Pesticides as Endocrine Disruptors in South Africa: Laboratory and Field Studies

Report to the
WATER RESEARCH COMMISSION

by

JH van Wyk[1], E Archer[1], OO Babalola[1], JC Truter[1], E Jansen van Rensburg[1] & J Dabrowski[2]

[1]Stellenbosch University
[2]CSIR

WRC Report No. 1932/1/14
ISBN 978-1-4312-0532-5

April 2014

Obtainable from

Water Research Commission
Private Bag X03
Gezina, 0031

orders@wrc.org.za or download from www.wrc.org.za

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EXECUTIVE SUMMARY

Introduction

Globally, evidence have been mounting to support the concern that there are many environmental chemicals (pollutants) interacting (directly or indirectly) with the endocrine systems of organisms, including humans, resulting in modulation/disruption of hormonal control (Gore, 2007; Frye et al., 2012; Zoeller et al., 2012). These so-called, Endocrine Disrupting Contaminants (EDCs), per definition, include exogenous chemicals (or mixtures of chemicals) that interfere with the normal hormone action in some way, resulting in physiological disruption of embryonic development and adult life through changes in the neuroendocrine, reproductive, thyroid and immune systems (Bergman et al., 2013; Heindel et al., 2013). Therefore, subtle modulation of the endocrine system by xenobiotics can potentially lead to a diverse range of health effects, for example, infertility, reduced fertility, sex determination and developmental disorders, malformations, obesity, neurological disorders and several other diseases.

It is not surprising to find that aquatic organisms are regarded the most affected, since more than 100 000 chemicals are thought to be discharged directly into freshwater and marine environments (Trudeau and Tyler, 2007). Globally, the concern is that EDCs may be linked to declining fish and amphibian populations (Carey and Bryant, 1995; Davidson et al., 2001; Withgott, 2002; Hayes et al., 2006; Kidd et al., 2007).

Since the initial reports and the resulting information explosion, environmental agencies (USEPA and EU) and other organizations (OECD, GWRC, WHO, IPCS) were mandated to review the situation, develop and validate tests to screen chemicals for endocrine disruption activity. In South Africa, the Water Research Commission (WRC) initiated an Endocrine disruption focus area within cross-thrust, linking Water and Health programmes. Although the initial focus was on environmental contaminants potentially affecting mimicking female hormonal action (oestrogenic activity) (Van Wyk et al., 2005; De Jager et al., 2010), research was eventually extended to anti-oestrogenic activity as well as effects on the androgenic and thyroid systems (Van Wyk, 2013).

Pesticides as Pollutants and EDCs

One of the key concerns, recently listed by the World Health Organization (WHO) in a comprehensive report on EDCs, was the relative small number of studies available on the increasing number of chemicals suspected (800-1000) to interfere with hormone systems (Bergman et al., 2013; TEDX, 2014). Pesticides have been used widely for non-agricultural purposes, for example, herbicides are used to eradicate

alien plant species outside their countries of origin (Binns et al., 2001; Common-Ground, 2003; Hosking and Du Preez, 2004).

Today pesticides are present in most compartments of the environment and detected in ground water as well as surface water, used as source for drinking water (Plakas and Karabelas, 2012). The WHO report (Bergman et al., 2013) refers to epidemiological evidence linking pesticides to health concerns for humans, including malformations, neuro-developmental disorders, effects on the developing immune system, male and female reproduction, prostate cancer, thyroid cancer as well as breast cancer.

South Africa is the highest agriculture produce-producing country on the African continent (Bollmohr et al., 2008; Dalvie et al., 2009a; Ansara-Ross et al., 2012; Mensah et al., 2013; Dabrowski et al., 2014). With the high demand in food productivity due to increased population and increased food quality, pesticide use in South Africa represents 60% of the pesticide market in Africa (Dabrowski et al., 2014). Approximately 180 different pesticide active ingredients are commercially available in South Africa, with more than 500 registered trade names in use (Pan, 2014).

McKinlay et al., (2008) listed a total of 127 pesticides as having an endocrine disruptive mode of action, but not giving any specifics regarding mechanism of action. Mnif et al., (2011) listed 105 pesticides of which 46% were insecticides, 21% herbicides, and 31% fungicides. Burger and Nel (2008) listed 15 priority pesticides to consider as EDCs in South Africa. In the report of Dabrowski et al., (2014), they categorized only 22% of 152 pesticide active ingredients listed, as possible EDCs, but mentioned a few (11) that they felt confident as being potential EDCs in South Africa. However, most of these studies rely on database data (Lyons, 2010; Footprint, 2014), not differentiating between modes of action or pesticide type. In spite of the limited information in all classes, reports can be found relating to interaction with the reproductive and thyroid endocrine systems (McKinlay et al., 2008; Mnif et al., 2011).

Single Chemical vs. Mixtures

In the agricultural sector, single or different agricultural crops located near each other are regularly treated with different pesticide types (insecticides, nematocides, herbicides, fungicides) (Bollmohr and Schulz, 2009; Dabrowski et al., 2011; 2014), and potentially treated with chemicals having similar mechanisms of endocrine disrupting effects (therefore additive) modes of action or dissimilar modes of action (therefore independent or antagonistic). Backhaus and Faust, (2012) pointed out that different chemicals in environmentally relevant aquatic mixtures may not have similar effects on endocrine systems and may therefore interact differently than expected laboratory mixture studies. Although it is important to confirm the suggested

mechanism of action of single compounds (contaminants), it is also important to predict (based on their individual effects) and validate the behaviour of chemicals in mixtures. It is mostly assumed that a dose-addition response will result, so-called additivity null hypothesis (Boone, 2008; Christiansen et al., 2009; 2012; Ermler et al., 2011). However, this may not always be the case and predictability (risk modelling) based on the additivity hypothesis have been questioned.

EDC studies in South Africa and Vertebrate models

Few studies in South Africa directly link, individual or combinations of pesticides, to specific endocrine endpoints or mechanisms of action (Van Wyk et al., 2003; Van Wyk et al., 2005; Van Wyk, 2013). Specific endocrine (hormonal) activity have been shown to be present in selected water sources, either associated with treated waste water sources or intense farming or industrial areas (Swart and Pool, 2007, 2009a, b). To date, a handful of local studies showed activity in all three endocrine disruption modes of action groups, oestrogenicity (Hurter et al., 2001; Van Wyk et al., 2005; Esterhuyse et al., 2009; Masgoret et al., 2009), androgenicity (Van Wyk et al., 2003; Aneck-Hahn et al., 2007; McClusky et al., 2007; Marchand et al., 2009; Mlambo et al., 2009) and thyroid modulation (Van Wyk, 2013).

In South Africa we are fortunate to have two well-studied sentinel aquatic species, the African clawed frog, *Xenopus laevis* (also a recognized international model)(Kloas and Lutz, 2006; Lutz et al., 2008; Grim et al., 2009; Hayes et al., 2010; Opitz and Kloas, 2010; Tietge et al., 2010; Van Wyk, 2013) and a local freshwater fish, *Oreochromis mossambicus* (Mozambique tilapia) (Nussey et al., 1995; Van Dyk et al., 2007; Esterhuyse et al., 2008; Esterhuyse et al., 2009; Mlambo et al., 2009; Esterhuyse et al., 2010; Truter et al., 2014).

In summary, South Africa is the African country with the highest use of pesticides and although no information is available regarding possible species losses as reported internationally for most of the aquatic vertebrate groups, concern is that the health of wildlife and humans may be compromised because of exposure to pesticides in the freshwater aquatic environment. There is a need for more directed laboratory studies as well as field studies, combining *in vitro* screening, *in vivo* exposures and wild caught animals (long term exposure). Moreover, different mechanisms of action need to be included in screening tests.

Rationale

The approach in this study was to use two model species, the amphibian, *Xenopus laevis* and the fresh water fish, *Oreochromis mossambicus*, to address some of the gaps in our knowledge of locally used pesticides as endocrine modulators. In this study, the focus was on selected chemicals from the insecticide (oestrogenic activity), fungicide (androgenic activity) and herbicide (developmental, thyroid,

disruption) sub-groupings. Following laboratory exposure studies, selected field studies (Stellenbosch area, Upper Olifants River and Loskop and Flag Boshielo dams (reservoirs) in the Olifants River system were conducted to link possible endocrine disruption manifestation to laboratory data on pesticide exposures.

