THE DEVELOPMENT AND VALIDATION OF BIOASSAYS TO DETECT ESTROGENIC AND ANTI-ANDROGENIC ACTIVITY USING SELECTED WILDLIFE SPECIES

JH van Wyk • EJ Pool • E Hurter • AJ Leslie

WRC Report No. 926 & 1253/1/05

Water Research Commission

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Report to the Water Research Commission

by

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WRC Report No.: 926 & 1253/1/05

ISBN: 1-77005-349-2
Set No: 1-77005-347-6

AUGUST 2005

The Report is obtainable from:

Water Research Commission
Private Bag X03
Gezina
0031

This report emanates from two research projects entitled:

WRC K5/926: "An Assessment of the extent of estrogenic activity in Western Cape water resources."

and

WRC K5/1253: "Endocrine Disrupting contaminants (EDCs) in South African water resources: Development and validation of in vitro and in vivo bioassays to detect endocrine interaction and to characterize physiological disruption in non-mammalian animals"

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EXECUTIVE SUMMARY

On a global scale, human, agriculture and industrial activities, historically and recently, have released large quantities of manmade chemicals into the environment. During the nineties, after several keystone papers were published on the potentially adverse effects on human and wildlife health by various environmental contaminants, for example, DDT, ethinylestradiol (contraceptive) and several other estrogen mimics, public concern increased dramatically. It has been suggested that several of these environmental chemicals may modulate the endocrine system, specifically associated with the developmental and reproductive systems of wildlife and humans. The endocrine disruption hypothesis soon emerged, stating that: "Synthetic, and some naturally occurring, chemical substances in the environment are disrupting the normal functions of the endocrine system and its hormones in humans and wildlife" (Society of Environmental Toxicology and Chemistry (SETAC) 2000). In response, international environmental agencies were mandated by their governments to identify endocrine disrupting contaminants (EDCs) by developing screening and testing programmes to supplement chemical detection of known EDCs. Subsequently, the field of EDC research received increased attention. Soon the list of potential endocrine active substances (endocrine modulators) increased.

Endocrine disruption is not an adverse effect *per se*, but rather a mode of action. Chemicals originating from a far reaching range emerged, for example, phyto (plant)-estrogens, natural hormones (e.g., 17ß-estradiol), pharmaceuticals, including synthetic hormones, industrial chemicals (e.g., PCBs in lubricants, coolants, bisphenol A and nonylphenol in polymer and plastic products), agrichemicals (e.g., pesticides, herbicides fungicides) and several consumer products (e.g. food packaging and detergents). From the literature it is clear that although research regarding the extent and effects of EDCs on the quality of life, for wildlife and humans, is progressing fast in most developed countries of the world, relatively little attention, beyond the listing of potential EDCs, has been given to the potential hazard of endocrine disruptors. Although several keystone case studies accessioned the potential health hazard to wildlife the link to human health remains controversial. Wildlife abnormalities reported, include feminization of fish when exposed to sewage effluents, altered sex determination and differentiation in reptiles, including turtles and alligators, limb deformities in amphibians, masculinization in bivalves and gastropods, DDE induced eggshell thinning in raptors, and abnormalities and declined fertility in mammal species, including small mammals, lambs, panthers and aquatic mammals.

The link to human health relates to several aspects regarding male and female reproductive biology, including low sperm counts, increased incidence of testicular cancer and reproductive abnormalities of the male reproductive system (e.g., cryptorchidism, hypospadias), increased risk for breast cancer, ovarian abnormalities and abnormalities of the female reproductive system.

South Africa, although a developing country, but being a user of most of the potential EDCs, including several persistent organic pollutants (POPs), the most well-known being DDT, did not escape this concern. The first WRC funded project, mainly a literature review, regarding estrogen and estrogen mimicking substances in the water environment was published in 2000.

This study recommended that screening and testing methodology be developed or optimized, *in vitro* and *in vivo* bioassays as well as analytical methods for the specific detection of suspected EDCs in the aquatic environment and consumer products. Several reports also mentioned disruption of normal reproductive hormone profiles in several wildlife species, although in a South African context, few studies were available reporting normal values for local wildlife species, including the well studied African clawed frog, *X. laevis*. Another obvious shortfall was the lack of validated biomarkers to assess the disruption of the androgenic system (e.g., anti-androgenic effects).

It was against this backdrop that this research evolved, on the one hand with the focus on learning more about potential endpoints/biomarker systems as well as potential endogenous bio-indicator species on the other hand, but also to explore the available validated endpoints using internationally recognized bio-indicator species to begin to assess the extent of EDC activity in South African water resources. Most of the initial international focus was on the disruption of the male and female reproductive systems, but it was soon recognized that the disruption of the thyroid system should also be a major concern, specifically during early development of organisms. *Xenopus laevis*, was internationally selected (USA-EPA, Japan-EPA, EU and OECD) as a model system, using the control of the metamorphosis (tadpole to frog) phenomenon by the thyroid system as potential biomarker complex. Although the use of local wildlife species in toxicological assessment of the local aquatic health has a long history in South Africa, few studies and reports refer to the non-lethal, subtle effects, mainly because of a general lack of well researched biomarker systems in local species.

Most of the research reported on here, is the culmination of a long and difficult process that started with studies to understand the basic reproductive biology of the aquatic amphibian, *X. laevis* collected from its natural aquatic environment as well as other potential bio-indicator species (e.g. turtles and crocodiles). As the study proceeded, analytical methods had to be developed and new biomarkers identified and studied. The main aim being to eventually establish or contribute towards a battery of EDC tests that could be used in a cost-effective way in South Africa as part of the envisaged toxicology monitoring programme mandated by the National Water Act (Act No 36 of 1998) and employed in local ecological impact assessment. In order to also serve the needs of water suppliers the development of *in vitro* tests were included, as well as, set-up and validation of internationally used bioassays using exotic species under local conditions. This report must be seen against the background of being part of the initial South African EDC initiative when hardly any research had been conducted in this field, specifically, research regarding novel bioassays using indigenous wildlife species and being cost-effective for a developing country.

The project, WRC 926: "An assessment of the extent of estrogenic activity in Western Cape water resources" was followed by project, WRC 1253: "Endocrine Disrupting contaminants (EDCs) in South African water resources: Development and validation of *in vitro* and *in vivo* bioassays to detect endocrine interaction and characterize physiological disruption in non-mammalian animals". Since the research associated with these projects link very closely, it was decided to combine the reports.

The assessment of chemical interaction with the vertebrate endocrine system, especially the reproductive system, including female hormone, estrogen, and male (androgenic) hormones rely on relevant bio-indicator species and specific and sensitive biomarker systems reflecting the levels of such interaction. This research represents a first attempt to utilize local endemic vertebrate species as bio-indicators investigating the potential of specific biomarkers such as the hepatic produced yolk precursor, vitellogenin (Vtg) to determine environmental estrogenic activity.The estrogen (female hormone) inducible proteins (EIP), including the yolk precursor, vitellogenin (Vtg), produced in the liver under control of female hormone, estrogen, was studied as potential end-point for estrogen activity. The African clawed frog (*Xenopus laevis*), internationally used as a model species, was selected as bio-indicator. In this study, Vtg was isolated and anti-Vtg polyclonal as well as anti-EIP monoclonal antibodies produced in rabbits and mice. These antibodies were validated and used to set-up ELISA bioassays to quantitatively estimate Vtg induction in *X. laevis* frogs.

To understand the dynamics of the natural reproductive cycles in potential local bio-indicator fresh water species, including *Xenopus laevis* and the turtle, *Pelomedusa subrufa*, seasonal studies were conducted for the first time on these species.

These base-line seasonal data are a prerequisite for using the selected local species in environmental monitoring programmes. The results show that although *X. laevis* may breed throughout the year a peak in reproductive activity occur in spring and summer months. Reproduction in the fresh water turtles is a seasonal phenomenon and male and female cycles may not be well synchronised. The potential of using local fresh water turtles as bio-indicators of historical EDC exposures and effects of sex determination was confirmed.

Although males don't normally exhibit high levels of circulating estrogens, they have estrogen receptors in the liver and therefore could be used in Vtg induction studies. The development of ELISAs for *Xenopus* Vtg and total EIPs for the first time allowed that frogs could be used as biomonitors for estrogen induction by environmental chemicals. Subsequently, *X. laevis* frogs was used in, *in situ* (caged) studies (21 day exposure) in natural water bodies in three different agricultural areas (vine, fruit and wheat) in the Western Cape to assess possible estrogenic activity in these water resources. In the grape producing area limited estrogen activity was measured along the river as well as on a seasonal basis (4-5 times throughout the year). In the fruit producing area cages were mostly placed in man-made water bodies (dams) receiving run-off from orchards. Limited estrogenicity was measured over the study period. However, in the wheat producing area estrogenicity was measured on several occasions in several sites. Because these laboratory exposures require large numbers of male frogs and to limit the number of live male frog needed to *in situ* caged exposure studies, a *in vitro* liver slice culture bioassay methodology was developed and validated, in which the liver of a single male was used in a tissue culture exposure set-up to study the induction of EIPs and Vtg.. Increased sensitivity was obtained by using liver tissue of males pre-exposed to estrogen in the laboratory. This assay was extensively used to study estrogen activity in water collected from the same agricultural areas as well as other water resources, including sewage effluents, and drinking water.

Limited estrogenic activity was found in several sites, although not in a consistent pattern. However the potential of using the *in vitro* frog liver culture assay as a screening tool for estrogenic activity has been demonstrated.

In order to expand the battery of tests, additional biomarkers for estrogen activity were investigated. For example, estrogen inducible lipo-proteins in *X. laevis* and estrogen induced proteins in cultured chicken cell-line. To expand the use of Vtg as biomarker for estrogen activity, an ELISA determining Vtg (using commercial Vtg antibodies) in tilapia (*Oreochromis mossambicus*) was set-up and validated. In addition a novel universal Vtg antibody was validated and used in a universal ELISA (UniVtg) that could be used for several Vertebrate species to study Vtg induction. This assay was used in several fish species, the frog, *X. laevis*, the chicken, crocodiles and fresh water turtles to measure plasma Vtg levels. This assay eliminates the expensive production of species specific anti-Vtg antibodies for ELISA determinations.

Since endocrine disruption also includes modulation of the male reproductive system, including anti-androgenic activity and thyroid system, bioassays to asses the effect of potential EDCs on these systems were initiated. For this, the androgenic control and histology of the breeding glands in the skin of male *X. laevis* frogs were studied. An initial controlled exposure study, including known anti-androgenic compounds as well as suspected ones, was conducted to evaluate the use of these glands as biomarkers for androgenic effects.

In this study, however, only histological changes proved to be reliable bio-markers of anti-androgenic effects, rather than biochemical aspect of the secretion produced by these structures. The exact nature of the secretion of these glands could not be characterized and needs further study.

Following C18 solid phase extraction protocol of environmental water samples, liver slices were exposed to a range of different water samples, including drinking water, water from bore-holes, rivers and dams in urban and rural agriculture areas, final sewage effluents, and several synthetic chemicals suspected to have estrogenic activity. Results show that if cytotoxic effects are eliminated the liver slice bioassay is a relatively sensitive screen for estrogenic activity in water samples. Although this assay only represents a screening tool, several water samples were found to be positive, in that significant increases in Vtg production by liver slices were measured after a six day incubation period. This bioassay was used in a study regarding the effectiveness of treatment in the Windhoek sewage reclamation plant (direct system) in Namibia. It was found that the activated charcoal filters removed most of the estrogenic activity detected in the untreated sewage. Results also showed some estrogen activity in the source water originating from bore-holes, suggesting some ground water contamination.

These projects were the first to investigate the potential of several biomarkers for estrogenic, anti-androgenic and thyroid related EDC activity using local and international bio-indicator wildlife models. The studies confirmed that male breeding glands in *X. laevis* are under control of androgens and not affected by gonadotrophic activity directly. It was also confirmed that compounds like, DDE and the fungicide, vinclozolin, indeed have anti-androgenic effects in male frogs, including the possible inhibition of breeding behaviour.

Preliminary results also confirmed that metamorphosis of *X. laevis* tadpoles (under control of thyroid hormones) has potential to be used as biomarker to assess EDC activities related to disrupting the thyroid system.

Although the recently revised National Water Act (Act No 36 of 1998), passed by the South African government, is designed to protect our natural water resources, specifically catchments areas, no regulations regarding EDC activity are in place and this research contributes towards the establishment of a battery of tests to assess EDC activity in local water resources or chemicals known to contaminate natural water resources. Moreover, this programme also successfully highlighted the use of biomarkers related to the male androgenic system as well as the sex determination and differentiation developmental systems. In addition, the potential of using the functional involvement of the thyroid endocrine gland in the early developmental and metamorphosis programmes in amphibians as biomarker system for studying the interaction with the thyroid systems proved valuable.

APPENDIX

The following research outputs generated by these projects are available in the literature or on request. The peer reviewed scientific articles submitted for publication, in press or published in local or international scientific journals contain further details on the work summarized in this report.

A) CONFERENCE PRESENTATIONS

Van Wyk, J.H., Leslie, A.J., & Pool, E.J. 1999. *Xenopus laevis* as a bio-indicator for endocrine-disrupting contaminants in South African water resources. Tenth Meeting of the African Amphibian Working Group, Stellenbosch, South Africa (6-9 June 1999).

Pool, E.J., van Wyk, J.H., & Leslie, A.J. 1999. The monitoring of water quality using a whole blood culture assay: the inflammatory activity of riverine and drinking water. Ninth International Symposium on Toxicity Assessment, Pretoria, South Africa, (26 September-1 October 1999).

