarian homogenate (table 3.1). These PFs also correspond to the intensity of each of the major pVn subunits shown in figure 3.1 further supporting the efficacy of the purification. However, further investigation is required to determine whether the purified proteins are the major subunits of Vn.

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Table 3.1. Purification table for Vn purified from the ovaries of vitellogenic *P. perlatus* females. This was accomplished by mechanical homogenisation, ammonium sulphate precipitation, and dialysis. The OD of each major subunit was determined by densitometry with ImageJ (described in section 3.2.3.5).

			OD of 98 kDa subunit		OD of 78 kDa subunit		OD of 75 kDa subunit	
Sample	**Protein (mg/ml)**	**Volume (ml)**	**OD/µg protein**	**PF**	**OD/µg protein**	**PF**	**OD/µg protein**	**PF**
Crude ovarian homogenate	10.72	0.83	794.76	1.00	1 739.16	1.00	1 722.60	1.00
Purified vitellin (pVn)	2.70	1.37	5 925.32	7.46	7 777.64	4.47	2492.79	1.45

3.3.2 Characterisation of pVn

3.3.2.1 SDS-PAGE

Haemolymph is the plasma component of the circulatory system of some arthropods and consists of a number of proteins (Horn & Kerr, 1969; Fredrick & Ravichandran, 2012). The main constituent is the copper-containing respiratory protein, haemocyanin, and is it present in the haemolymph of both male and female crustaceans (Horn & Kerr, 1969; Zatta, 1984). In the lanes representing the male and female haemolymph, bands were observed at approximately 75 kDa (figure 3.1). The molecular weights suggest that these proteins are most likely haemocyanins as it corresponds to those of crustacean haemocyanins ranging from 72-79 kDa, with an average of 75 kDa (Salvato *et al.*, 1972; Markl *et al.*, 1979; Markl & Decker, 1992; Chen *et al.*, 2007).

Moreover, the haemolymph is also a vehicle for the female-specific protein VTG, synthesised in the hepatopancreas (HP) of crustaceans and transported to the ovaries via the haemolymph (Yang *et al.*, 2000). In the female haemolymph lane, a protein of approximately 188 kDa was found, whereas, this protein was absent from the male haemolymph lane (figure 3.1). A female-specific protein of a similar molecular weight has been reported in the haemolymph of various other female crustaceans (Lee & Puppione, 1988; Spaziani *et al.*, 1995; Pateraki & Stratakis, 1997; Abdu *et al.*, 2000; Kawazoe *et al.*, 2000; Yehezkel *et al.*, 2000; Okumura *et al.*, 2004; Tiu *et al.*, 2009). Thus, the 188 kDa protein is likely the high molecular weight subunit of VTG but further characterisation would be required to confirm this.

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Once VTG reaches the ovaries, it is taken up by the ovaries through receptor-mediated endocytosis (RME). Inside the ovaries, VTG is cleaved into various subunits known as vitellins, and these make up the major yolk proteins (Yang *et al.*, 2000). The number of Vn subunits in crustaceans differ between species and also vary based on the method of purification that was used (Chen & Kuo, 1998; Kawazoe *et al.*, 2000; Longyant *et al.*, 2000; Tsukimura, 2001). Vitellin was purified from the ovaries of *P. perlatus* females by ammonium sulphate precipitation and the success was assessed by SDS-PAGE. In the lane representing pVn, three bands were observed with molecular weights corresponding to approximately 75 kDa, 78 kDa, and 98 kDa (figure 3.1). These molecular weights are in agreement with the size of Vn subunits that has been reported for other crustaceans (Qiu *et al.*, 1997; Avarre *et al.*, 2003; Girish *et al.*, 2014). Similarly, purification of Vn from the ovaries of the Chinese mitten crab, *Eriocheir sinensis*, yielded Vn subunits with molecular weights of 74 kDa and 97 kDa, and the Vn subunits of the redclaw crayfish, *Cherax quadricarinatus*, was reported to be 75 kDa and 95 kDa (Serrano-Pinto *et al.*, 2003; Chen *et al.*, 2004). The proteins isolated from the ovary of *P. perlatus* in this study were thus in agreement with the previous data obtained for other crustaceans.

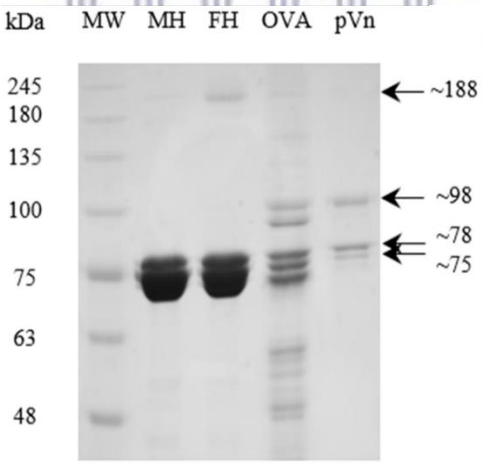

Figure 3.1. Analysis of Vn purified from the ovaries of *P. perlatus* by SDS-PAGE (7.5%). Molecular weight marker (MW), male haemolymph (MH), female haemolymph (FH), unpurified ovarian homogenate (OVA), and purified vitellin (pVn). The arrows (from the top) indicate VTG at approximately 188 kDa and Vn subunits at approximately 98 kDa, 78 kDa, and 75 kDa.

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3.3.2.2 Native PAGE

Gel electrophoresis was performed under non-denaturing and non-reducing conditions to determine the native molecular weight of *P. perlatus* Vn. Two proteins of a similar molecular weight were found, one in the female haemolymph and the other in the lane representing pVn (figure 3.2). The presence of this protein in the female haemolymph and its absence from the male haemolymph indicates that this might be a female-specific protein. It has been reported that the female-specific protein, VTG, is comprised of 2 polypeptides and usually consists of subunits of approximately 80 and 180 kDa in size (Yehezkel *et al.*, 2000). Zmora *et al.*, (2007) reported a similar result where two subunits with a calculated mass of 78.5 and 207.3 kDa were transported in the haemolymph to the ovaries. Inside the ovaries, only the 207.3 kDa subunit was cleaved into smaller subunits, while the 78.5 kDa subunit remains intact. These proteins then constitute Vn. Auttarat *et al.*, (2006) also reported VTG as having a 78 kDa subunit in the female haemolymph.

In the current study, it is difficult to discern whether a subunit of approximately 78.5-80 kDa was found exclusively in the female haemolymph (figure 3.1) However, as the VTG polypeptides are transported to the ovaries, the presence of the 78 kDa subunit in the lane representing pVn is in accordance with the abovementioned studies (figure 3.1). Moreover, the presence of the approximate 188 kDa female-specific protein as seen in the SDS-PAGE is also in accordance with these previous studies (figure 3.1). Therefore, the female-specific protein found in the female haemolymph on the native PAGE has been calculated to be of approximately 266 kDa (figure 3.2).

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Vitellogenin and Vn have a similar electrophoretic mobility (Lee & Watson, 1994; Pateraki & Stratakis, 1997; Auttarat *et al.*, 2006). In decapod crustaceans, the native form of Vn has a molecular weight that ranges between 283 and 600 kDa (Tsukimura, 2001). Okumura *et al.*, (2004) estimated Vn of the humpback shrimp, *Pandalus hypsinotus*, to be 295 kDa by gel filtration chromatography. Purification of Vn from the kuruma prawn, *Penaeus japonicus*, by gel filtration and ion exchange chromatography, revealed a native molecular weight of 530 kDa (Kawazoe *et al.*, 2000). Thus, the band that was found in the lane representing pVn from *P. perlatus* indicates an approximate molecular weight of 266 kDa and also suggests that it is the native form of Vn (figure 3.2).

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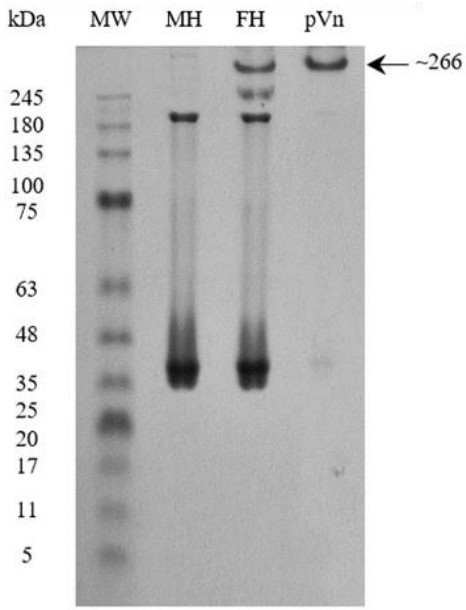

Figure 3.2. Native PAGE (7.5%) of *P. perlatus* haemolymph samples and pVn stained with EZBlue. Molecular weight marker (MW), male haemolymph (MH), female haemolymph (FH), purified vitellin (pVn). The arrow indicates VTG and pVn at approximately 266 kDa.

3.3.2.3 Lipid staining: Oil Red O

Vitellogenin and Vn are both lipidated proteins and an Oil Red O lipid stain was used to identify these lipoproteins (Lee *et al.*, 1997; Chen & Kuo, 1998). A native PAGE was performed and the separated proteins were transferred to a nitrocellulose membrane to stain the lipids. This assay showed that the 266 kDa bands in both the lanes representing the female haemolymph and pVn were lipidated (figure 3.3). Moreover, no positive bands were detected in the male haemolymph, suggesting the identities of these proteins to be VTG and Vn, respectively.

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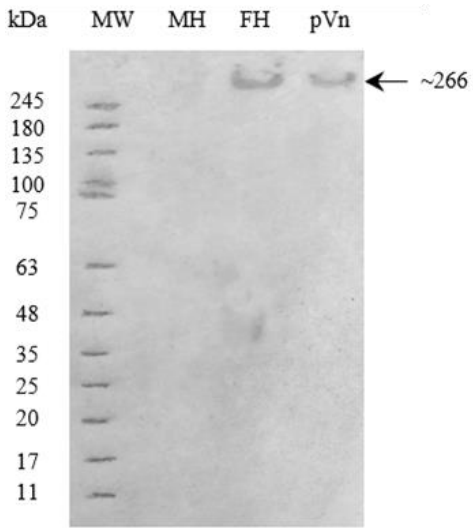

Figure 3.3. Oil Red O lipid stain of *P. perlatus* haemolymph samples and pVn from a 7.5% native PAGE. Molecular weight marker (MW), male haemolymph (MH), female haemolymph (FH), purified vitellin (pVn). The arrow indicates VTG and pVn at approximately 266 kDa.

3.3.2.4 Concanavalin A lectin blot

Vitellogenin and Vn are both glycosylated proteins and this post-translational modification was used to identify them (Lee *et al.*, 1997; Chen & Kuo, 1998). After proteins were transferred to a nitrocellulose membrane from a native PAGE, the membrane was used to do a Con-A lectin blot to detect for any glycosylated proteins in their native conformation. The Con-A positively reacted with the 266 kDa bands in both the lanes representing the female haemolymph and pVn, indicating that these proteins were glycosylated (figure 3.4). Similar to the previous results, no positive bands were detected in the male haemolymph, supporting the identities of these proteins as VTG and Vn, respectively. Collectively, these results strongly suggest that Vn was successfully purified from the ovary of *P. perlatus* and that it can be used for the production of mAbs.

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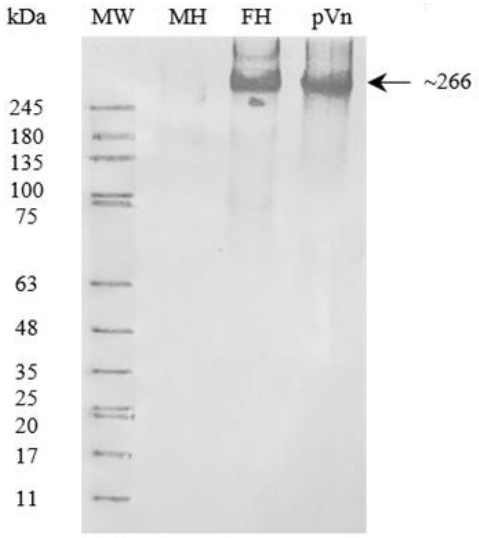

Figure 3.4. Concanavalin A lectin blot of *P. perlatus* haemolymph and pVn from a 7.5% native PAGE. Molecular weight marker (MW), male haemolymph (MH), female haemolymph (FH), purified vitellin (pVn). The arrow indicates VTG and pVn at approximately 266 kDa.

3.3.3 The production of polyclonal antiserum against pVn

The function of the immune system is to defend the host against foreign materials, i.e. anything that is not recognised as the host's own tissues, or 'self'. These foreign materials, called antigens, are able to react with and bind to components of the immune system, such as antibodies. Antigens are able to react with the immune system but may not stimulate an immune response unless they are sufficiently immunogenic. Immunogens are compounds that have certain characteristics that cause them to be recognised as foreign by the immune system and can, consequently, induce an immune response. Accordingly, all immunogens are antigenic but not all antigens are immunogenic. Factors that influence the immunogenicity of an antigen include foreignness, chemical nature and complexity, and molecular weight (Murphy, 2011).

Foreign compounds stimulate the immune system as they are 'non-self' and, therefore, considered harmful to the host. The host's immune system is tolerant toward its own tissues but compounds with a greater phylogenetic or species difference between the host's self-proteins and the antigen induce a stronger immune response (Murphy, 2011). Vitellin is a yolk protein produced in egg-laying organisms and is not found in mammals. Thus, upon immunisation with pVn, the mice's immune system recognised pVn as foreign and effectively induced an immune response. This was evident by the consecutive increase in the concentration of polyclonal antibodies from the pVn-immunised mice (figure 3.5). However, this increase in antibody production is most likely attributed to a combination of the foreignness, molecular

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mass and chemical nature of Vn. Therefore, it is not surprising that immunisation of the mice with pVn induced an effective immune response.

Generally, high molecular weight antigens favour an antibody-driven immune response (Young & Atassi, 1982; Vasanthakumari, 2007; Hermanson, 2008). Most low molecular weight antigens, known as haptens, require conjugation to large carrier molecules to increase their molecular weight, thereby inducing an immune response (Strynadka *et al.*, 1988; Schaaper *et al.*, 1989; Houen *et al.*, 2003). Vitellin and its precursor VTG are very high molecular weight proteins that range from 283 kDa – 700 kDa and according to the literature cited above, it will make a good candidate for antibody production (Lee *et al.*, 1997; Pateraki & Stratakis, 1997; Tsukimura, 2001; Ferré *et al.*, 2012).

In addition to molecular weight, the chemical nature and complexity of an antigen also contribute to its ability to evoke an effective immune response. Among macromolecules, proteins are the most immunogenic. Their immunogenicity is attributed to the vast heterogeneity that can come from the arrangement of amino acids to make up a protein antigen (Stanley, 2002). Conversely, homopolymeric compounds, like nylon and Teflon®, are nonimmunogenic as they do not form higher order structures like proteins do (Stevens, 2009). Both VTG and Vn are proteins but their post-translational modifications add to their chemical complexity and further contribute to their immunogenicity. They are lipidated, glycosylated, and contain conjugated carotene moieties (Wallace *et al.*, 1967; Chen *et al.*, 2004; Roth *et al.*, 2010). It is important to note that it is unlikely that each of these factors alone made pVn immunogenic, however, the same cannot be said for VTG as its immunogenicity was not part of this study. It is more plausible to conclude that the combination of phylogenetic difference, a high molecular weight, and chemical complexity was responsible for the induction of an effective antibody response against pVn.

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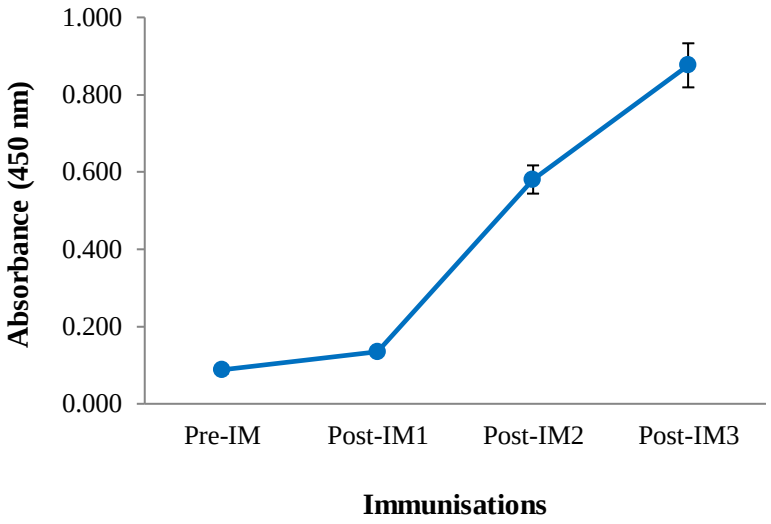

Figure 3.5. Change in pVn-antisera over time, collected prior to each immunisation, i.e. pre-immunisation serum (Pre-IM), post-immunisation serum 1 (Post-IM1), post-immunisation 2 serum (Post-IM2), and post-immunisation 3 serum (Post-IM3). Sera were diluted (1:8 000, v/v) in diluent. Each data point represents the mean ± SEM of a duplicate measurement.

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3.3.4 Success of mAb production

After fusion, the hybridomas were plated into five 96-well plates and after each screening, the wells that had an OD higher than the positive control were designated as positive wells. These were subsequently cloned and rescreened. The hybridoma colonies were cloned four times and yielded six hybridoma clones that produced mAbs with high specificity and affinity for pVn. The success of cloning was determined for each of the six highest clones (tables 3.2-3.7). The affinity and specificity of each of the six highest clones were also determined so as to establish which clone would be best suited for a pVn and VTG detection system (figures 3.6-3.11). The percentage yield after each cloning is a reflection of how effective the previous cloning was. It is expressed as the number of wells that were identified as positive by the screening after each cloning. A higher percentage yield indicates that the previous cloning was effective.

All the clones demonstrated an increased percentage yield after each cloning (tables 3.2-3.7). This indicates that each of the cloning attempts was successful in isolating the hybridomas responsible for good antibody production against pVn. Clone 2H4A3B6H3 demonstrated the highest number of positive wells after the third cloning, but not all of the highest clones were isolated as effectively. This means that the hybridoma colony responsible for producing the anti-pVn mAb was more effectively isolated and is more homogenous than the other hybridoma

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colonies. It is evident by the presence of ten positive wells that were identified after the third screening, while the other clones did not yield as many positive wells after the third cloning.

Some of the other clones also demonstrated a high number of positive wells after each cloning (tables 3.2 and 3.5). However, in addition to looking at the percentage yield of a clone, it is important to take into account the specificity and affinity that the clone demonstrated for pVn and VTG. In light of that, clone 2H4A3B6H3 exhibited a good affinity for pVn, an affinity for VTG similar to that of pVn, and no affinity for the male haemolymph. Other clones such as 2H4A3E4H11 did not detect pVn and VTG in the female haemolymph as effectively (figure 3.10). Furthermore, this clone also reacted with proteins in the male haemolymph, which means that it will detect proteins that it should not. Therefore, clone 2H4A3E4H11 would not be suitable for a pVn and VTG detection system. Clone 2H4A3B6H3 was grown in large culture so as to concentrate and partially purify the mAb produced by this clone. This mAb was further characterised so that it can be used in a pVn and VTG detection system.

Table 3.2. The success of isolation of hybridoma clone 2H4A3B4F7 by cloning. Wells with the highest specificity and affinity, as determined by ELISA, were selected for cloning. Wells that had an OD higher than the positive control were designated as positive wells. These wells were selected for cloning and each well was cloned into fourty seven new wells. The percentage of wells screened positive after cloning represent the number of positive wells expressed as a percentage of the number of wells that were plated from the previous screening.

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Clone: 2H4A3B4F7	**Number of wells plated**	**Number of wells that had growth and were selected for screening**	**Number of wells that screened positive**	**Percentage of wells screened positive (%)**	**OD of well selected for cloning**
After fusion (At 1st screening)	96	96	6	6.25	3.06
After 1st cloning (At 2nd screening)	47	6	1	16.67	3.01
After 2nd cloning (At 3rd screening)	47	12	4	33.33	0.84
After 3rd cloning (At 4th screening)	47	5	5	100	2.80
SUMMARY	237	119	16	13.45	2.43 (Average OD)

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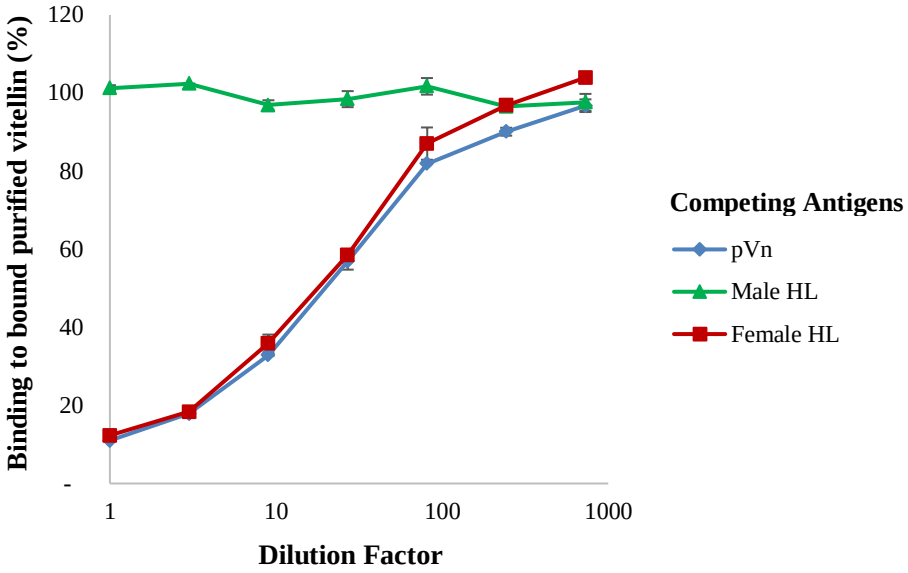

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Figure 3.6. Competition ELISA of anti-pVn mAb produced by clone 2H4A3B4F7 for bound purified vitellin (pVn, 10 µg/ml) and 3-fold diluted competing antigens. Competing antigens used were: male haemolymph (▲) (male HL, 100 µg/ml), female haemolymph (■) (female HL, 100 µg/ml), and purified vitellin (◆) (pVn, 20 µg/ml) as standard. Each data point represents the mean ± SEM of a triplicate measurement.