Objectives and Aims

The specific aims for this study included:

Aim 1
To identify relevant pesticide mixtures from two or three selected agricultural areas (winter and summer rainfall areas) to be used in laboratory exposure studies as well as field studies.

Aim 2
To select appropriate endpoints and sentinel aquatic species to evaluate ED activity of the selected mixtures.

Aim 3
To confirm the effects of laboratory studies in the selected aquatic environments.

Aim 4
To summarize the status of knowledge regarding the effects of pesticide mixtures in aquatic ecosystems, relate this knowledge to practical predictive models and its future application in environmental management and Ecological Risk Assessment programmes.

Methods and Results

Laboratory studies

Insecticides as EDCs

In this study we used two well-known *in vitro* assays to screen selected pesticides (mostly insecticides and fungicides chosen from previous studies conducted in the Louwrens and Hex river catchments (Dalvie et al., 2003) as well as the lower Olifants River catchment (Oberholster et al., 2012) for effects on oestrogen binding to the human oestrogen receptor (hER). We screened four individual pesticides (Azinphos-methyl, Endosulfan, Carbaryl, Chlorpyrifos) as well as two-way mixture combinations to test the mode-of-action (dose) addition hypothesis.

We confirmed oestrogenic activity in two of the four pesticides, Carbaryl and Endosulfan. Azinphos-methyl showed hardly any interaction with the oestrogen receptor and although Chlorpyrifos did show some interaction it seems to be rather weak. Cocco (2002) indicated that Carbaryl has been shown to be weakly oestrogenic in *in vitro* studies and that it has been shown to negatively impact fertility

in animal studies. Andersen et al. (2002) showed that Endosulfan increased cell proliferation and ER transactivation in MCF7 cells.

There were some unexpected interactions between insecticides when present together in a mixture. Most of the mixtures seem to have an additive effect. However, from the current data it appears that only Carbaryl and Azinphos-methyl have an interaction that is more than just additive and Carbaryl and Chlorpyrifos in mixture showed an antagonistic interaction. This study showed that although complex mixture exist in the aquatic environment, binary mixtures of pesticides could be helpful to test the additive hypothesis and therefore be useful in risk assessment modelling.

Herbicides as animal toxicants and EDCs

In order to extend our knowledge regarding the potential endocrine modulation effects of locally used herbicides on aquatic amphibians (as aquatic models), we focussed on selected chemicals (six herbicide formulations) used in the Working-for-Water (WfW) programme to control aquatic alien plant species. Initial studies to determine developmental stage dependent (three stages, embryo, larval and post-metamorphic) lethal concentrations (LC_{50}) and potential induction of malformations in developing African clawed frog (*Xenopus laevis*) tadpoles were conducted. Two important results emerged from these studies: 1) that in the case of certain herbicides (Roundup, Midstream and Basta) larval (NF stage 48) tadpoles showed lower LC_{50} concentrations than the expected environmental concentrations (EEC) if applied correctly as well as lower LC_{50} as recorded for fish (traditional aquatic representative) and 2) that differential stage dependent sensitivity exist for developing tadpoles (larval stage proved to be the most sensitive), when compared with early developing embryos (96 hours old; FETAX assay) or metamorphosing tadpoles.

The present study suggested that Roundup, Midstream and Basta formulations are embryotoxic, while Arsenal, Enviro Glyphosate and Kilo Max showed relative low toxicity to the *X. laevis* embryos. Kilo Max, Roundup, Basta, and Arsenal formulations revealed significant growth disruption. In terms of teratogenicity, Midstream formulation showed a strong teratogenic potential, while Enviro Glyphosate showed positive teratogenic potential at relative high exposure concentrations.

In light of the above results, we proceeded to evaluate the effect of herbicides on the thyroid hormonal system (involved in controlling early development and metamorphosis) using the internationally validated *Xenopus* metamorphosis assay (XEMA) to screen several herbicide formulations at different concentrations for thyroid disruption. In this study we showed that four herbicide formulations (Arsenal, Midstream, Basta and Roundup) affected tadpole growth and that herbicides

formulations like Midstream, Basta, Arsenal, Roundup and Kilo Max differentially inhibited the natural progression of developmental stages in *X. laevis* tadpoles, and may therefore be considered thyroid disruptive. However, more research is needed to confirm these effects at environmental relevant concentrations.

In addition, the present study showed varied results in terms of effects on the sex ratio; Midstream resulted in a female bias at concentrations below expected environmental concentrations, Kilo Max had a similar effect but at higher concentrations only, and Basta, Arsenal, Enviro Glyphosate and Roundup formulations showed varied effects on sex ratios. Diverse morphological abnormalities in the gonads were recorded for all six herbicide formulations, Midstream resulting in relatively high abnormality index (32.5-60%). Although the effects of these responses on adult reproduction are largely unknown, these may be early warning signs/predictions of compromised reproductive success.

Finally we exposed (28 days, semi-static) adult male African clawed frogs to three different, non-lethal concentrations of two selected herbicide formulations, Midstream (Diquat dibromide) and Arsenal (Imazapyr). The Midstream formulation reduced the body mass of exposed male frogs following a 28-day exposure, compared to the control but also decreased the size of the testes and gonadosomatic index. Arsenal did not have a similar effect and no evidence could be found suggesting effects on spermatogenesis. Plasma testosterone did not vary significantly among exposed males, although a slight decrease in the Midstream exposed males may suggest anti-androgenic activity. Plasma vitellogenin (yolk precursor produced in the liver under oestrogenic control) as a biomarker for oestrogenic activity varied among exposure groups although no single exposure group clearly confirmed oestrogenic activity. Some variation was also noted in plasma thyroxine (T_4) concentration in the males exposed to the lowest Midstream concentration. In the Arsenal exposed male frogs T_4 showed some decrease. Although T_4 may be associated with reproduction, no clear effect was found. Overall, adult male African clawed frogs did not show clear adverse reproductive or thyroid effects following exposure to Midstream and Arsenal herbicide formulations (even-though the exposure concentrations were rather high, but non-lethal). This preliminary research confirmed that differential sensitivity between life-stages should be considered when doing experimental exposures in the laboratory.

<u>Fungicides as EDCs</u>

Fungicides have been known for their effects on androgenic systems, and we pursued the potential to use the African Clawed frog (*Xenopus laevis*) as model to study/assess locally-used fungicides (selected from agricultural-use lists) as (anti-) androgenic endocrine disruptors. In this study, we described and validated male breeding glands, located the skin (epidermis) on the fore-legs of mature frogs, as androgen dependent secondary sexual organs controlled either directly through

interaction with the androgen receptor (AR) or indirectly through dehydrotestosterone (DHT) transformed from testosterone to DHT by the enzyme 5α-reductase (5αR). We confirmed that the male breeding gland activity in *X. laevis* is under androgenic control. Using either androgenic inhibitors, AR antagonist (Flutamide) and 5αR antagonist (dutasteride), and known anti-androgenic fungicides, Vinclozolin and Mancozeb, all significantly inhibited the normal activity of male breeding glands along with disrupting effects on the primary male reproductive system (spermatogenesis and testosterone). We also showed that breeding glands can be inducted prematurely in metamorphosing tadpoles with developing fore-legs when exposed to exogenous androgens (mimicking xenobiotic androgenic compounds in water). A recombinant yeast androgen binding assay (YAS), containing an AR linked reporter system was used to characterize the androgenic and anti-androgenic activity of selected fungicides (Vinclozolin, Folpet, Procymidone, Dimethomorph, Flusilazole, Fenarimol and Mancozeb). This study confirmed the dose-dependent inhibition of dehydrotestosterone (DHT) binding to AR by most of the selected fungicides. When compared to Vinclozolin, Mancozeb proved to be a more potent inhibitor of AR binding. We proceeded by testing the additive hypothesis for EDCs with similar effects by combining fungicides in binary mixtures. Although, in general these mixtures proved to be potent anti-androgenic combinations, the dose addition hypothesis was not supported in certain combinations. The basis of the non-additivity of commonly-used agricultural pesticide mixtures needs further study in order to eventually apply predictive risk assessment models to complex environmental mixtures. This result was confirmed following the exposure (21 days) of adult male *X. laevis* frogs to individual and a binary mixture of two fungicides (Mancozeb and Folpet). However, in this study, anti-androgenic effects was limited to lowered plasma testosterone concentrations and breeding gland activity in males, suggesting that although primary male reproduction may not be compromised, secondary sexual characters, may have been affected subtly, therefore, potentially affecting mating success.