Pool, E.J., van Wyk, J.H., & Leslie, A.J. 1999. An Enzyme Linked Immunosorbent Assay (ELISA) for measuring vitellogenin, a biomarker for xenobiotic estrogens. Ninth International Symposium on Toxicity Assessment, Pretoria, South Africa, (26 September-1 October 1999). Won the Best Poster Prize

Van Wyk, J.H. 2000. Health Related Water Research: Chemical Programme: Framework for New WRC programme on Endocrine Disrupting Contaminants. WRC Strategic Workshop, Pretoria, (October. 2000).

Van Wyk, J.H. 2001.Endocrine Disruptor Research at Stellenbosch University: Biomarker development, validation and implementation for assessment of EDC activities in natural water resources in South Africa. Workshop on South Africa-Finland Environmental Research Cooperation. RAU (12-18 May 2001).

Van Wyk, J.H. 2001. A Review of the proposed Framework for Research on Endocrine Disruptors to be supported in the Health Related Water Research Field of The Water Research Commission (WRC) of South Africa. Workshop on South Africa-Finland Environmental Research Cooperation. RAU (12-18 May 2001).

Van Wyk, J.H. 2001. An Overview of the Endocrine Disruptor Research conducted at Stellenbosch University: Biomarker development, validation and implementation for assessment of EDC activities in natural water resources in South Africa. WISA Meeting, Pretoria (14 May 2001).

Hurter, E., Pool, E.J., Van Wyk, J.H. 2001. Description and validation of a sensitive in vitro bioassay for detecting environmental estrogenicity using *ex vivo Xenopus laevis* liver slices. 11th Annual Meeting of SETAC Europa, Madrid, Spain, (May 2001).

Van Wyk, JH., Leslie, A.J. Pool, EJ. 2001. Amphibian nuptial glands as biomarkers for environmental anti-androgens: Morphological changes during exposure experiments with *Xenopus laevis*. International Comparative Morphology Conference, Jena, Germany (23 July 2001).

Strydom, A.V., Van Wyk, J.H. Leslie, A.J. 2001. Reproductive cycle of male African helmeted turtle, Pelomedusa subrufa (Family Pelomedusidae). Symposium of Zoological Society of Southern Africa., University of Port Elizabeth (July 2001).

Strydom, A.V., Van Wyk, J.H. Leslie, A.J. 2001. Reproductive cycle of female African helmeted turtle, *Pelomedusa subrufa* (Family Pelomedusidae). Symposium of Herpetological Association of Africa, University of Stellenbosch, (September 2001).

Pool, E.J. Van Wyk, J.H. 2001. Biomarker assays to screen for estrogenic EDCs in fishes and amphibians. SETAC-USA, Boston, (November 2001).

Van Wyk, J.H. 2002. Endocrine disruptors in the aquatic environment: The South African Perspective. IWA World Congress Melbourne 2002, Endocrine Disruptors Workshop, Melbourne, Australia, (7-12 April 2002).

Pool, E.J, van Wyk, J.H. 2002. The use of IL-6 induction as a human biomarker for inflammatory agents in water. IWA 3rd World Water Congress, Melbourne, Australia (9-12 April 2002).

Pool, E.J. Van Wyk, J.H. 2002. IL-6 secretion by human whole blood cultures as biomarker for immune system disruption. SETAC Conference 12th Annual Meeting. Vienna, Austria, (12-16 May 2002).

Hurter, E., Pool, E.J., van Wyk, J.H. 2003. Description,validation and implementation of a sensitive in vitro bioassay for detecting environmental estrogenicity using *ex vivo Xenopus laevis* liver slices. Pesticides in non-target agricultural environments. Joint European-Southern African International Conference, Cape Town, South Africa, (21-23 January, 2003).

Van Wyk, J.H., Pool, E., Leslie, A.J., Hurter, E. 2003. An assessment of estrogenic activity in three agricultural areas (vine, fruit & wheat growing) in the Western Cape, South Africa. Pesticides in non-target agricultural environments. Joint European-Southern African International Conference, Cape Town, South Africa, (21-23 January, 2003).

Van Wyk, J.H. 2003. Endocrine disruptor research activities at the Ecophysiology Laboratory, Department of Zoology, University of Stellenbosch: Past, Present, and Future. WRC Strategic Planning Workshop on EDC Research, Morgenhof, Stellenbosch, (May 2003).

Van Wyk, J.H. 2003. The African clawed frog, *Xenopus laevis*, as model to study endocrine disruptors in the South African aquatic environment. EcoToxicoGenomics Symposium, Okazaki, Japan, (October, 2003).

B) WORKSHOP PARTICIPATION

- WRC Johannesburg International Airport, Johannesburg,2001.
- WRC Strategic Planning Workshop, Pretoria, 2001.
- Finland Environmental Research Cooperation. RAU, 12-18 May 2001.
- WRC Strategic Planning Workshop on EDC Research, Morgenhof, Stellenbosch, May, 2003.
- USA- Environmental Protection Agency (EPA) on "Use of anuran models in endocrine disruption and reproductive toxicology research" Duluth, Minnesota, USA, June 23 - 25, 2003.
- OECD (Environment, Health and Safety Div), Paris, France: "Ad hoc Expert group on Amphibian testing" Duluth, Minnesota, USA, June 26- 28, 2003

C) PEER REVIEWED SCIENTIFIC MANUSCRIPTS

Pool, E.J., van Wyk, J.H. & Leslie, A.J. 2000. The monitoring of water quality using a whole blood culture assay: the inflammatory activity of riverine and drinking water. J. Immunoassay 21:387-399.

Hurter, E., Pool, E.J., van Wyk, J.H.. 2002. Description and validation of a sensitive in vitro bioassay for detecting environmental estrogenicity using *ex vivo Xenopus laevis* liver slices. Ecotoxicology and Environmental Safety 53: 178-187.

Pool, E.J., van Wyk, J.H., Hermann, M, Ivessab, N.E., Hurter, E. 2002. The development of a chicken APOII quantification ELISA. J. of Immunoassay and Immunochemistry 23: 439-449.

Pool, E.J., Jagels, C., van Wyk, J.H., Jagels, P. 2003. The use of IL-6 induction as a human biomarker for inflammatory agents in water. Water Science and Technology 47: 71-75.

Van Wyk, J.H., Pool, E.J., Leslie, A.J. 2003. The effects of anti-androgenic and estrogenic disrupting contaminants on the breeding gland (nuptial pad) morphology, plasma vitellogenin levels and plasma testosterone levels in male *Xenopus laevis*. Archives Environmental Contamination and Toxicology 44: 247-256.

Pool, E.J., van Wyk, J.H., Hurter, E., Faul, A Enzyme-linked immunosorbent assays for measuring vitellogenin in the Southern African, aquatic vertebrates *Xenopus laevis* and *Oreochromis mossambicus*. Water SA. (In Press).

Hurter, E., van Wyk, J.H., Leslie, A., Pool, E.J. Estradiol levels in a natural population of *Xenopus laevis* and the correlation with plasma vitellogenin and ovarian cycles. General Comparative Endocrinology (In Press).

Strydom, A.V., van Wyk, J.H., Leslie, A.J. Male Reproductive Cycle of the fresh water turtle, *Pelomedusa subrufa*. Journal of Herpetology (In Press).

Hayes, J. Hurter, J., Van Wyk, J.H. Estrogenic activity and cytotoxicity of selected rivers of the Western Cape. Water Research (In Press)

Hurter, E., Pool, E., van Wyk, J.H. Monoclonal antibodies against estrogen-specific proteins in *Xenopus laevis* plasma. Comparative Biochemistry Physiology (In Press).

Strydom, A.V., van Wyk, J.H., Leslie, A.J. Female Reproductive Cycle of the fresh water turtle, *Pelomedusa subrufa*. African Zoology (Submitted).

Van Wyk, J.H., Leslie, A.J. Pool, E.J. The male reproductive cycle of natural occurring African clawed frogs, *Xenopus laevis*. General and Comparative Endocrinology (Submitted).

Hurter, E., Pool, E.J., van Wyk, J.H. Screening of drinking and agricultural water for potential estrogenic activity. Journal of Water Health (Submitted).

Van Wyk, J.H., Leslie, A.J. Pool, E.J., Guillette, L.J. The effects of environmental chemicals, DDT, DDD, and Endosulfan on sex determination and steroidogenesis in the Nile crocodile (*Crocodylus niloticus*). Environmental Sciences (Submitted).

Van Wyk, J.H., Leslie, A.J., Pool, E. An Assessment of Estrogenic activity in three Agricultural areas in the Western Cape: Part I. Vine growing area (Hex River Catchment). Water SA (Submitted).

Van Wyk, J.H., Leslie, A.J., Pool, E. An Assessment of Estrogenic activity in three Agricultural areas in the Western Cape: Part II. Fruit growing area (Grabouw Area). Water SA (Submitted).

Van Wyk, J.H., Leslie, A.J., Pool, E. An Assessment of Estrogenic activity in three Agricultural areas in the Western Cape: Part III. Wheat growing area (Caledon/Napier Area). Water SA (Submitted).

D) CAPACITY BUILDING OUTCOMES

Postdoctoral fellows

Dr A.J. Leslie (1998-2000)

Seasonal reproductive cycles of selected bio-indicator species and seasonal caging of adult male *Xenopus laevis* frogs in three different agricultural areas.

Dr E.J. Pool (1999-2001; 2003)

Development and validation of vitellogenin ELISA detection systems as tools for studies regarding endocrine disruption contaminants

Student reports and dissertations

- A description of early (hatchling stage) gonad development in the Nile crocodile (*Crocodylus niloticus*). Ms. A. Strydom, BSc(hons)(1998).
- The estrogenic effect of selected agricultural chemicals, using the African clawed frog (*Xenopus laevis*) as a bio-indicator. Ms. A. Strydom BSc(hons)(1998).
- An assessment of estrogenic and anti-androgenetic activity in three selected sewage outlets in the Western Cape Province. Mr. R. Albertus BSc(hons)(1999).
- *Oreochromis mossambicus* (Mozambique tilapia) as bioindicator species for detecting xenoestrogens in South African aquatic systems. Mr. A. K. Faul BSc(hons)(2000).
- Nuptial pads of *Xenopus laevis* as potential biomarkers for detecting anti-androgenic activity in aquatic systems. Ms. L. Burger BSc(hons)(2000).

- The effect of endocrine disruptors (DDT & DDE) on the reproductive system of male *Xenopus laevis*. Anagnostu, M., Dawson, E.K., Fraser, M.L., Gould, S.A., Meijer, H., Morris, H.C., Sclanders, L., Withers, M.J. Zoology 344 research project (2000).
- The seasonal reproductive cycle of the fresh water turtle, *Pelomedusa subrufa*. Ms. A. Strydom. MSc (1999-2001).
- The effect of sex steroids and endocrine-disrupting contaminants on primary sexual differentiation in the African clawed frog, *Xenopus laevis*. Ms. M. Fraser BSc (hons) (2001).
- Assessment of fish bio-indicators of river health in rivers of the south-western Cape. Mr. J.B. Hayes MSc (2002).
- Oestrogenic effects of o,p'-DDT on the African clawed frog (*Xenopus laevis*) and the role of the skin as a barrier to pesticides. Bell, C., Cruse, D.G., Harmse, L., Greve, M., Mennen, H.W. Zoology 344 research project (2002).
- Xenobiotic estrogens and wastewater, the effects it has on the male African clawed frog, *Xenopus laevis*. Hough, M., Hugo, H., Maule, J., Oliver, F., Spies, T.. Swanepoel, C. Zoology 344 research project (2002)
- An evaluation of the *Xenopus laevis* liver slice model to study the toxic effects of microcystin. Ms. N. Coates MSc (University of Port Elizabeth; 2003).
- The natural endocrine cycles associated with the female reproductive system in the aquatic frog *Xenopus laevis*, with special reference to the application of the yolk precursor, vitellogenin as a bio-indicator for detecting estrogenic contaminants in the environment. Mr. E. Hurter PhD (2003).
- The effects of sodium fluoride upon the thyroid function of the developing *Xenopus laevis* tadpole. Boyes, L.J., Gouws, E.J., Irlich, U.M., Mostert, M.M., Van den Heever, M. Zoology 344 research project (2003).
- An Assessment of Endocrine Disruption Activities in the Kuils-Eerste River system in the Western Cape, South Africa. Ms. S. Fourie. MSc (2003/2004).

Technical Support

- Mrs M. Sauerman (University of Stellenbosch)
- Ms F. Gordon (University of Stellenbosch)
- Mr P. Beneke (University of Stellenbosch)
- Mr H. Davids (University of Stellenbosch)

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- Ms A. Strydom (MSc Student and Research Assistant)
- Ms L. Burger (MSc Student and Research Assistant)
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- Ms M. Fraser (BSc-honours Student)
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ARCHIVING OF DATA

All reports, manuscripts, dissertations, and abstracts will be deposited in the Web site on EDC research associated with Prof J.H. van Wyk, University of Stellenbosch

ACKNOWLEDGEMENTS

Water Research Commission Project Steering Committees

The financing of the projects by the Water Research Commission and the contributions of the members of the Steering Committees are gratefully acknowledged.

WRC Project K5/926: "An Assessment of the extent of estrogenic activity in Western Cape water resources."