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Table 3.3. The success of isolation of hybridoma clone 2H4A3B6H3 by cloning. Wells with the highest specificity and affinity, as determined by ELISA, were selected for cloning. Wells that had an OD higher than the positive control were designated as positive wells. These wells were selected for cloning and each well was cloned into fourty seven new wells. The percentage of wells screened positive after cloning represent the number of positive wells expressed as a percentage of the number of wells that were plated from the previous screening.

Clone: 2H4A3B6H3	**Number of wells plated**	**Number of wells that had growth and were selected for screening**	**Number of wells that screened positive**	**Percentage of wells screened positive (%)**	**OD of well selected for cloning**
After fusion (At 1st screening)	96	96	6	6.25	3.06
After 1st cloning (At 2nd screening)	47	6	1	16.67	3.01
After 2nd cloning (At 3rd screening)	47	12	4	33.33	1.16
After 3rd cloning (At 4th screening)	47	10	10	100	3.05
SUMMARY	237	124	21	16.94	2.57 (Average OD)

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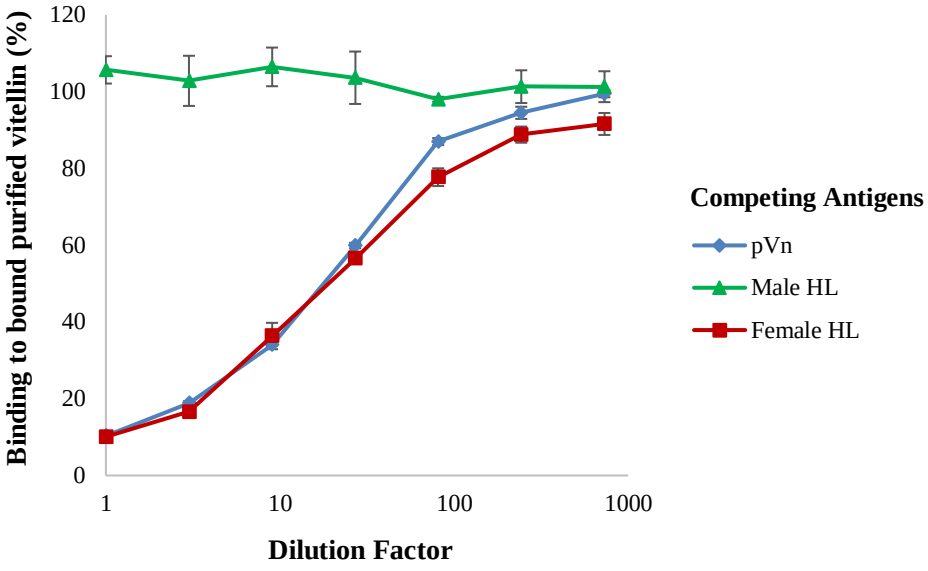

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Figure 3.7. Competition ELISA of anti-pVn mAb produced by clone 2H4A3B6H3 for bound purified vitellin (pVn, 10 µg/ml) and 3-fold diluted competing antigens. Competing antigens used were: male haemolymph (▲) (male HL, 100 µg/ml), female haemolymph (■) (female HL, 100 µg/ml), and purified vitellin (◆) (pVn, 20 µg/ml) as standard. Each data point represents the mean ± SEM of a triplicate measurement.

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Table 3.4. The success of isolation of hybridoma clone 2H4A3E4H11 by cloning. Wells with the highest specificity and affinity, as determined by ELISA, were selected for cloning. Wells that had an OD higher than the positive control were designated as positive wells. These wells were selected for cloning and each well was cloned into fourty seven new wells. The percentage of wells screened positive after cloning represent the number of positive wells expressed as a percentage of the number of wells that were plated from the previous screening.

Clone: 2H4A3E4H11	**Number of wells plated**	**Number of wells that had growth and were selected for screening**	**Number of wells that screened positive**	**Percentage of wells screened positive (%)**	**OD of well selected for cloning**
After fusion (At 1st screening)	96	96	6	6.25	3.06
After 1st cloning (At 2nd screening)	47	6	1	16.67	3.01
After 2nd cloning (At 3rd screening)	47	12	4	33.33	1.02
After 3rd cloning (At 4th screening)	47	3	3	100	2.92
SUMMARY	237	117	14	11.97	2.50 (Average OD)

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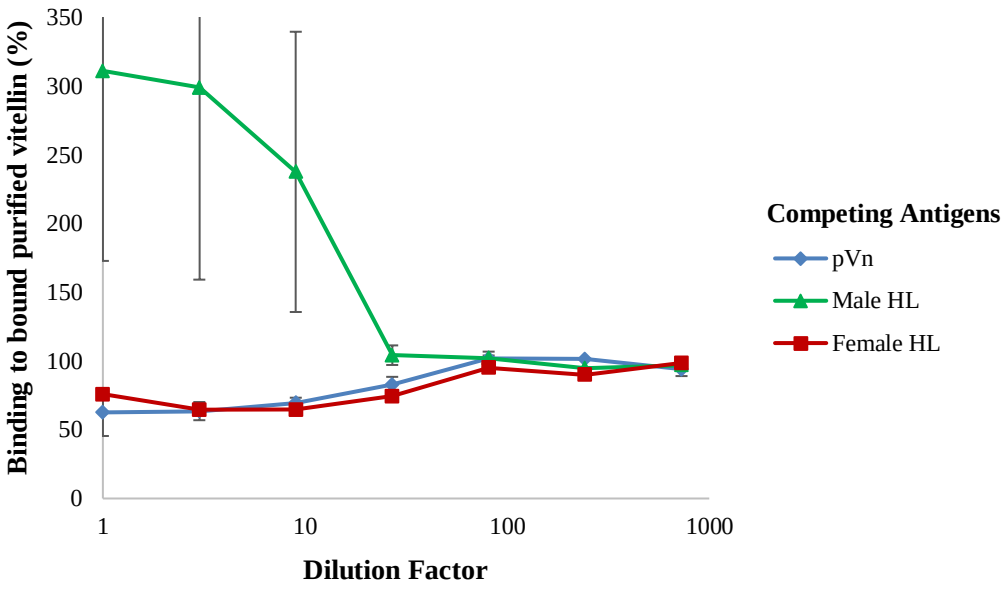

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Figure 3.8. Competition ELISA of anti-pVn mAb produced by clone 2H4A3E4H11 for bound purified vitellin (pVn, 10 µg/ml) and 3-fold diluted competing antigens. Competing antigens used were: male haemolymph (▲) (male HL, 100 µg/ml), female haemolymph (■) (female HL, 100 µg/ml), and purified vitellin (◆) (pVn, 20 µg/ml) as standard. Each data point represents the mean ± SEM of a triplicate measurement.

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Table 3.5. The success of isolation of hybridoma clone 2H4A3E6A6 by cloning. Wells with the highest specificity and affinity, as determined by ELISA, were selected for cloning. Wells that had an OD higher than the positive control were designated as positive wells. These wells were selected for cloning and each well was cloned into fourty seven new wells. The percentage of wells screened positive after cloning represent the number of positive wells expressed as a percentage of the number of wells that were plated from the previous screening.

Clone: 2H4A3E6A6	**Number of wells plated**	**Number of wells that had growth and were selected for screening**	**Number of wells that screened positive**	**Percentage of wells screened positive (%)**	**OD of well selected for cloning**
After fusion (At 1st screening)	96	96	6	6.25	3.06
After 1st cloning (At 2nd screening)	47	6	1	16.67	3.01
After 2nd cloning (At 3rd screening)	47	12	4	33.33	1.00
After 3rd cloning (At 4th screening)	47	9	9	100	2.89
SUMMARY	237	123	20	16.26	2.49 (Average OD)

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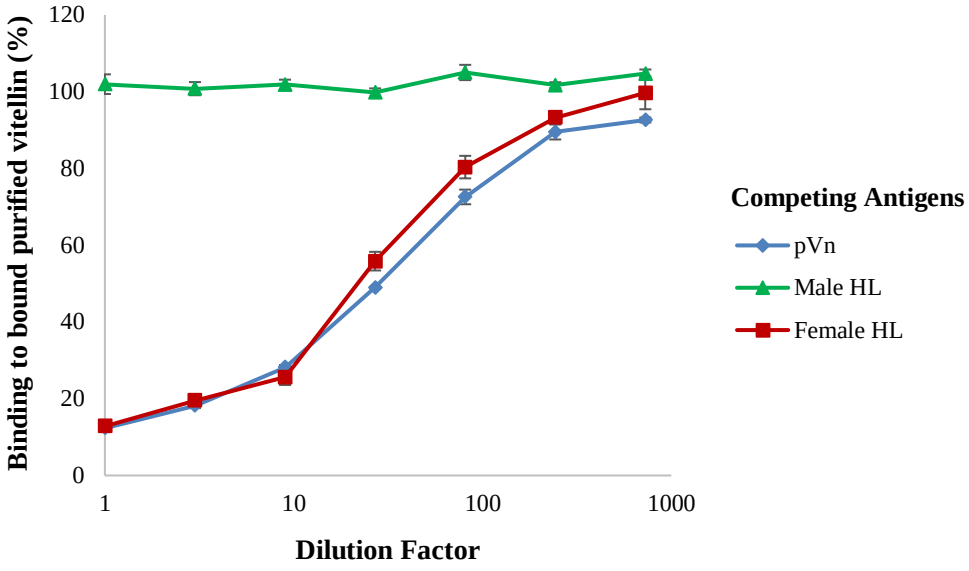

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Figure 3.9. Competition ELISA of anti-pVn mAb produced by clone 2H4A3E6A6 for bound purified vitellin (pVn, 10 µg/ml) and 3-fold diluted competing antigens. Competing antigens used were: male haemolymph (▲) (male HL, 100 µg/ml), female haemolymph (■) (female HL, 100 µg/ml), and purified vitellin (◆) (pVn, 20 µg/ml) as standard. Each data point represents the mean ± SEM of a triplicate measurement.

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Table 3.6. The success of isolation of hybridoma clone 3E1F3D9H3 by cloning. Wells with the highest specificity and affinity, as determined by ELISA, were selected for cloning. Wells that had an OD higher than the positive control were designated as positive wells. These wells were selected for cloning and each well was cloned into fourty seven new wells. The percentage of wells screened positive after cloning represent the number of positive wells expressed as a percentage of the number of wells that were plated from the previous screening.

Clone: 3E1F3D9H3	**Number of wells plated**	**Number of wells that had growth and were selected for screening**	**Number of wells that screened positive**	**Percentage of wells screened positive (%)**	**OD of well selected for cloning**
After fusion (At 1st screening)	96	96	10	10.42	2.84
After 1st cloning (At 2nd screening)	47	10	2	20	2.68
After 2nd cloning (At 3rd screening)	47	2	1	50	1.17
After 3rd cloning (At 4th screening)	47	2	2	100	3.34
SUMMARY	237	110	15	13.64	2.51 (Average OD)

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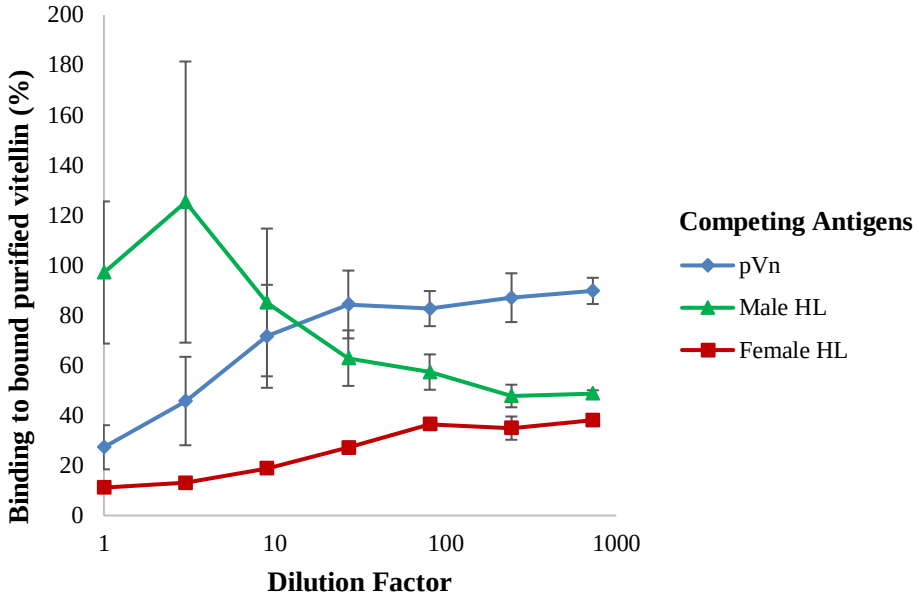

Figure 3.10. Competition ELISA of anti-pVn mAb produced by clone 3E1F3D9H3 for bound purified vitellin (pVn, 10 µg/ml) and 3-fold diluted competing antigens. Competing antigens used were: male haemolymph (▲) (male HL, 100 µg/ml), female haemolymph (■) (female HL, 100 µg/ml), and purified vitellin (◆) (pVn, 20 µg/ml) as standard. Each data point represents the mean ± SEM of a triplicate measurement.

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Table 3.7. The success of isolation of hybridoma clone 5E7E8B2C8 by cloning. Wells with the highest specificity and affinity, as determined by ELISA, were selected for cloning. Wells that had an OD higher than the positive control were designated as positive wells. These wells were selected for cloning and each well was cloned into fourty seven new wells. The percentage of wells screened positive after cloning represent the number of positive wells expressed as a percentage of the number of wells that were plated from the previous screening.

Clone: 5E7E8B2C8	**Number of wells plated**	**Number of wells that had growth and were selected for screening**	**Number of wells that screened positive**	**Percentage of wells screened positive (%)**	**OD of well selected for cloning**
After fusion (At 1st screening)	96	96	8	8.33	2.54
After 1st cloning (At 2nd screening)	47	8	1	12.50	3.01
After 2nd cloning (At 3rd screening)	47	2	1	50	0.74
After 3rd cloning (At 4th screening)	47	1	1	100	2.34
SUMMARY	237	107	11	10.28	2.16 (Average OD)

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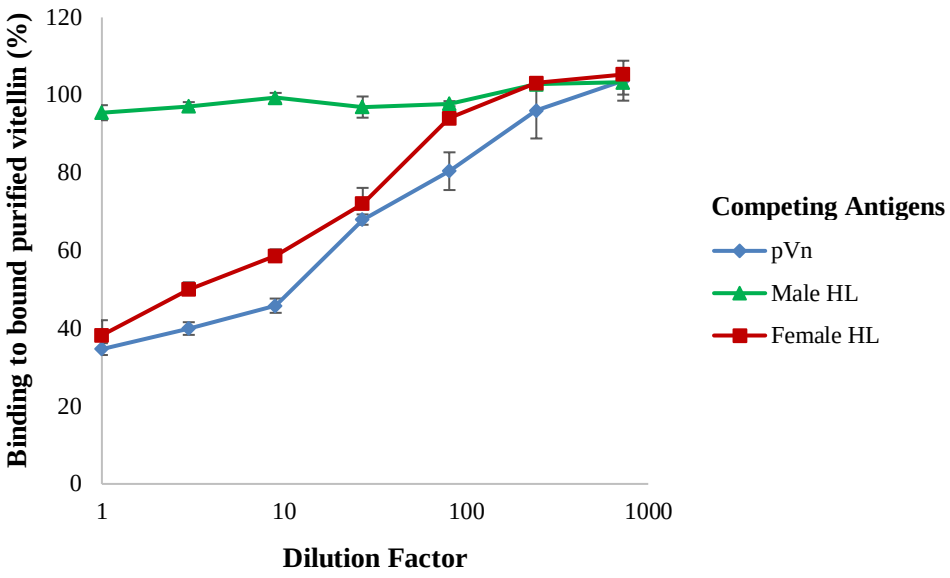

Figure 3.11. Competition ELISA of anti-pVn mAb produced by clone 5E7E8B2C8 for bound purified vitellin (pVn, 10 µg/ml) and 3-fold diluted competing antigens. Competing antigens used were: male haemolymph (▲) (male HL, 100 µg/ml), female haemolymph (■) (female HL, 100 µg/ml), and purified vitellin (◆) (pVn, 20 µg/ml) as standard. Each data point represents the mean ± SEM of a triplicate measurement.

3.3.5 Specificity of the anti-pVn mAb from clone 2H4A3B6H3 for Vn and VTG: western blot

To confirm the specificity that the anti-pVn mAb from clone 2H4A3B6H3 exhibited for Vn and VTG in the ELISA, the haemolymph and pVn samples were run on a native PAGE and proteins were electrophoresed onto a nitrocellulose membrane. A western blot was performed with the pVn-antiserum and anti-pVn mAb to determine if the antibodies could detect pVn and VTG. Western blotting demonstrated that the pVn-antiserum were able to detect pVn and also detected a single band in the haemolymph of *P. perlatus* females (figure 3.12). The western blot with the anti-pVn mAb showed that those same two bands were also detected by the mAb (figure 3.13). These results are in accordance with other studies that have also demonstrated the immunological similarity between Vn and VTG, showing that antibodies against Vn can detect Vn and VTG (Lee *et al.*, 1997; Longyant *et al.*, 2000; Tsukimura *et al.*, 2000; Okumura *et al.*, 2004; Auttarat *et al.*, 2006). No bands were detected in the male haemolymph for either western blots. This corroborates the previous results that the two bands detected in the western blot correspond to VTG in the female haemolymph and Vn in the ovaries.

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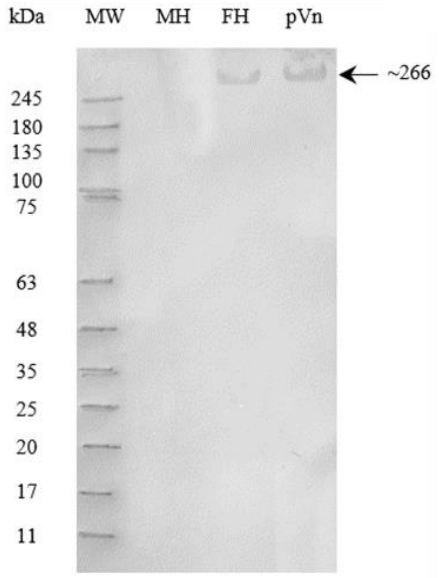

Figure 3.12. Western blot of *P. perlatus* haemolymph and pVn using the polyclonal pVn-antiserum. Antiserum was diluted (1:8 000, v/v) in diluent. Molecular weight marker (MW), male haemolymph (MH), female haemolymph (FH), purified vitellin (pVn). The arrow indicates VTG and pVn at approximately 266 kDa.

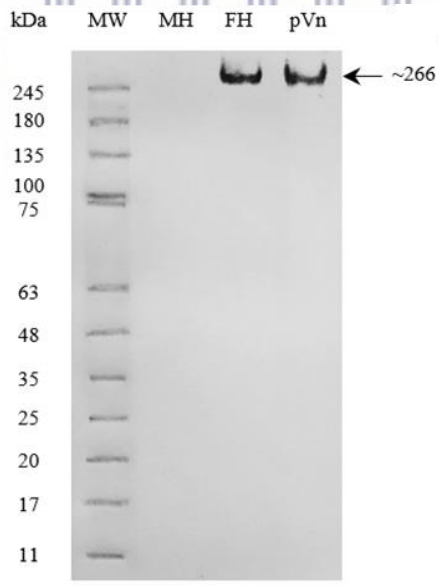

Figure 3.13. Western blot of *P. perlatus* haemolymph and pVn using the anti-pVn mAb from a 7.5% native PAGE. Anti-pVn mAb was diluted (0.1 µg/ml) in diluent. Molecular weight marker (MW), male haemolymph (MH), female haemolymph (FH), purified vitellin (pVn). The arrow indicates VTG and pVn at approximately 266 kDa.

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3.3.6 Concentration and partial purification of the anti-pVn mAb from clone 2H4A3B6H3

A number of purification methods exist to purify antibodies from a heterogeneous solution (Kuhlmann, 2008). The intended use of the antibody affects the degree to which the antibody can be purified. This, in turn, determines the method of purification. For example, immunochemical techniques that make use of only one antibody labelled with an enzyme, require highly purified antibodies. Conversely, techniques that use more than one antibody, where the secondary antibody is labelled with an enzyme, does not require such a high degree of purity (Kuhlmann, 2008). For this reason, it must also be noted that under certain circumstances it is advantageous to purify an antibody only partially in that certain proteins in the solution can stabilise the antibody. The anti-pVn mAb produced by the hybridoma clone 2H4A3B6H3 was concentrated and partially purified from culture medium (40 ml) to a concentrate (0.475 ml) by means of ammonium sulphate precipitation (table 3.8).