Field studies

One of the aims of this project was to link laboratory studies regarding biomarkers associated with endocrine disrupting activity of pesticides to field studies in which water is screened for endocrine activity (*in vitro* and *in vivo* approaches) or where aquatic vertebrates, fish and amphibians, are collected from water bodies and studied for signs of endocrine disruption.

<u>Stellenbosch region</u>

In the first field study, we selected several impoundments (dams; n = 10) located in an intensive farmed area, mostly located down-slope of surrounding vines and orchards (Stellenbosch area, Western Cape). The main focus of this study was to screen for reproductive activity, specifically (anti-) androgenic and oestrogenic activity (on a seasonal basis), but also to include biomarkers related to potential

thyroid disruption. For this study we used the African clawed frog (*X. laevis*) occurring naturally in local water bodies (collected from a subset of four sites) as model system. Using recombinant Yeast assays (YES and YAS), ELISA hormone determination kits and a minced testis assay (steroidogenesis), we showed that hormones and hormonal modulation activity were present in several of the selected impoundments. We confirmed anti-androgenic activity in five of the ten sites and androgenic activity in three of the ten sites (during the summer season). Steroidogenic inhibition (using the minced testis assay) was confirmed in a single site and oestrogenic activity in two of the sites. However, studying several biomarkers in wild-caught frogs from four selected impoundments, showed that predicted gonadal endocrine disruption activity, especially (anti-) androgenic activity, in laboratory studies did not always correlate with biomarkers (breeding gland development) displaying (anti-) androgen activity in wild-caught male frogs. Although, frogs from individual sites did show signs of endocrine disruption, the reproductive health implications are unclear. In one site we also recorded a significant down-regulation of an associated thyroid gene. These findings confirm the importance of presenting both *in vitro* as well as *in vivo* endpoints in environmental screening efforts.

Olifants River

The Olifants River is an important South African river, being a major source of agricultural irrigation water and drinking water to humans and livestock, but also regarded as one of the most polluted rivers in South Africa. In this study we linked to a larger study concerning the water quality (Oberholster et al., 2010), and selected six study sites based on their representation of different land-use areas and subsequently followed a three-way screening approach. First, we used ELISAs to determine human female hormone (17-β-estradiol, E_2) and the synthetic female hormone (17α-ethinylestradiol, EE_2) concentrations, associated with natural human hormones and contraceptives in the water, secondly, screening for (anti-) oestrogenicity (binding to ER) and (anti-) androgenicity (binding to AR) using recombinant yeast assays (Sohoni and Sumpter, 1998), and finally exposed juvenile Mozambique tilapia (*Oreochromis mossambicus)* (22 days-post-fertilization) fish to water collected at the selected sites, followed by an assessment of changes in expression of six different genes (mRNA transcription) associated with the endocrine system, using real-time RT-qPCR methods.

The upper Olifants River catchment, showed human oestrogen contamination, suggesting contamination from waste water treatment plant (WWTP) sources. The human oestrogen hormone (E_2) concentrations were high at two sites, exceeding the levels observed from WWTP effluents in the Western Cape (Swart and Pool, 2007; Manickum et al., 2010). The ER yeast screen indicated oestrogenic activity at a single location downstream of a WWTP, and anti-androgenic activity at two locations within an agricultural region. The anti-androgenic activity is likely associated with

pesticides (fungicides), reportedly acting as anti-androgens (Orton et al., 2011). In spite of the Olifants River catchment being known for the diversity of receiving-effluent types, limited effects were observed in the expression of a number of endocrine-linked genes in juvenile tilapia fish exposed to surface water collected throughout the catchment. The lack of responses in reproductive linked genes is surprising seeing that oestrogen concentrations exceeding the reproduction predicted no-effect concentration (PNEC) for fish (Caldwell et al., 2012) were measured at all six the localities sampled. Our results therefore suggest that the *O. mossambicus* juveniles (22 dpf) were insensitive to exogenous oestrogen exposure. A statistical analysis indicated an association of the waste-water treatment works with oestrogenicity and oestrogen concentrations. No grouping in the agriculture impacted or downstream sites were evident, suggesting no clear links in the responses of the genes under investigation and land-cover regions when anti-androgenicity, oestrogenicity and oestrogen loads were accounted for.

Loskop Dam

In the third study, the focus was on two impoundments in the Olifants River catchment, Loskop Dam and Flag Boshielo Dam. Mozambique tilapia (*Oreochromis mossambicus*) in Loskop Dam is characterized by obesity, lipid peroxidation and yellow fat disease (pansteatitis). Fish collected from Flag Boshielo Dam (downstream from Loskop Dam in the Olifants River catchment) generally does not show the same degree of obesity and incidence of pansteatitis. Since the thyroid hormonal system is known to be an integral part of growth, development and metabolic activity in humans and most vertebrates, including fish, in this study the focus was on the potential disruption of the thyroid as an explanation for the high incidence of obesity and pansteatitis in tilapia. We followed a two-tiered approach; the first study was based on adult fish collected from Loskop Dam and Flag Boshielo Dam to describe the thyroid status of these fish. In the second study we evaluated possible altered expression of selected genes (associated with thyroid and adrenal (stress related) systems) in adult tilapia fish collected from Loskop Dam and two other locations for comparison purposes. To confirm potential contaminant induced thyroid and adrenal modulation and induction of obesity by obesogens (exogenous contaminants driving a positive energy balance, by targeting for example *peroxisome-proliferator receptor gamma* (*pparγ*) signalling), we exposed juvenile tilapia to water collected from Loskop Dam (riverine, transitional and lacustrine zones).

The fish collected from Loskop Dam were significantly larger that fish collected from Flag Boshielo Dam and the incidence of pansteatitis was confirmed in Loskop Dam fish. Thyroid status differed between Loskop Dam and Flag Boshielo fish. The plasma concentrations of the thyroid hormone, T_3, were significantly elevated in the Loskop Dam fish, but plasma thyroxin (T_4) levels did not differ significantly. Thyroid histology showed enlarged thyroid follicles (but lower coloidal area) in fish from

Loskop Dam, with greater epithelial cell height and an abundance of visible vascuolisations (droplets) in the peripheral coloid. Indications were therefore that the fish from Loskop Dam displayed active thyroids, although not extensively to be classified as being hyperactive. In comparison, fish from Flag Boshielo, showed reduced thyroid activity with lower plasma T_3 levels. After considering additional data (plasma cholesterol, triglycerides, liver lipid content and liver cell histology) linked to nutritional status, indications are that the higher thyroid activity associated with Loskop Dam fish may be partly due to over-nutrition, rather than causing it. These data therefore does not suggest a direct link between thyroid disruption and incidence of pansteatitis in Loskop Dam tilapia.

In the additional fish sourced, thyroid hormone receptor alpha (*trα*) expression was increased in Loskop Dam fish (as compared to fish from an aquaculture facility maintained in borehole water or irrigation canal water. The expression of the enzyme, type 2 deiodinase (*dio2*) (enzyme responsible for conversion of T_4 to T_3) mRNA was increased in Loskop Dam fish. Confirming the initial results, pansteatitis was not associated with variation in the expression profiles of any of the thyroid and adrenal genes studied. Moreover, expression of the obesity related gene, *pparγ* also did not vary significantly among sites. The observed increased in gene expression (*trα* and *dio2*) may be related to the active thyroid and higher T3 levels in the plasma recorded for the Loskop Dam fish. The general lack of association of fish condition factor, and the expression of genes (expressed in the brain) linked to the thyroid gland, adrenal gland, and obesity suggest that the pansteatitis and obesity may not be a direct response to endocrine disruption of these systems. The juvenile exposure studies confirmed these results but furthermore showed that the nutritional content of the water (filtered vs. unfiltered) significantly affected the expression *dio2* (higher expression in water with higher algae content). This finding independently suggested that differential thyroid status in this case could be a response to nutritional status. These results correspond to the suggestion that the difference in thyroid activity found in fish from Flag Boshielo and Loskop Dam could be diet related (Flag Boshielo fish being more food stressed).