- Mrs A.P.M. Moolman (Water Research Commission) Chairperson
- Mr H.M. du Plessis (Water Research Commission)
- Mr O.C.E. Hempel (Water Research Commission) Committee Secretary
- Dr K.C.D. Hamman (Cape Nature Conservation)
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- Mr J.F. Goede (SASOL Industries)
- Mr S. Pieterse (Cape Metropolitan Council)
- Mr H.R. Meinhardt (ARC-Plant Protection Research Institute, Agriculture Research Council, Pretoria)
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- Mr M. Makwela (National Department of Health)
- Dr H. Slater (SRK Consulting)
- Prof N.H. Casey and Dr J. Meyer (Faculty of Veterinary Science, University of Pretoria)
- Dr F.K. Siebrits (Agriculture Research Council)
- Mr J.N. Rossouw (Ninham Shand Consulting Engineers (Pty) Ltd)
- Prof O.S. Fatoki (Venda University)
- Prof J.O. Okonkwo (Technikon Pretoria)
- Dr H.L. Malan (Fresh Water Research Unit, University of Cape Town)
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- Mr S. Pieterse (Cape Metropolitan Council)
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The project team wish to gratefully acknowledge the co-operation, contributions and support of the following individuals and institutions:

- University of Stellenbosch for additional funding and the use of infrastructure and facilities.
- Support staff of the Department of Zoology, University of Stellenbosch, Mrs M. Sauerman; Ms F. Gordon; Mr P. Beneke; Mr H. Davids.
- Students and student assistants making valuable contributions to all projects
- The Hex River Irrigation Board for information and assistance during fieldwork.
- Cape Provincial Nature Conservation for permits.
- Western Province Blood Transfusion Services for the loan of a laminar flow cabinet.
- National Research Foundation for post-graduate bursaries and free standing Post-Doctoral Fellowships to Dr A. Leslie and Dr E.J. Pool. Also for capacity building bursary to Mr N. Dooling.
- Cape Metropolitan Council, specifically Mr S. Pieterse.
- City of Windhoek, specifically Mr J. Menge.
- Rand Water, specifically Dr H. du Preez.
- Prof J. van Vuren, Department of Zoology, Rand Afrikaans University, Johannesburg.
- Dr J. Myburg, Faculty of Veterinary Science, University of Pretoria.
- Dr A. Kuhn and Dr S. Jooste, Department of Water Affairs and Forestry.
- Mr R. Albertus, Saschem, SASOL.
- Mr R. Meinhardt, Plant Protection Research Institute, Agriculture Research Council.
- Prof L. London, Department of Community Health, University of Cape Town.
- Prof R. Bornman and Prof T. de Jager, Faculty of Medical Sciences, University of Pretoria.
- Ms J.L. Slabbert, Environmentek, CSIR, Pretoria.
- Prof D. Marais, Faculty of Medical Sciences, University of Cape Town
- Mr J. Levings, Two-A-Day Co-operative, Grabouw.
- Ms S.K.C. Peal, Forensic Chemistry Laboratory, Department of Health, Cape Town.
- Mr K. Hartman, UNIFRUCO/Cape Span for valuable information on pesticide usage in the agriculture industry.
- Van der Byl Farm, Bredasdorp/ Napier.
- Farmers that allowed the collection of water samples and placing of frog cages.

1. INTRODUCTION

Human, agriculture and industrial activity over the past decade has released large quantities of manmade chemicals into the environment. Since Rachal Carson's insights and predictions in her book, "Silent Spring", published in 1962, concern regarding the potential adverse effects of environmental contaminants on wildlife and human health is still increasing (Carson 1962; Colborn *et al.* 1993; Cadbury 1998; Guillette and Gunderson 2001). Although the initial focus of health concerns was on lethal, carcinogenic and extreme teratogenic manifestations, the publication of the book "Our Stolen Future" (Colborn *et al.* 1996) highlighted evidence that suggested possible disruption of the developmental and reproductive systems associated with exposure to increasing natural hormones as well as a wide range of synthetic chemicals and their by-products in the environment. This increased awareness led to numerous investigations and scientific reviews on the potential subtle, non-lethal effects of environmental contaminants on wildlife and humans (Guillette 1994; Colborn 1995; Jobling *et al.* 1995; Colborn 1996; Ankley *et al.* 1998; Colborn 1998; Reiter *et al.* 1998; Dodge 1998; Knobel *et al.* 1999). Concerns were strengthened with the increasing incidence of cancer and reproductive and developmental anomalies, for example, increasing incidence of breast, testicular and prostate cancer, cryptorchidism, diminishing sperm counts, developmental anomalies in reproductive organs. Moreover, the effects of early exposure to these environmental chemicals, either *in ovo* or intra-uterine, may not become apparent in humans for the first 10-15 years of life, until puberty occurs (Harrison *et al.* 1997).

Following early evidence or suggestions of endocrine disruption by environmental contaminants, the endocrine disruption hypothesis was formulated as: "Synthetic, and some naturally occurring, chemical substances in the environment are disrupting the normal functions of the endocrine system and its hormones in humans and wildlife" (Society of Environmental Toxicology and Chemistry (SETAC) 2000). Therefore, from this hypothesis, a chemical substance that interferes with, or has adverse effects on, the production, distribution, or function of hormones are referred to as an "Endocrine Disruptor" (ED) or "Endocrine Disrupting Contaminant" (EDC).

Sometimes these substances are also termed "Endocrine Modulators" or "Endocrine Active Substances" (EAS) to differentiate the more subtle changes they elicit, compared to substances that disrupt the endocrine system and cause dramatic effects (Society of Environmental Toxicology and Chemistry (SETAC) 2000) (Knobel *et al.* 1999). Endocrine disruption or endocrine disrupting contaminants (EDCs), are terms more often used, in spite of arguments that the term "endocrine disruptor" suggests a negative outcome and therefore does not include modulation of the endocrine system.

The endocrine disruptor hypothesis and the early definition of an endocrine disruptor proposed by organizations like the USA-EPA seems rather general and lacking understanding of specific modes of action. Many intricate regulatory mechanisms and biochemical pathways remain incompletely understood (Juberg 2000).

Humans and wildlife regulate homeostasis in an ever-changing environment. This is accomplished through a complicate exchange between nervous and endocrine systems. The endocrine system is the chemical communication system of the body and is involved in the regulation of most physiological functions including intracellular communication. The endocrine system includes several glands such as the pituitary and hypothalamus, gonads, thyroid, parathyroids, adrenal, pancreas and liver. The classical lifecycle of hormones include aspects of production, release, transport, metabolism, binding to a cellular receptor in the target tissue, biochemical and physiological response and elimination of circulating hormones. A wide range of developmental processes is under hormonal control, including sexual determination and differentiation. It is generally accepted now; that the endocrine system is mostly viewed in a rather simplistic way and that the field of endocrinology has greatly expanded and are no longer clearly defined as in the past. Moreover, the interaction of environment and endocrine system, although well studied, remains a field with many unanswered questions.

It has become increasingly apparent in the scientific literature that the endocrine system is particularly sensitive to very low levels of certain environmental

chemicals (Willingham 2001; Welshons *et al.* 2003). The basic endocrine disrupter hypothesis, therefore, predicts that certain environmental chemicals mimic the action of hormones or compromise the endocrine system to disrupt the normal physiological events causing physiological disruption in the exposed human or animal.

The origin of the endocrine disrupter hypothesis may be traced to a few sentinel reports that drew the attention of the scientific and lay communities:

(1) The publication of "Our Stolen Future" (Colborn *et al.* 1996) and "The feminization of nature: our future at risk " (Cadbury 1998) as sequels to Carson's "Silent Spring" (1962). Although these publications focused on "environmental estrogens" it is now realized that estrogen mimics are only a small proportion of pollutants acting as endocrine modulators/disrupters released into the environment;

(2) The reproductive anomalies reported in the daughters of mothers treated with diethylstilbestrol (DES) in the 1950s and 1960s to combat miscarriage. In affected women, adverse health affects included cancer, endometriosis, and breast cancer, and men experienced undescended testicles. These reports suggested that embryos and foetuses are more sensitive to the impacts of EDCs than adults and that the effects might not be seen until later in life or even until the next generation (Society of Environmental Toxicology and Chemistry (SETAC) 2000; Juberg 2000);

(3) Reproductive abnormalities reported in alligators studied in Lake Apopka (Florida, USA), a lake contaminated with DDT (Guillette *et al.* 1994; Guillette 1994);

(4) Reports of feminization in caged juvenile trout exposed in rivers receiving industrial and sewage effluents. Feminization in other aquatic animals, including snails, fish and birds was also reported. Marine snails exposed to tributyl tin, a compound of antifouling paint on boats showed changes in their reproductive organs (Society of Environmental Toxicology and Chemistry (SETAC) 2000);

(5) A study which reported a decrease in sperm counts in men from industrialized countries (Sharpe and Skakkebaek 1993; Carlsen *et al.* 1995).

Collectively these early reports created concern that environmental chemicals at seemingly low concentrations have the potential to interfere with the proper functioning of hormones in wildlife and humans.

Although initial studies mostly reported on aspects of feminization (estrogenic or anti-estrogenic activity), subsequent research showed that the male (androgenic) and thyroid endocrine systems may be important target areas for EDC effects.

It is clear that the important question of whether low levels of exposure to EDCs pose any significant health risk or not, will only be answered after gaining more knowledge on the long-term effects of interaction of environmental chemicals with components of the endocrine system. However, based upon the recognition of the potential scope of the problem and the precautionary principle, the possibility of effects on the health of wildlife and human individuals and populations, and the reported persistence of identified EDC compounds in the environment, research on EDCs was identified as a high-priority research topic within most international environmental agencies, including the USA-EPA (Reiter *et al.* 1998; Kavlock 1999), member countries of the European Union (EU), Japanese Environmental Agency, Organization for Economic Cooperation and Development (OECD).

Because of the concern that existing toxicological testing guidelines included endpoints that neither showed sensitivity to endocrine modulation/disruption, nor were indicative of the underlying biological effects, calls were made for serious revision of testing guidelines. In response, most developed countries initiated extensive research and monitoring programmes or passed laws to mandate environmental protection agencies to develop and validate screening programmes to evaluate chemicals for estrogenic and other endocrine activity. For example, the USA-EPA was required to convene an advisory group (Endocrine Disruptor Screening and Testing Advisory Committee, EDSTAC) to develop a screening and testing programme (EDSTP) for EDCs that would include screening for adverse effects to wildlife and humans. It was recognized that wildlife are an inherently valuable element of ecosystems and their well-being can be an indication for overall health of the environment in which humans live.

It was recommended by EDSTAC that three primary hormone systems be included in the EDSTP, estrogen, androgen and thyroid (EAT). EDSTAC also recommended that a tiered approach would be most effective in utilizing the available resources to detect EDC activity and quantify their effects.

Tier 1 screening was designed to detect chemicals that interacted with the EAT hormonal system. Tier 2 testing was designed to answer specific questions regarding whether the chemical substance or mixture had adverse effects on the EAT and characterize and quantify those. EDSTAC therefore recognized three elements of a specific sequence,

i) priority setting, by selection of chemicals for screening,

ii) ii) screening to detect chemicals or mixtures that interact with the endocrine system and

iii) iii) testing to characterize and quantify the nature of the endocrine disrupting activities.

Although EDSTAC initially agreed on a battery of screens and tests, it was suggested that other tests should be evaluated as more information becomes available. It was also underlined that screening and testing should be relevant to both human and wildlife as well as predict ecological effects. While the EDSTP flowing from the EDSTAC recommendations was aimed towards screening and testing chemicals, the Safe Drinking Water and Food Quality Protection Acts were subsequently amended to include EDC screening. Complimenting the activities of the EPA, the OECD initiated a framework for testing and assessment of EDCs. An Endocrine Disruptors Testing and Assessment Task Force (EDTA) was established in 1997 to develop a harmonized testing strategy and set OECD test guidelines. The EDTA Task Force decided to specifically focus on the coordination of the validating of existing and new screening and testing methods.

The environment, especially the aquatic and soil environments, serve as sinks for a diversity of pollutants. Although great advances have been made to determine concentrations of certain chemical compounds in the environment, it is no longer possible or feasible to, as a starting point, analyse for all pollutants at low concentrations. It is well-known that the environmental hazard of a chemical does not only depend on its concentration in the abiotic environment (O'Connor and Paul 2000; Reinecke *et al.* 2004). While specific chemicals may be relative easy

to detect, for example pesticides, the diversity of chemicals originating from industrial wastes, bioavailability of chemicals, long-term bioaccumulation, formation of breakdown metabolites and possibilities of synergistic interaction among compounds, makes the interpretation of such data problematic. The use of biomarkers in specific bioassays have been employed widely to ensure a more integrated approach (Kime 1998; Reinecke *et al.* 2004). Biomarker response is linked to a specific endpoint, for example, morphological, biochemical or molecular endpoints. The sensitivity of endpoints will depend on the category of endpoints selected. The anticipated order of endpoint sensitivity would be most likely expressed as molecular tests through biochemical tests to morphological changes. Although the morphological endpoints may be less sensitive, these may provide understanding of the actual physical outcome in the organism (Touart 2002).

EDSTAC recommended that a battery of bioassays should be used to show interaction with sex steroids and thyroid hormone systems, and this battery of test should include assessment of both potential human health effects and effects in wildlife. Although specific criteria were set to be recognized when developing bioassays for EDC screening and testing, it has been pointed out that tests to be used should have well-defined endpoints and that results gained must support further research of effects of EDCs on populations, communities and ecosystems. Moreover, seen from an African (developing vs. developed country) perspective, it has been recognized that cost-effective testing is needed and that a range of endemic wildlife models be included to eventually facilitate the assessment of the quality of water resources and water catchment areas.

The EDSTAC advisory committee to the USA-EPA recommended that the potential interaction of environmental substances with sex steroids and thyroid hormone function should be evaluated. It has been suggested that a screening battery be employed to assess potential human health effects and effects on wildlife (Kavlock 1999). It is acknowledged that a large number of the manmade chemicals exist and potentially many should be evaluated for endocrine modulatory activity. Therefore, EDSTAC suggested that high through-put screening bioassays be developed, validated and implemented to assist the

prioritization process. For this purpose, published information or cellular-based, receptor mediated gene transcription assays for chemicals acting either as agonists or antagonists for estrogen, androgen and thyroid receptors will be used to evaluated environmental chemicals. Following-on from the initial screening and prioritization stage, EDSTAC suggested a tiered screening and testing approach to gain more specific information on the interaction with or effects on endocrine systems:

Tier 1 screening focuses on assessment and priority setting of chemicals for which there is insufficient scientific data. The bioassays recommended, include three *in vitro* assays and five *in vivo* assays. The *in vitro* assays are estrogen and androgen receptor binding or transcriptional activation assays and a steroidogenesis assays using minced testes. The five *in vivo* assays include a rodent 3-day uterotrophic assay; a rodent 20-day pubertal female assay for effects on thyroidal function, a male rodent 5-7 day Hershberger Assay, a frog metamorphosis assay for thyroid effects, and a fish gonadal recrudescence or vitellogenin production assay.