The low protein concentration of the culture supernatant could be attributed to the large volume in which the proteins were found. However, the low protein concentration is more likely a consequence of having cultured clone 2H4A3B6H3 in medium without FBS. The clone was initially cultured from cryo-stock in FBS-supplemented medium but subsequently weaned off FBS and grown in serum-free medium. The considerable decrease in volume from 40 ml to 0.475 ml with a concomitant increase in protein concentration suggests that the proteins became more concentrated as a consequence of the decreased volume. However, factors such as the fold purification and percentage yield should also be considered.

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The combination of fold purification and percentage yield indicates how useful a purification step was. Steps with a low fold purification and a low yield should be reconsidered in future purifications, whereas steps with a high fold purification and high yield are effective steps in purification and could be used again in future. Having said that, the low fold purification and low percentage yield of the purified product indicates that the purification of the mAb was not effective (table 3.8). The titre is expressed as the number of units per microlitre and indicates that more units remained after the purification but it provides no indication as to whether these units retained their activity or not (table 3.8). This increase in the number of units creates the expectation of a corresponding increase in activity. However, when looking at both the total activity and specific activity after purification, the data suggests that the activities of the antibody decreased as a result of the purification (table 3.8).

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There are advantages and disadvantages to each of the methods that can be used to purify antibodies from heterogeneous solutions (Kuhlmann, 2008). Ammonium sulphate precipitation is a technique commonly used to remove antibodies from a solution as it is simple, cheap, and convenient. However, as high molecular weight proteins are purified along with the antibodies, this technique does not yield high purity antibody preparations (Kuhlmann, 2008). It is, therefore, advised that this technique should not be used as a single step but rather in conjunction with other purification methods to remove these high molecular weight proteins if a higher degree of purity is needed. However, in some cases, a high degree of purity is not advantageous as certain "contaminating" proteins (e.g. albumin) function to stabilise the antibody and avoids aggregation (Kuhlmann, 2008). The loss of total activity and specific activity suggests that this was a possibility during the purification of the mAb from clone 2H4A3B6H3. Some proteins that stabilise the mAb, and by extension contribute to the retention of its activity could have been lost during the purification. This could explain the decrease in the activity of the mAb.

The purified product was further diluted by the addition of an equal volume of glycerol for storage purposes at -20°C, thereby decreasing the concentration to 151.48 µg/ml (data not shown). A titration curve from the aliquot of each major step revealed that the anti-pVn mAbs could be used at a concentration of 0.1 µg/ml (data not shown).

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Table 3.8. Purification table showing the fold purification and percentage yield of the anti-pVn mAb after concentration and partial purification from the culture supernatant of hybridoma clone 2H4A3B6H3. This was accomplished by centrifugal filter unit, ammonium sulphate precipitation, and dialysis.

	Protein (µg/ml)	**Volume (µl)**	**Total protein (µg)**	**Total activity (units)**	**Titre (units/µl)**	**Specific activity (units/µg)**	**Purification (fold)**	**Percentage Yield (%)**
Culture supernatant	7.13	40 000	285.20	6 419 152.92	160.48	22 507.55	1	100
Partially purified anti-pVn mAb	302.97	475	143.91	2 025 702.30	4 264.64	14 076.17	0.63	32

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3.3.7 Characterisation of the anti-pVn mAb from clone 2H4A3B6H3

3.3.7.1 Sensitivity for Vn: competition ELISA

A competition ELISA was performed to determine the sensitivity of the anti-pVn mAb. This was done by adding soluble pVn as a competing antigen to a pVn-coated 96-well plate, allowing the anti-pVn mAb to compete between the bound and soluble form of pVn. The sensitivity of the ELISA (calculated as the amount of bound pVn which gave 90% binding) was 156 ng/ml and demonstrated a practical operating range between 150 ng/ml and 5 000 ng/ml of pVn (figure 3.14). This means that the anti-pVn mAb is sensitive enough to detect pVn between 150 ng/ml and 5 000 ng/ml. Other studies have also established ELISAs for the quantification of crustacean VTG and Vn with sensitivities comparable to the ELISA from the current study. Lee & Watson, (1994) developed an ELISA for the blue crab, *Callinectes sapidus*, which demonstrated a sensitivity of 148 ng/ml. The ELISA developed by Chen *et al.*, (2004) for the Chinese mitten crab, *Eriocheir sinensis*, could also detect Vn effectively at 7.8 ng. However, these studies used pAbs to establish their ELISAs.

The complexity of antigens in a natural immune response stimulates a number of B cells, culminating in the production of a number of pAbs (Mak & Saunders, 2005). Polyclonal antibodies are produced by different B-lymphocytes and, therefore, each pAb exhibits a different sensitivity, specificity, and affinity for the cognate antigen. Compared to mAbs, pAbs exhibit a higher sensitivity (Zola, 1987). The principle advantage of mAbs is that they are produced by the same B cell and are, therefore, identical in sensitivity, specificity, and affinity for the cognate antigen (Oberdörster *et al.*, 2000; Lipman *et al.*, 2005). Hence, the comparable sensitivities between the anti-pVn mAb from this study and the pAbs from other studies should not suggest that the production of the mAbs was an unnecessary expense. Rather, the rationale behind the production of the anti-pVn mAbs should be justified owing to the batch-to-batch variability in the sensitivities and specificities of pAbs, while mAbs offer a more reproducible sensitivity and specificity (Murphy, 2011). Some studies, albeit to a lesser extent, have generated mAbs against Vn in other crustaceans and these mAbs have demonstrated sensitivities similar to the ones generated in this study (Derelle *et al.*, 1986; Longyant *et al.*, 2000; Sripiromrak *et al.*, 2014).

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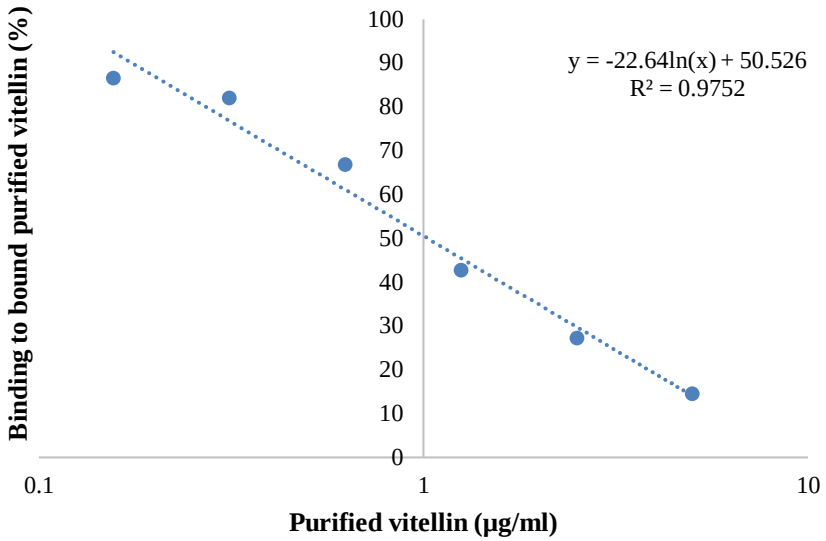

Figure 3.14. The sensitivity of the anti-pVn mAbs for pVn as demonstrated in a competition ELISA between bound pVn and soluble pVn. A linear relationship was found between 150-5 000 ng/ml after log transformation of the x-axis.

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3.3.7.2 Cross-reactivity with VTG: competition ELISA

Another competition ELISA was performed to determine if VTG in the female haemolymph could compete with soluble pVn for binding to the mAb. Similar to the previous ELISA, competing antigens were added but in the form of male and female haemolymph. The results demonstrated that the mAb exhibited a specificity for the female haemolymph parallel to that of their specificity for pVn (figure 3.15). From this, it is evident that VTG in the female haemolymph can compete with pVn by inhibiting the binding of the mAb to the bound pVn. Thus, the mAb was able to detect VTG in the female haemolymph. In support of this, previous studies have established that Vn and VTG are immunologically indistinguishable and that neither are found in male crustaceans (Meusy, 1980; Lee & Watson, 1994; Yehezkel *et al.*, 2000; Tsukimura, 2001; Xuereb *et al.*, 2011; Sripiromrak *et al.*, 2014). Moreover, no VTG was detected in the male haemolymph. This was evident by the 95-100% detection of only the bound pVn by the mAb, indicating that there was no VTG in the male haemolymph for the mAb to detect and bind to. This result demonstrates that the anti-pVn mAb could detect Vn and could also cross-react with and detect VTG in the female haemolymph.

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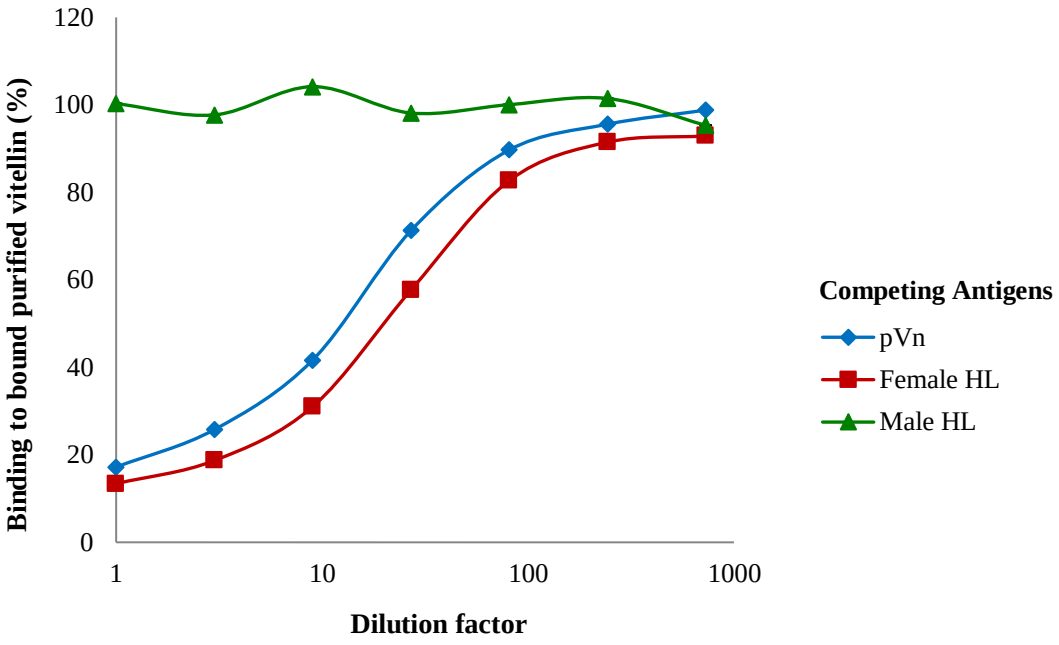

Figure 3.15. Competition ELISA of anti-pVn mAb for bound purified vitellin (pVn, 10 µg/ml) and 3-fold diluted competing antigens. Competing antigens used were: male haemolymph (▲) (male HL, 100 µg/ml), female haemolymph (■) (female HL, 100 µg/ml), and purified vitellin (◆) (pVn, 20 µg/ml) as standard. Each data point represents the mean ± SEM of a triplicate measurement.

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3.4 CONCLUSION

Vitellin is the major yolk protein found in the ovaries of oviparous organisms. It was purified from the ovaries of the freshwater crab, *P. perlatus*, by ammonium sulphate precipitation. The purification was successful as only the three major subunits of 75, 78, and 98 kDa remained after purification when compared to the unpurified ovarian homogenate. With a native molecular weight of approximately 266 kDa, the identity of the purified product was further supported to be Vn. Further characterisation by Oil Red O lipid staining and Con-A lectin blotting confirmed that the purified product was pVn. Moreover, the immunisation of BALB/c mice with pVn was effective as evident by the consecutive increase in anti-pVn polyclonal antibodies. A total of 700 wells of hybridoma colonies were screened for mAb production against pVn of which 97 wells screened positive through four repetitions of screening and cloning. Ultimately, hybridoma clone 2H4A3B6H3 was selected based on its high affinity and specificity for pVn and used for characterisation. The anti-pVn mAb produced by clone 2H4A3B6H3 could detect pVn in a range of 150-5 000 ng/ml. In a competition ELISA between soluble pVn and VTG, this mAb could detect VTG in the female haemolymph of *P. perlatus* whereas no VTG was detected in the male haemolymph. This result was confirmed by a western blot of the native form of VTG and pVn.

The results demonstrate that the anti-pVn mAb produced by hybridoma clone 2H4A3B6H3 is able to detect VTG in the female haemolymph as well as pVn from *P. perlatus*. As a result, this mAb can be used to quantify VTG and Vn in the tissues of *Potamonautes* spp. Furthermore, they can also be of use in a potential rapid detection assay to assess the effects of oestrogenic EDCs on VTG synthesis.

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3.5 REFERENCES

1. Abdu, U., Yehezkel, G. & Sagi, A. (2000). Oocyte development and polypeptide dynamics during ovarian maturation in the red-claw crayfish *Cherax quadricarinatus*. *Invertebrate Reproduction & Development,* 37, 75-83.

2. Ansar, W. & Ghosh, S. (2013). Monoclonal antibodies: A tool in clinical research. *Indian Journal of Clinical Medicine,* 4, 9-21.

3. Auttarat, J., Phiriyangkul, P. & Utarabhand, P. (2006). Characterization of vitellin from the ovaries of the banana shrimp *Litopenaeus merguiensis*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 143, 27-36.

4. Avarre, J. C., Michelis, R., Tietz, A. & Lubzens, E. (2003). Relationship between vitellogenin and vitellin in a marine shrimp (*Penaeus semisulcatus*) and molecular characterization of vitellogenin complementary DNAs. *Biology of Reproduction,* 69, 355-364.

5. Bowman, C. J., Kroll, K. J., Hemmer, M. J., Folmar, L. C. & Denslow, N. D. (2000). Estrogen-induced vitellogenin mRNA and protein in sheepshead minnow (*Cyprinodon variegatus*). *General and Comparative Endocrinology,* 120, 300-313.

6. Chen, H. Y., Ho, S. H., Chen, T. I., Soong, K., Chen, I. M. & Cheng, J. H. (2007). Identification of a female-specific hemocyanin in the mud crab, *Scylla olivacea* (Crustacea: Portunidae). *Zoological Studies,* 46, 194-202.

7. Chen, L., Jiang, H., Zhou, Z., Li, K., Li, K., Deng, G. Y. & Liu, Z. (2004). Purification of vitellin from the ovary of Chinese mitten-handed crab (*Eriocheir sinensis*) and development of an antivitellin ELISA. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 138, 305-311.

8. Chen, Y. N. & Kuo, C. M. (1998). Purification and characterization of vitellin from the freshwater giant prawn, *Macrobrachium rosenbergii*. *Zoological Studies (Taipei),* 37, 126-136.

9. Christiansen, L. B., Pedersen, K. L., Pedersen, S. N., Korsgaard, B. & Bjerregaard, P. (2000). *In vivo* comparison of xenoestrogens using rainbow trout vitellogenin induction as a screening system. *Environmental Toxicology and Chemistry,* 19, 1867-1874.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

10. Clotfelter, E. D., Bell, A. M. & Levering, K. R. (2004). The role of animal behaviour in the study of endocrine-disrupting chemicals. *Animal Behaviour,* 68, 665-676.

11. Derelle, E., Grosclaude, J., Meusy, J. J., Junera, H. & Martin, M. (1986). ELISA titration of vitellogenin and vitellin in the freshwater prawn, *Macrobrachium rosenbergii*, with monoclonal antibody. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 85, 1-4.

12. Ding, X., Nagaraju, G., Novotney, D., Lovett, D. & Borst, D. (2010). Yolk protein expression in the green crab, *Carcinus maenas*. *Aquaculture,* 298, 325-331.

13. Downs, C. A., Fauth, J. E. & Woodley, C. M. (2001). Assessing the health of grass shrimp (*Palaeomonetes pugio*) exposed to natural and anthropogenic stressors: A molecular biomarker system. *Marine Biotechnology,* 3, 380-397.

14. Ercal, N., Gurer-Orhan, H. & Aykin-Burns, N. (2001). Toxic metals and oxidative stress part I: Mechanisms involved in metal-induced oxidative damage. *Current Topics in Medicinal Chemistry,* 1, 529-539.

15. Farombi, E., Adelowo, O. & Ajimoko, Y. (2007). Biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in African catfish (*Clarias gariepinus*) from Nigeria Ogun River. *International Journal of Environmental Research and Public Health,* 4, 158-165.

16. Ferré, L. E., Medesani, D. A., García, C. F., Grodzielski, M. & Rodríguez, E. M. (2012). Vitellogenin levels in hemolymph, ovary and hepatopancreas of the freshwater crayfish *Cherax quadricarinatus* (Decapoda: Parastacidae) during the reproductive cycle. *International Journal of Tropical Biology and Conservation,* 60, 253-261.

17. Fredrick, W. S. & Ravichandran, S. (2012). Hemolymph proteins in marine crustaceans. *Asian Pacific Journal of Tropical Biomedicine,* 2, 496-502.

18. Gagné, F. & Blaise, C. (2000). Organic alkali-labile phosphates in biological materials: A generic assay to detect vitellogenin in biological tissues. *Environmental Toxicology,* 15, 243-247.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

19. García-Orozco, K. D., Vargas-Albores, F., Sotelo-Mundo, R. R. & Yepiz-Plascencia, G. (2002). Molecular characterization of vitellin from the ovaries of the white shrimp *Penaeus* (*Litopenaeus*) *vannamei*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 133, 361-369.

20. Ghekiere, A., Verslycke, T. & Janssen, C. (2006). Effects of methoprene, nonylphenol, and estrone on the vitellogenesis of the mysid *Neomysis integer*. *General and Comparative Endocrinology,* 147, 190-195.

21. Girish, B., Swetha, C. & Reddy, P. S. (2014). Hepatopancreas but not ovary is the site of vitellogenin synthesis in female freshwater crab, *Oziothelphusa senex senex*. *Biochemical and Biophysical Research Communications,* 447, 323-327.

22. Haeba, M. H., Hilscherová, K., Mazurová, E. & Bláha, L. (2008). Selected endocrine disrupting compounds (vinclozolin, flutamide, ketoconazole and dicofol): Effects on survival, occurrence of males, growth, molting and reproduction of *Daphnia magna*. *Environmental Science and Pollution Research,* 15, 222-227.

23. Hallgren, P. (2009). *Strategies for monitoring of endocrine disrupting chemicals in aquatic environment.* Ph.D. thesis, Lund University.

24. Hamza-Chaffai, A. (2014). Usefulness of bioindicators and biomarkers in pollution biomonitoring. *International Journal of Biotechnology for Wellness Industries,* 3, 19-26.

25. Hannas, B. R., Wang, Y. H., Thomson, S., Kwon, G., Li, H. & LeBlanc, G. A. (2011). Regulation and dysregulation of vitellogenin mRNA accumulation in daphnids (*Daphnia magna*). *Aquatic Toxicology,* 101, 351-357.

26. Havrilesky, L. J., Whitehead, C. M., Rubatt, J. M., Cheek, R. L., Groelke, J., He, Q., Malinowski, D. P., Fischer, T. J. & Berchuck, A. (2008). Evaluation of biomarker panels for early stage ovarian cancer detection and monitoring for disease recurrence. *Gynecologic Oncology,* 110, 374-382.

27. Hermanson, G. T. (2008). *Bioconjugate techniques,* 2nd Ed, San Diego, USA: Academic Press, 746.

http://etd.uwc.ac.za/

28. Horn, E. C. & Kerr, M. S. (1969). The hemolymph proteins of the blue crab, *Callinectes sapidus* - I. Hemocyanins and certain other major protein constituents. *Comparative Biochemistry and Physiology*, 29, 493-508.

29. Houen, G., Olsen, D., Hansen, P., Petersen, K. & Barkholt, V. (2003). Preparation of bioconjugates by solid-phase conjugation to ion exchange matrix-adsorbed carrier proteins. *Bioconjugate Chemistry*, 14, 75-79.

30. Iguchi, T., Irie, F., Urushitani, H., Tooi, O., Kawashima, Y., Roberts, M., Norrgren, L. & Hutchinson, T. (2005). Availability of *in vitro* vitellogenin assay for screening of estrogenic and anti-estrogenic activities of environmental chemicals. *Environmental Sciences: An International Journal of Environmental Physiology and Toxicology*, 13, 161-183.

31. Kawazoe, I. C., Jasmani, S. A., Shih, T. U. W., Suzuki, Y. U. & Aida, K. A. (2000). Purification and characterization of vitellin from the ovary of kuruma prawn, *Penaeus japonicus*. *Fisheries Science*, 66, 390-396.

32. Kefaloyianni, E., Gourgou, E., Ferle, V., Kotsakis, E., Gaitanaki, C. & Beis, I. (2005). Acute thermal stress and various heavy metals induce tissue-specific pro-or anti-apoptotic events via the p38-MAPK signal transduction pathway in *Mytilus galloprovincialis* (Lam.). *Journal of Experimental Biology*, 208, 4427-4436.

33. Kidd, K. A., Blanchfield, P. J., Mills, K. H., Palace, V. P., Evans, R. E., Lazorchak, J. M. & Flick, R. W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences*, 104, 8897-8901.

34. Kuhlmann, W. D. (2008). Purification of antibodies by the use of biochemical and immunochemical techniques. *Cell-Marker: Immune sera, antibodies* [Online], 3, 10. Available at: http://www.immunologie-labor.com/cellmarker_files/IET_antibody_04.pdf, [Accessed: 17/01/2017].