CONCLUSIONS

1) The aims of this project was largely reached, however the linking of endocrine disruption caused by pesticides to ecological risk assessment models proved to be a bridge too far.

2) Pesticides, including insecticides, fungicides and herbicides, could interact with reproductive as well as thyroid endocrine systems, and a single chemical may interact with more than one system at a time. Modes of action associated with Insecticides and herbicides varied and individual chemicals or formulations needs comprehensive testing to predict the potential for endocrine disruption. Fungicides

on the other hand were mostly associated with anti-androgenic activity, either by inhibiting binding of male hormone to its receptor (AR) or by inhibiting the activity of the enzyme 5α-reductase.

3) Laboratory studies proved valuable to perform first tier screening for potential endocrine disruption activity of individual pesticides and mixtures of pesticides. However, *in vivo* exposure experiments are needed to validate modes of action and understand real health implications.

4) Mixture exposure experiments, on the basis of the dose addition hypothesis, may be helpful to make accurate predictions if the mode of action on the particular endocrine system is the same. But, when conducting *in vivo* exposures this prediction did not always hold.

5) Field collection of sentinel species proved to be valuable, but remains a complex system to understand and confirm direct links to endocrine disrupting activity being the origin of compromised physiological systems.

RECOMMENDATIONS

- More South African used pesticides should be studied for endocrine disrupting activity to understand the dose response relationships, before embarking on predicting health risk.

- Clearly, pesticides as potential endocrine disruptors needs more research; specifically focused on understanding the details of interaction with the diversity of facets presented by the endocrine system.

- More research is needed regarding the behaviour of pesticides in mixture, to understand the interaction of these chemicals when attempting to predict endocrine disruption when dealing with mixtures.

- Pesticide-linked epidemiology should be investigated by including endocrine disruption research.

- The capacity to determine environmental concentrations of pesticides is needed in South Africa.

- More research regarding potentially affected wildlife populations is needed.

- Pesticides as developmental and reproductive modulators needs more study, if we intend to eventually develop ecological or health risk models to manage the use of these chemicals in South Africa.

- Linkage and cross-cutting research projects including pesticides should be facilitated.

ACKNOWLEDGEMENTS

The authors would like to thank the Reference Group of the WRC Project K5/1932 for the assistance and the constructive discussions during the duration of the project:

Mr B Madikizela	Water Research Commission (Chairperson)
Mrs APM Moolman	Water Research Commission (Past Chairperson)
Mrs AEC Burger †	Consultant
Prof JHJ van Vuren	University of Johannesburg
Prof H Bouwman	North-West University
Prof P Swart	Stellenbosch University
Prof EJ Pool	University of the Western Cape
Dr SJ Jooste	Department of Water Affairs
Dr R Pieters	North-West University
Mrs JM Hloyi	Cape Metro Scientific Services
Mr S Pieters	Cape Metro Scientific Services
Dr EJ Ncube	Rand Water
Dr B Newman	CSIR
Mr Odusanya	Department of Water Affairs
Mr E van der Walt	Agriculture Research Council

Mrs J Basson	Stellenbosch University for financial management
Me F Gordon	Stellenbosch University for technical support in the laboratory
Mr H Witbooi	Stellenbosch University for technical support in the laboratory

Stellenbosch University is acknowledged for financial support and use of their facilities and infrastructure.

Department of Water Affairs and Forestry, Working for Water programme for donating herbicide formulations for study in this project.

Dr NH Aneck-Hahn and Ms MC van Zijl, University of Pretoria for training and use of their laboratory.

Prof EJ Pool, University of the Western Cape, for training and use of his laboratory.

Dr P Oberholster, CSIR and leader of the Olifants River project, for his guidance and resources.

TABLE OF CONTENTS

EXECUTIVE SUMMARY iii
Introduction iii
Pesticides as Pollutants and EDCs iii
Single Chemical vs. Mixtures iv
EDC studies in South Africa and Vertebrate models v
Rationale v
Objectives and Aims vi
Methods and Results vi
Laboratory studies vi
Insecticides as EDCs vi
Herbicides as animal toxicants and EDCs vii
Fungicides as EDCs viii
Field studies ix
Stellenbosch region ix
Olifants River x
Loskop Dam xi
CONCLUSIONS xii
RECOMMENDATIONS xiii
ACKNOWLEDGEMENTS xvii
TABLE OF CONTENTS xix
LIST OF FIGURES xxiv
LIST OF TABLES xxxii
LIST OF ABBREVIATIONS xxxiv
1. BACKGROUND, INTRODUCTION AND OBJECTIVES 1
1.1. General: Endocrine Disruption 1
1.2. Potential mode of action (MOA) of EDCs 3
1.2.1. Oestrogenicity 3
1.2.2. Androgenicity 5
1.2.3. Thyroid modulation 6
1.3. Mixtures and Mode of Actions 9
1.4. Agrichemicals 11
1.5. Non-specific toxicity threat of agrichemicals (stage dependent toxicity and the Frog Embryonic Teratogenic Assay (FETAX)) 12
1.6. Agrichemical use in South Africa 14
1.7. Aquatic Vertebrate Models for EDC Studies in South Africa 15
1.8. EDC Research Progress and Needs in South Africa (Pesticides) 16
2. Laboratory Studies: Summary of results 19
2.1. Pesticides: Oestrogenic activity: *In vitro* screening and mixture studies 19
2.1.1. Aims 19
2.1.2. Methods 19
Pesticide selection 19
T47D-kBluc assay (oestrogenicity and anti-oestrogenicity) 19
2.1.3. Results 20
Single Chemicals 20

Mixtures 22
2.1.4. Conclusions **26**
2.1.5. Location of the full version: **26**
2.2. Herbicides as biological toxicants and endocrine disruptors **27**
2.2.1. Specific aims of this project **27**
2.2.2. Materials and Methods **27**
Herbicides 27
Xenopus laevis as model 27
Xenopus laevis care and breeding of tadpoles 27
2.2.3. Exposure procedures **28**
Acute toxicity testing 28
FETAX Testing 28
The *Xenopus* Metamorphosis Assay (XEMA) 28
Juveniles (post-metamorphic) 29
Adult male exposures 30
2.2.4. Statistical analyses **30**
2.2.5. Results and Discussion **31**
Acute toxicity of *X. laevis* tadpoles 31
Non-lethal and teratogenic impacts 33
Embryotoxicity/Mortality 33
Growth effects 34
Malformations and teratogenic Index (TI) 34
Xenopus Metamorphosis Assay (XEMA) 36
Morphometric endpoints 36
Developmental stage endpoint 38
Thyroid histopathology endpoints 40
Juvenile Results 42
Percentage sex ratios 42
Gonadal Malformations 42
Midstream formulation 44
Basta formulation 44
Arsenal formulation 44
Kilo Max formulation 44
Roundup formulation 44
Enviro Glyphosate 45
Adult Male exposure 48
2.2.6. Conclusions **49**
Acute Toxicity (96-hour LC_{50} test) 49
FETAX (Embryotoxicity test) 50
Xenopus Metamorphosis Assay (XEMA) – Thyroid disruption 50
Post-metamorphic juvenile exposure (sexual development) 51
Adult male exposure (reproductive endocrine disruption) 51
2.2.7. Location of the full version: **52**
2.3. Fungicides as endocrine disruptors **52**
2.3.1. Specific aims of this project **52**
2.3.2. Materials and Methods **52**
Breeding gland description and validation 52
In vitro screening of fungicides 53