Tier 2 testing is designed to characterize more defined responses, and therefore includes endpoints that will give decisive evidence whether or not a tested chemical may be an endocrine disruptor. It has been suggested that Tier 2 tests include several wildlife species and are longer-term tests. The battery of tests suggested, include a two-generation mammalian reproductive toxicity assay, an avian reproductive toxicity assay, a fish life cycle toxicity assay, an invertebrate life cycle toxicity assay, and an amphibian development and reproductive assay. It has been suggested that the weight of evidence approach will be used to evaluate the results obtained from these tests. Following Tier 2 testing positive chemicals will be tested for hazard assessment.

Several specialist workshops concluded that a continued search for better biomarkers, sensitive and specific, linking with specific mode of actions, are needed (Reiter *et al.* 1998; Kavlock 1999). It is therefore not surprising to see additional tests being described, validated and used in screening programmes, for example, the fish vitellogenin assay or variations thereof. However, although

chemical screening and testing is important for determining the potential hazardous effect in the environment, biomarker endpoints in indigenous aquatic animals will allow for long-term studies to assess impact on individuals and populations, and should not be excluded. More basic research is needed to study endocrine endpoints and different mode of actions in local indigenous species. Sentinel species, for example, *Xenopus laevis* and several exotic fresh water fish species (Medaka, Zebrafish) may be employed in the laboratory for screening specific chemicals or water samples. However, natural population effects can only be assessed by studying local indigenous species.

Although the usefulness of wildlife as sentinels for human health still remains uncertain, circumstantial evidence strongly suggest links to exposure to EDCs (Harries *et al.* 1997; Li and Li 1998; Sharpe 1998). Vertebrate bio-indicator species, ranging from fish to mammals are used as sentinels to study the effects and potential hazards that EDCs may hold for humans and wildlife populations. The similarities in the vertebrate endocrine systems and the fact that humans share the environment with wildlife species allow such extrapolations. The reproductive disorders in wildlife reported to date involve, early sexual developmental anomalies, reduced fertility, reduced hatchability, reduced viability of offspring, impaired hormonal function, modified sexual behaviour and demascularization or feminization of males (Van der Kraak 1998; Kime 1998). Some of the concerns about endocrine disruption effects in humans are based on reports of hormonally regulated cancers and other hormone sensitive events (for example sperm production) (Juberg 2000). However, in spite of the earlier case studies and reports, many scientists remain critical, maintaining that a lack of consistent evidence makes it difficult to relate EDCs to adverse health effects in humans (Juberg 2000). From a human health perspective, wildlife bio-indicators could be used in exposure bioassays confirming EDC exposure rather that implicating long-term endpoint disruption. The implementation of the "precautionary principle" could then be considered. It is widely acknowledged that differential sensitivity to EDCs of different life history stages and reproductive strategies in many species occur and that in many more species more basic research is needed.

Over 200 species are either known or are suspected to have been affected by EDCs (Miyamoto and Burger 2003) and in most of these, the endocrine system is often poorly understood.

Aquatic animals are a group exposed to a large array of anthropogenic chemicals and because they are exposed, mostly, to lower levels of complex mixtures over a long time scale, they can act as valuable biomonitors for EDC activity in the environment, both on individual and population level. Although fish has been regarded as the aquatic group most at threat (Kime 1998), amphibians are receiving more attention since it has been acknowledged that a global decline of amphibians is occurring and that EDC effects could be partly responsible for such declines (Kloas *et al.* 1999; Lutz and Kloas 1999; Kloas 2002; Mosconi *et al.* 2002). Invertebrates on the other hand, also hold potential for use in screening programmes, especially determining effects of pesticides on non-target species. However, it is clear from the lack of knowledge regarding the invertebrate endocrine system and how the link will be made to vertebrates and humans, that this group has largely been neglected as bio-indicators for EDC activity.

Most examples of EDC effects in wildlife have been reported from Europe, North America, Japan and Australasia, but this may simply be a reflection of the current global distribution of research effort and funding on EDCs (Miyamoto and Burger 2003). Several non-endemic fish species routinely used in EDC screening studies, for example, carp, catfish, trout and mosquito fish occur in South African water bodies and river systems. The African clawed frog, *Xenopus laevis* on the other hand occurs naturally in Southern Africa and is used extensively in international EDC screening and testing programmes, however, little research has been conducted locally. Therefore, local aquatic bio-indicators species, reasonably well-studied internationally could be used locally in the interim in screening programmes.

Many chemicals, both natural and synthetic, have been implicated to mimic the actions of estrogens (Dodge 1998; Knobel *et al.* 1999). Presently, more than 1500 environmental chemicals are known, many of which have been shown to exhibit estrogenic activity (Kime 1998; Knobel *et al.* 1999). Environmental

estrogens are a structurally diverse group of chemicals that includes natural estrogens and its derivates, contraceptives, organochlorine pesticides, herbicides, polychlorinated biphenols (PCBs) and alkylphenols from the plastic and detergent industries (Dodge 1998). Matters may be complicated by natural occurring phyto-estrogens and otherwise harmless compounds like fertilisers that may act synergistically when in mixture with EDCs (Preziosi 1998; Porter *et al.* 1999). Moreover, in the case of persistent chemicals that bio-accumulate in adipose tissue, contaminant transfer to offspring may predispose them so that on exposure to EDCs at low environmental concentrations adverse effects result. Since the chemical structure of known estrogenic mimics is so diverse, potential estrogenicity is difficult to predict. Many of these substances were discovered only by chance on exposure to the chemical. In the environment a cocktail of potential estrogenic and non-estrogenic compounds occurs, and although in low concentrations, may through synergistic mechanisms cause endocrine disruption (Preziosi 1998; Porter *et al.* 1999). Because environmental estrogens mostly have anthropogenic origins, highest concentrations would be expected to occur near urbanised (residential and industrial) or agriculture intensive areas. Although the sources of pollution and routes of exposures may be diverse, many of these compounds find their way into rivers via Sewage Treatment Works (STWs)(Johnson *et al.* 2002), point-discharges, diffuse sources and major environmental spills. A recent USA Geological Survey study investigated 15 biogenic and synthetic hormones and sterols as well as 35 household and industrial wastewater products in 30 USA streams and rivers (Kolpin *et al.* 2002). The European Union (EU) lists 33 priority compounds (Water Framework Directive, September 2000), but accepting that a vast number of substances need to be evaluated further. The OECD listed 20 EDCs in SPEED'98 but extended the list with an additional 65 chemicals to be considered. The USA-EPA lists a number of chemicals requiring priority action (Johnson *et al.* 2002). In South Africa, 30 potential EDCs (including hormones, pesticides, herbicides, and various industrial chemicals) have been prioritized to be detected analytically in low concentrations in water samples (Burger 2003). Because most of the initial focus was on estrogenic activity, more chemicals will certainly be added to include those with androgenic and thyroid effects.

It is clear from the literature that research regarding the extent and effects of EDCs on the quality of life, for wildlife and humans, is progressing fast in most developed countries of the world, including the USA, Europe and Japan.
In Africa, specifically South Africa, relatively little attention, beyond the listing of potential EDCs, has been given to the potential hazard of endocrine disruptors. From these listings it becomes clear that most of the potential EDCs listed, could be expected to be present in South African water resources (London and Myers 1995a; Meintjies *et al.* 2000). The continued use of several persistent organic pesticides (POPs), for example, DDT, a classical EDC, to combat malaria in the northern provinces in South Africa, raises many questions amongst scientists in the developed world. The use of agrochemicals for pest and weed control, including the Western Cape where a large agriculture activity is settled has shown to be substantial (London and Myers 1995a; London and Myers 1995b; London *et al.* 2000). The increased use of pesticides in certain river catchments and subsequent bio-accumulation of these pesticides in endemic, edible fish species may hold a significant health hazard for humans eating the fish (Kime 1998; Heath and Claassen 1999; Kleinkauf *et al.* 2004). Although pesticides (chemical concentrations) in local aquatic systems have been studied by several researchers, no study reported on endocrine effects of modulations specifically. Moreover, increasing industrial and urban development as well as the expanding of small-scale farming will inevitably intensify the threat of EDC contamination and activity in South African water resources. Though treated, industrial and/or domestic sewage eventually discharged into natural river catchments and in certain areas, downstream water utilization for human drinking water consumption may not be uncommon. Moreover, South Africa, being a relatively dry country, will in future experience more pressure to, similar to the City of Windhoek in Namibia, use reclamation of domestic waste waters to supplement limited sources of natural water for drinking and irrigation.

Although the recently revised National Water Act (Act No 36 of 1998), passed by the South African government, is designed to protect our natural water resources, specifically catchment areas, no regulations regarding EDC activity are in place. The potential or real hazard of EDCs in the South African water resources is

further confounded by a lack of, or non-availability of epidemiological data linking EDCs to human or wildlife health.

Despite increased research effort in the field of environmental biotechnology, hardly any new or indigenous biotechnology regarding EDC screening and testing has been developed and validated in South Africa. However, even-though the development and validation of EDC bioassays is a fast growing field internationally, the simple transplantation of such technology to South Africa has not excelled, mainly because it is not a financially viable option. Moreover, the use of endemic biomarkers and biomonitor species will ensure the added bonus of studying endemic wildlife populations in long-term studies. On the other hand, the lack of basic biological and ecological data regarding indigenous wildlife species limit such an approach and local laboratory assessment/screening studies will have to rely on international employed bio-indicator species and be limited to laboratory exposure studies in the interim.

It was against this backdrop that the current research effort evolved, on the one hand with the focus on learning more about potential endogenous bio-indicator species but on the other hand to use existing biotechnology and internationally recognized bio-indicator species and well characterized and validated biomarkers to begin to assess the extent of EDC activity in South African water resources.

2. REVIEW OF RESEARCH ACHIEVEMENTS

(SETTING AND ACCOMPLISHMENT OF OBJECTIVES)

Original objectives and the eventual achievements thereof are summarized below with reference to further details published in peer reviewed national and international scientific journals, unpublished MSc and PhD theses, and unpublished student research project reports. Theses and reports are available on request.

2.1 WRC PROJECT 926: "AN ASSESSMENT OF THE EXTENT OF ESTROGENIC ACTIVITY IN WESTERN CAPE WATER RESOURCES."

Objectives:

- To study the normal reproductive cycles of *Xenopus laevis* and the turtle species, *Pelomedusa subrufa* in order to gain baseline information for comparative ecotoxicological studies.
- To study the vitellogenin response in the frog, *Xenopus laevis* when exposed to selected xenobiotic estrogens.
- To investigate the practical implementation of vitellogenin as a biomarker for xenobiotic estrogens using either fish, amphibian or turtle model systems.
- To determine whether estrogenic activity occur in water resources in the Western Cape. If so then continue to:
- establish predicative models of exposure based on different effluents or exposures;
- determine the effect of dilution with respect to distance from discharge point and/or seasonal variables.
- To determine whether there is evidence of estrogen activity in drinking water in selected study areas.
- To make recommendations regarding Environmental Quality Standards and conservation of endemic wildlife, potentially effected by unnatural estrogenic activity in water resources.
- To facilitate capacity building and technological transfer for the benefit of South Africa, i.e. ensuring quality of water resources.

All objectives have been accomplished as defined above. When the study started, no validated vitellogenin (Vtg) ELISA protocol was available for *X. laevis*. Since the development, screening and validation of *X. laevis* anti-Vtg antibodies is a time consuming exercise, the presence of plasma Vtg could not be checked in pre-exposure frogs. The research regarding the seasonal *in situ* exposure of caged frogs in the selected agriculture regions, however, proceeded and tissues and blood samples were stored until the analytical tools were ready for use. In hind-sight, it was found that male frogs sacrificed as pre-exposure controls occasionally showed high levels of plasma Vtg, suggesting estrogenic activity in wild caught males from their natural habitat. As the study progressed more research needs were identified and therefore added to the objectives.

This was understandable, since most of the research planned did not have specific, well defined end-goals, mainly because new biomarkers were to be developed. For example, following the sixteen month *in situ* exposure of caged frogs in three different agriculture areas the number of wild caught frogs that were sacrificed as well as the number of exposure cages we lost due to theft, flooding or drought prompted the team to look at alternative *in vitro* exposure of *X. laevis* liver slices rather than individuals. While working with *X. laevis* and realizing the importance of other EDCs, for example, anti-androgenic effects due to exposure to fungicides, guided the project in the direction of investigating the potential of male breeding glands as a potential biomarker.

The importance of base-line physiological information for *X. laevis* from its native waters has recently again been emphasized by organizations to the likes of the USA-EPA and OECD. In view of this call, major progress has been made, with the completed projects. Similarly, the biomarker development represents new contributions and allows for cost effective screening of water resources and suspected EDCs without the need to import expensive *in vitro* technology.

However, since the first project (WRC Project 926) contributed valuable information and biotechnology as well as explored the potential of *in situ* caged aquatic exposures (similar to the UK fish studies) several aspects had to be followed-up in order to contribute to a battery of EDC screens to be considered for use in an envisaged toxicology monitoring programme mandated by the National Water Act (Act No 36 of 1998).

Therefore, WRC Project 1253 followed. However, this was also the time when the Water Research Commission realized the importance of a clear research framework regarding EDCs in water resources, and WRC Projects, 926 and 1253 must be seen as initial research efforts.