35. Kumagai, Y., Mizukado, S., Nagafune, J., Shinyashiki, M., Homma-Takeda, S. & Shimojo, N. (1997). Post-transcriptional elevation of mouse brain Mn-SOD protein by mercuric chloride. *Brain Research*, 769, 178-182.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

36. Lee, C. Y. & Watson, R. D. (1994). Development of a quantitative enzyme-linked immunosorbent assay for vitellin and vitellogenin of the blue crab *Callinectes sapidus*. *Journal of Crustacean Biology,* 14, 617-626.

37. Lee, F. Y., Shih, T. W. & Chang, C. F. (1997). Isolation and characterization of the female-specific protein (vitellogenin) in mature female hemolymph of the freshwater prawn, *Macrobrachium rosenbergii*: Comparison with ovarian vitellin. *General and Comparative Endocrinology,* 108, 406-415.

38. Lee, R. F. & Puppione, D. L. (1988). Lipoproteins I and II from the hemolymph of the blue crab *Callinectes sapidus*: Lipoprotein II associated with vitellogenesis. *Journal of Experimental Zoology,* 248, 278-289.

39. Lipman, N. S., Jackson, L. R., Trudel, L. J. & Weis-Garcia, F. (2005). Monoclonal versus polyclonal antibodies: Distinguishing characteristics, applications, and information resources. *ILAR Journal,* 46, 258-268.

40. Locatello, L., Matozzo, V. & Marin, M. G. (2009). Biomarker responses in the crab *Carcinus aestuarii* to assess environmental pollution in the Lagoon of Venice (Italy). *Ecotoxicology,* 18, 869-877.

41. Longyant, S., Sithigorngul, P., Thammapalerd, N., Sithigorngul, W. & Menasveta, P. (2000). Characterization of vitellin and vitellogenin of giant tiger prawn *Penaeus monodon* using monoclonal antibodies specific to vitellin subunits. *Invertebrate Reproduction & Development,* 37, 211-221.

42. Lye, C. M., Bentley, M. G. & Galloway, T. (2008). Effects of 4-nonylphenol on the endocrine system of the shore crab, *Carcinus maenas*. *Environmental Toxicology,* 23, 309-318.

43. Mak, T. W. & Saunders, M. E. (2005). *The immune response: Basic and clinical principles,* Massachusetts, USA: Academic Press, 122-128.

44. Markl, J. & Decker, H. (1992). Molecular structure of the arthropod hemocyanins. Mangum, C. P. (ed.), *In: Blood and Tissue Oxygen Carriers.* Berlin, Germany: Springer Berlin Heidelberg, 326.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

45. Markl, J., Hofer, A., Bauer, G., Markl, A., Kempter, B., Brenzinger, M. & Linzen, B. (1979). Subunit heterogeneity in arthropod hemocyanins: II. Crustacea. *Journal of Comparative Physiology*, 133, 167-175.

46. Martinović, D., Hogarth, W. T., Jones, R. E. & Sorensen, P. W. (2007). Environmental estrogens suppress hormones, behavior, and reproductive fitness in male fathead minnows. *Environmental Toxicology and Chemistry*, 26, 271-278.

47. Metzger, D. C., Pratt, P. & Roberts, S. B. (2012). Characterizing the effects of heavy metal and *Vibrio* exposure on Hsp70 expression in *Crassostrea gigas* gill tissue. *Journal of Shellfish Research*, 31, 627-630.

48. Meusy, J. (1980). Vitellogenin, the extraovarian precursor of the protein yolk in Crustacea: A review. *Reproduction Nutrition Développement*, 20, 1-21.

49. Micheletti, C., Critto, A. & Marcomini, A. (2007). Assessment of ecological risk from bioaccumulation of PCDD/Fs and dioxin-like PCBs in a coastal lagoon. *Environment International*, 33, 45-55.

50. Murphy, K. P. (2011). *Janeway's immunobiology*, 8th Ed, New York, USA: Garland Science, 717-719 & 730-731.

51. NRC (1999). *Monoclonal antibody production*, Washington, USA: National Academy Press, 10-11.

52. Oberdörster, E., Rice, C. D. & Irwin, L. K. (2000). Purification of vitellin from grass shrimp *Palaemonetes pugio*, generation of monoclonal antibodies, and validation for the detection of lipovitellin in Crustacea. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 127, 199-207.

53. Okumura, T., Yoshida, K. & Nikaido, H. (2004). Ovarian development and hemolymph vitellogenin levels in laboratory-maintained protandric shrimp, *Pandalus hypsinotus*: Measurement by a newly developed time-resolved fluoroimmunoassay (TR-FIA). *Zoological Science*, 21, 1037-1047.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

54. Okuno, A., Katayama, H. & Nagasawa, H. (2000). Partial characterization of vitellin and localization of vitellogenin production in the terrestrial isopod, *Armadillidium vulgare*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 126, 397-407.

55. Park, K., Kwak, T. S. & Kwak, I. S. (2014). Vitellogenin gene characterization and expression of Asian paddle crabs (*Charybdis japonica*) following endocrine disrupting chemicals. *Ocean Science Journal,* 49, 127-135.

56. Pateraki, L. E. & Stratakis, E. (1997). Characterization of vitellogenin and vitellin from land crab *Potamon potamios*: Identification of a precursor polypeptide in the molecule. *Journal of Experimental Zoology*, 279, 597-608.

57. Qiu, Y., Ng, T. & Chu, K. (1997). Purification and characterization of vitellin from the ovaries of the shrimp *Metapenaeus ensis* (Crustacea: Decapoda: Penaeidae). *Invertebrate Reproduction & Development*, 31, 217-223.

58. Revathi, P., Iyapparaj, P., Munuswamy, N. & Krishnan, M. (2012). Vitellogenesis during the ovarian development in freshwater female prawn *Macrobrachium rosenbergii* (De Man). *International Journal of Aquatic Science*, 3, 13-27.

59. Ricciardi, F., Matozzo, V., Binelli, A. & Marin, M. G. (2010). Biomarker responses and contamination levels in crabs (*Carcinus aestuarii*) from the Lagoon of Venice: An integrated approach in biomonitoring estuarine environments. *Water Research,* 44, 1725-1736.

60. Roth, Z., Parnes, S., Wiel, S., Sagi, A., Zmora, N., Chung, J. S. & Khalaila, I. (2010). N-glycan moieties of the crustacean egg yolk protein and their glycosylation sites. *Glycoconjugate Journal,* 27, 159-169.

61. Salvato, B., Sartore, S., Rizzotti, M. & Magaldi, A. G. (1972). Molecular weight determination of polypeptide chains of molluscan and arthropod hemocyanins. *FEBS Letters,* 22, 5-7.

62. Schaaper, W., Lankhof, H., Puijk, W. & Meloen, R. (1989). Manipulation of antipeptide immune response by varying the coupling of the peptide with the carrier protein. *Molecular Immunology,* 26, 81-85.

http://etd.uwc.ac.za/

63. Scholz, S., Kordes, C., Hamann, J. & Gutzeit, H. O. (2004). Induction of vitellogenin *in vivo* and *in vitro* in the model teleost medaka (*Oryzias latipes*): Comparison of gene expression and protein levels. *Marine Environmental Research*, 57, 235-244.

64. Schueler, F. W. (1946). Sex hormonal action and chemical constitution. *Obstetrical & Gynecological Survey*, 1, 538-539.

65. Segner, H., Caroll, K., Fenske, M., Janssen, C., Maack, G., Pascoe, D., Schäfers, C., Vandenbergh, G., Watts, M. & Wenzel, A. (2003). Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: Report from the European IDEA project. *Ecotoxicology and Environmental Safety*, 54, 302-314.

66. Serrano-Pinto, V., Vazquez-Boucard, C. & Villarreal-Colmenares, H. (2003). Yolk proteins during ovary and egg development of mature female freshwater crayfish (*Cherax quadricarinatus*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 134, 33-43.

67. Shay, J. W. (1987). Mechanisms of cell fusion and selection in the generation of hybridomas. Bartal, A. H. & Hirshaut, Y. (eds.), *In: Methods of Hybridoma Formation.* New Jersey, USA: Humana Press, 67-70.

68. Sluczewski, A. & Roth, P. C. J. (1948). Effects of androgenic and estrogenic compounds on the experimental metamorphoses of amphibians. *Gynecology and Obstetrics*, 47, 164-176.

69. Smeets, J. M., Rankouhi, T. R., Nichols, K. M., Komen, H., Kaminski, N. E., Giesy, J. P. & van den Berg, M. (1999). *In vitro* vitellogenin production by carp (*Cyprinus carpio*) hepatocytes as a screening method for determining (anti) estrogenic activity of xenobiotics. *Toxicology and Applied Pharmacology*, 157, 68-76.

70. Snyder, S. A., Westerhoff, P., Yoon, Y. & Sedlak, D. L. (2003). Pharmaceuticals, personal care products, and endocrine disruptors in water: Implications for the water industry. *Environmental Engineering Science*, 20, 449-469.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

71. Spaziani, E., Wang, W. & Novy, L. (1995). Serum high-density lipoproteins in the crab *Cancer antennarius* - IV. Electrophoretic and immunological analyses of apolipoproteins and a question of female-specific lipoproteins. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 111, 265-276.

72. Sripiromrak, A., Chaivisuthangkura, P., Longyant, S. & Sithigorngul, P. (2014). Using vitellin monoclonal antibodies to assess the vitellogenesis-inhibiting hormone activity of *Macrobrachium rosenbergii*. *ScienceAsia,* 40, 157-167.

73. Stanley, J. (2002). *Essentials of immunology and serology,* 1st Ed, Boston, USA: Cengage Learning, 15-16.

74. Stevens, C. D. (2009). *Clinical immunology and serology: A laboratory perspective,* 3rd Ed, Philadelphia, USA: F.A. Davis, 41.

75. Stokes, W. S. (2004). Selecting appropriate animal models and experimental designs for endocrine disruptor research and testing studies. *ILAR Journal,* 45, 387-393.

76. Strynadka, N., Redmond, M., Parker, J., Scraba, D. & Hodges, R. (1988). Use of synthetic peptides to map the antigenic determinants of glycoprotein D of herpes simplex virus. *Journal of Virology,* 62, 3474-3483.

77. Sung, H. J., Na, S. S., Seul, K. J., Jeon, H. S., Park, J. Y. & Cho, J. Y. (2014). Clinically applicable diagnostics assay development for lung cancer biomarker, SAA. *Journal of Proteomics & Bioinformatics,* 7, 303-309.

78. Thompson, J. R. & Banaszak, L. J. (2002). Lipid-protein interactions in lipovitellin. *Biochemistry,* 41, 9398-9409.

79. Tiu, S. H. K., Hui, H. L., Tsukimura, B., Tobe, S. S., He, J. G. & Chan, S. M. (2009). Cloning and expression study of the lobster (*Homarus americanus*) vitellogenin: Conservation in gene structure among decapods. *General and Comparative Endocrinology*, 160, 36-46.

80. Tsukimura, B. (2001). Crustacean vitellogenesis: Its role in oocyte development. *American Zoologist,* 41, 465-476.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

81. Tsukimura, B., Bender, J. & Linder, C. (2000). Development of an anti-vitellin ELISA for the assessment of reproduction in the ridgeback shrimp, *Sicyonia ingentis*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 127, 215-224.

82. Vasanthakumari, R. (2007). *Textbook of microbiology,* 1st Ed, New Delhi, India: BI Publications Pvt Ltd, 103.

83. Vazquez-Boucard, C. G., Levy, P., Ceccaldi, H. J. & Brogren, C. H. (2002). Developmental changes in concentrations of vitellin, vitellogenin, and lipids in hemolymph, hepatopancreas, and ovaries from different ovarian stages of Indian white prawn *Fenneropenaeus indicus*. *Journal of Experimental Marine Biology and Ecology,* 281, 63-75.

84. Wallace, R. A., Walker, S. L. & Hauschka, P. V. (1967). Crustacean lipovitellin. Isolation and characterization of the major high-density lipoprotein from the eggs of decapods. *Biochemistry,* 6, 1582-1590.

85. Xuereb, B., Bezin, L., Chaumot, A., Budzinski, H., Augagneur, S., Tutundjian, R., Garric, J. & Geffard, O. (2011). Vitellogenin-like gene expression in freshwater amphipod *Gammarus fossarum* (Koch, 1835): Functional characterization in females and potential for use as an endocrine disruption biomarker in males. *Ecotoxicology,* 20, 1286-1299.

86. Yang, W. J., Ohira, T., Tsutsui, N., Subramoniam, T., Huong, D. T. T., Aida, K. & Wilder, M. N. (2000). Determination of amino acid sequence and site of mRNA expression of four vitellins in the giant freshwater prawn, *Macrobrachium rosenbergii*. *Journal of Experimental Zoology*, 287, 413-422.

87. Yehezkel, G., Chayoth, R., Abdu, U., Khalaila, I. & Sagi, A. (2000). High-density lipoprotein associated with secondary vitellogenesis in the hemolymph of the crayfish *Cherax quadricarinatus*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 127, 411-421.

88. Young, C. R. & Atassi, M. Z. (1982). Antibodies to sperm-whale myoglobin evoked by free synthetic peptides of an antigenic site. *Immunological Communications,* 11, 9-16.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

89. Zatta, P. (1984). Zinc transport in the haemolymph of *Carcinus maenas* (Crustacea: Decapoda). *Journal of the Marine Biological Association of the United Kingdom,* 64, 801-807.

90. Zmora, N., Trant, J., Chan, S. M. & Chung, J. S. (2007). Vitellogenin and its messenger RNA during ovarian development in the female blue crab, *Callinectes sapidus*: Gene expression, synthesis, transport, and cleavage. *Biology of Reproduction,* 77, 138-146.

91. Zola, H. (1987). *Monoclonal antibodies: A manual of techniques,* 1st Ed, Boca Raton, USA: CRC Press, 5-9.

92. Zola, H. & Thomson, P. R. (2001). *Monoclonal antibodies: Diagnostic uses,* Chichester, UK: John Wiley & Sons Ltd, 1-2.

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CHAPTER 4: SEASONAL STUDY

4.1 INTRODUCTION

4.1.1 Reproductive strategies

Effective energy management ensures that an organism balances energy allocated for growth, reproduction, and survival. By allocating energy for growth, the organism ensures its own survival, while the allocation of energy for reproduction allows the survival of its offspring (Kunz & Orrell, 2004). Life history traits, traits that determine how and how frequently reproduction can occur, govern the allocation of energy to the physiological processes of growth and reproduction (Kunz & Orrell, 2004). Semelparity and iteroparity are two reproductive strategies that illustrate the trade-off in energy allocation between growth and reproduction. In semelparous organisms, all available energy is directed towards a single reproductive effort, followed by death. Conversely, iteroparous organisms allocate energy to both growth and reproduction and are, therefore, able to reproduce more than once (Kunz & Orrell, 2004).

As opposed to males, females invest a considerable amount of energy in reproduction (Lindenfors *et al.*, 2002). The energetic investment of males is more directed towards physical attributes such as courtship, territorial defence, and competition. In comparison, females direct resources toward egg production and nurturing of the offspring (Andersson, 1994). Nutrients required for the development of the offspring is supplied by the mother and can be provided either directly through the placenta or in the form of yolk. The former is the mode of reproduction known as matrotrophy, while the latter is called lecithotrophy (Lodé, 2012). Lecithotrophy is a form of reproduction employed by oviparous organisms to provide nutrients to their developing offspring. This requires the formation of yolk, which is stored in the eggs of the female until it is needed for the development and sustenance of the embryo (Auttarat *et al.*, 2006; Phiriyangkul *et al.*, 2007; García *et al.*, 2008; Lodé, 2012).

4.1.2 Vitellogenesis and ovarian maturation

Vitellogenesis is an integral part of the reproductive cycle of oviparous females and is characterised by the accumulation of yolk proteins in developing oocytes. It occurs in two stages, namely primary and secondary vitellogenesis. During primary vitellogenesis, the yolk

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is synthesised by the ovaries in an autologous manner and stored in vesicles. Briefly, the endoplasmic reticulum differentiates and vesicles start to form that become filled with the primary yolk protein, vitellin (Vn) (Tsukimura, 2001). Thereafter, during secondary vitellogenesis, vitellogenin (VTG), the precursor to Vn, is additionally synthesised at extra-ovarian sites and is taken up by the ovaries through receptor-mediated endocytosis (RME). The rapid, exogenously synthesised VTG accumulates in the ovaries and appears as large yolk globules (Subramoniam, 2011).

With the progression of vitellogenesis, the ovaries mature and undergo colour changes, corresponding to a particular stage of vitellogenesis. Previtellogenic ovaries are translucent to opaque white in colour. At the start of primary vitellogenesis, ovaries take on a pale yellow colour. As yolk production increases, the ovaries progressively become orange with the onset of secondary vitellogenesis. Ovaries at the end of secondary vitellogenesis can appear brown to dark brown prior to spawning or oviposition. After spawning, the ovaries return to an early vitellogenic state in preparation for the next reproductive cycle and appear yellow to light orange (Tsukimura *et al.*, 2002; Quinitio *et al.*, 2007). Ovarian maturation is accompanied by enlargement of the ovaries, due to oocyte proliferation, and a concomitant increase in oocyte diameter, as a result of yolk production (Reddy *et al.*, 2006; Ye *et al.*, 2010b; Nagaraju, 2011). Based on morphological and histological criteria, ovarian maturation has been divided into 5 stages (Quinitio *et al.*, 2007; Ferreira *et al.*, 2012; Revathi *et al.*, 2012).

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Ovarian maturation and vitellogenesis occur concurrently and are good indicators of female reproductive activity. During primary vitellogenesis, the oocytes gradually enlarge over a period of months, while, during secondary vitellogenesis, a rapid enlargement occurs which culminates in oviposition (Ferreira *et al.*, 2012). Vitellin is actively produced during the female reproductive cycle and it has been reported that the concentration of VTG in the haemolymph also increases during vitellogenesis (Yehezkel *et al.*, 2000; Revathi *et al.*, 2012).

4.1.3 Vitellogenin as a measure of female reproductive activity

Studies often rely on indirect, qualitative methods of determining vitellogenesis based on gonadal maturation. This includes observing macroscopic changes in ovarian weight, colour, and morphology, as well as microscopic parameters such as oocyte size (López Greco & Rodríguez, 1999; Quinitio *et al.*, 2007). However, some studies employ quantitative methods of measuring vitellogenesis by expressing the gonads and hepatopancreas (HP) as an index of

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the total body weight. This is referred to as the gonadosomatic index (GSI) and hepatosomatic index (HPSI). Although this is a more direct approach, it is somewhat cumbersome and not as rapid, reproducible, and accurate as immunological methods. The use of antibodies to quantify proteins offers the unique advantage of identifying the analyte of interest in a crude homogenate. Enzyme-linked immunosorbent assays (ELISAs) have been established for Vn in a number of crustaceans, and due to their immunological similarity, these antibodies can be used for the quantification of VTG in the haemolymph and Vn in the ovaries (Derelle *et al.*, 1986; Lee & Watson, 1994; Tsukimura *et al.*, 2000; Vazquez-Boucard *et al.*, 2002; Chen *et al.*, 2004; Sripiromrak *et al.*, 2014).

Studies have demonstrated that VTG levels correlate to the reproductive condition of crustaceans. Revathi *et al.*, (2012) has shown that the levels of VTG and Vn increase with the progression of ovarian maturation in the freshwater prawn *Macrobrachium rosenbergii*. While investigating levels of VTG during the reproductive cycle of the freshwater crayfish, *Cherax quadricarinatus*, it was found that VTG levels increased with the progression of the reproductive cycle (Ferré *et al.*, 2012). In the Indian white prawn, *Fenneropenaeus indicus*, both Vn and VTG were also shown to increase sequentially as the ovaries matured (Vazquez-Boucard *et al.*, 2002). Others have reported similar findings (Shafir *et al.*, 1992; Quinitio *et al.*, 1994; Yehezkel *et al.*, 2000). Thus, VTG concentrations in the haemolymph can be used as a measure of female reproductive activity.

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4.1.4 Site of vitellogenesis

Yolk synthesis occurs in a biphasic manner; first, endogenously within the ovaries, and subsequently in extra-ovarian tissues, such as the hepatic tissue (Subramoniam, 2011). Numerous studies have been conducted to determine the primary site of VTG synthesis (Tsukimura, 2001). It has been reported that the HP is the primary site of VTG synthesis in brachyurans from the suborder Pleocyemata (Jayasankar *et al.*, 2002; Chan *et al.*, 2005; Tiu *et al.*, 2009). Conversely, in the suborder Dendrobrachiata, which includes mainly the penaeids, the HP and the ovaries have been reported to contribute equally to VTG production (Tsang *et al.*, 2003; Kung *et al.*, 2004). However, in Pleocyemata, there have also been reports of VTG expression in both the HP and the ovaries (Li *et al.*, 2006; Zmora *et al.*, 2007; Jia *et al.*, 2013). All things considered, the literature indicates that it is possible for VTG to be expressed in

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multiple tissues and that the primary site of synthesis varies between species, even within Crustacea.

4.1.5 Regulation of vitellogenesis

Vitellogenesis is influenced by numerous hormones, such as neuropeptides, terpenoids, steroid hormones, and, in male crustaceans, the androgenic gland hormone (AGH).

Located in the eyestalks of crustaceans, the X-organ sinus gland (XO-SG) complex regulates vitellogenesis antagonistically through the neuropeptides vitellogenesis/gonad-inhibiting hormone (VIH/GIH) and vitellogenesis/gonad-stimulating hormone (VSH/GSH). Through eyestalk ablation and *in vitro* assays, VIH has been shown to negatively regulate vitellogenesis (Tsutsui *et al.*, 2007; Treerattrakool *et al.*, 2008). Conversely, research conducted on the effects of GSH suggest a stimulatory effect on vitellogenesis (Takayanagi *et al.*, 1986; Sarojini *et al.*, 1995; Fingerman, 1997).