Adult male frog exposure to fungicides 55
Circulating hormone and vitellogenin (VTG) determinations 56
RNA isolation and cDNA synthesis 56
RT-qPCR 57
2.3.3. Statistical Procedures 57
2.3.4. Results 57
Breeding glands of *Xenopus laevis* males 57
Adult male *X. laevis* exposures 57
Plasma hormone analyses 64
Spermatogenesis 65
Pre-metamorph tadpole breeding gland development response 66
Juvenile (froglet) *Xenopus laevis* breeding gland development response 68
In Vitro screening of regular-used fungicides 69
Adult male frog exposure to fungicides 72
Plasma hormone analysis 72
Breeding gland morphology 76
RT-qPCR analyses 78
2.3.5. Conclusions 79
3. Field studies 81
3.1. Agriculture dams in Stellenbosch area, Western Cape: Using *in vitro* and *in vivo* biomarkers (African clawed frog, *Xenopus laevis*). 81
3.1.1. Aims 81
3.1.2. Materials and Methods 81
3.1.3. Statistical procedures 84
3.1.4. Results and Conclusions 84
Recombinant yeast androgen- and oestrogen receptor screens of impoundments 84
Cell viability and minced testis assay 86
Breeding gland morphology of male *X. laevis* frogs and comparison between impoundment sites 88
Spermatogenesis comparison of male *X. laevis* frogs between impoundment sites 89
Plasma steroid hormone levels of male *X. laevis* frogs between impoundment sites 91
Thyroid disruption of male *X. laevis* frogs between impoundment sites 94
3.1.5. Conclusions 94
3.1.6. Location of full version 95
3.2. The application of *in vitro* and *in vivo* techniques to evaluate endocrine disruption activity in the Olifants River, a major South African river. 95
3.2.1. Aims 95
3.2.2. Materials and Methods 96
Enzyme-linked Immunosorbent Assays (ELISAs) 97
In vitro recombinant yeast assay 97
In vivo fish exposures 98
3.2.3. Statistical analysis 98
3.2.4. Results 99
Steroid hormone analyses 99
Yeast Reporter Gene Assays 101

Gene expression 102
3.2.5. Conclusions **104**
3.2.6. Location of full version **105**
3.3. Potential disruption of the Thyroid and Adrenal endocrine axes in the Mozambique tilapia (*Oreochromis mossambicus)* population Loskop Dam: I. Thyroid health in wild caught fish from Loskop Dam and Flag Boshielo Dam **105**
3.3.1. Aims **105**
3.3.2. Materials and Methods **105**
Study Site 105
Fish Collection 106
Plasma Thyroid Hormones 107
Histomorphology of Thyroid Follicles 107
3.3.3. Statistical procedures **107**
3.3.4. Results **108**
Fish Summary 108
Plasma Thyroid Hormones 109
Histomorphology of Thyroid Follicles 110
3.3.5. Conclusions **112**
3.3.6. Location of full version **113**
3.4. Potential disruption of the Thyroid and Adrenal endocrine axes in the Mozambique tilapia (*Oreochromis mossambicus*) population Loskop Dam: II. Molecular markers associated with the thyroid, adrenal glands and lipids storage systems **113**
3.4.1. Aims **113**
3.4.2. Materials and Methods **114**
Adult fish collection 114
Juvenile fish exposures 114
Chemical analyses 115
Phytoplankton identification 115
3.4.3. Statistical analysis **115**
3.4.4. Results **116**
Adult fish 116
Juvenile exposures 120
Metal analysis 123
Phytoplankton classification 124
3.4.5. Conclusions **125**
3.4.6. Location of full version **126**
4. PROJECT CONCLUSIONS **127**
5. RECOMMENDATIONS **129**
6. LIST OF REFERENCES **131**

APPENDIX A: CAPACITY BUILDING AND RESEARCH OUTPUTS **157**
Student dissertations 157
Manuscripts in preparation, submitted or published 157
Conference contributions 159

LIST OF FIGURES

Figure 1: *Concentration-response curves for the positive control (17β-estradiol, E_2) and the pesticides, Carbaryl (CA; **A**), Chlorpyifos (CL; **B**) and Endosulfan (EN; **C**), inducing oestrogen receptor (ER) transactivation in T47D human breast cancer cells. Data points represent the mean of duplicate samples.*

Figure 2: *Concentration-response curves for the positive control (17β-estradiol, E_2) and binary pesticide mixtures, Carbaryl-Chlorpyifos (CACL; **A**), Chlorpyifos-Azinphos-methyl (CLAZ; **B**), inducing the oestrogen receptor (ER) transactivation in T47D human breast cancer cells. Data points represent the mean of duplicate samples.*

Figure 3: *Concentration-response curves for the positive control (17β-estradiol, E_2) and binary mixtures pesticides, Endosulfan-Carbaryl (ENCA; **A**), Endosulfan-Chlorpyifos (ENCL; **B**) and Carbaryl-Endosulfan (CAEN; **C**), inducing the oestrogen receptor (ER) transactivation in T47D human breast cancer cells. Data points represent the mean of duplicate samples.*

Figure 4*: The 96-hour LC_{50} indexes determined for three different X. laevis developmental stages exposed to six herbicide formulations. The 96-hour LC_{50} comparison suggests marked variation among exposed chemicals as well as among developmental stage. In general, NF-stage 48 tadpoles exposed to the six herbicide formulations, proved to be the most sensitive stage of the three developmental stages in the X. laevis developmental life cycle*.

Figure 5*: Examples of malformations associated with the various formulations used.*

Figure 6: *Stage differentiation of X. laevis exposed for 21 days to a range of different concentrations of the Arsenal formulation compared to the control (Ctrl). Asterisks indicate significant difference from the control. The developmental stage was reduced across all the three exposure concentrations, but significantly different at 3.5 mg/L (Dunn's test, $p < 0.05$) compared to the control.*

Figure 7*: Stage differentiation following exposure (21 days) to different concentrations of the Midstream formulation compared to the control (Ctrl). Asterisks indicate significant difference from the control. The mean developmental stage was significantly reduced at concentrations of 0.11 and 0.14 mg/L (Dunn's test, $p < 0.05$) compared to the control.*

Figure 8: *Stage differentiation following 21 days of exposure to different concentrations of the Kilo Max formulation compared to the control. Asterisks indicate significant difference from the control. The tadpole developmental stages were reduced in dose dependent manner across the exposure concentrations. The stages were significant differences at concentrations of 190 mg/L and 280 mg/L (Dunn's test, $p < 0.05$) compared to the control.*

Figure 9: *Stage differentiation following exposure for 21 days to different concentrations of the Basta formulation. Developmental stages of tadpoles exposed to graded concentrations of Basta formulations were reduced generally across the exposure concentrations but did not prove to be significantly different from the mean developmental stage of the control (Dunn's test, $p > 0.05$).*

Figure 10: *Macroscopic view of normal male (a) and female (b) gonads of juvenile, post-metamorphic, X. laevis frogs.*

Figure 11: *Examples of the morphological malformations recorded in reproductive gonads of X. laevis frogs, following the exposure to the selected herbicides.*

Figure 12: *Cross sections (400x) of the NP area on the ventral forearm of male X. laevis frogs containing breeding glands. Treatment groups: (**A**) 100 i.u. hCG (control); (**B**) 100 ug/g Flutamide + 100 i.u. hCG, (**C**) 5 µg/g Dutasteride + 100 i.u. hCG and (**D**) 50 µg/g Dutasteride + 100 i.u. hCG. Labels: KH, keratinized hooks; EP, epidermis; BG, breeding gland. Scale bar: 100µm.*

Figure 13: *Variation in the overall size (area) of breeding glands (**A**; $µm^2$ ± SD) and breeding gland epithelium height (**B**; µm, ±SD) among male X. laevis frogs exposed to hCG (control), Flutamide (AR antagonist) and two concentrations of Dutasteride (5αR antagonist). Six breeding glands and the length of ten epithelial cells within each breeding gland were measured per frog (n = 6) in each exposure group. The asterisk indicates a significant difference of treatment groups compared to control frogs (ANOVA; Tukey's HSD post hoc test: $p < 0.05$). Treatment groups: hCG control = 100 i.u. hCG; Flutamide = 100 µg/g body mass + 100 i.u. hCG; Dutasteride 5 µg = 5 µg/g body mass + 100 i.u. hCG; Dutasteride 50 µg = 50 µg/g body mass + 100 i.u. hCG.*