Summary of research results to achieve the objectives:

2.1.1 The vitellogenin response in the frog, *Xenopus laevis* when exposed to selected xenobiotic estrogens.

2.1.2 Practical implementation of vitellogenin as a biomarker for xenobiotic estrogens using either fish, amphibian or turtle model systems.

Vitellogenin (Vtg), a serum phospholipoprotein, precursor of egg yolk, produced in the liver under estrogen control, is a well-recognized biomarker for detecting environmental estrogens. The main objective of this study was to isolate Vtg and to develop Vtg-enzyme immunosorbent assays (ELISAs) for the African clawed frog, *X. laevis*, fresh water fish, *O. mossambicus* (tilapia) and fresh water turtle, *P. subrufa*.

Methodology

For the *X. laevis* Vtg ELISA, polyclonal anti-Vtg antibodies were raised in rabbits, isolated, validated and incorporated in a Vtg ELISA, for the tilapia, commercially available sea bream (*Sparus aurata*) anti-Vtg antibodies were purchased and incorporated in a Vtg ELISA. Plasma Vtg levels in the turtle were determined using a newly developed universal vertebrate Vtg ELISA (UniVtg).

Results and Discussion

Both the frog and fish ELISAs are highly reproducible and sensitive. The inter- and intra-assay coefficients of variation are <5 % for both assays. The linear range of the *X. laevis* ELISA is 62,5 $\mu g.l^{-1}$ to 2000 $\mu g.l^{-1}$, while that for the tilapia, *O. mossambicus* ELISA is 20 to 160 $\mu g.l^{-1}$. The *X. laevis* ELISA was used to screen: females and males during the natural reproductive cycle, males following *in situ* field exposure, in cages, in three agricultural regions, *in vitro* liver culture supernatants for Vtg production upon estrogen or environmental water exposure as well as evaluating animals obtained from the field for potential xenoestrogen exposure. The tilapia (*O. mossambicus)* Vtg ELISA was used to screen males during water exposure experiments. In both cases, the results indicated that stock animals were previously exposed to estrogenic compounds. These results further showed that the assays developed could be employed successfully during Tier 1 *in vivo* (frogs and fish) and *in vitro* (frog liver slices exposed in culture) estrogenicity screening of water samples. Screening of wild caught frogs before experimental/exposure studies, to eliminate frogs from contaminated collecting sites will now be possible. The tilapia Vtg ELISA will be of great benefit to researchers, specifically those in Southern Africa, as the assay can be used to screen both natural and aquaculture farm populations for potential estrogenic EDC exposure.

The details of this work have been submitted for publication (Hurter *et al.* 2002; Hurter *et al.* 2003; Pool *et al.* 2003) and presented at conferences.

2.1.3 Development and validation of a sensitive *in vitro* bioasssay for detecting environmental estrogenicity using *Xenopus laevis* liver slices in culture (Added Objective).

A sensitive *in vitro* bioassay for detecting environmental estrogens and estrogen mimics was developed using a *X. laevis* liver slice tissue culture protocol.

The initiative for this *in vitro* bioassay came while conducting this research project and was not included in the original study. The objective being to limit *in vivo* exposures for screening purposes and to screen larger numbers of samples using an increased sensitive assay.

Methodology

Vitellogenin synthesis by *X. laevis* liver slices in culture was used as bio-assay system for estrogenic activity in water samples. Sensitisation of the assay for estrogens and environmental mimics was accomplished by employing liver tissue from animals after *in vivo* pre-exposure to estrogen. Effects of various tissue culture factors were investigated in order to obtain optimum conditions for the bioassay.

Results and Discussion

Optimum conditions for culturing liver slices were established. Pre-treatment of male frogs to estrogen significantly increased sensitivity of liver slices for estrogen. However, it was found that endogenous vitellogenin and/or estrogen could be 'washed out' of liver tissue in culture and that not only liver slices from uncontaminated males, but also estrogen pre-treated males and females (non-vitellogenic reproductive stage) can successfully be used in this bio-assay. Estrogenicity was detected, using this *in vitro X.* laevis liver slice culture assay, in drinking water, sewage effluent, lake- and dam water and results confirmed the value of this *in vitro* bioassay as a Tier 1 screen. The fact that an *ex vivo* liver slice is used includes all the dynamics of a responding tissue rather that just interaction of the EDC with the estrogen receptor like in most receptor binding assays.

In a published paper we presented an optimised protocol for using this *in vitro* bioassay to detect estrogen activity in environmental water samples (Hurter *et al.* 2002). Several conference papers were also presented.

2.1.4 Potential use of *Xenopus laevis* male breeding glands (nuptial pads) as biomarker system for anti-androgenic effects (Added Objective).

Breeding glands (nuptial pads) are areas, on digits and fore-arms, characterized by epidermal keratin hooks and dermal glands opening on the epidermis surface in male frogs. The presence of the epidermal hooks and secretory activity of the breeding glands are suggested to be androgen dependent and suspected to play an important role during mating.

Chemical pollutants released into the aquatic environment by humans could potentially disrupt the normal hormonal control pathways and functioning of these glands. Recently, specific anti-androgenic activity by certain EDCs, including DDE and several fungicides, have been shown in mammalian studies. In this study, we investigated the potential of breeding (nuptial) gland activity to be employed as a biomarker system to screen for anti-androgenic activity by certain EDCs.

Methodology

Histological techniques were used to study the morphology of the breeding glands of male frogs. Morphometric measurements of the glands and the secretory epithelium were used to assess changes in secretory activity of these skin glands following exposure to different relevant hormonal controls and selected environmental chemicals. Plasma concentrations of reproductive hormones in exposed males were also measured using specific hormone ELISAs.

Results and Discussion

Results indicate that the pharmaceutical anti-androgen, flutamide, did significantly ($P<0.05$) effect the androgen-dependent breeding (nuptial) glands as well as plasma testosterone concentrations in male *X. laevis*. Results further confirm that the dicarboximide fungicide, vinclozolin, mimic the anti-androgenic action of flutamide. Vinclozolin, however, did not significantly effect the plasma testosterone concentration. These preliminary data clearly showed that several aspects of the male breeding glands need more research in order to fully develop the potential of these androgenic endpoints for bioassay application.

Therefore, it is recommended that research concerning *X. laevis* male breeding glands should be continued.

The details of this work was published (van Wyk *et al.* 2003) and presented at conferences. A more comprehensive research project was established towards a Masters degree.

2.1.5 Study of the normal reproductive cycles of *Xenopus laevis* and the freshwater turtle, *Pelomedusa subrufa,* to gain baseline information for comparative ecotoxicological studies.

The main objective was to conduct base-line studies regarding the reproductive cycles of selected, potential, aquatic bio-indicator species. The rationale being that knowledge about natural reproductive cycles is important (vital) base-line information needed before employing an endemic species as bio-indicator/biomonitor for EDCs in the environment. The initial indigenous aquatic species selected, as potential bio-indicators (biomonitors), were the African clawed frog, *X. laevis* and the freshwater turtle, *P. subrufa*.

Xenopus laevis is kept in captivity and breed all over the world, however, very little information is available regarding its natural reproductive cycles, including the reproductive hormones and circulating yolk precursor, vitellogenin (Vtg), levels.

Methodology

To obtain seasonal morphological and physiological data, ELISA analytical methods had to be developed (vitellogenin) and validated for use with *X. laevis* plasma (hormones, estrogen, progesterone and testosterone). Monthly samples of naturally occurring *X. laevis* were obtained from the wild, autopsied and along with an assessment of gonadal activity, spermatogenesis and oogenesis, we measured and reported circulating hormone and Vtg levels for the first time. In addition, the seasonal variation in the breeding (nuptial) glands in male frogs was also studied. It was important to confirm that the breeding glands in males varied in association with androgenic activity.

Freshwater turtles (***Pelomedusa subrufa***) were more difficult to obtain than *X. laevis* and specimens were collected with special baited funnel traps. The fresh water turtle, *P. subrufa,* occurs wide-spread throughout Southern Africa and could easily be bled without killing it. Although turtles were sacrificed for autopsy to learn more about the dynamics of the gonadal cycles, individuals were also released after bleeding to be recaptured on a next occasion. Gonadal tissues were subjected to histological analyses and circulating levels of the reproductive

hormones measured with hormone ELISAs. It was the first time that ELISAs were validated and standardized to measure the circulating hormone levels in a South African fresh water turtle. Since it was beyond the scope of this project to develop specific anti-vitellogenin antibodies for *P. subrufa*, the need for developing an universal Vtg ELISA (see UniVtg ELISA development) that could be used in a whole range of vertebrate species without loss of sensitivity and specificity was identified. The success of this development allowed for the characterization of the seasonal plasma profile of vitellogenin in females but also in males exposed to water with estrogenic activity.

Results and Discussion

Xenopus laevis: The female gonadosomatic index varied significantly during the year with minimum ovarian mass measured in March and maximum in September (spring). Although the number of large vitellogenic ovarian follicles varied throughout the year, vitellogenic follicles were present during all the months of the year. Plasma estrogen levels also varied during the year reaching a peak in June (winter) and low values in March. Plasma Vtg varied significantly during the year with peak levels measured in September (spring). Plasma Vtg profiles corresponded with the occurrence of vitellogenic follicles exhibiting highest during the months of September, October and November.

These data suggest that the ovary is capable to produce vitellogenic follicles throughout the year but that a peak in vitellogenic activity occurs in spring through early summer.

The male reproductive cycle was characterised by maximum gonadosomatic index (GSI) during the November (early summer). Plasma testosterone levels, however, reached peak concentration during winter (July).

Spermatogenic activity occurred throughout the year but peak spermiogenesis coincided with maximum GSI during November. Noteworthy, was the significant variation in plasma vitellogenin (Vtg) concentration in male frogs. Male frogs collected during the months, March'98, April'98, January'99 and February'99 exhibited increased Plasma Vtg levels compared to the other months.

Although the Plasma Vtg levels are low compare to that measured in females, <1ug/ml vs. 3ug/ml, the increased Vtg levels in males could be attributed to environmental estrogenicity in the water bodies where males were collected from.

Details of the female reproductive cycle have been presented in a PhD thesis (Hurter 2004) and a paper submitted for publication (Hurter *et al.* 2004c). Details regarding the male cycle have been submitted for publication (van Wyk *et al.* 2004b)

Pelomedusa subrufa: Male and female reproductive cycles were found to be seasonal, however, cycles were not well synchronized. Spermatogenesis was distinctly seasonal and the timing of events corresponded to what has been described for other fresh water turtles. Males exhibited a post-nuptial pattern of spermatogenesis in which spermatogenesis starts in summer, after emergence from hibernation and spring/early summer mating. Peak testicular volume and maximum spermiogenic activity occur in late summer and early autumn. Testicular regression commenced in late autumn through winter until the next recrudescence in summer. Spermatozoa were abundant in the ductus epididymidis throughout the year. Plasma testosterone concentrations peaked once during the testicular cycle, typically coinciding with spermiogenesis in late summer, early autumn. Epithelial cell height of the ductus epididymidis showed significant seasonal variation, and peak secretory activity coincided with spermiogenic activity and high circulating testosterone concentrations in late summer, early autumn. Like most other reptiles that exhibit a post-nuptial spermatogenic cycle, male and female reproductive cycles were asynchronous and all indications are that mating behaviour in summer could be regarded as dissociated from the normal testosterone control. The female reproductive cycle was characterized by vitellogenesis starting in spring, ovulation in early summer and egg-laying in mid-summer.

Although more research on the general ecophysiology of this species is needed, the fact that aquatic turtles show environmental sex determination and males showed a vitellogenic response when exposed to estrogen underlined the potential of this species in future studies regarding EDC activity.

Details of this work have been presented as a MSc thesis (Strydom 2001) and two papers submitted for publication (Strydom *et al.* 2004a; Strydom *et al.* 2004b) and presented at conferences.

2.1.6 Use of caged frogs to determine estrogenic activity occurs in water in selected areas in the Western Cape.

Since many pesticides used in agriculture have been suggested to contribute estrogenic properties, the hypothesis that water resources in agricultural areas receiving run-off or drift spray are estrogenic was tested. Three different areas were selected to represent dominant farming activities with different pesticide usage. In this study caged frogs, *X. laevis*, were placed in rivers or dams in the selected study areas. For the purpose of internal control, cages were also placed in adjacent uncultivated areas. Following exposure, frogs were sacrificed and plasma Vtg determined using the *X. laevis* Vtg ELISA described above (Pool *et al.* 2004) and compared with a pre-exposure control group as well as frogs exposed at a control site in the study area.

Hex River Valley (predominantly grape farming):

Methodology

Cages with adult male frogs (n=10) were placed at nine (9) different sites in the Hex River and tributaries (to include different potential inflows and dilution effects downstream) for periods of 21 days on seven (7) occasions between October 1998 and September 1999 (seasonal variation).

Results and Discussion

Frog recoveries varied between 41% - 81% at each site and 47% - 70% between dates (exposure events). An overall recovery of 58% was obtained.

Variation in mean plasma Vtg levels of recovered frogs varied significantly among dates and sites. In most cases, however, did the mean Vtg levels in the exposed males not differ significantly from that of the pre-exposed control frogs. Following caged exposures during October 1998, mean Vtg levels in male frogs at two sites showed increased plasma Vtg levels when compared with the pre-exposed control frogs.

Frogs from one (1) site (April 1999) showed increased Vtg levels when compared with the other sites but not the pre-exposed control frogs. Data show that the wild collected pre-exposed control male frogs on occasions had high plasma Vtg levels and that at most of the sites, except at one site, plasma Vtg levels actually decreased during the exposure period ("wash-out"). In all exposure events the Condition Index (CI) of frogs taken before and after exposure, did not changed significantly. It was clear that although estrogenic activity was recorded, no consistent pattern occurred. Comparing different sites along the river, no indication of accumulation or dilution effect was observed.