Methyl farnesoate (MF) is a sesquiterpene that is structurally homologous to the insect juvenile hormone involved in vitellogenesis. Consequently, MF has been suggested to have a comparable function in crustaceans. Several studies on various crustaceans have reported that MF might be involved in vitellogenesis (Laufer *et al.*, 1987; Nagaraju *et al.*, 2003; Nagaraju *et al.*, 2004).

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Ecdysteroids, ecdysone and 20-hydroxyecdysone, are steroid hormones that are involved in moulting and have also been suggested to function in vitellogenesis. However, studies have reported conflicting results (Chaix & De Reggi, 1982; Gunamalai *et al.*, 2004; Tiu *et al.*, 2006; Kappalli *et al.*, 2012; Shyamal *et al.*, 2014). This suggests that, although ecdysteroids may have a role in vitellogenesis, the exact function is still unclear and it appears to differ between taxa (Subramoniam, 2000).

The presence of vertebrate-type steroids, oestrogens and progesterone in crustaceans, has been known for some time but the function(s) remain unclear. Studies have focused on finding a correlation between vertebrate steroid titres and the progression of the reproductive cycle (Warrier *et al.*, 2001; Kirubagaran *et al.*, 2005; Gunamalai *et al.*, 2006). Yano & Hoshino, (2006) and Coccia *et al.*, (2010) have attempted to induce vitellogenesis by exposing crustaceans to vertebrate steroids. Recently, Li *et al.*, (2014a) demonstrated that 17β-oestradiol decreased the expression of GIH mRNA in the eyestalks of *Litopenaeus vannamei*, suggesting a possible stimulatory mechanism for oestrogen on vitellogenesis.

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The VTG gene is present in male crustaceans but it is negatively regulated by AGH; a hormone produced by the androgenic gland (AG) found only in male crustaceans. A few studies have demonstrated this suppressive effect of AGH on VTG synthesis through andrectomy of males and AG implantation into females (Suzuki *et al.*, 1990; Sagi *et al.*, 1999; Manor *et al.*, 2004).

4.1.6 Influence of environmental factors on vitellogenesis

Brachyuran crabs exhibit an iteroparous reproductive cycle and breeding can occur throughout the year (continuous) or seasonally (Sudha & Anilkumar, 1996; Cobo, 2002a; Cobo & Fransozo, 2003). The breeding period is the period in which the highest percentage or proportion of ovigerous females can be found (Litulo, 2005). Environmental factors have been reported to influence the reproductive periodicity of crustaceans (Cobo, 2002a; Rosa & Nunes, 2002; Pereira Rodrigues de Lira *et al.*, 2013). Studies have found that crabs from tropical and subtropical areas exhibit a continuous breeding cycle (Cobo, 2002a; Litulo, 2005). A higher frequency of ovigerous female crabs has been reported during warmer months (Ituarte *et al.*, 2004; Pereira Rodrigues de Lira *et al.*, 2013). Rainfall was also found to correlate highly with the frequency of ovigerous females (Litulo, 2004). Other factors such as the lunar cycle and photoperiod have also been reported to influence the reproductive cycle (López Greco & Rodríguez, 1999; Cobo & Fransozo, 2003). However, as Liu & Li, (2000) remarked, studies that provide information of the reproduction of freshwater crabs is "scanty".

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4.1.7 Potamonautes perlatus (P. perlatus)

Potamonautes perlatus is a freshwater crab that is also known as the Cape River crab. It is endemic to the Western Cape but can also be found in areas North of East of Clanwilliam and has been reported to occur along the Buffalo River (Hill & O'Keeffe, 1992; Daniels, 2003). *P. perlatus* belongs to the most widely distributed genus of freshwater crabs in Africa and *Potamonautes* species can be found throughout sub-Saharan Africa, except for the Saharan desert and the Maghreb (Cumberlidge, 1999).

4.1.8 Rationale and aim of the study

The reproductive cycle of *P. perlatus* females has not yet been investigated. Thus, the aim of this study is to determine the reproductive cycle of *P. perlatus* by investigating the seasonal

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synthesis of VTG and Vn in the HP and ovaries. This study will use the anti-pVn mAbs produced in chapter 2 to quantify the levels of VTG and Vn to determine the seasonal synthesis thereof in *P. perlatus*. This study will also make use of the haemolymph to monitor VTG levels, which confers the advantage of using a non-injurious technique. Research into the reproductive cycle would also indicate the optimal time of year to sample females for studies on vitellogenesis. This research could also be potentially useful in determining the impact of pollutants on vitellogenesis.

4.2 MATERIALS AND METHODS

4.2.1 Crab collection

Six male and six female *Potamonautes perlatus* crabs, of approximately the same size, were collected every three to four months from Jonkershoek, Stellenbosch (GPS coordinates: 33°56'25.9" S 18°53'24.3" E). The sampling period was carried out from May 2014 – March 2015, where each collection corresponded approximately to each one of the four seasons. The total body weight of each crab was measured the day after capture. The haemolymph, HP, and gonads were also collected the day after capture.

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4.2.2 Haemolymph collection and preparation

Haemolymph was collected from the sinuses at the base of the second walking leg and mixed with 3.2% sodium citrate (Vacuette, Austria) as an anticoagulant. A protease inhibitor cocktail (Sigma-Aldrich, USA) was prepared in phosphate buffered saline (PBS; Lonza, Belgium) (1:200, v/v) and a volume was added to equal that of the haemolymph. The haemolymph-mixture was centrifuged at 2 469 relative centrifugal force (rcf) for 5 mins to remove the haemocytes. Thereafter, the haemocyte pellet was discarded and the remaining haemolymph was stored in aliquots at -80°C.

4.2.3 Tissue preparation

After the haemolymph was collected, the crabs were cryo-anaesthetized by subjecting them to -80°C for 3 mins, after which, the HP, gills, and gonads were collected. At the collection of the organs, the mass of each organ was also measured and recorded. These measurements were used to determine the organ indices HPSI for the HP, and GSI for the gonads. The indices were

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calculated by weighing the respective organs and expressing them as a percentage of the total body mass. The following formula was used:

$$\text{Index (GSI or HPSI)} = \frac{\text{Organ, wet mass}}{\text{Body mass}} \times 100$$

Haemolymph samples were further diluted (1:50, v/v) with PBS containing protease inhibitors to determine the total protein concentration. The PBS protease inhibitor cocktail was also added to the ovaries and the HP to give 100 mg tissue per ml of buffer. The ovaries and HP were then mechanically homogenised (Tissuemiser, Fisher Scientific). In addition to the protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF; 1 mM) was added to the HP homogenates.

4.2.4 Protein determination and native polyacrylamide gel electrophoresis (PAGE)

Samples were analysed as described in sections 3.2.2 and 3.2.3.2 for protein determination and native polyacrylamide gel electrophoresis (PAGE) respectively. For the native PAGE, the following protein amounts were applied per lane of the gel: male and female haemolymph (2.5 µg), ovarian homogenates (0.25 µg), HP homogenates (3 µg), and pVn (0.5 µg).

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4.2.5 Western blot

Proteins from the native PAGE gels were electrophoretically transferred onto a 0.45 µm supported nitrocellulose membrane (Santa Cruz Biotechnology, USA). The transfer was performed at room temperature in transfer buffer (10% (v/v) methanol in 25 mM Tris, 192 mM Glycine) at 30 V and 250 mA over 125 mins with a semi-dry transfer unit (TE70X Hoefer). To confirm a successful transfer, the membranes were stained with amido black (0.1% (w/v) amido black, 50% (v/v) methanol, 10% (v/v) acetic acid) and subsequently destained with amido black destain solution (20% (v/v) methanol and 7.5% (v/v) acetic acid).

After confirming a successful transfer, membranes were blocked with 2% (v/v) human serum albumin (HAS) in PBS at room temperature for 2 hours. Thereafter, the anti-pVn mAb (0.1 µg/ml) produced by hybridoma clone 2H4A3B6H3 was prepared in diluent and membranes were incubated overnight at 4°C with this antibody. The following day, membranes were washed 3 times with wash buffer in intervals of 15 mins. After that, membranes were incubated with rabbit-anti-mouse immunoglobulin G (IgG) HRP conjugate (1:5 000, v/v) for 1 hour.

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Subsequently, membranes were washed 4 times with wash buffer at 15 min intervals and tetramethylbenzidine (TMB) (KPL, USA) substrate was added for the visualisation of positive bands.

4.2.6 Densitometry and statistical analysis

ImageJ (version 1.50i) was used to perform densitometry. Densitometry was used to semi-quantify the VTG content of the HP and haemolymph and the Vn content of the ovaries of each sample throughout the sampling period. Each measurement was expressed as a percentage of the control and repeated five times to allow for the calculation of statistical differences.

SigmaPlot (v12.0) was used for statistical analysis. Data was analysed by One-Way Analysis of Variance (ANOVA) with the Holm-Sidak test as a multiple comparison test and confirmed with an unpaired t-test. A P-value of less than 0.05 was considered statistically significant. All data is presented as the mean ± standard error of the mean (SEM). Graphpad Prism (version 5.01) was used to construct statistical figures.

4.3 RESULTS & DISCUSSION

A monoclonal antibody (mAb) was previously developed against Vn purified from the ovaries of *P. perlatus* females (chapter 3). This anti-pVn mAb was used in western blots for the semi-quantification of VTG and Vn in *P. perlatus*. This study ran from May (2014) – March (2015) where male and female *P. perlatus* crabs were collected approximately every three to four months from the Jonkershoek area in Stellenbosch. All female crabs were sampled for haemolymph, HP, and ovaries, while the male crabs were only sampled for haemolymph. These tissue samples were subsequently assayed by western blot with the anti-pVn mAb for VTG and Vn as a measure of the reproductive cycle of female *P. perlatus*. The results have been represented in chronological order as the specific time points in which the crabs were collected, i.e. May (2014) – March (2015). The seasons correspond only to the months in which the crabs were collected: summer (November), autumn (March), winter (May), and spring (August).

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4.3.1 Physical parameters

Morphological changes in organs associated with the reproductive process have been correlated to the progression of the reproductive cycle (Quinitio *et al.*, 2007; Revathi *et al.*, 2012). Accordingly, some crude, qualitative measurements such as the colour of the ovaries, and the HPSI and GSI were used to determine the reproductive state of an organism (Lee & Chang, 1997; Revathi *et al.*, 2012).

4.3.1.1 Weight

Prior to tissue sampling, all crabs were weighed, and somatic indices GSI and HPSI were determined after tissue sampling as measures of reproductive activity (table 4.1). The lowest mean weight was found in November (2014), while the highest mean weight was found in March (2015). A gradual decrease in the mean weight of female crabs was observed from May (2014) to November (2014). The mean weight in November (2014) was significantly lower than the mean weights in May (2014) ($P = 0.003$), August (2014) ($P = 0.034$) and March (2015) ($P = 0.002$).

4.3.1.2 Gonadosomatic index (GSI)

The ovaries of the female crabs were weighed and expressed as a percentage in relation to their body weight, reported as the GSI (table 4.1). March (2015) was reported to have the lowest GSI, while the highest GSI was found in August (2014). It should be noted that in November (2014), only two of the six females were found to have discernible ovaries and, therefore, have a recorded GSI. A significant difference was found between the GSI in August (2014) and in March (2015) ($P \leq 0.001$).

4.3.1.3 Hepatosomatic index (HPSI)

Like the gonads, the HP were also weighed and expressed as the HPSI (table 4.1). The lowest HPSI was observed in May (2014), after which it peaked in August (2014) and remained fairly constant until March (2015). The HPSI in May (2014) was significantly lower compared to March (2015) ($P = 0.001$) and August (2014) ($P = 0.007$).

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Table 4.1. Physical parameters measured for female *P. perlatus* crabs for the sampling period of May (2014) – March (2015). Data is presented as the mean ± SEM. The sample size for every collection was n = 6, except for November (2014) where n = 2.

Physical parameters	May (2014)	August (2014)	November (2014)	March (2015)
Females				
Mean weight (grams)	25.05 ± 0.9641*	22.65 ± 0.9916*	18.41 ± 1.4134*	25.35 ± 0.9856*
Mean GSI (%)	2.41 ± 0.6326	3.50 ± 0.3666*	1.64 ± 1.1950	1.19 ± 0.1162*
Mean HPSI (%)	4.38 ± 0.3602*	6.41 ± 0.4743*	6.00 ± 0.8447	6.36 ± 0.2654*

*indicates significant ($P < 0.05$) differences between groups. Significant differences between groups are explained in the text. GSI – gonadosomatic index, HPSI – hepatosomatic index.

4.3.2 The reproductive cycle of P. perlatus: semi-quantification of VTG content in tissue samples by western blot

Circulating levels of VTG have been shown to correspond to the reproductive state of an oviparous organism and have thus been employed as a measure of reproductive activity in these organisms (Lee & Chang, 1997; Ferré *et al.*, 2012; Revathi *et al.*, 2012). Therefore, the biochemical marker VTG was used in conjunction with the morphological changes to provide a more accurate and representative indication of the reproductive state of *P. perlatus* during the collection period. Western blots were performed with the anti-pVn mAb to semi-quantify the VTG content in the various tissues of *P. perlatus* females for the entire sampling period. The VTG content in each of the tissues was expressed relative to the control that was confirmed to have VTG in the haemolymph and Vn in the ovaries (chapter 3).

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4.3.2.1 Hepatopancreas

In some crustaceans, VTG is synthesised in the HP (Subramoniam, 2011). After synthesis, VTG undergoes post-translational modifications such as glycosylation, lipidation, and conjugation to carotenoids. It is then rapidly processed and secreted into the haemolymph (Pateraki & Stratakis, 1997). In the current study, there were inconsistent detections of VTG in the HP of *P. perlatus* (figures 4.1 and 4.2). A similar report has been attributed to factors such as rapid processing after its synthesis and the inability of the antibodies to access the epitopes in the different forms of the protein (Zmora *et al.*, 2007). These factors could have made it difficult to detect VTG in the HP at the protein level. Other studies have also reported difficulty

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in detecting VTG in the HP, where the proteins from the HP were found to be smaller than 100 kDa, or the antibodies were unable to detect VTG in the HP by immunoblotting (Chan *et al.*, 2005; Auttarat *et al.*, 2006; Zmora *et al.*, 2007; Hiransuchalert *et al.*, 2013). Consequently, some studies have taken to measuring VTG mRNA as an indicator of vitellogenesis (Jayasankar *et al.*, 2002; Zmora *et al.*, 2007; Hiransuchalert *et al.*, 2013).

Auttarat *et al.*, (2006) suggested that the protease content of the HP could be too high and might not be completely inhibited by the serine protease inhibitor PMSF. However, Lee & Chang, (1997) prepared ovarian and HP extracts with and without 2 mM PMSF. The authors reported no difference in the protein concentration of both ovarian and HP extracts with and without PMSF. As the authors used double the concentration of PMSF than was used in the current study, it seems unlikely that neither a high protease content nor the insufficient inhibition of proteases might have been responsible for the lack of detection of VTG in the HP. Due to the inconsistency in the detection of VTG in the HP of *P. perlatus*, the VTG content of the HP was not quantified in this study.

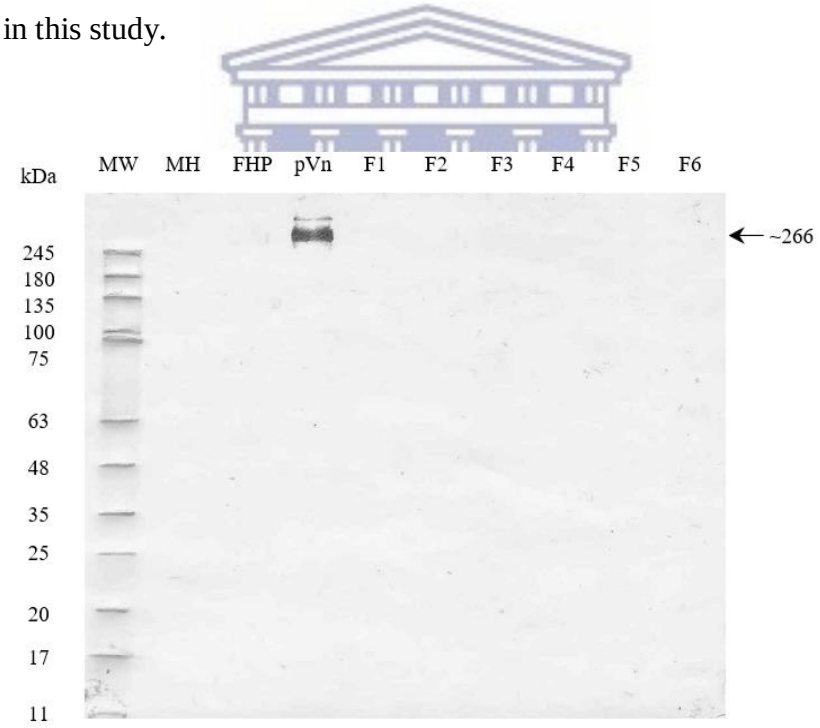

Figure 4.1. Western blot of *P. perlatus* female HP samples using the anti-pVn mAb for the sampling point: August (2014). Molecular weight marker (MW), male haemolymph (MH), female HP (FHP), purified vitellin (pVn), female HP samples 1-6 (F1-6). The arrow indicates VTG and pVn at approximately 266 kDa.

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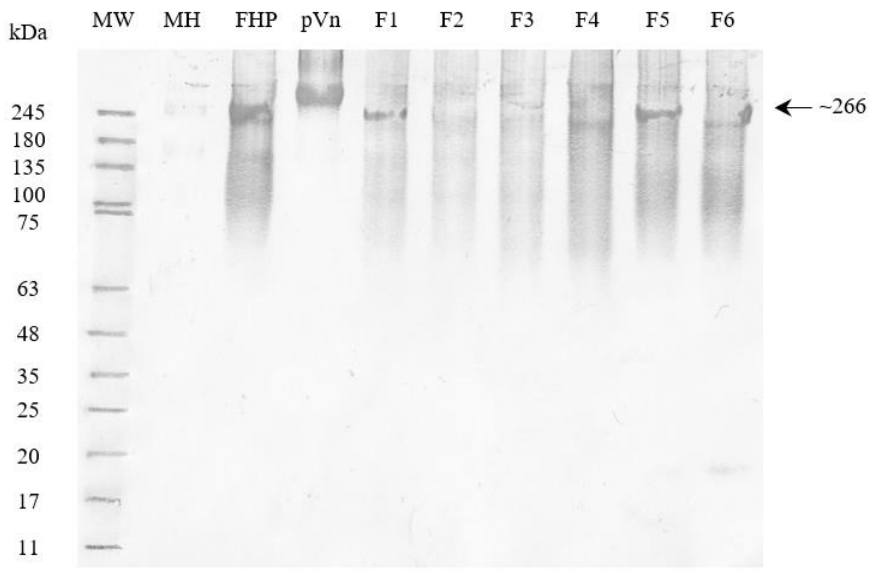

Figure 4.2. Western blot of *P. perlatus* female HP samples using the anti-pVn mAb for the sampling point: November (2014). Molecular weight marker (MW), male haemolymph (MH), female HP (FHP), purified vitellin (pVn), female HP samples 1-6 (F1-6). The arrow indicates VTG and pVn at approximately 266 kDa.

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4.3.2.2 Haemolymph

After synthesis, VTG is rapidly secreted from the HP into the haemolymph. The highest VTG content in the haemolymph was found in August (2014), while the lowest was found in November (2014) (figure 4.7). Throughout the sampling period, the VTG content in the males was considerably lower than in any of the females. There was no significant ($P = 0.383$) difference in the VTG content of the females throughout the sampling period. There were, however, significant differences between the VTG content of the males and females in May (2014) ($P = 0.0345$), August (2014) ($P = 0.0127$), and March (2015) ($P = 0.0363$).

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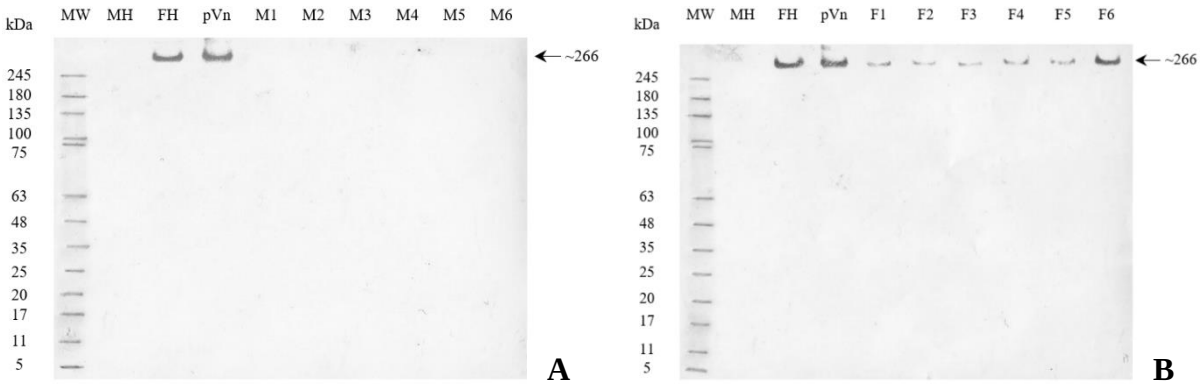

Figure 4.3. Western blot of *P. perlatus* male (A) and female (B) haemolymph samples using the anti-pVn mAb for the sampling point: May (2014). Molecular weight marker (MW), male haemolymph (MH), female haemolymph (FH), purified vitellin (pVn), male haemolymph samples 1-6 (M1-6), female haemolymph samples 1-6 (F1-6). The arrow indicates VTG and pVn at approximately 266 kDa.