Figure 14: *Transmission electron micrographs of regions of a stimulated (hCG-induced; A, B, C, D, E) and inhibited (Flutamide-exposed; F, G, H) breeding gland situated on the front extremities of male X. laevis frogs. **A:** Columnar secretory cell (SC) epithelium of a stimulated gland, showing irregular-shaped secretory granules (SG) and a nucleus situated at the basal portion of the cell. **B & C:** Clearly visible rough endoplasmatic reticulum (rer) and mitochondria (mi) situated in the perinuclear portion of the SC. **D:** Apical portion of the SC showing microvilli (mv) located on the membrane of the cells leading towards the gland lumen (Lu). **E:** Merocrine secretion of a SG being released into the Lu. **F & G:** SCs of an inhibited gland (Flutamide-exposure) showing less developed SGS and a nucleus which is pyknotic in shape. **H:** Apical region of an inhibited gland SC showing SGs which are smaller in size and some SGs which differ in secretory content.*

Figure 15: *Immunohistochemical visualization of the distribution of AR in the NP area of adult male X. laevis frogs exposed to an hCG control (**A** & **B**), negative stained control (**C** & **D**), Flutamide pharmaceutical (**E** & **F**) and Mancozeb fungicide (**G** & **H**). The negative stained control (**C** & **D**) represents skin sections stained with PBA only in the absence of the PG-21 primary antibody. Less immunolocalization of AR can be observed in the Flutamide and Mancozeb-exposed frogs (**E, F, G** & **H**) compared to the hCG control (**A** & **B**). Labels: BG, breeding gland; KH, keratinised hooks. Scale bar: 200 µm.*

Figure 16: *Variation in plasma testosterone (T) and dihydrotestosterone (DHT) concentrations (ng/ml ± SD) among male X. laevis frogs (N = 6) exposed to hCG (control), Flutamide and two concentrations of Dutasteride. The asterisk indicates a significant difference of treatment groups compared to control frogs (ANOVA: $F_{3,16} = 100.7$; Tukey's HSD post hoc test: $p < 0.05$). Treatment groups: Control = 100 i.u. hCG; Flutamide = 100µg/g body mass + 100 i.u. hCG; Dutasteride 5 µg = 5 µg/g body mass + 100 i.u. hCG; Dutasteride 50 µg = 50 µg/g body mass + 100 i.u. hCG.*

***Figure 17**: Variation in the ratio of plasma DHT/T among male X. laevis frogs exposed to hCG (control), Flutamide and two concentrations of Dutasteride. The asterisk indicates a significant difference of treatment groups compared to control frogs (ANOVA: $F_{3,16} = 48.34$; Tukey's HSD post hoc test: $p < 0.05$). Treatment groups: Control = 100 i.u. hCG; Flutamide = 100 µg/g body mass + 100 i.u. hCG; Dutasteride 5 µg = 5 µg/g body mass + 100 i.u. hCG; Dutasteride 50 µg = 50 µg/g body mass + 100 i.u. hCG.*

***Figure 18**: Variation in the average percentage (% ±SD) of germ cell cysts and spermatozoa per seminiferous tubules in male X. laevis testes exposed to hCG (control), Flutamide and two concentrations of the 5αR inhibitor Dutasteride. The asterisk indicates a significant difference from the hCG treated control (ANOVA, $p < 0.05$; Tukey's HSD post hoc test, $p < 0.05$).1° SPG, primary spermatogonia; 2° SPG, secondary spermatogonia; SPC, spermatocyte; SPT, spermatid; SPZ, spermatozoa.*

***Figure 19**: Development of BGs and epidermal KHs in pre-metamorphic X. laevis tadpoles (NF 59-62) given either a DMSO supplement (**A**) or a 17α-MT supplement of 30 µg/L (**B**), 100 µg/L (**C**) or 300 µg/L (**D, E** & **F**). Some of the sub-epidermal glands in tadpoles from the 300 µg/L 17α-MT treatment showed a mixed-type of gland (**D** & **E**, arrows). Staining of skin sections with PAS clearly differentiate between breeding- and mucous glands in the 300 µg/L treatment (**F**). Labels: MG, mucous gland; BG, breeding gland; KH, keratin hooks; EP, epidermis. Scale bar: A, B, C, D, F (100 µm); E (40 µm).*

***Figure 20:** Occurrence of the breeding gland density per mm NP surface area (**A**) and epidermal KH density (**B**) per mm NP surface area in pre-metamorphic tadpoles (NF stage 56-59; n = 10) exposed to different concentration ranges of 17α-MT. Values are expressed as mean (± SD).*

***Figure 21**: Skin sections of the NP area in juvenile X. laevis frogs (five days post-metamorphosis) given a daily androgen supplement of 0 ug/g food (**A** & **B**) or 600 ug/g food (**C** & **D**) for 21 days. Labels: MG, mucous gland; BG, breeding gland; KH, keratin hooks; EP, epidermis; Lu, lumen; SC, secretory cell. Scale bar: 100 µm.*

***Figure 22**: AR antagonistic dose-response of the test pesticides regularly used in agriculture in the Western Cape. Values are expressed as a percentage relative to the model AR antagonistic fungicide Vinclozolin.*

***Figure 23:** AR antagonistic dose-response of equimolar binary mixtures of the test pesticides. Values are expressed as a percentage relative to the model AR antagonistic control fungicide, Vinclozolin.*

***Figure 24:** Concentrations of plasma testosterone (T) of adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Test chemicals were exposed in the water using dimethylsulfoxide (DMSO) as solvent. The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, $p < 0.05$) from frogs in the control exposure.*

Figure 25: *Concentrations of plasma dihydrotestosterone (DHT) of adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Test chemicals were exposed in the water using dimethylsulfoxide (DMSO) as solvent. The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, $p < 0.05$) from frogs in the control exposure.*

Figure 26: *Concentrations of plasma oestradiol (E_2) of adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides mancozeb and folpet (0.25 + 0.05 mg/L respectively). Test chemicals were exposed in the water using dimethylsulfoxide (DMSO) as solvent. The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, $p < 0.05$) from frogs in the control exposure.*

Figure 27: *Relative fold change in plasma VTG concentrations of adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Test chemicals were exposed in the water using dimethylsulfoxide (DMSO) as solvent. The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, $p < 0.05$) from frogs in the control exposure.*

Figure 28: *Ratios of plasma dihydrotestosterone (DHT) relative to plasma testosterone (T) of adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Test chemicals were exposed in the water using dimethylsulfoxide (DMSO) as solvent. The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, $p < 0.05$) from frogs in the control exposure.*

Figure 29: *Ratios of plasma oestradiol (E_2) relative to plasma testosterone (T) of adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Test chemicals were exposed in the water using dimethylsulfoxide (DMSO) as solvent. The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, $p < 0.05$) from frogs in the control exposure.*

Figure 30: *Variation in the overall size (area) of breeding glands ($\mu m^2 \pm SD$) among adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Six breeding glands and the length of ten epithelial cells within each breeding gland were measured per frog (n = 6) in each exposure group. The asterisk indicates a significant difference of treatment groups compared to control frogs (ANOVA; Tukey's HSD post hoc test: $p < 0.05$).*

Figure 31: *Variation in overall breeding gland epithelium height (µm, ±SD) among adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Six breeding glands and the length of ten epithelial cells within each breeding gland were measured per frog (n = 6) in each exposure group. The asterisk indicates a significant difference of treatment groups compared to control frogs (ANOVA; Tukey's HSD post hoc test: $p < 0.05$).*

Figure 32: *The mRNA expression (mean ± SD) of thyroid receptor β b (trβ-b) and type 2 deiodinase (dio2) in the liver tissue of adult X. laevis exposed to Folpet (0.1 mg/L), Mancozeb (0.5 mg/L or a Mancozeb-Folpet mixture (0.25 mg/L Mancozeb; 0.05 mg/L Folpet) (n = 5 to 7 frogs per treatment).*

Figure 33: *Localities of water samples taken from water impoundments located close to agricultural practices. Site 1 was selected as relatively secluded from agricultural practices, surrounded by natural vegetation and not receiving runoff from converging slopes whereas the other sites (Site 1-3 and 5-10) were located at the bottom of slopes converging to a specific impoundment (dam) within intensive agricultural activity, mostly vineyards. Sampling was done during late summer and early winter periods. Ten adult male X. laevis frogs (n = 10) were also captured at sites 1, 2, 3 and 4 for biomarker investigations.*