Grabouw Area (predominantly fruit farming)

Methodology

Cages with male frogs (n=10) were placed at ten (10) different sites (different run-off patterns) for periods of 21 days on eight (8) occasions between July 1998 and August 1999.

Results and Discussion

Frog recoveries at each site varied between 18% - 73% and 15% - 58% between dates (exposure events). An overall recovery of 38% was obtained. Throughout this study, only three (3) sites showed frogs with increased mean Vtg plasma levels, indicative of significant estrogenic activity. Frogs kept in cages at the Theewaterskloof Dam site during June 1999, showed plasma Vtg levels as high as normally measured in females. In all exposure events the Condition Index (CI) of frogs taken before and after exposure, did not changed significantly. It was clear that although estrogenic activity was recorded occasionally, no consistent pattern occurred.

Caledon/Napier Area (predominantly wheat farming)

Methodology

Cages with male frogs (n=10) were placed at six (6) different sites for periods of 21 days on seven (7) occasions between September 1998 and August 1999.

Results and Discussion

Frog recoveries at each site varied between 18% - 73%, and 15% - 58% between dates (exposure events). An overall recovery of 38% was obtained. Variation in mean plasma Vtg levels of recovered frogs varied significantly among both date and sites. Male frogs recovered from cages during August 1998, at several sites, showed increased Vtg levels. In December 1998, one male frog survivor from a cage showed very high Vtg levels when compared with the pre-exposure frogs. Frogs from other sites had lower Vtg levels than this specific individual frog, but also had higher Vtg levels than the pre-exposed control frogs. Male frogs from most sites showed increased Vtg levels during the April 1999 exposure, although only one group proved to be statistically significant when compared to the pre-exposed control frogs. All male frogs recovered from the August 1999 exposure, only 20% of sample, showed significantly increased Vtg levels.

Details of this work have been submitted for publication (van Wyk *et al.* 2004a) and presented at an international conference.

2.1.7 *In vitro* screening of drinking water for evidence of estrogenic activity, in selected study areas

The objective of this study was to use an *in vitro* bioassay (*X. laevis* liver culture assay) to screen for estrogenicity in water resources.

Methodology

For this study the newly developed *X. laevis* liver slice *in vitro* assay was used (see above). Initially the *X. laevis* Vtg ELISA employing the polyclonal *Xenopus* anti-Vtg antibodies was used to screen selected water samples for estrogenic activity.
However, as the monoclonal-produced antibodies against the whole estrogen-induced protein complement became available (see paragraph 2.2.2.2), the screening was repeated and the results compared. Drinking water, agricultural water and reclaimed water (City of Windhoek) was screened. A total of 48 samples were included.

Results and Discussion

The results from the in vitro assay showed that this screening tool has the sensitivity to locate estrogenic activity in water samples, including drinking water. It was also established that the prior solid phase extraction (C18) will remove most of the toxins that potentially could result in false negatives.

See paragraph 2.2.3.1 and Hurter *et al.* (2004b) for details.

2.2 WRC PROJECT 1253: "ENDOCRINE DISRUPTING CONTAMINANTS (EDCS) IN SOUTH AFRICAN WATER RESOURCES: DEVELOPMENT AND VALIDATION OF *IN VITRO* AND *IN VIVO* BIOASSAYS TO DETECT ENDOCRINE INTERACTION AND CHARACTERIZE PHYSIOLOGICAL DISRUPTION IN NON-MAMMALIAN ANIMALS.

Objectives:

- To conduct a desktop literature review to establish the state of the art both internationally and locally in order to develop a framework for research on endocrine disruptors in South Africa.
- To develop and validate bioassays to eventually be employed in a battery of bioassays for hazard and risk assessment of EDCs in water resources:
- Develop monoclonal antibodies against estrogen-sensitive plasma proteins.
- Preliminary investigations regarding *X. laevis* estrogen sensitive plasma lipids and lipoproteins.
- Development and validation of an universal vitellogenin (UniVtg) bioassay.
- Male nuptial skin glands in *X. laevis* as biomarker for androgenicity.
- Preliminary investigations into sex determination and metamorphosis in *X. laevis* tadpoles as biomarkers for estrogenicity and thyroid function.
- To screen selected water sources for EDC activity.

The objectives of WRC Project 1253 were set broadly and it was intended to be an extension/continuation of WRC Project 926. Initially Project 1253 included participation of several other institutions, however, these partners selected to withdraw. The initial objectives were subsequently amended to focus more on method development, specifically focussing on bio-assays including non-mammalian bio-indicator species, i.e. extending Project 926. The original set of biomarkers and bioassays to be researched were trimmed to include mostly the expansion of the new biomarkers using endemic species, as identified during Project 926.

These amended objectives were largely accomplished. The later WRC EDC Programme (1402) realized the objective regarding a literature review and the development of a research framework for EDCs in South Africa, as well as the

objective to conduct a comparative study to assess the water handling and extraction procedures used by the different research laboratories.

Summary of research results to achieve the objectives of project 1253:

2.2.1 To conduct a desktop literature review to establish the state of the art both internationally and locally in order to develop a framework for research on endocrine disruptors in South Africa.

The research fields related to EDC research are very dynamic and an exponential growth in the number of research papers continues, especially several books and review articles. The EDC literature concerning the research conducted in both these projects has been reviewed in a number of publications that emanated from the current projects as well as graduate reports and post-graduate dissertations.

2.2.2 Development and validation of bioassays to detect EDCs in water resources. (for use in a battery of tests)

2.2.2.1 Preliminary investigations regarding frog and avian estrogen sensitive plasma lipids and lipoproteins

(a) Plasma lipoprotein profiles as biomarker for estrogenicity

In search of additional estrogen sensitive biomarkers, the plasma cholesterol, -phospholipid and –triglyceride concentrations in natural populations of male and female *X. laevis* were investigated. The effect of estrogen exposure on these were evaluated for use as biomarkers for EDC contamination, specifically estrogenic activity. The various lipoproteins (VLDL, LDL and HDL) were compared between sexes and the effect of estrogen exposure upon these also studied.

Methodology

Adult male and female frogs, *X. laevis*, were exposed to estrogen and subsequently bled.

Polyacrylamide gradient gel electrophoresis was used to visualize the different lipoproteins. Plasma lipid profiles were determined using enzymatic, colorimetric diagnostic kits.

Results and Discussion

Cholesterol and phospholipid concentrations were higher in females. Little LDL is present in *X. laevis*, especially in males. There was generally greater variation of HDL in the female plasma compared to the males. The male plasma samples showed a level of conformity that was absent in the females. Estradiol induced changes in lipoprotein composition and lipid concentrations in both sexes. Lipid and lipoproteins can be employed as biomarkers for EDC exposure.

The details of this work were presented in a PhD thesis (Hurter 2003).

(b) The development of a chicken Apolipoprotein (APO II) (apoII) quantification ELISA.

Birds are also oviparous and biomarkers associated with estrogen induced vitellogenesis may also be used in estrogenicity assessment studies as part of a battery of tests to include a broad range of vertebrate species. In this regard the application of high density lipoproteins for estrogenicity screening were investigated. The objective was to used an available anti- Apo II chicken anti-body to set-up and validate an ELISA for avian plasma Apo II lipoprotein determination. The hepatic apoB gene acquired estrogen-responsiveness at day 6.5 and its hormone-dependent expression increased throughout development in concert with the estrogen-responsive expression of the apoII gene.

This bioassay could then complement the measurements of plasma vitellogenin (using the UniVtg ELISA for vertebrates) in a battery of estrogenicity screens using avian models.

Methodology

Blood was collected from egg-laying chickens and roosters by venipuncture. Plasma samples were fractionated using SDS-PAGE method on 15% acrylamide gels under non-reducing conditions. The separated proteins in the gel were transferred to a nitrocellulose membrane. After transfer the membrane was incubated with rabbit anti-chicken Apo II antiserum and incubated with sheep anti-rabbit peroxidase complex. The anti-Chicken Apo II antibody concentration was optimized for use in an ELISA. A direct ELISA and an indirect ELISA for Chicken Apo II were subsequently set-up and validated.

Results and Discussion

Polyclonal antibodies raised against chicken Apo II apoII was characterised for its use in Western blotting and ELISA detection systems of Apo II in chicken plasma. The antibody has a high avidity and specificity for Apo II. Western blots show that the antibody reacts with a single band at 15 kDa. The antibody was used for setting up both direct and indirect ELISA assays for Apo II. The indirect ELISA has a broader detection range (10 - 1600 U/ml) than the direct ELISA (10 - 100 U/ml). It was found that both ELISA systems discriminate very well between vitellogenic (laying hen) and non-vitellogenic (rooster) plasma. The indirect ELISA, due to its broad detection range, can potentially be used for monitoring female reproductive cycles, accidental and environmental exposure of males to estrogen and for Apo II secretion by cultures hepatocytes and hepatomas.

The details of this research have been published (Pool *et al.* 2002).

2.2.2.2 Development of *Xenopus laevis* monoclonal anti-Vtg antibodies against estrogen-sensitive plasma proteins.

Although in WRC Project 926 polyclonal anti-Vtg antibodies were raised and incorporated in a *X. laevis* Vtg ELISA (Pool *et al.* 2003) it was decided to raise specific monoclonal anti-bodies against the whole complement of estrogen inducible proteins, to include proteins like Vtg, Zona radiata (ZR)

proteins and other potential end-points of estrogenic control. It was clear that the *X. laevis in vitro* liver slice culture bioassay had the potential to be used routinely for estrogenicity screening in South Africa and therefore would require large amounts of antibody. Monoclonal antibody production is better suited for use in ELISAs routinely.

Methodology

In order to accomplish this goal, monoclonal antibodies were raised against estrogen-specific proteins. Instead of using isolated proteins as immunogens, whole plasma from estrogen-treated animals was used.

Results and Discussion

The monoclonal antibodies reliably detected those proteins that could serve as biomarkers for estrogens and estrogen-mimics in the environment. There were three monoclonal antibodies of interest. Characterisation showed that two of these were specific for two different estrogen-induced proteins, one in the 220 kDa range and another in the 160 kDa range. These proteins were detected in plasma samples, as well as in liver slice culture medium. The third monoclonal antibody was non-specific and might serve as an indicator of cytotoxicity. These antibodies were used in combination with a liver slice bioassay to indicate its application in the screening of environmental water samples.

The detail of this work can be found in a published paper (Hurter *et al.* 2004a), a PhD thesis (Hurter 2003) and conference presentations.

2.2.2.3 Development and validation of a universal vitellogenin (UniVtg) bioassay.

One of the major criticisms for the use of Vtg as biomarker for estrogenicity is the fact that the Vtg phospho-lipoprotein complex is species specific and therefore requires the development of specific polyclonal or monoclonal anti-Vtg antibodies. Therefore, anti-Vtg antibodies must be raised in rabbits or mice for every species to be used as bio-indicator. Apart from the ethical issues (concerns), it remains a financially expensive operation. In this study

the prospects of using an universal vertebrate Vtg ELISA was researched. The production of specific anti-Vtg, antibodies for other potential bio-indicator species, including the fresh water turtle, *P. subrufa* and the crocodile, *C. niloticus* was therefore delayed pending the outcome of setting up and validating such an ELISA.

Methodology

A monoclonal antibody (UniVtg) was obtained and used in the set-up and validation of an universal vertebrate Vtg ELISA. Plasma obtained from estrogen treated males or vitellogenic females of various egglaying vertebrates were used to validate the UniVtg ELISA.

Results and Discussion

The UniVtg ELISA detected vitellogenin (Vtg) in plasma of several egglaying vertebrate species, including, *O. mosambicus* (tilapia fresh water fish), *C. gariepensis* (African sharptooth catfish), *X. laevis* (African clawed frog), *C. niloticus* (Nile crocodile), *P. subrufa* (Fresh water turtle) and *G. domesticus* (chicken). Western blotting showed (confirmed) that the antibody detects a single protein band, while no protein bands were detected in the plasma of untreated (estrogen negative) males. It was therefore shown that the antibody recognised Vtg from all the vertebrate species investigated. Standard curves for Vtg using this antibody shows that the antibody detects a highly conserved area on the Vtg molecule. The standard curves obtained for the different species were very similar. The UniVtg ELISA was compared to the species specific ELISA available for *X. laevis*. This comparison showed a strong positive correlation between these two assays (R=0.925). Although the UniVtg ELISA was not as sensitive or specific as the *X. laevis* Vtg ELISA using an monoclonal anti-Vtg antibody, it has the potential to be used for screening for plasma Vtg as an estrogen response system in environmental screening programmes.

Moreover, the UniVtg ELISA will also allow researchers studying natural reproductive cycles and plasma Vtg in males of lesser known vertebrate species, including endemic aquatic species, to make plasma Vtg

determinations, otherwise only possible if species specific anti-Vtg anti bodies are commercially available or raised at great costs.

The details of this work have been published (Pool and van Wyk 2004) and the UniVtg ELISA has been used to study seasonal changes in Vtg levels in the freshwater turtle, *Pelomedusa subrufa* (Strydom *et al.* 2004a). The development of this universal assay was also presented at an International conference.

2.2.2.4 Hormonal control of male *Xenopus laevis* breeding glands in and the use of these skin glands as biomarker for androgenicity.

As a follow-on of the first paper (van Wyk *et al.* 2003) the objective of this project was to extend the research on the breeding glands of male *X. laevis* to characterize and validate this androgenic endpoint as a potential biomarker for anti-androgenic EDCs. In previous studies, including the preliminary study (Van Wyk *et al.* 2003), histological parameters were used to assess the activity of the breeding glands. With this project we hoped to gain more detailed knowledge about the hormonal control, chemical nature of the secretion, role of the glands in mating and the impacts of different EDCs known for their anti-androgenic activity. The key role player in this study, following the completion of a BSc-honors research project (Burger 2000), had intentions of using this study for a MSc degree. Unfortunately the researcher/student left for the UK and failed to return to complete the project. Attempts to recruit a suitable candidate to carry on with the project were not successful. The study, however, was launched successfully and initial data obtained.