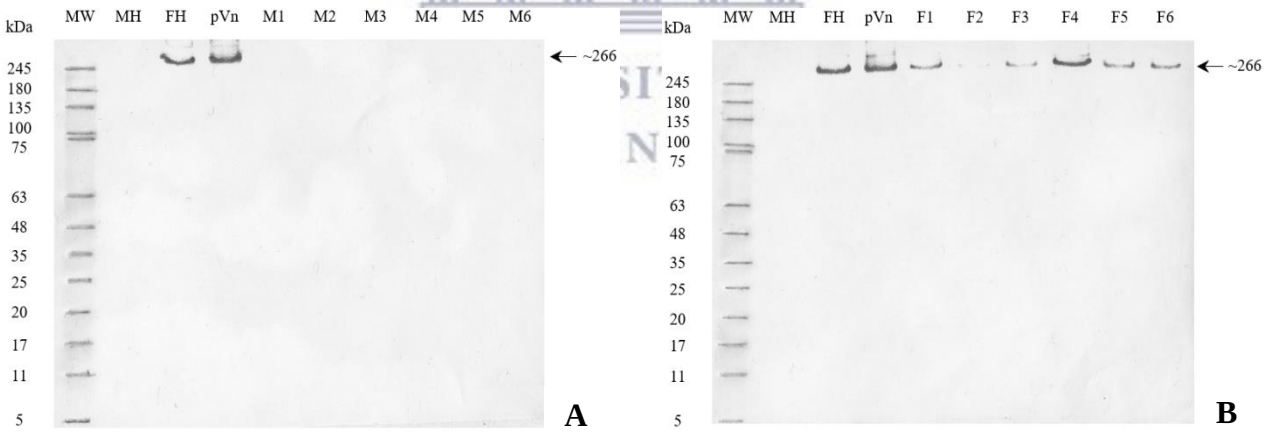

Figure 4.4. Western blot of *P. perlatus* male (A) and female (B) haemolymph samples using the anti-pVn mAb for the sampling point: August (2014). Molecular weight marker (MW), male haemolymph (MH), female haemolymph (FH), purified vitellin (pVn), male haemolymph samples 1-6 (M1-6), female haemolymph samples 1-6 (F1-6). The arrow indicates VTG and pVn at approximately 266 kDa.

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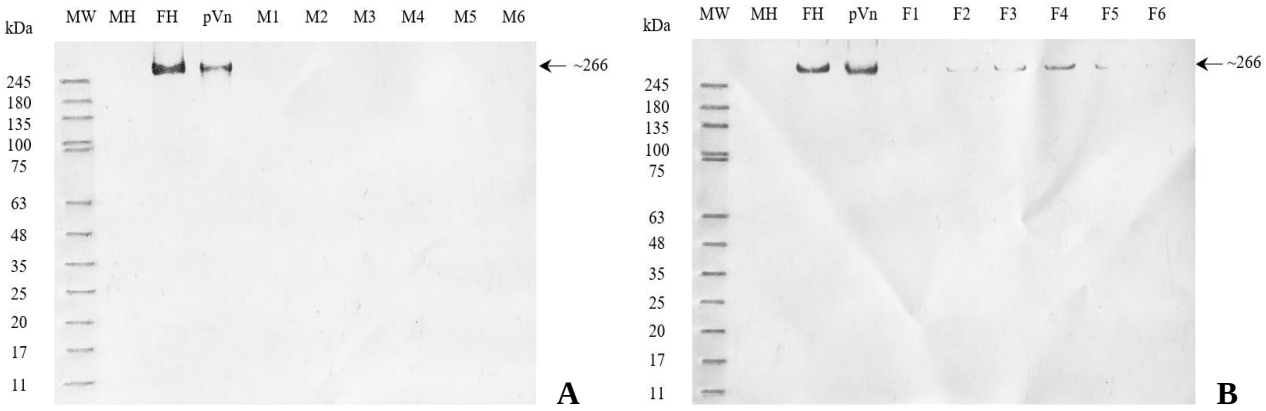

Figure 4.5. Western blot of *P. perlatus* male (A) and female (B) haemolymph samples using the anti-pVn mAb for the sampling point: November (2014). Molecular weight marker (MW), male haemolymph (MH), female haemolymph (FH), purified vitellin (pVn), male haemolymph samples 1-6 (M1-6), female haemolymph samples 1-6 (F1-6). The arrow indicates VTG and pVn at approximately 266 kDa.

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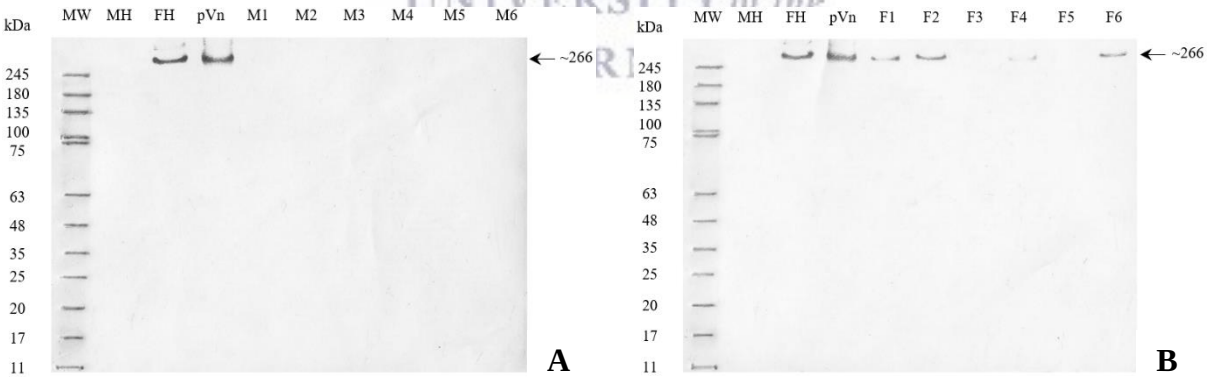

Figure 4.6. Western blot of *P. perlatus* male (A) and female (B) haemolymph samples using the anti-pVn mAb for the sampling point: March (2015). Molecular weight marker (MW), male haemolymph (MH), female haemolymph (FH), purified vitellin (pVn), male haemolymph samples 1-6 (M1-6), female haemolymph samples 1-6 (F1-6). The arrow indicates VTG and pVn at approximately 266 kDa.

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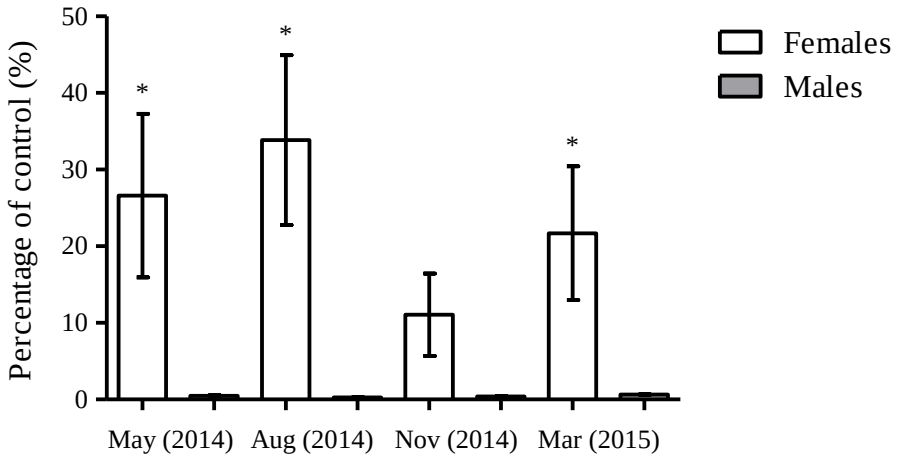

Figure 4.7. Vitellogenin content of the haemolymph samples from the western blot data as determined by densitometry with ImageJ (described in section 4.2.6). Samples from each group were expressed as a percentage of the control. Data is presented as the mean ± SEM of n = 5. *indicate significant differences ($P < 0.05$) in the VTG content between male and female haemolymph for the respective sampling points. Significant differences between groups are explained in the text.

4.3.2.3 Ovaries

Vitellogenin in the haemolymph is taken up by the ovaries where it is broken down into various subunits that make up the major yolk protein, Vn. The lowest Vn content in the ovaries was recorded in November (2014), while the highest was found in August (2014) (figure 4.12). Significant differences were found between August (2014) and May (2014) ($P = 0.0002$), November (2014) ($P = 0.002$), and March (2015) ($P = 0.0003$); and November (2014) and March (2015) ($P = 0.014$). No significant differences were found between May (2014) and November (2014) and March (2015). Furthermore, it should be noted that in November (2014) only two of the six samples were found to have discernible ovaries. This is likely an indication of the reproductive state of the majority of samples collected from that period. It can be inferred that the other four samples without any visible ovaries had likely taken on an opaque-white to pale yellow colour, resembling the colour of the HP. Thus, it can be argued that the ovaries of those four samples resembled a pre- or early primary vitellogenic state, or atretic ovaries (Quinitio *et al.*, 2007; Antunes *et al.*, 2010).

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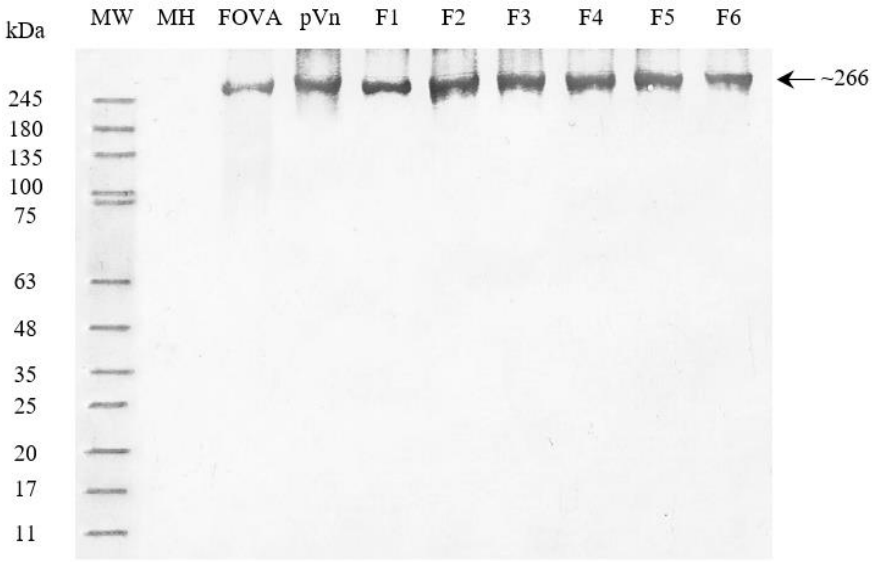

Figure 4.8. Western blot of *P. perlatus* female OVA samples using the anti-pVn mAb for the sampling point: May (2014). Molecular weight marker (MW), male haemolymph (MH), female ovary (FOVA), purified vitellin (pVn), female OVA samples 1-6 (F1-6). The arrow indicates VTG, pVn, and Vn at approximately 266 kDa.

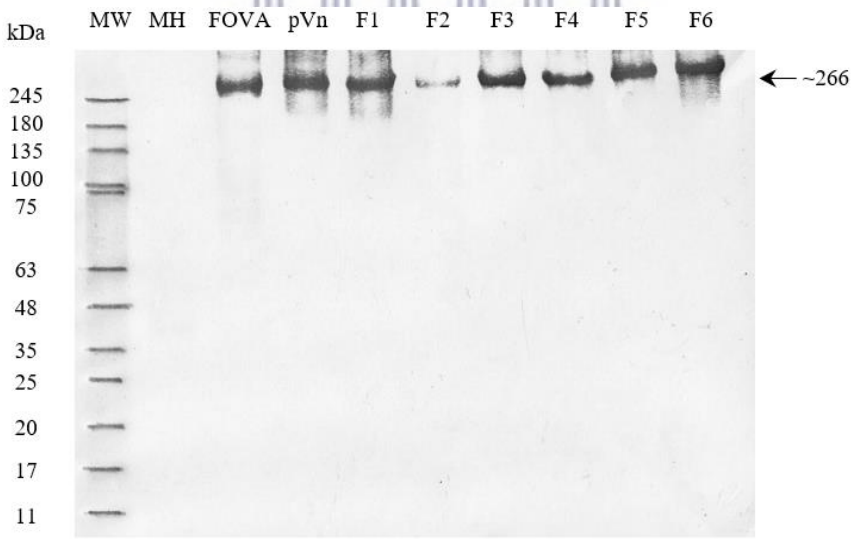

Figure 4.9. Western blot of *P. perlatus* female OVA samples using the anti-pVn mAb for the sampling point: August (2014). Molecular weight marker (MW), male haemolymph (MH), female ovary (FOVA), purified vitellin (pVn), female OVA samples 1-6 (F1-6). The arrow indicates VTG, pVn, and Vn at approximately 266 kDa.

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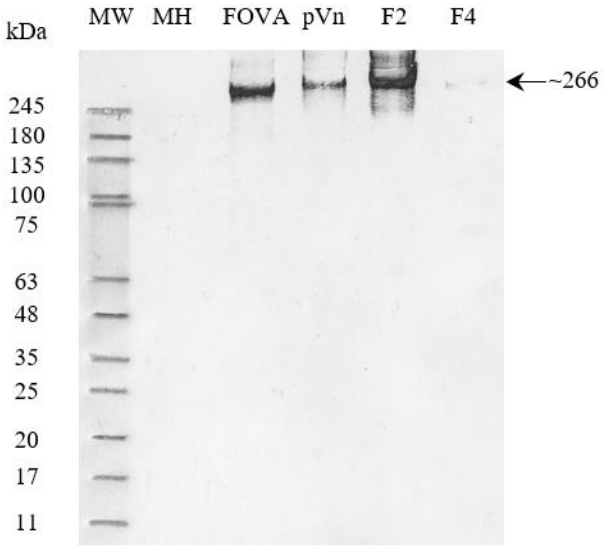

Figure 4.10. Western blot of *P. perlatus* female OVA samples using the anti-pVn mAb for the sampling point: November (2014). Molecular weight marker (MW), male haemolymph (MH), female ovary (FOVA), purified vitellin (pVn), female OVA samples 2 and 4 (F2, 4). The arrow indicates VTG, pVn, and Vn at approximately 266 kDa.

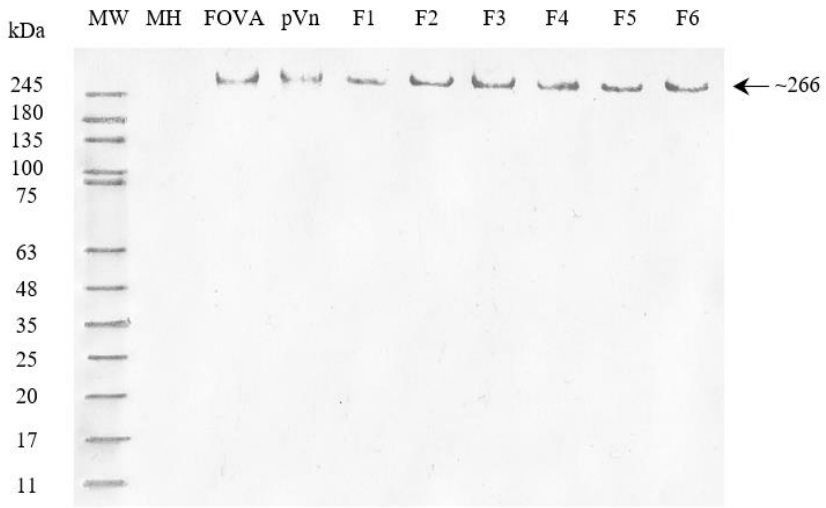

Figure 4.11. Western blot of *P. perlatus* female OVA samples using the anti-pVn mAb for the sampling point: March (2015). Molecular weight marker (MW), male haemolymph (MH), female ovary (FOVA), purified vitellin (pVn), female OVA samples 1-6 (F1-6). The arrow indicates VTG, pVn, and Vn at approximately 266 kDa.

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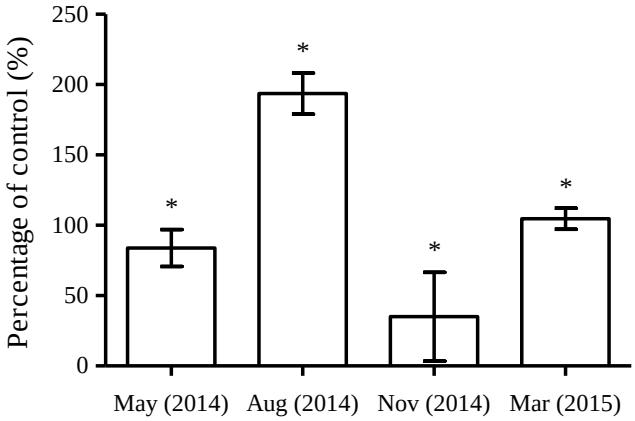

Figure 4.12. Vitellin content of the OVA samples from the western blot data as determined by densitometry with ImageJ (described in section 4.2.6). Samples from each group were expressed as a percentage of the control. Data is presented as the mean ± SEM of n = 5. *indicates significant ($P < 0.05$) differences between groups. Significant differences between groups are explained in the text.

Liu & Li, (2000) described the availability of information on the reproduction of freshwater crabs as "scanty". From the background reading into this study, this description can be corroborated. During vitellogenesis, VTG levels can fluctuate. Initially, VTG is synthesised in the HP during primary vitellogenesis with a concurrent increase in haemolymph levels. This process occurs slowly and extends over several months (Chen *et al.*, 2004). During this phase, the ovaries undergo morphological changes and the GSI gradually increases as the circulating VTG is taken up from the haemolymph and incorporated into the ovaries (Lee & Chang, 1997; Zmora *et al.*, 2007; Ferré *et al.*, 2012). With the onset of secondary vitellogenesis, VTG and Vn levels are reportedly similar in the HP and the ovaries, while there is a peak in the haemolymph levels. Near the end of the reproductive cycle, prior to oviposition, the HP and haemolymph levels of VTG decrease while Vn concentrations remain high in the ovaries from the incorporation of circulating VTG (Lee & Chang, 1997; Bembe, 2009). At this time, the ovaries rapidly increase in size and vitellogenesis culminates in oviposition (Chen *et al.*, 2004). The fluctuations in the physical parameters, as well as the VTG and Vn levels in *P. perlatus*, followed a similar pattern.

To reiterate, the time points at which the crabs were collected have been grouped according to seasons. Thus, the reproductive cycle of *P. perlatus* will be discussed in terms of the different seasons rather than in chronological order so as to make the comparison between collection points easier and to simplify the discussion. Each season will be discussed in comparison to

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the next season. The seasons have been allocated according to the following time intervals so as to correspond only to the months in which the crabs were collected: summer (November vs March), autumn (March vs May), winter (May vs August), and spring (August vs November). Oviposition was inferred to have taken place just before November (2014) and thus, the discussion will start at summer. As the HP is proposed to be the common site of VTG synthesis, each section will start with the discussion of parameters associated with the HP. As the haemolymph receives the VTG produced by the HP and transports it to the ovaries, the haemolymph parameters will be discussed next, followed by the parameters of the ovaries as the target tissue for VTG.

4.3.2.4 Summer: November vs March

In this study, it was inferred that oviposition had occurred shortly before summer, near the end of spring (Lee & Chang, 1997; Tsukimura *et al.*, 2002). Based on that premise, it can be argued that primary vitellogenesis started in summer and that an increase in VTG synthesis can be expected in the HP. However, the HPSI was stable (6.00 ± 0.845% to 6.36 ± 0.265%) and demonstrated no significant fluctuation (table 4.1). Therefore, at this point, the inconsistency in the VTG content of the HP together with the stable HPSI offers no clear role for the HP in the reproductive cycle of *P. perlatus*.

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At the same time, the VTG content in the haemolymph only increased moderately (11.1 ± 5.373% to 21.7 ± 8.719%) (figure 4.7). This increase in the haemolymph VTG content suggests that VTG is synthesised by either the HP or the ovaries and is rapidly secreted into the haemolymph. Regardless, this increase in the haemolymph VTG content also suggests that the previous reproductive cycle had recently ended, which then created the need for VTG to be synthesised in preparation for the commencement of the next cycle.

Simultaneously, there was a significant (P = 0.014) increase (35.0 ± 31.583% to 104.7 ± 7.49%) in the Vn content of the ovaries (figure 4.12). However, at this point in the reproductive cycle the uptake of VTG from the haemolymph was not sufficient to increase the ovarian weight as the GSI was also fairly stable (1.64 ± 1.195% to 1.19 ± 0.116%) (table 4.1). The marked increase in Vn content at the end of the summer indicates that the ovaries function in the sequestration and subsequent processing of circulating VTG into Vn. It also suggests that the ovaries might be a possible site of vitellogenesis in *P. perlatus*, rather than merely incorporating VTG into the tissue. It could be an indication of autologous Vn production by

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the ovaries during primary vitellogenesis in a contributing role (Subramoniam, 2011). However, autologous Vn production in the ovaries would increase the protein content of the ovary and, consequently, increase the GSI. As the GSI remained stable, it is difficult to say whether this was truly the case.