Figure 34: *Androgenic activity in water samples collected from impoundments situated close to agricultural practices (Sites 2-10) and an impoundment isolated from agricultural practices (Site 1) in the YAS. Samples were taken during summer and winter seasons and expressed relative to untreated (blank) cells in the assay. The concentration shown for DHT represents the* EC_{50} *concentration of DHT in the standard curve (dashed line, 6.25 nM). Expression of water samples under the detection limit of the assay were shown as not detected (nd). The asterisk indicates significant difference from the blank (untreated) cells.*

Figure 35: *Anti-androgenic activity in water samples collected from impoundments situated close to agricultural practices (Sites 2-10) and an impoundment isolated from agricultural practices (Site 1) in the anti-YAS. Samples were taken during summer and winter seasons and expressed relative to untreated (blank) cells in the assay (bottom dashed line). The concentration of Vinclozolin (Vin) represents the generated* IC_{50} *in the assay (4.94 nM). Expression of water samples under the detection limit of the assay were shown as not detected (nd). The asterisk indicates significant difference from the* EC_{50} *of the DHT spike (top dashed line).*

Figure 36: *Oestrogenic activity in water samples collected from impoundments situated close to agricultural practices (Sites 2-10) and an impoundment isolated from agricultural practices (Site 1) in the YES. Samples were taken during summer and winter seasons and expressed relative to untreated (blank) cells in the assay. The concentration shown for* E_2 *represents the* EC_{50} *concentration of* E_2 *in the assay (5.39 nM). Expression of water samples under the detection limit of the assay were shown as not detected (nd). The asterisk indicates significant difference from the blank (untreated) cells.*

Figure 37: *Cell viability of the minced testis cell suspension treated with the water catchment samples taken during summer and winter periods. Values are expressed as the measured formazan formation in the XTT assay in percentage of absorbance (% ± SD) relative to control treated (blank) cells. The asterisk indicates significant difference from the blank cells (ANOVA; Tukey's HSD test,* $p < 0.05$*.).*

Figure 38: *Effect of water samples at the catchment sites during summer and winter sampling on testosterone secretion (ng/ml ± SD) in the rat minced testis culture. Cells were exposed to the water samples obtained from the various impoundments. Samples were screened in duplicates and values are expressed as average testosterone concentration in the culture medium. The asterisk indicates significant difference from untreated (blank) cells (ANOVA; Tukey's HSD test,* $p < 0.05$*.).*

Figure 39: *Morphological variation in breeding gland area (**A**) and breeding gland secretory cell epithelium height (**B**) in adult male X. laevis frogs from the different water impoundments. Male frogs (n = 6) from a water impoundment isolated from agricultural practice (Site 1) was compared to frogs from water impoundments situated in close proximity of agricultural practices (Sites 2, 3 & 4).*

Figure 40: *Breeding gland morphology (400x magnification) of adult male X. laevis frogs (n = 6) captured from a water impoundment surrounded by natural vegetation and not receiving runoff from converging slopes with the impoundment isolated from agricultural practice (**A**; Site 1, control) and water impoundments receiving water from catchments in close proximity to agricultural crops (**B**, Site 2; **C** Site 3; **D**, Site 4). Labels: kh, keratinised hooks; ep, epidermis; bg, breeding gland. Scale bar: 50 µm.*

Figure 41: *Variation in the average percentage (% ± SD) of germ cell cysts and spermatozoa per seminiferous tubules in male X. laevis testes (n = 6) obtained from water impoundments isolated from agricultural practice (Site 1, Fig. 28) and close to agricultural practices (Sites 2, 3 & 4, Fig. 28). The asterisk indicates a significant difference from frogs in water impoundments isolated from agricultural practice (Site 1, Fig. 5.1) (ANOVA; Tukey's HSD post hoc test, $p < 0.05$). SPG, spermatogonia; SPC, spermatocyte; SPT, spermatid; SPZ, spermatozoa.*

Figure 42: *Variation in spermatogenic germ cell types between adult male X. laevis frogs captured from a water impoundments isolated from agricultural practice (**A** & **B**; Site 1) and water impoundments situated close to agricultural practices (**C** & **D**, **E** & **F**, **G** & **H**; Sites 2, 3 & 4 respectively). Magnification: 100x (A, C, E, G); 400x (B, D, F, H). Scale bar: 100 µm.*

Figure 43: *Concentrations of plasma T (**A**), DHT (**B**), DHT:T ratio (**C**), E_2 (**D**), fold change in plasma VTG expression (**E**) and E_2:T ratios (**F**) of adult male X. laevis frogs (n = 6 per impoundment site). Frogs were captured from a water impoundment not receiving runoff from converging slopes, surrounded by natural vegetation and isolated from agricultural practices (Site 1, control) and water impoundments receiving water from catchments in close proximity to agricultural practices (Sites 2, 3 & 4). The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, $p < 0.05$) from frogs in the control water impoundment (Site 1).*

Figure 44: *The mRNA expression (mean ± SD) of thyroid receptor β b (trβ-b) and of type 2 deiodinase (dio2) in the liver tissue of adult X. laevis individuals captured from three impoundments (dams) in the Stellenbosch agricultural area (N = 5 to 7 frogs per treatment). Dissimilar characters indicate statistically significant differences (Tukey's HSD post hoc test, $\alpha = 0.05$). Significant differences are indicated by asterisks (Student's t-test).*

Figure 45: *Map of the upper Olifants River catchments with land use and the current sampling locations indicated.*

Figure 46: *Mean 17β-estradiol (E_2) concentrations (ng/L) measured in water collected from a selection of localities within the upper Olifants River catchment during May, June, September and November 2011. Error bars denote the standard deviation (SD) of technical repeats within immunoassays.*

Figure 47: *Mean 17α-ethinylestradiol (EE_2) levels (ng/L) measured in water collected from six localities within the upper Olifants River catchment during May, June, September and November 2011. Error bars denote the standard deviation (SD) of technical repeats within immunoassays.*

Figure 48: *17β-Estradiol levels ng/L (**A**) across seasons (i.e., summer, autumn, winter and spring) (**B**) among six localities within the upper Olifants River catchment. Significant differences are indicated by dissimilar characters above figure bars. Locality (within the river) was a significant source of variation in E_2 concentrations measured across seasons ($H_{5,24}$ = 12.3, p = 0.03).*

Figure 49: *17α-Ethinylestradiol levels ng/L (**A**) across seasons (i.e., summer, autumn, winter and spring) (**B**) among six localities within the upper Olifants River catchment. Significant differences are indicated by dissimilar characters above figure bars. Locality (within the river) was a significant source of variation in EE_2 ($H_{5,24}$ = 13.73, p = 0.01, Kruskal-Wallis ANOVA).*

Figure 50: *Oestrogenic activity (expressed as 17β-estradiol equivalents) observed in surface water collected from six localities within the upper Olifants River catchment during summer. The error bar indicates inter-assay variability as standard deviation.*

Figure 51: *Anti-androgenic activity (expressed as Flutamide equivalents) observed in surface water collected from six localities within the upper Olifants River catchment during summer. Error bars indicate inter-assay variability as standard deviation.*

Figure 52: *Expression of androgen receptor 1 (ar1), aromatase (cyp19a1b), glucocorticoid receptor 1 (gr1), mineralocorticoid receptor (mr), thyroid receptor α (trα) and trβ in juvenile Oreochromis mossambicus (22 dpf) exposed for 48 hours to C18 solid phase extracts of water collected from six localities within the upper Olifants River catchment, a buffered RO water control and solvent (DMSO) control. Expression was determined using RT-qPCR. Expression is expressed relative to the solvent control exposure group and was quantified using the Pfaffl method (Pfaffl, 2001) with β-actin as reference gene.*