Methodology

Detailed histological and electron microscopy comparisons between active and inhibited breeding glands were initiated. An experiment to study the role of pitiutary gonadotrophic hormones (using human chorionic gonadotropin, HCG, as mimic) in the control of gland activity was conducted. At the same time effective blocking of androgenic receptor sites and the effects of such

inhibition on plasma testosterone profiles were investigated (Burger 2000, and unpublished data). In another preliminary study, flutamide treated male *X. laevis* frogs as well as untreated control male frogs were presented to receptive female frogs. Mating behaviour and successful offspring production were noted.

Results and Discussion

The initial routine histological and electron microscopy studies showed that details of the breeding glands of *X. laevis* needs further study.

Epithelial measurements using normal histology sectioning and staining seem adequate to establish anti-androgenic effects of EDCs. It was established that glandular activity is under control of androgens and was not stimulated directly by HCG. The well-known anti-androgen pharmaceutical, flutamide, inhibited both HCG and androgen stimulation. One out of ten flutamide treated adult *X. laevis* males was successful with mating after HCG treatment, while all control, untreated males, mated and females produced fertile eggs. Therefore, preliminary data suggested that anti-androgen-like EDCs may affect successful mating in these frogs.

Because of several technical problems, the isolation of any unique (different from adjacent skin or skin from female frogs) secretion proteins or glycoproteins was unsuccessful and needs more research effort. A recent USA-EPA and OECD workshop on amphibians as bio-indicators in EDC research and monitoring programmes underlined the importance of research on male *X. laevis* breeding glands, particularly exploring the potential of these glands as androgenic biomarkers in EDC studies. The initial efforts and progress made in research on this biomarker made a valuable contribution towards this goal.

Details of the research to date were published (van Wyk *et al.* 2003) and reported in a BSc-honours research project report (Burger 2000). Recommendations for future research were recently published in a recent USA-EPA report (Ankley *et al.* 2003).

2.2.2.5 Preliminary investigations into sex determination and metamorphosis in *Xenopus laevis* tadpoles as biomarkers for estrogenicity and thyroid function.

The aim of this study was, firstly, to expose developing tadpoles (sexually undifferentiated) to a whole range of environmental chemicals, including female hormone, estrogen, to establish whether EDC effects on sex determination and differentiation could be assessed with such a bioassay. Secondly, to explore the practical use of "The *Xenopus* Metamorphosis Assay (XEMA)" suggested as a Tier 1 screen for thyroid effects by USEPA and OECD.

The rationale behind this bioassay being that chemically-induced modulation of the thyroid will translate into alterations in the process of metamorphosis.

Methodology

Xenopus laevis tadpoles were produced by mating adult males and females after hormonal treatment (HCG). Tadpoles were exposed to chemicals from hatching until metamorphosis and subsequently investigated to establish sexual development and differentiation. Exposure chemicals included, estrogen, testosterone, flutamide (anti-androgen), DDT, DDE and vinclozolin (fungicide with suspected anti-androgenic properties).

For the "*Xenopus* Metamorphosis Assay (XEMA)" study a semi-static exposure approach was used. The protocol used was described by Kloas, Opitz & Lutz (2002) and as recommended by the OECD and USA-EPA for screening purposes. As part of a preliminary study to assess the effects that sodium fluoride in drinking water may have on the thyroid function developing tadpoles (developmental stage 51) were exposed to thyroid hormone, thyroxine (T4)(Positive control), to propyl-2-thiouracil (PTU)(Inhibitor) and to buffered distilled water (Negative control). Using developmental stage of the tadpoles and thyroid histology after an exposure period of 21 days results were compared with that of other international laboratories. As a follow-up study, *X. laevis* tadpoles were exposed, using a semi-static exposure system,

to environmental water samples (MSc: Fourie 2004) collected from the Kuils River catchment within the borders of the City of Cape Town.

Results and Discussion

The results of the sex determination and differentiation study showed that the tadpoles raised in the Control groups showed a strong bias towards the female sexual development. This bias in the control groups did not allow for comparison with other chemical exposure groups and after several exposure/experimental attempts it was concluded that maternal estrogen transfer or contaminant transfer interfered with the exposure regime. It is clear that the understanding of several physiological aspects regarding sexual determination and differentiation needs more research.

The details of this project are available in the form of an unpublished BSc-honours research project (Fraser 2002).

The "*Xenopus* Metamorphosis Assay (XEMA)" study showed that thyroid hormone, thyroxine (T4) enhanced the normal progression of metamorphosis containing more advanced-staged tadpoles in this group. Similarly, propyl-2-thiouracil (PTU) (Inhibitor) significantly slowed normal metamorphic development compared to the negative control. Although, the semi-static exposure is relatively cheap and easy to set-up it turned out to be very labour intensive and future studies will have to consider a diluter and flow-through exposure system similar to one used by the USA-EPA and Japan Environmental Agency. The study, however, showed that capacity is available in South Africa to conduct such exposures. The XEMA study using environmental water samples collected from the Kuils River (Cape Metropolitan Council) was successful (MSc: Fourie 2004). Initial results showed significant stimulatory (hyperthyroidism activity) at certain sites in a river receiving a whole range of effluents as the result of anthropogenic activity along its banks. In practical terms the use of environmental water samples for a semi-static XEMA exposure were even more laborious than when exposing tadpoles to chemicals, since large volumes of water had to be collected and transported to the laboratory.

The details of these studies are available in an unpublished student project report (Boyes *et al.* 2003; Zoology undergraduate project) and a MSc thesis (Fourie 2004).

2.2.3 SCREENING SELECTED WATER SOURCES FOR EDC ACTIVITY

2.2.3.1 Screening water samples using the *Xenopus laevis* liver slice bioassay with Vtg ELIZA

This study served as implementation and practical evaluation of the *in vitro Xenopus* liver slice bioassay for environmental estrogens, specifically to be employed in a monitoring program as part of the WRC 1402 project and the need of industry and water suppliers to evaluate the current situation regarding EDCs in South African water resources.

Methodology

Water samples collected at selected sites were extracted using a solid phase (C18) extraction procedure. The *X. laevis* liver culture assay for detection of estrogen-induced synthesised vitellogenin was used according to the previously established protocol. Samples from drinking water, agricultural water, sewage effluents and sewage treatment works reclaimed water (City of Windhoek) resources were tested. The results of two *X. laevis* Vtg ELISA systems (using polyclonal and monoclonal anti-Vtg antibodies respectively) were compared.

Results and Discussion

Of a total of 48 samples, 12 sites tested positive, indicating estrogenic activity. These isolated samples testing positive came from a range of sites in the Western Cape and City of Windhoek distribution system and was not limited to one specific water type. Initially the *X. laevis* Vtg ELISA using the polyclonal Vtg antibodies were used but the analyses was repeated using the newly developed Vtg ELISA using the monoclonal anti-Vtg antibodies (selected to recognize estrogen inducible proteins). This comparison confirmed the superior sensitivity of the monoclonal antibodies. Since this initial employment of this *ex vivo Xenopus* liver culture bioassay, water

samples were screened on a routine basis for City of Windhoek water reclamation plant, SASOL Chemical Industries and water samples from suspected hotspot areas supplied by WRC project 1402. The principle and protocols of using *in vitro Xenopus* liver slices in bioassays was also introduced to a research group (University of Port Elizabeth) investigating potential bioassays to study microcystin toxicity in water samples (Coates, 2003, MSc thesis, University of Port Elizabeth).

The detail of this study can be found in a PhD thesis (Hurter 2003) and a paper submitted for publication (Hurter *et al.* 2004).

2.2.3.2 Screening water samples by *in vivo* exposure using frogs (*Xenopus laevis)* and tilapia fish (*Oreochromus mossambicus*) as bio-indicators

In vivo exposure of aquatic species to water samples is used widely in toxicology monitoring programmes. Although *in vitro* screening has the advantage of supplying an answer about a possible environmental hazard rapidly with increased sensitivity, *in vivo* exposure integrates the effect of potential hazards in a whole body scenario. The main disadvantages of such *in vivo* exposures include the loss of sensitivity and the possibility of false negatives because of the short-term nature of *in vivo* exposures. The goal of these preliminary studies was to investigate the practicalities around *in vivo* exposure of adult male *X. laevis* and to assess the potential of increased sensitivity regarding estrogenicity testing when using a tilapia species, *O. mossambicus* (Mozambique tilapia).

Methodology

Male *X. laevis* were exposed *in vivo* for six days in the laboratory to undiluted final sewage treatment works (STW) effluent, discharging into natural river systems. Following exposure, plasma Vtg determinations and other morphometric measurements were taken and compared with experimental controls. In a first study, three Western Cape towns were selected, Wellington, Paarl and Stellenbosch. In a follow-up study, frogs were injected

intra-peritoneal with STW solid phase extracts (C18) while others were housed in undiluted sewage water.

Adult male *O. mossambicus* were exposed to diluted (50%) and undiluted (100%) final STW effluents collected from the outflow to the Eerste River flowing through the town of Stellenbosch. Because of the sensitivity of the fish to STW effluents, fish were sampled after three days and six days. Positive (1ppm estrogen) and negative control groups were included.

Results and Discussion

Although frogs exposed to 1ppm Diethylstilbestrol (DES) as positive controls, responded by showing significantly increased plasma Vtg levels in male frogs, the male frogs exposed to undiluted STW effluents did not show significant increases in plasma Vtg. Breeding glands (nuptial pads) in males showed signs of deterioration, suggesting possible anti-androgenic activity (Albertus, 2001; unpublished BSc-hons. report).

Results from the injection study showed that greater sensitivity was obtained when injecting water extracts, which suggests that the skin of *X. laevis* could be an effective barrier to chemical uptake and therefore explain the lower sensitivity when compared to fish with large gill surface areas (Hough *et al.* 2002; Zoology undergraduate project), Zoology undergraduate project). Similar results were obtained when exposing frogs to the classical EDC, DDT (Bell *et al.*, 2002; Zoology undergraduate project).

When exposing male *O. mossambicus* to estrogen, significant increases in plasma Vtg levels were measured after three days of exposure. Male fish exposed to diluted and undiluted final STW effluent, however, showed no significant increase in plasma Vtg levels during exposure periods (Faul, 2000; BSc-hons research project).

2.2.3.3 Indigenous fish species as bio-indicators for estrogenic activity in three Western Cape rivers

The studies using Mozambique tilapia as fish bio-indicator for estrogenic activity in water resources showed that the superior sensitivity to estrogenic compounds, when compared to *in vivo* studies using *X. laevis* frogs, highlight the potential of these fish as bio-indicators. It was therefore decided to sample local tilapia fish from river systems in the Western Cape. This study was part of a larger study concerning the River Health Programme (RHP), Department of Water Affairs and Forestry (Roux 1999).

Methodology

Tilapia fish, *O. sparmanii* was collected alongside other local fresh water fish in three river systems (Palmiet, Lourens and Houtbay rivers) in the Western Cape. Water samples were also collected and screened for estrogenicity using the *X. laevis* liver culture assay. Plasma samples from male tilapia collected from the Lourens River were used to screen for Vtg presence.

Because specific anti-Vtg antibodies or universal Vtg antibodies were not available at the time, plasma samples of male fish were screened for Vtg using SDS gel electrophoresis. Vitellogenin presence was later confirmed by using a Western blot technique using the UniVtg and seabream antiVtg antibodies.

Results and Discussion

Estrogenicity screening of water samples taken in-stream and using the *in vitro Xenopus* Vtg liver assay did not detect any significant estrogenic activity in any of the three river systems. However, the tilapia collected from the Lourens River showed that a large proportion (60%) of the collected male fish showed the presence of Vtg in their plasma (Hayes, *et al.* 2004). These results show the different outcomes that may be obtained when conducting point estrogenicity screening compared to sampling of local animal species (long-term exposure).

Although this was a preliminary study, it was clear that until more specific tilapia anti-Vtg antibodies, including the UniVtg antibodies, are available, fish Vtg response studies will be limited to in the laboratory exposures using species like the OECD recommended species, *Danio rerio* (zebrafish), for which specific anti-Vtg antibodies are commercially avaliable (WRC project 1402C). Recent completed validation done for the newly developed UniVtg antibodies showed adequate sensitivity and specificity for tilapia Vtg (see Pool *et al.* 2004). This Vtg ELISA will allow for plasma Vtg screening in future studies concerned with species for which no specific Vtg ELISA are available.

3. CONCLUSIONS

It is noted that:

- Two sensitive, specific and reproducible Vtg ELISAs were successfully developed and validated for the aquatic frog, *Xenopus laevis* and the fresh water fish, *Oreochromis mossambicus* respectively. These ELISAs are the first of their kind to be developed locally. The fact that male egg-laying animals can produce Vtg makes this lipoprotein complex a good biomarker for estrogenic activity in the aquatic environment. Screening for estrogenic stimulation in experimentally exposed animals (*in vivo*) as well as natural occurring (wild) animals is an important tool/bioassay in any EDC related study. Vitellogenin ELISAs also allow biologists to study the seasonal dynamics of Vtg synthesis during the reproductive cycle in the species concerned.

- The development of an *in vitro Xenopus laevis* liver slice culture system to screen for estrogenic activity in water samples was successful and represent the first locally developed bioassay for the screening of estrogenicity employing intact tissue samples rather than single cell cultures. On a Tier 1 level of assessment, an *in vitro* bioassay is an important tool since fewer whole animal exposures are done and only positive samples could be selected for *in vivo* testing, Tier 2 level. Moreover, because of the removal of the barriers between internal and external environments, sensitivity increases and high through-put screening becomes possible.