From this season, the data suggests that the previous vitellogenic cycle had recently ended and culminated in oviposition. While certain parameters were stable during this part of the cycle, others had increased in preparation for the new vitellogenic cycle.

4.3.2.5 Autumn: March vs May

The HPSI decreased (6.359 ± 0.265% to 4.383 ± 0.36%) significantly (P = 0.001) and was accompanied by an increase in the haemolymph VTG content, albeit only slightly (21.7 ± 8.719% to 26.6 ± 10.677%) (table 4.1 and figure 4.7). Thus, it can be inferred that VTG synthesis in the HP was increased to compensate for the new vitellogenic cycle and is now rapidly being secreted into the haemolymph. The rapid secretion of VTG from the HP would lower the protein content of the HP and explain why there was a decrease in the HPSI.

The Vn content reflected a slight decrease (104.678 ± 7.49% to 83.855 ± 13.215%) (figure 4.12). Conversely, the GSI increased nearly two-fold (1.188 ± 0.116% to 2.413 ± 0.633%) (table 4.1). This could simply be a demonstration of a delayed reflection by the ovaries having taken up a large amount of the circulating VTG that was detected at the end of summer. This argument might also explain why the VTG content of the haemolymph from this season reflected only a slight increase compared to the moderate increase reported in summer. This increase in GSI would also support the possibility of autologous Vn production by the ovaries as was suggested in summer.

In this season, the decrease in the HPSI, accompanied by an increase in circulating VTG levels, suggest the rapid synthesis and subsequent secretion of VTG from the HP into the haemolymph. The marked increase in the GSI could simply reflect a delay in the removal of residual circulating VTG that was detected at the end of summer.

4.3.2.6 Winter: May vs August

The HPSI increased again (4.383 ± 0.360% to 6.407 ± 0.474%) significantly (P = 0.007) (table 4.1). The VTG content of the haemolymph also increased (26.602 ± 10.677% to 33.879 ±

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11.089%), reaching the highest VTG content throughout the cycle (figure 4.7). The Vn content in the ovaries demonstrated a significant ($P \leq 0.001$) increase (83.9 ± 13.215 to 193.6 ± 14.660) (figure 4.12) and was paralleled by an increase in the GSI (2.413 ± 0.633% to 3.495 ± 0.367%) (table 4.1). This strongly suggests that the ovaries took up circulating VTG and consequently now contain large amounts of Vn.

The recovery of the HPSI, the peak in haemolymph VTG content, along with the high Vn content and GSI, all indicate that primary vitellogenesis has ended during this season and marks the onset of secondary vitellogenesis (Ferré *et al.*, 2012; Revathi *et al.*, 2012). Furthermore, in the reproductive cycle of *P. perlatus*, the peak in the haemolymph VTG content during this season is consistent with reports that circulating VTG levels peak prior to oviposition (Tsukimura, 2001; Okumura *et al.*, 2004). From the data in this season, the highest level of activity in the reproductive cycle of *P. perlatus* takes place during winter and peaks at the end of winter, i.e. in August.

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4.3.2.7 Spring: August vs November

The HPSI stabilised again (6.407 ± 0.474% to 6.002 ± 0.845%), similar to what was observed in summer (table 4.1). In line with primary vitellogenesis ending in the previous season, the stabilisation of the HP could suggest that VTG is no longer being produced by the HP and that this cycle of vitellogenesis is coming to an end. There was a decline in the haemolymph VTG content (33.9 ± 11.089% to 11.1 ± 5.373%) which could be attributed to a halt in the production of VTG by the HP, together with the uptake of the remaining levels of circulating VTG by the ovaries (figure 4.7). The lack of a significant difference between the VTG content of the male and female haemolymph in this season suggests that VTG levels in the female haemolymph might be comparable to the levels in male haemolymph. Only at the start of the next reproductive cycle in summer VTG synthesis would increase again which, in turn, would lead to an increase in the VTG content of the haemolymph (figure 4.7). The Vn content of the ovaries decreased (193.6 ± 14.660% to 35.0 ± 31.583%) significantly ($P = 0.002$) and was accompanied by a decrease in GSI (3.50 ± 0.367% to 1.64 ± 1.195%), quite likely indicating that the reproductive cycle had ended and culminated in oviposition (table 4.1 and figure 4.12).

The significant decrease in the Vn content of the ovaries between spring and summer, along with the marked decrease in the GSI strongly suggests that oviposition had occurred in spring, shortly before November. Furthermore, the presence of discernible ovaries in only two of the

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specimens that were collected for the November (2014) sampling also supports the argument that vitellogenesis had ended shortly before November and that oviposition ensued. This is likely an indication of the reproductive state of the majority of samples collected from that period. It can be inferred that the other four samples without any apparent ovaries had likely taken on an opaque-white to pale yellow colour, resembling the colour of the HP. Thus, it can be argued that the ovaries of those four samples resembled a pre- or early primary vitellogenic state, or atretic ovaries (Quinitio *et al.*, 2007).

Notwithstanding, the evidence suggests the most plausible argument would be that the females had recently undergone oviposition and that the ovaries had become atretic or reverted to a primary vitellogenic state in preparation for the commencement of the next reproductive cycle. Moreover, the bright orange appearance (data not shown) of only one of the two discernible ovary samples from the November (2014) sampling showed that it was the only sample that was reproductively active. Whereas, the other ovary sample was pale and white to yellow in appearance (data not shown). This suggests that the latter specimen was also not reproductively active at the time or had recently undergone oviposition, further supporting the argument that vitellogenesis had ended and that oviposition occurs between August and November. Accordingly, the marked decrease in the GSI and Vn content of the ovaries along with the peak in the VTG content of the haemolymph, it can be inferred that oviposition occurred in the latter part of spring, just before November.

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The fluctuations in VTG and Vn levels over the reproductive cycle of *P. perlatus* from this study are comparable to those found in the reproductive cycle of the freshwater crayfish *Cherax quadricarinatus* (Ferré *et al.*, 2012). The HPSI and VTG concentration in the haemolymph were lowest during the late reproductive period, while the GSI and the ovarian Vn levels were highest during this period. Moreover, in the post-reproductive period, the HPSI increased again significantly, suggesting preparation for the commencement of the next reproductive cycle. Furthermore, the fluctuations of VTG in the haemolymph of the American lobster, *Homarus americanus*, during ovarian maturation are also comparable to the current study (Tsukimura *et al.*, 2002). The authors reported elevated levels of VTG in the haemolymph during primary vitellogenesis, which peaked during secondary vitellogenesis. The ovarian mass and oocyte size were also reported to increase most rapidly during secondary vitellogenesis. Avarre *et al.*, (2003) reported a similar trend of VTG mRNA expression in the HP of the marine shrimp, *Penaeus semisulcatus*. Previtellogenic levels were undetectable and markedly increased once

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the shrimp became vitellogenic and gradually decreased with the progression of vitellogenesis. This is similar to the trend of the VTG content in the HP in this study.

In accordance with the literature, the overall VTG content in the male haemolymph was significantly lower than that of the female haemolymph throughout most of the sampling period as VTG is a female-specific protein that is not produced in male oviparous organisms (figure 4.7) (Tsukimura, 2001; Xuereb *et al.*, 2011). As evident by the western blot data, the significant differences in VTG content between the males and females throughout most of the sampling period support the identity of VTG as a female-specific protein, with the exception of November (2014) (figure 4.7). However, as the latter difference was not significant, it can be inferred that the circulating VTG content in both male and female crabs for that time point was virtually similar. It could thus be argued that the haemolymph VTG levels of the females were low enough, almost to the extent that they mimic the levels normally found in the male haemolymph.

The data shows that the morphological parameters and fluctuations in VTG and Vn detected in this study correlate with the gonadal development and subsequent reproductive cycle of *P. perlatus* as described earlier. That is the initial synthesis of VTG, possibly in the HP, accompanied by a steady increase in circulating VTG levels. Thereafter, the ovaries underwent morphological changes and increased in size as circulating VTG was incorporated into the ovaries and converted to Vn, in turn, causing an increase in the GSI (Lee & Chang, 1997; Zmora *et al.*, 2007; Ferré *et al.*, 2012). This occurred gradually, over the course of several months from summer to winter (Chen *et al.*, 2004). The recovery of the HPSI and peaks in the VTG content of the haemolymph and Vn content of the ovaries during winter marked the end of primary vitellogenesis and the onset of secondary vitellogenesis. Thus, secondary vitellogenesis was characterised by a stable HPSI, high levels of circulating VTG, and a high GSI and Vn content in the ovaries (Lee & Chang, 1997; Bembe, 2009). The reproductive cycle ended shortly thereafter in spring, which saw the culmination of vitellogenesis in oviposition (Chen *et al.*, 2004).

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4.3.3 Site of vitellogenesis

The site of vitellogenesis has been a controversial issue for some time. Reports indicate that in some crustacean species, VTG is synthesised solely in the HP, while in others VTG is synthesised in both the HP and the ovaries, depending on the species (Subramoniam, 2011). In

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the current study, it was not possible to quantify the VTG content of the HP due to the instability of the protein. However, significant fluctuations in the HPSI during autumn and winter suggests that the HP might, in fact, contribute to vitellogenesis in the reproductive cycle of *P. perlatus* (table 4.1). Furthermore, the presence of VTG in the female haemolymph indicates that it could be synthesised by the HP and subsequently secreted into the haemolymph.

It is also possible that the ovaries play a minor, more supportive role in VTG synthesis rather than simply functioning in the sequestration and subsequent processing of circulating VTG into Vn (Avarre *et al.*, 2003). The rapid increase noted in the ovarian Vn content in autumn suggests that the ovaries might be responsible for autologous Vn production by the ovaries during primary vitellogenesis. It is thus likely that the HP and ovaries might both contribute to the synthesis of VTG as found in other crustaceans belonging to the suborder of Pleocyemata (Zmora *et al.*, 2007). It is possible that the difference lies in the relative contribution from each tissue (Zmora *et al.*, 2007). However, this would require further investigation to confirm.

4.3.4 Environmental influence on the reproductive cycle of P. perlatus

Brachyuran crabs are iteroparous organisms with highly diverse reproductive patterns. They are able to reproduce either periodically, i.e. seasonal, or throughout the year, i.e. continuous (Pereira Rodrigues de Lira *et al.*, 2013). A continuous reproductive cycle in brachyuran crabs is attributed to the relatively stable environmental conditions. Conversely, in a seasonal reproductive cycle, breeding is limited to a specific season only when the environmental conditions are favourable (da Silva Castiglioni *et al.*, 2007). In seasonal iteroparity, higher survival rates in offspring were likely experienced during a certain period of the year, while survival was likely experienced throughout the year and is observed with continuous iteroparity (Pereira Rodrigues de Lira *et al.*, 2013). This reflects the effects that environmental factors have on the reproductive timing and period of organisms where reproductive events commence only when conditions are favourable (Boolootian *et al.*, 1959; Litulo, 2005; Swetha *et al.*, 2011).

The breeding period of a population has been defined as the time period during which ovigerous or vitellogenic organisms can be found in that population (Cobo, 2002b). At the end of the breeding period, oviposition or spawning occurs and indicates that the reproductive period has now been completed (Tsukimura *et al.*, 2002). Thereafter, the organism transitions to a post-

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reproductive state wherein the ovaries revert back to a previtellogenic state from which vitellogenesis will start again as the next reproductive cycle commences (Tsukimura *et al.*, 2002; Ferré *et al.*, 2012).

South Africa has subtropical and Mediterranean climates. The part of South Africa where this study was conducted, namely the Western Cape, has a Mediterranean climate. This kind of climate is generally characterised by hot, dry summers with mild winters. Regions with this kind of climate have a relatively narrow temperature range, particularly those that are located near large bodies of water. This is especially true of Cape Town; located next to the sea with many bodies of water throughout the region, there is little variation in the monthly mean of temperatures. Winters are wet and mild in temperature and summers are warm and dry. Although temperature and other abiotic factors were not recorded at the time of each crab collection, in South Africa the months of September and October are generally regarded as warm months owing to the transition from spring to summer.

In accordance with literature, comparing the collectively low VTG content in the haemolymph, the low Vn in the ovaries, and low GSI during summer, to the higher levels of VTG in the haemolymph, high ovarian Vn content, and high GSI in spring, it suggests that *P. perlatus* oviposits late in spring, just before summer (figures 4.7 and 4.12) (Lee & Chang, 1997). With no other marked decreases in the measured parameters, the data also suggests that *P. perlatus* oviposits only once a year and is reproductively active throughout the rest of the year. This indicates that *P. perlatus* exhibits a continuous reproductive cycle and oviposits only during the warm months of spring when environmental conditions such as temperature and rainfall might be more favourable for oviposition.

Oviposition, or spawning, under similar environmental conditions, has also been reported for other crustaceans where a higher percentage of ovigerous females were found during the months with warm temperatures, compared to the months with higher, hotter temperatures. Litulo, (2005) found a higher percentage (40-70%) of ovigerous females when the water temperature was warm (26-28°C). Conversely, fewer ovigerous females were found (30-40%) when the water temperature was warmer (28-30°C). Also, the freshwater crab, *Barytelphusa cunicularis*, demonstrated a reproductive periodicity similar to that of *P. perlatus* in the current study. Pathre & Meena, (2010) determined the reproductive periodicity of *B. cunicularis* and found the highest percentage of female crabs with mature ovaries between June and August, females with spent ovaries in November and December, and females with immature ovaries in January and February.

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Moreover, although spring in Cape Town is not classified as a rainy season, there is a higher occurrence of rainfall in spring than in summer, which is why summers in Cape Town are often described as hot and dry. Similar to the current study, Liu & Li, (2000) reported that the freshwater crab, *Candidiopotamon rathbunae*, spawns shortly after the rainy season but before the dry season. This was suggested to be a tactic employed by freshwater crabs found in areas of high precipitation so as to ensure the survival of juveniles by allowing them to grow and mature shortly after the rainy season (Liu & Li, 2000). The authors go on to suggest that this strategy increases the juveniles' chances of survival and minimises injury when the rainy season comes. Although Cape Town is not a region characterised by extremely high precipitation, the occasional occurrence of high rain during winter is not uncommon. This kind of strategy might similarly be employed by *P. perlatus*; spawning or oviposition after the rainy season might increase the survival rates of juveniles by allowing them time to mature until the next rainy season.

With the exception of summer, the levels of VTG in the haemolymph and those of Vn in the ovaries gradually increased throughout the cycle of seasons as would be expected during vitellogenesis, suggesting that females were reproductively active throughout the year. Moreover, all samples had orange ovaries indicative of reproductive activity. Thus, it can be inferred from the data that *P. perlatus* exhibits a continuous reproductive pattern as reproductively actively active females were found in all the seasons except summer. This environment of mild levels of rain, compared to heavy rains, along with warm temperatures, as opposed to hot, might create a favourable environment for oviposition. Under these conditions, juvenile crabs might be allowed to grow and mature sufficiently so as to enable them to survive potentially hazardous conditions during the rainy season.

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4.4 CONCLUSION

Organs associated with the reproductive process of female crabs undergo morphological changes with the progression of the reproductive cycle. Accordingly, qualitative measurements, such as the HPSI and GSI were used along with the quantification of the biochemical marker VTG to determine the reproductive cycle of *P. perlatus* females. In this study, the VTG content of the HP could not be determined due to the instability of VTG in the HP, which lead to inconsistencies in detection. However, the significant fluctuations in the HPSI during autumn and winter suggests the involvement of the HP in vitellogenesis, similar to other crustaceans that belong to suborder Pleocyemata. The marked decreases in the VTG content of the haemolymph, Vn content in the ovaries, as well as that of the GSI during spring, indicate that oviposition most likely occurred late in spring. The fluctuations in the physical parameters of organs and of VTG and Vn in the tissues involved in the reproductive cycle correlate to the reproductive cycle of *P. perlatus* females. This data also suggests that *P. perlatus* oviposits only once a year during a warmer period and after the rainy season. It is possible that conditions during that period are most favourable for oviposition and might also ensure a higher survival rate for the offspring. Moreover, the presence of reproductively active females in all seasons, except for summer, suggests that *P. perlatus* females in this region exhibit a continuous reproductive cycle.

A continuous reproductive cycle means that *P. perlatus* females can be sampled throughout most of the year for the purpose of reproductive studies, with the exception of summer. This demonstrates the suitability of *P. perlatus* females as bioindicators for oestrogenic environmental pollutants in that reproductively active females with detectable levels of VTG and Vn can be sampled throughout most of the year. Therefore, studies investigating environmental pollutants with the potential to inhibit vitellogenesis can be conducted during most of the year. However, the occurrence of oviposition in spring should not suggest that studies cannot be conducted during that time. Research into the potential induction of vitellogenesis by environmental pollutants could be done during summer after spawning and before the start of the next reproductive cycle. It might simply be that studies with this aim might not reach comprehensive conclusions from the samples obtained during spring, owing to the transition or recess in reproductive activity. Alternatively, given the complexity of factors involved in the regulation of vitellogenesis, studies directed towards the investigation of oestrogenic environmental pollutants with the potential to induce VTG, can consider using *P. perlatus* males instead due to the fact that males do not synthesise VTG.

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4.5 REFERENCES

1. Andersson, M. B. (1994). *Sexual selection,* Princeton University, USA: Princeton University Press, 146.

2. Antunes, G. d. F., Do Amaral, A. P. N., Ribarcki, F. P., Wiilland, E. d. F., Zancan, D. M. & Vinagre, A. S. (2010). Seasonal variations in the biochemical composition and reproductive cycle of the ghost crab *Ocypode quadrata* (Fabricius, 1787) in Southern Brazil. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology,* 313, 280-291.

3. Auttarat, J., Phiriyangkul, P. & Utarabhand, P. (2006). Characterization of vitellin from the ovaries of the banana shrimp *Litopenaeus merguiensis*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 143, 27-36.

4. Avarre, J. C., Michelis, R., Tietz, A. & Lubzens, E. (2003). Relationship between vitellogenin and vitellin in a marine shrimp (*Penaeus semisulcatus*) and molecular characterization of vitellogenin complementary DNAs. *Biology of Reproduction,* 69, 355-364.

5. Bembe, S. E. (2009). *Reproductive physiology of the female blue crab, Callinectes sapidus: Spawning induction and vitellogenesis.* M.Sc. thesis, University of Maryland.

6. Boolootian, R., Giese, A., Farmanfarmaian, A. & Tucker, J. (1959). Reproductive cycles of five west coast crabs. *Physiological Zoology,* 32, 213-220.

7. Chaix, J. C. & De Reggi, M. (1982). Ecdysteroid levels during ovarian development and embryogenesis in the spider crab *Acanthonyx lunulatus*. *General and Comparative Endocrinology,* 47, 7-14.

8. Chan, S. M., Mak, A. S., Choi, C. L., Ma, T. H., Hui, J. H. & Tiu, S. H. (2005). Vitellogenesis in the red crab, *Charybdis feriatus*: Contributions from small vitellogenin transcripts (CfVg) and farnesoic acid stimulation of CfVg expression. *Annals of the New York Academy of Sciences,* 1040, 74-79.

http://etd.uwc.ac.za/

9. Chen, L., Jiang, H., Zhou, Z., Li, K., Li, K., Deng, G. Y. & Liu, Z. (2004). Purification of vitellin from the ovary of Chinese mitten-handed crab (*Eriocheir sinensis*) and development of an antivitellin ELISA. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 138, 305-311.

10. Cobo, V. J. (2002a). Breeding period of the arrow crab *Stenorhynchus seticornis* from Couves Island, South-Eastern Brazilian coast. *Journal of the Marine Biological Association of the UK*, 82, 1031-1032.

11. Cobo, V. J. (2002b). Breeding period of the spider crab *Mithraculus forceps* (A. Milne Edwards) (Crustacea, Majidae, Mithracinae) in the South-Eastern Brazilian coast. *Revista Brasileira de Zoologia*, 19, 229-234.

12. Cobo, V. J. & Fransozo, A. (2003). External factors determining breeding season in the red mangrove crab *Goniopsis cruentata* (Latreille) (Crustacea, Brachyura, Grapsidae) on the São Paulo State Northern coast, Brazil. *Revista Brasileira de Zoologia*, 20, 213-217.

13. Coccia, E., De Lisa, E., Di Cristo, C., Di Cosmo, A. & Paolucci, M. (2010). Effects of estradiol and progesterone on the reproduction of the freshwater crayfish *Cherax albidus*. *The Biological Bulletin*, 218, 36-47.

14. Cumberlidge, N. (1999). *The freshwater crabs of West Africa: Family Potamonautidae*, Paris, France: IRD Editions, 77-79, 128 & 334.

15. da Silva Castiglioni, D., Negreiros-Fransozo, M. L. & Flores Cardoso, R. C. (2007). Breeding season and molt cycle of the fiddler crab *Uca rapax* (Brachyura, Ocypodidae) in a subtropical estuary, Brazil, South America. *Gulf and Caribbean Research*, 19, 11-20.

16. Daniels, S. R. (2003). Examining the genetic structure among populations of the common Cape river crab *Potamonautes perlatus* from river systems in South Africa reveals hydrographic boundaries. *Journal of Crustacean Biology*, 23, 936-950.

17. Derelle, E., Grosclaude, J., Meusy, J. J., Junera, H. & Martin, M. (1986). ELISA titration of vitellogenin and vitellin in the freshwater prawn, *Macrobrachium rosenbergii*, with monoclonal antibody. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 85, 1-4.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

18. Ferré, L. E., Medesani, D. A., García, C. F., Grodzielski, M. & Rodríguez, E. M. (2012). Vitellogenin levels in hemolymph, ovary and hepatopancreas of the freshwater crayfish *Cherax quadricarinatus* (Decapoda: Parastacidae) during the reproductive cycle. *International Journal of Tropical Biology and Conservation,* 60, 253-261.