- Advances were made in the preliminary development of *X. laevis* male breeding (nuptial) glands as biomarkers for screening for anti-androgenic activity in water samples. This allows for the possibility to in future employ a single bio-indicator species to screen for both estrogenicity and androgenicity.

- Employing the newly developed *Xenopus* Vtg ELISA to monitor Vtg production in male frogs exposed to natural water resources at different

sites in the three agriculture area in the Western Cape revealed that estrogenic activity may occur from time to time. Estrogenic activity was more frequently observed in caged frogs in the Caledon/Napier area (wheat farming) than the Grabouw (fruit farming) or Hex River (grape farming) areas. Although no clear pattern of occurrence was found, increased estrogenicity correlated with periods of heavy spraying. However, more short-term screening of water and sediment is necessary to substantiate possible patterns in the occurrence of estrogenic activity in water resources. This study showed that although caging of animals in aquatic environments has its own problems, this kind of *in situ* exposure allows for real assessment of EDC activity in the particular water resource at the time of exposure.

- Tilapia fish and *X. laevis* frogs exposed to diluted and undiluted sewage effluents from three STWs, Stellenbosch, Paarl and Wellington, did not exhibit estrogenic activity. Using the *X. laevis* Vtg liver culture bioassay, however, it was found that the effluents from the Stellenbosch STW were positive on one occasion. Because of general toxicity in STW effluents, routine C18 solid phase extraction procedure was employed when screen in such samples.

- Seasonal base-line studies indicated that *X. laevis* frogs are capable to breed throughout the year but in nature, heightened reproductive activity, spermiogenesis and vitellogenesis occurred in the spring-summer period.

- Seasonal reproductive studies on fresh water turtles revealed that the African helmeted turtle (*Pelomedusa subrufa*) exhibit a typical post-nuptial cycle and these male and female cycles are poorly synchronized. Fresh water turtles, with its large body size and wide spread distribution, showed great potential to be employed in the assessment of estroginicity in aquatic systems. Moreover, this species has all the potential for future studies regarding EDC effects on early sex determination and differentiation.

- On several occasions, wild caught male frogs, *X. laevis*, collected for *in vivo* exposure or seasonal studies and wild caught male turtles, *Pelomedusa subrufa* exhibited high plasma Vtg levels. This could be attributed to chemicals in the natural water resources with estrogenic activity.

  The implications of such unnatural high plasma Vtg levels in males are yet to be studied. These incidences, however, suggest that estrogenic activity in water resources and catchment areas may be real, affecting aquatic animals in the long-term. Screening studies will have to test/screen wild caught frogs/fish before they are used in caged or other experimental exposure studies. It was found that if experimental males are kept in the laboratory for at least 14 days, plasma Vtg levels will decrease to normal values, a so-called "wash-out" of Vtg background.

- Estrogen inducible proteins other than Vtg were investigated as possible supplementary endpoints to be screen for estrogenic activity in *X. laevis*. Preliminary studies showed that estrogen treatment significantly affected the lipoprotein composition in the blood and that this endpoint has potential to be researched in future.

- Using polyclonal antibodies and indirect ELISA was set-up and validate to detect apoII lipoproteins in chickens. This ELISA has the potential to be used to detect estrogenic activity by measuring apoII secretion by hepatocytes and hepatomas cultures. Due to its broad detection range, the apoII ELISA can potentially be used for monitoring female reproductive cycles, accidental and environmental exposure of males. It is already been used in medical research in Austria.

- The detection of an estrogenic response in male and female *X. laevis* is a well-known endpoint. Because of this, and the potential to use estrogen induced antibodies in routine screening of water samples, monoclonal antibodies were raised. Instead of using isolated proteins as immunogens, whole plasma from estrogen-treated animals was used. A *X. laevis* specific

and sensitive ELISA was set-up and validated. This product has commercial potential.

- In light of the increased demand for anti-Vtg antibodies for use in species specific Vtg ELISAs an universal Vtg antibody for vertebrates was characterized and validated. This antibody was subsequently used for setting up an ELISA that detects Vtg in several oviparous species *Oreochromus mosambicus* (tilapia), *Clarias gariepensis* (African sharptooth catfish), *Xenopus laevis* (frog), *Pelomedusa subrufa* (freshwater turtle) *Crocodylus niloticus* (crocodile) and *Gallus domesticus* (chicken). This newly developed universal Vtg antibody and Vtg ELISA will be particularly useful if species specific Vtg antibodies are not available in South Africa. It will therefore allow for screening of estrogenic activity in endemic aquatic vertebrates collected from local water resources or in aquatic animals used as bio-indicators in experimental studies.

- The breeding gland activity in male, *X. laevis* was found to be under androgenic control and a pharmaceutic anti-androgen, flutamide, inhibited gland activity, also in combination with a gonadotropic hormone (Human chorionic gonadotropin, HCG). The sensitivity of male breeding glands to anti-androgenic compounds, including the fungicide, vinclozolin and pesticide, DDT breakdown product, DDE, were confirmed. Although breeding gland specific proteins could not been isolated, anti-androgenic activity significantly inhibited mating behaviour in breeding experiments. The potential of male breeding glands as biomarker for anti-androgenic activity in water has been confirmed but need more research to characterize endpoints to be used in future bioassays.

- Sex determination and differentiation as biomarkers for reproductive disruption early in the development of *X. laevis* were investigated. The female bias in sex determination, even in control tadpoles, suggests either exposure contamination through food and water or maternal transfer of estrogenic compounds (natural or synthetic). Although these endpoints have potential as biomarkers, clearly more research is needed regarding the

endocrine control of sex determination and differentiation in developing *X. laevis* tadpoles.

- The *X. laevis* metamorphosis assay (XEMA) has recently been proposed as a screen for EDC effects on the thyroid system. In a preliminary exposure it has been demonstrated that the XEMA protocol can be applied locally and is ready to be used in studies investigating EDC effects on the thyroid endocrine system.
- This research programme contributed *in vitro* and *in vivo* bioassays for use in screening of environmental water samples, including drinking water. *X. laevis* males kept in cages or exposed in laboratory tanks were used. Although robust and hardy experimental animals, the sensitivity to environmental estrogens was not in the low concentration range reported for fish species. The *in vitro X. laevis* liver culture bioassay, however, was found to be sensitive, easy to use and could detect low estrogenic activity. With *in vitro* screening of water samples, we identified the need to use bioassays to demonstrate cytotoxic activity in order to rule out the possibility of false negatives.

- Estrogenicity was detected in different types of water, including drinking water, sewage effluents, industrial water and rivers receiving agriculture run-off. The *in vitro Xenopus* liver culture Vtg bioassay was successfully used to monitor different points in the City of Windhoek reclamation plant on a seasonal basis. Although source water, bore-hole and reclaimed water, occasionally tested positive for estrogenicity, treatment through activated charcoal filtering successfully removed all estrogenicity from the final water.

- Estrogenicity was also detected in several rivers in the Western Cape as well as water bodies receiving industrial effluents. However, no pattern could be ascertained and more frequent screening is necessary to understand the occurrence of estrogenicity in these water sources. It is also important that water resources screening positively for estrogen activity are tested more thoroughly using *in vivo* exposures before reaching any conclusion regarding EDC contamination.

4. RECOMMENDATIONS

4.1 Bioassay development: International transfer of knowledge

International environmental agencies, including the USA-EPA, OECD and Japan Environmental Agency, has embarked on a EDC screening and testing programme that employ a battery of bioassays on tier 1 and tier 2 levels. Endocrine modulations regarding the androgenic, estrogenic and thyroid systems are the focus areas in these programmes. Future research in South Africa, should in a well organized manner, investigate the use of these recommended bioassays, specifically the capacity for running the screens, the practicalities in terms of costs, infrastructure and cost of effectiveness. Specific validation or ring testing is necessary to establish these tests locally.

4.2 Bioassay development: New cost-effective biotechnology.

4.2.1 Other endocrine systems.

Although more focus has been on estrogenicity, more attention must be given to other important endocrine systems or related systems. Bioassay research to understand the dynamics of endocrine modulator activity is needed for the male androgenic system, the thyroid system and the neuro-endocrine system. Associated immune modulatory research is also needed.

4.2.2 Bio-indicator species

It is well recognized that a diversity of bio-indicator species are needed to understand the possible variations in mode of action and therefore disrupting effects of EDCs. Aquatic species, including fish, amphibians and turtles are well suited to be used as bio-indicators but the possible inclusion of other vertebrates, including crocodiles, birds and mammals will strengthen the approach of screening and testing for EDC activity and to understand the potential exposure routes. Although most of the focus has been on vertebrate species, mainly because of similarities to the human endocrine system, the potential of including invertebrate bio-indicators must be explored. Invertebrates may be effectively used in high-throughput tier 1

screening programmes. However, very limited knowledge regarding the endocrine system of invertebrates exists, especially local species.

Endemic bio-indicator species included in locally developed bioassays will give the added benefit in that natural population and aquatic ecosystems monitoring will be possible. Long-term catchment quality and receiving waters health could then be monitored. Ecosystem and wildlife health are important indicators for human risk especially considering that persistent organic compounds have been used in the past and are still being used, for example, DDT to combat malaria. To use local species, more base-line research data are needed on the relevant endocrine systems of potential bio-indicator species.

4.2.3 Biomarkers

Basic biomarker research, to discover, develop and validate new biomarker systems, especially linked to lesser-known biochemical pathways must continue. Apart from using whole body, organ, tissue or one cell response systems, sub-cellular genomic systems needs more attention. Importantly, more emphasis is also needed to understand and investigate the disruption of the endocrine system through disruption of enzymes involved in steroid synthesis and metabolism.

Vitellogenin production, under control of estrogens, as biomarker was the main focus in this study is indeed being used internationally as a biomarker for estrogenic activity in aquatic systems. Since biomarkers must also be used to determine whether the health of the organism or human being has been compromised, additional biomarkers that relate directly to these aspects need to be developed.

Gene regulation holds great potential for future screening programmes. To understand the molecular basis of estrogenic actions and effects of hormonally active chemicals on developing organisms, the links between exposure levels to EDCs, gene responses and adverse effects need to be understood. For this approach, estrogen-responsive genes and critical developmental windows of various animal species need to be identified

Tier 2 testing, i.e. partial life-cycle and full life-cycle testing has hardly started in South Africa and needs urgent attention. The goal of Tier 2 testing is to determine the consequences to the organism of the endocrine modulator activities identified during Tier 1 and to understand the dose-response relationships. This must be done in a larger context of testing focussing on reproductive and developmental toxicity potential. These tests are longer-term studies, two generations including effects on fertility, mating, embryonic development, sexual determination and differentiation. It could also be designed to encompass critical life stages in partial life-cycle testing. Examples would include, fish life cycle and amphibian (*Xenopus laevis*) life cycle or partial life cycle testing.

4.3 Effective removal of EDC activity.

Imported bioassays and local developed bioassays must be employed to study the effective removal of EDC activity during water treatment. Analytical chemistry and specific immunological assays must be developed to understand the dynamics of EDCs in complex mixtures of wastewater. Cost effective local developed bioassays are needed for routine monitoring of treated water. It must be recognised that large proportion of the African population do not receive drinking water from state of the art treatment plants and research is needed to investigate cost effective ways to remove EDC activity from water and to conduct cost effective monitoring programmes.

4.4 Assessment of environmental health, including catchment health.

Long-term monitoring of EDC activity in important catchment areas must be initiated. The subtle long-term implications, far removed from the well-known dose-response approach needs more study. Specific controls and contaminated areas also need to be studied on a longer term. Wildlife model systems, especially long-lived aquatic species, could play an important role. The significance of EDC activity in exposed wildlife populations urgently needs some study, since these studies could predict what will happen in affected human populations, especially those still using untreated water from natural resources.

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Insecticide contamination of the water environment in malaria endemic areas of KwaZulu-Natal (SA)

BL Sereda; HR Meinhardt

The agricultural use of pyrethroids and other insecticides registered for crop protection increased in the emergent farmer sectors in KwaZulu-Natal. These areas are protected by the malaria control programme in which DDT and delmethrin are used for indoor spraying of dwellings and constitute a worst–case scenario potential contamination of the water environment. Cases of pyrethroid and organophosphate resistance in the malaria vector Anopheles species were detected. Insecticide resistance in malaria vectors can originate from the selection of mosquitoes to agricultural pesticides.

The research objectives were to establish the patterns of agricultural pesticide used by emergent farmers in two areas, namely Ubombo and Ingwavuma to develop a protocol for sampling, analysis of insecticides and interpretation whereby areas at risk can be investigated for resistance potential within a short period. The residues of the insecticides in water sources near the communities were determined and selected for resistance in malaria vector larvae.

The study area was selected as being the most polluted with agricultural insecticides and anti-malaria chemicals. A questionnaire was used to gather information on a list of pesticides used in the area so as to select the pesticides for residue analysis. Water and sediment samples were collected and analysed for the residues.

Results of the analyses show insecticide contamination of the water environment in the investigation area from pyrethroid, organophosphate, organochloride and carbamate chemical groups. Game reserves selected for control areas supposedly not contaminated were found to be contaminated.

It is believed that the major selection pressure for the development of mosquito resistance exists in the study area and it is crucial to ascertain the relative contribution of the different insecticide classes to the development of resistance. The identification of pyrethroid and organophoshate resistance reported in the study area is of great concern and poses severe consequences in designing an efficient mosquito vector programme. The detection of DDT in the samples shows contamination of the water sources resulting from anti-malaria control interventions and is a serious health threat not only in South Africa, but also internationally, taking into account that DDT is on the Stockholm Convention (2001) list of the 12 POP pollutants that circulate globally through air and water.

Report Number: 1119/1/03 **ISBN No: 1 86845 928 4**

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