19. Ferreira, M., Resende, B., Lima, M., Santos, S. & Rocha, R. (2012). The stage of ovarian development affects organ expression of vitellogenin as well as the morphometry and ultrastructure of germ cells in the freshwater prawn *Macrobrachium amazonicum* (Heller, 1862). *Theriogenology*, 78, 981-990.

20. Fingerman, M. (1997). Roles of neurotransmitters in regulating reproductive hormone release and gonadal maturation in decapod crustaceans. *Invertebrate Reproduction & Development,* 31, 47-54.

21. García, F., Cunningham, M., Garda, H. & Heras, H. (2008). Embryo lipoproteins and yolk lipovitellin consumption during embryogenesis in *Macrobrachium borellii* (Crustacea: Palaemonidae). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 151, 317-322.

22. Gunamalai, V., Kirubagaran, R. & Subramoniam, T. (2004). Hormonal coordination of molting and female reproduction by ecdysteroids in the mole crab *Emerita asiatica* (Milne Edwards). *General and Comparative Endocrinology,* 138, 128-138.

23. Gunamalai, V., Kirubagaran, R. & Subramoniam, T. (2006). Vertebrate steroids and the control of female reproduction in two decapod crustaceans, *Emerita asiatica* and *Macrobrachium rosenbergii*. *Current Science,* 90, 119-123.

24. Hill, M. & O'Keeffe, J. (1992). Some aspects of the ecology of the freshwater crab (*Potamonautes perlatus,* Milne Edwards) in the upper reaches of the Buffalo River, Eastern Cape Province, South Africa. *Southern African Journal of Aquatic Science,* 18, 42-50.

25. Hiransuchalert, R., Thamniemdee, N., Khamnamtong, B., Yamano, K. & Klinbunga, S. (2013). Expression profiles and localization of vitellogenin mRNA and protein during ovarian development of the giant tiger shrimp *Penaeus monodon*. *Aquaculture,* 412, 193-201.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

26. Ituarte, R. B., Spivak, E. D. & Luppi, T. A. (2004). Female reproductive cycle of the South-Western Atlantic estuarine crab *Chasmagnathus granulatus* (Brachyura, Grapsoidea, Varunidae). *Scientia Marina*, 68, 127-137.

27. Jayasankar, V., Tsutsui, N., Jasmani, S., Saido-Sakanaka, H., Yang, W. J., Okuno, A., Hien, T. T. T., Aida, K. & Wilder, M. N. (2002). Dynamics of vitellogenin mRNA expression and changes in hemolymph vitellogenin levels during ovarian maturation in the giant freshwater prawn *Macrobrachium rosenbergii*. *Journal of Experimental Zoology*, 293, 675-682.

28. Jia, X., Chen, Y., Zou, Z., Lin, P., Wang, Y. & Zhang, Z. (2013). Characterization and expression profile of vitellogenin gene from *Scylla paramamosain*. *Gene*, 520, 119-130.

29. Kappalli, S., Supriya, N. T., Krishnakumar, V., Anilkumar, G. & Chang, E. S. (2012). Hemolymph ecdysteroid titers in a brachyuran crab (*Uca triangularis*) that concomitantly undergoes molting and reproduction. *Zoological Studies*, 51, 966-976.

30. Kirubagaran, R., Peter, D. M., Dharani, G., Vinithkumar, N. V., Sreeraj, G. & Ravindran, M. (2005). Changes in vertebrate-type steroids and 5-hydroxytryptamine during ovarian recrudescence in the Indian spiny lobster, *Panulirus homarus*. *New Zealand Journal of Marine and Freshwater Research*, 39, 527-537.

31. Kung, S. Y., Chan, S. M., Hui, J. H. L., Tsang, W. S., Mak, A. & He, J. G. (2004). Vitellogenesis in the sand shrimp, *Metapenaeus ensis*: The contribution from the hepatopancreas-specific vitellogenin gene (MeVg2). *Biology of Reproduction*, 71, 863-870.

32. Kunz, T. H. & Orrell, K. S. (2004). Reproduction, energy costs of. Cleveland, C. J. (ed.), *In: Encyclopedia of Energy*. Oxford, USA: Elsevier, 423-425.

33. Laufer, H., Borst, D., Baker, F. C., Carrasco, C., Sinkus, M., Reuter, C. C., Tsai, L. W. & Schooley, D. A. (1987). Identification of a juvenile hormone-like compound in a crustacean. *Science*, 235, 202-205.

34. Lee, C. Y. & Watson, R. D. (1994). Development of a quantitative enzyme-linked immunosorbent assay for vitellin and vitellogenin of the blue crab *Callinectes sapidus*. *Journal of Crustacean Biology*, 14, 617-626.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

35. Lee, F. Y. & Chang, C. F. (1997). The concentrations of vitellogenin (vitellin) and protein in hemolymph, ovary and hepatopancreas in different ovarian stages of the freshwater prawn, *Macrobrachium rosenbergii*. *Comparative Biochemistry and Physiology Part A: Physiology,* 117, 433-439.

36. Li, G., Chen, H., Deng, S., Ye, M., Jiang, S., Chan, S. & Zhu, C. (2014a). *In vivo* and *in vitro* inhibitory action of 17β-estradiol and environmental estrogen 4-nonylphenol on gonad-inhibiting hormone (GIH) expression in the eyestalks of *Litopenaeus vannamei*. *Genetics and Molecular Research,* 14, 14056-14065.

37. Li, K., Chen, L., Zhou, Z., Li, E., Zhao, X. & Guo, H. (2006). The site of vitellogenin synthesis in Chinese mitten-handed crab *Eriocheir sinensis*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 143, 453-458.

38. Lindenfors, P., Tullberg, B. S. & Biuw, M. (2002). Phylogenetic analyses of sexual selection and sexual size dimorphism in pinnipeds. *Behavioral Ecology and Sociobiology,* 52, 188-193.

39. Litulo, C. (2004). Reproductive aspects of a tropical population of the fiddler crab *Uca annulipes* (H. Milne Edwards, 1837) (Brachyura: Ocypodidae) at Costa do Sol Mangrove, Maputo Bay, Southern Mozambique. *Hydrobiologia,* 525, 167-173.

40. Litulo, C. (2005). External factors determining the reproductive periodicity in a tropical population of the hairy crab *Pilumnus vespertilio* (Decapoda: Brachyura: Pilumnidae). *The Raffles Bulletin of Zoology,* 53, 115-118.

41. Liu, H. C. & Li, C. W. (2000). Reproduction in the freshwater crab *Candidiopotamon rathbunae* (Brachyura: Potamidae) in Taiwan. *Journal of Crustacean Biology,* 20, 89-99.

42. Lodé, T. (2012). Oviparity or viviparity? That is the question... . *Reproductive Biology,* 12, 259-264.

43. López Greco, L. S. & Rodríguez, E. M. (1999). Annual reproduction and growth of adult crabs *Chasmagnathus granulata* (Crustacea, Brachyura, Grapsidae). *Cahiers de Biologie Marine,* 40, 155-164.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

44. Manor, R., Aflalo, E. D., Segall, C., Weil, S., Azulay, D., Ventura, T. & Sagi, A. (2004). Androgenic gland implantation promotes growth and inhibits vitellogenesis in *Cherax quadricarinatus* females held in individual compartments. *Invertebrate Reproduction & Development,* 45, 151-159.

45. Nagaraju, G. P. C. (2011). Reproductive regulators in decapod crustaceans: An overview. *The Journal of Experimental Biology,* 214, 3-16.

46. Nagaraju, G. P. C., Reddy, P. R. & Reddy, P. S. (2004). Mandibular organ: Its relation to body weight, sex, molt and reproduction in the crab, *Oziotelphusa senex senex* Fabricius (1791). *Aquaculture,* 232, 603-612.

47. Nagaraju, G. P. C., Suraj, N. & Reddy, P. S. (2003). Methyl farnesoate stimulates gonad development in *Macrobrachium malcolmsonii* (H. Milne Edwards) (Decapoda, Palaemonidae). *Crustaceana,* 76, 1171-1178.

48. Okumura, T., Yoshida, K. & Nikaido, H. (2004). Ovarian development and hemolymph vitellogenin levels in laboratory-maintained protandric shrimp, *Pandalus hypsinotus*: Measurement by a newly developed time-resolved fluoroimmunoassay (TR-FIA). *Zoological Science,* 21, 1037-1047.

49. Pateraki, L. E. & Stratakis, E. (1997). Characterization of vitellogenin and vitellin from land crab *Potamon potamios*: Identification of a precursor polypeptide in the molecule. *Journal of Experimental Zoology,* 279, 597-608.

50. Pathre, R. & Meena, P. (2010). Breeding cycle and fecundity of the freshwater crab, *Barytelphusa cunicularis* (Decapoda, Potamonidae). *World Journal of Zoology*, 5, 96-102.

51. Pereira Rodrigues de Lira, J. J., dos Santos Calado, T. C. & Leitão Câmara de Araújo, M. d. S. (2013). Breeding period in the mangrove crab *Goniopsis cruentata* (Decapoda: Grapsidae) in North-East Brazil. *International Journal of Tropical Biology and Conservation,* 61, 29-38.

http://etd.uwc.ac.za/

52. Phiriyangkul, P., Puengyam, P., Jakobsen, I. B. & Utarabhand, P. (2007). Dynamics of vitellogenin mRNA expression during vitellogenesis in the banana shrimp *Penaeus* (*Fenneropenaeus*) *merguiensis* using real-time PCR. *Molecular Reproduction and Development,* 74, 1198-1207.

53. Quinitio, E. T., De Pedro, J. & Parado-Estepa, F. D. (2007). Ovarian maturation stages of the mud crab *Scylla serrata*. *Aquaculture Research,* 38, 1434-1441.

54. Quinitio, E. T., Hara, A., Yamauchi, K. & Nakao, S. (1994). Changes in the steroid hormone and vitellogenin levels during the gametogenic cycle of the giant tiger shrimp, *Penaeus monodon*. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology,* 109, 21-26.

55. Reddy, P. R., Kiranmayi, P., Kumari, K. T. & Reddy, P. S. (2006). 17α-Hydroxyprogesterone induced ovarian growth and vitellogenesis in the freshwater rice field crab *Oziotelphusa senex senex*. *Aquaculture,* 254, 768-775.

56. Revathi, P., Iyapparaj, P., Munuswamy, N. & Krishnan, M. (2012). Vitellogenesis during the ovarian development in freshwater female prawn *Macrobrachium rosenbergii* (De Man). *International Journal of Aquatic Science,* 3, 13-27.

57. Rosa, R. & Nunes, M. L. (2002). Changes in organ indices and lipid dynamics during the reproductive cycle of *Aristeus antennatus*, *Parapenaeus longirostris*, and *Nephrops norvegicus* (Decapoda) from the Portuguese South coast. *Crustaceana,* 75, 1095-1105.

58. Sagi, A., Khalaila, I., Abdu, U., Shoukrun, R. & Weil, S. (1999). A newly established ELISA showing the effect of the androgenic gland on secondary-vitellogenic-specific protein in the hemolymph of the crayfish *Cherax quadricarinatus*. *General and Comparative Endocrinology,* 115, 37-45.

59. Sarojini, R., Nagabhushanam, R. & Fingerman, M. (1995). Mode of action of the neurotransmitter 5-hydroxytryptamine in stimulating ovarian maturation in the red swamp crayfish, *Procambarus clarkii*: An *in vivo* and *in vitro* study. *Journal of Experimental Zoology,* 271, 395-400.

http://etd.uwc.ac.za/

60. Shafir, S., Tom, M., Ovadia, M. & Lubzens, E. (1992). Protein, vitellogenin, and vitellin levels in the hemolymph and ovaries during ovarian development in *Penaeus semisulcatus* (de Haan). *The Biological Bulletin,* 183, 394-400.

61. Shyamal, S., Sudha, K., Gayathri, N. & Anilkumar, G. (2014). The Y-organ secretory activity fluctuates in relation to seasons of molt and reproduction in the brachyuran crab, *Metopograpsus messor* (Grapsidae): Ultrastructural and immunohistochemical study. *General and Comparative Endocrinology,* 196, 81-90.

62. Sripiromrak, A., Chaivisuthangkura, P., Longyant, S. & Sithigorngul, P. (2014). Using vitellin monoclonal antibodies to assess the vitellogenesis-inhibiting hormone activity of *Macrobrachium rosenbergii*. *ScienceAsia,* 40, 157-167.

63. Subramoniam, T. (2000). Crustacean ecdysteroids in reproduction and embryogenesis. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 125, 135-156.

64. Subramoniam, T. (2011). Mechanisms and control of vitellogenesis in crustaceans. *Fisheries Science,* 77, 1-21.

65. Sudha, K. & Anilkumar, G. (1996). Seasonal growth and reproduction in a highly fecund brachyuran crab, *Metopograpsus messor* (Forskal) (Grapsidae). *Hydrobiologia,* 319, 15-21.

66. Suzuki, S., Yamasaki, K. & Katakura, Y. (1990). Vitellogenin synthesis in andrectomized males of the terrestrial isopod, *Armadillidium vulgare* (Malacostracan Crustacea). *General and Comparative Endocrinology,* 77, 283-291.

67. Swetha, C., Sainath, S., Reddy, P. & Reddy, P. (2011). Reproductive endocrinology of female crustaceans: Perspective and prospective. *Journal of Marine Science: Research & Development,* 3, 1-13.

68. Takayanagi, H., Yamamoto, Y. & Takeda, N. (1986). An ovary-stimulating factor in the shrimp, *Paratya compressa*. *Journal of Experimental Zoology*, 240, 203-209.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

69. Tiu, S. H., Hui, J. H., Mak, A. S., He, J. G. & Chan, S. M. (2006). Equal contribution of hepatopancreas and ovary to the production of vitellogenin (PmVg1) transcripts in the tiger shrimp, *Penaeus monodon*. *Aquaculture,* 254, 666-674.

70. Tiu, S. H. K., Hui, H. L., Tsukimura, B., Tobe, S. S., He, J. G. & Chan, S. M. (2009). Cloning and expression study of the lobster (*Homarus americanus*) vitellogenin: Conservation in gene structure among decapods. *General and Comparative Endocrinology,* 160, 36-46.

71. Treerattrakool, S., Panyim, S., Chan, S. M., Withyachumnarnkul, B. & Udomkit, A. (2008). Molecular characterization of gonad-inhibiting hormone of *Penaeus monodon* and elucidation of its inhibitory role in vitellogenin expression by RNA interference. *FEBS Journal,* 275, 970-980.

72. Tsang, W. S., Quackenbush, L. S., Chow, B. K., Tiu, S. H., He, J. G. & Chan, S. M. (2003). Organization of the shrimp vitellogenin gene: Evidence of multiple genes and tissue specific expression by the ovary and hepatopancreas. *Gene,* 303, 99-109.

73. Tsukimura, B. (2001). Crustacean vitellogenesis: Its role in oocyte development. *American Zoologist,* 41, 465-476.

74. Tsukimura, B., Bender, J. & Linder, C. (2000). Development of an anti-vitellin ELISA for the assessment of reproduction in the ridgeback shrimp, *Sicyonia ingentis*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 127, 215-224.

75. Tsukimura, B., Waddy, S. L., Vogel, J. M., Linder, C. J., Bauer, D. K. & Borst, D. W. (2002). Characterization and quantification of yolk proteins in the lobster, *Homarus americanus*. *Journal of Experimental Zoology,* 292, 367-375.

76. Tsutsui, N., Ohira, T., Kawazoe, I., Takahashi, A. & Wilder, M. N. (2007). Purification of sinus gland peptides having vitellogenesis-inhibiting activity from the whiteleg shrimp *Litopenaeus vannamei*. *Marine Biotechnology,* 9, 360-369.

http://etd.uwc.ac.za/

77. Vazquez-Boucard, C. G., Levy, P., Ceccaldi, H. J. & Brogren, C. H. (2002). Developmental changes in concentrations of vitellin, vitellogenin, and lipids in hemolymph, hepatopancreas, and ovaries from different ovarian stages of Indian white prawn *Fenneropenaeus indicus*. *Journal of Experimental Marine Biology and Ecology,* 281, 63-75.

78. Warrier, S. R., Tirumalai, R. & Subramoniam, T. (2001). Occurrence of vertebrate steroids, estradiol 17β and progesterone in the reproducing females of the mud crab *Scylla serrata*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 130, 283-294.

79. Xuereb, B., Bezin, L., Chaumot, A., Budzinski, H., Augagneur, S., Tutundjian, R., Garric, J. & Geffard, O. (2011). Vitellogenin-like gene expression in freshwater amphipod *Gammarus fossarum* (Koch, 1835): Functional characterization in females and potential for use as an endocrine disruption biomarker in males. *Ecotoxicology,* 20, 1286-1299.

80. Yano, I. & Hoshino, R. (2006). Effects of 17β-estradiol on the vitellogenin synthesis and oocyte development in the ovary of kuruma prawn (*Marsupenaeus japonicus*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 144, 18-23.

UNIVERSITY of the
WESTERN CAPE

81. Ye, H., Song, P., Ma, J., Huang, H. & Wang, G. (2010b). Changes in progesterone levels and distribution of progesterone receptor during vitellogenesis in the female mud crab (*Scylla paramamosain*). *Marine and Freshwater Behaviour and Physiology,* 43, 25-35.

82. Yehezkel, G., Chayoth, R., Abdu, U., Khalaila, I. & Sagi, A. (2000). High-density lipoprotein associated with secondary vitellogenesis in the hemolymph of the crayfish *Cherax quadricarinatus*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 127, 411-421.

83. Zmora, N., Trant, J., Chan, S. M. & Chung, J. S. (2007). Vitellogenin and its messenger RNA during ovarian development in the female blue crab, *Callinectes sapidus*: Gene expression, synthesis, transport, and cleavage. *Biology of Reproduction,* 77, 138-146.

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CHAPTER 5: RECOMMENDATIONS

When recording events over a certain period of time, it is best to take systematic recordings so as to track the progression of the events more accurately over the time period. Other studies that have investigated the breeding cycle or reproductive periodicity of crustaceans sampled on a monthly basis. This provides a more progressive and comprehensive view of the fluctuations that might occur and allows for a more detailed and comprehensive analysis of the events being studied. I, thus, recommend that sampling occur on a monthly basis so that variations between months can be noted more accurately and a more definitive inference can be made with regards to the time of oviposition.

Studies have reported correlations between the environmental factors such as rainfall, temperature, photoperiod, the lunar cycle and the reproductive cycle of crustaceans. In this study, however, parameters such as temperature and rainfall were not recorded at the time of sampling. In future, such parameters should be recorded so that more definitive and accurate inferences can be made with regards to establishing the reproductive period as well as the environmental conditions that surround that period.

The sample size is a crucial component of research. It is important to have a sufficiently large sample size so as to decrease the probability of observed effects being attributed to chance and/or individual variability. A larger sample size will allow for any differences that may occur between experimental groups to be confirmed and validated as truly significant differences. In this way, larger sample sizes can increase the validity of the results. Regarding the study of intermediate metabolism in crustaceans, it has been remarked that it is difficult to establish a standard metabolic profile of crustaceans as they exhibit high inter- and intra-specific variability. It was further remarked that this variability could be attributed to various factors, including habitat, feeding state, sexual maturity (especially in females), and seasonality as they determine a differential metabolic response. Although metabolic profiles were not established in this study, the comment is applicable to the sample size used in this study. A high level of variability was noted between the samples in some of the parameters measured which could be minimised considerably with the advent of a larger sample size.

Detection of the precursor form of vitellogenin (VTG) in the hepatopancreas (HP) is a challenge and has only been detected in primary cultures of the HP. Hepatopancreatic cells are cultured *in vitro* and the precursor form of VTG (pre-VTG) is then secreted into the medium from which it can be analysed. However, this is set in an *in vitro* environment and cannot be

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applied to *in vivo* environment. Suggestions as to why the *in vivo* detection of pre-VTG presents a challenge include the high enzymatic activity of the HP; steric hindrance of the antigenic site which makes the site inaccessible to antibody binding; and rapid processing and secretion of the pre-VTG into the haemolymph which leaves very low levels of detectable levels of VTG in the HP. The theory on high enzymatic activity of the HP, and therefore possible digestion of the antigenic site by enzymes, has been refuted by another study. That study reported no difference in the protein concentration with and without the commonly used serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF).

Furthermore, the mAbs produced in the current study were generated against pVn. It is possible that cleavage of VTG in the ovaries generates new epitopes, or alters existing epitopes. Therefore, it is possible that the structure or conformation of pre-VTG in the HP might prevent interaction between the anti-pVn mAbs as the epitopes might be hidden and inaccessible by steric hindrance. I suggest that a common epitope be identified between pre-VTG, VTG in the haemolymph, and Vn. Some bioinformatics tools exist that can identify homologous amino acid sequences while others can be used to predict whether these sequences can be used to generate antibodies against. This approach is known as peptide antibody production and in this way, it might be possible to produce a single mAb that can detect pre-VTG, VTG, and Vn.

Regardless of the above mentioned, it is also a possibility that on a protein level pre-VTG is simply not stable enough to be detected in the HP homogenates. Many studies have investigated VTG production in the HP by measuring VTG mRNA instead. I, therefore, recommend considering the measurement of pre-VTG by quantification of mRNA in the HP.

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