Figure 53: *A Redundancy Analysis triplot indicating the (dis)similarities among six sampling locations within the upper Olifants River catchment with regard to the expression of O. mossambicus trα, trβ, ar1, gr1, mr and cyp19a1b expression in combination with 17β-oestradiol, ethinylestradiol concentrations and oestrogenicity and androgenicity as indicated by the yeast oestrogen- and anti-androgen screens respectively. The mean of gene expression per treatment group was applied in the analysis.*

Figure 54: *Map showing the location of Loskop Reservoir (LR) and Flag Boshielo Reservoir (FBR) on the Olifants River. Black circles indicate the location of established water quality monitoring points at both reservoirs (Dabrowski et al., 2013; 2014).*

Figure 55: *Plasma T_3 (a) and T_4 (b) concentrations, and T_3/T_4 ratio (c) measured in Oreochromis mossambicus sampled from Loskop Reservoir (LR) and Flag Boshielo Reservoir (FBR). Bars represent mean values ± S.E., with the number of individuals per mean shown within bars.*

Figure 56: *Photomicrographs (H&E) of thyroid follicle sections collected from Oreochromis mossambicus.* ***A****. follicles in fish from Loskop Reservoir were abnormally enlarged with greater surface area (40x magnification);* ***B****. hypertrophy and hyperplasia of the follicular epithelium in fish from LR (100x magnification);* ***C****. increased number of epithelial-colloid interface follicular vacuoles in fish from LR (200x magnification);* ***D****. typical variation in size, colloid staining intensity, resting epithelium and low vacuolar activity of follicles in fish from FBR (100x magnification).*

Figure 57: *Lake Loskop and the lake's position within South Africa, as well as the location of a further O. mossambicus population in the North West Province from which fish were obtained. Three collection points within Lake Loskop from where water applied in fish exposures and chemical analysis was collected are indicated. Map credits: Google Inc.*

Figure 58: *The mRNA expression of thyroid receptor α (trα), thryroid receptor β (trβ), type 2 deiodinase (dio2), thyroid stimulating hormone β (tshβ), glucocorticoid receptor 1 (gr1), glucocorticoid receptor 2 (gr2), mineralocorticoid receptor (mr) and peroxisome-proliferator receptor γ (pparγ) in the (total) brain tissue of adult O. mossambicus captured from Loskop reservoir (n = 10) and reference fish maintained in a small impoundments supplied by borehole water (n = 8) and irrigation canal water (n = 8) respectively. β-actin was applied as normalizer. Dissimilar characters indicate statistically significant differences (Fisher's LSD post hoc test, α = 0.05).*

Figure 59: *Examples of the brain cavities of pansteatitis suffering O. mossambicus captured from Loskop reservoir (****a****) and pansteatitis free fish from an alternative populations (****b****).*

Figure 60: *The expression of thyroid receptor α (trα), thryroid receptor β (trβ), type 2 deiodinase (dio2), thyroid stimulating hormone β (tshβ), glucocorticoid receptor 1 (gr1), glucocorticoid receptor 2 (gr2), mineralocorticoid receptor (mr) and peroxisome-proliferator receptor γ (pparγ) in the (total) brain tissue of adult O. mossambicus captured from Loskop reservoir. The fish diagnosed with pansteatitis in the abdominal fat "pansteatitis" (n = 7) were compared to those with no visible signs of pansteatitis "normal" (n = 3). β-actin was applied as normalizer. No significant differences were observed among the "normal" and "pansteatitis" groups (Student's t-test, α = 0.05).*

Figure 61: *Expression of (****A****) thyroid receptor α (trα) and (****B****) thyroid receptor β (trβ) in juvenile Oreochromis mossambicus (32 dpf) (whole body homogenates) exposed for 48h to water from the lacustrine, transitional and riverine sections of Lake Loskop. Significant differences are indicated by asterisks (Student's t-test or Man-Whitney U-test). The numbers within the horizontal boxes indicate the number of fish representing each treatment.*

Figure 62: *Expression of (****A****) type 2 deiodinase (dio2) and (****B****) peroxisome- proliferator receptor γ (pparγ) in juvenile Oreochromis mossambicus (32 dpf) (whole body homogenates) exposed for 48h to water from the lacustrine, transitional and riverine sections of the Loskop reservoir. Significant differences are indicated by asterisks (Student's t-test or Man-Whitney U-test). The numbers within the horizontal boxes indicate the number of fish representing each treatment.*

LIST OF TABLES

Table 1: *Four insecticides selected for oestrogenic assessment as individual chemicals or as binary mixtures.*

Table 2: *Calculated Oestradiol (E_2) EC_{50} values, Chemical EC_{50} values, Relative Potency (RP) and Relative Induction Efficiency (RIE) for the selected insecticides.*

Table 3: *Xenopus laevis tadpoles NF-stages, exposure concentrations and calculated 96-hr LC_{50} values for the selected herbicide formulations. The LC_{50} values are in bold and the upper and lower ranges are in brackets.*

Table 4: *The exposure concentrations (mg/L), LC_{50}, EC_{50}, Teratogenic Index (TI), and minimum concentration that inhibit growth (MCIG) for the herbicide formulations (95 % CI in brackets).*

Table 5: *Summary of all measured endpoints with the standard deviations for the six herbicide formulation after 21 day exposure.*

Table 6: *Summary data of histopathology morphometrics of the six herbicide formulation used in a 21 day XEMA exposure assessment. Bold values with asterisk indicate significantly different values compared to control (0 mg/L) exposed tadpoles (Dunn's test, $p < 0.05$).*

Table 7: *Numbers and percentage of morphological abnormalities and sex ratios at various concentrations of the herbicide formulations.*

Table 8: *Summary of the exposure results (mean ±SD) following a 28-day exposure to different concentrations of the two selected herbicides, Midstream and Arsenal. Group means differing significantly from the Control (0) are marked with asterisk (Dunn's test, $p < 0.05$).*

Table 9: *Test chemicals and their suggested mechanisms of endocrine disrupting action. Test chemicals were used in the current study to evaluate anti-androgenic (inhibition of AR binding) activity of binary mixtures as well. Selected compounds were diluted to obtain 12 different concentrations, before added to the yeast cultures along with 6.25 nM DHT. Vinclozolin and DHT served as positive controls for the anti-YAS and YAS respectively. Pesticide active ingredients indicated with an asterisk (*) are anti-fungal pesticides.*

Table 10: *The IC_{50} of test compounds and their isobole coefficients in the mixtures as well as the relative potency (RP) of the pesticides to the control fungicide, Vinclozolin. Isobole coefficients for the mixtures with a value > 1 indicates antagonistic activity, value < 1 indicates synergism and a value = 1 indicates additivity. Predicted IC_{50} concentrations for the binary pesticide mixtures assuming concentration addition (CA) or independent action (IA) is also estimated.*

Table 11: *Summary incidences of significant interaction with the steroid receptors (hAR and hER) and testosterone production using a rat minced testis assay. S: Summer, W: Winter. The + sign indicate significant variance from untreated (blank) cells used in the respective assays (ANOVA, $p < 0.05$).*

Table 12: *Summarised catch statistics for Oreochromis mossambicus sampled from Loskop (LR) and Flag Boshielo (FBR) Reservoirs in 2011 including the sex ratio (M:F), weight and total length (TL; Mean ± SD).*

Table 13: *Mean (±SE) histopathological and morphometric descriptor values for thyroid follicles evaluated in Oreochromis mossambicus from Loskop Reservoir (n = 30) and Flag Boshielo Reservoir (n = 30).*

Table 14: *Spearmans rank correlation coefficients (R) and p-values of relationships between nutritional and thyroid parameters measured in Oreochromis mossambicus from Loskop Reservoir and Flag Boshielo Reservoir. Bold font indicates that the correlation coefficient is significant ($p < 0.05$).*

Table 15: *Generalized Linear Model Analysis of Covariance (GLM ANCOVA) of the expression of: (**A**) thyroid receptor α (trα), thyroid receptor β (trβ) and type 2 deiodinase (dio2); (**B**) peroxisome-proliferator receptor γ (pparγ) and thyroid stimulating hormone β (tshβ); (**C**) glucocorticoid receptor 1 (gr1), glucocorticoid receptor 2 (gr2) and mineralocorticoid receptor (mr) in the (total) brain tissue of adult O. mossambicus captured from Loskop reservoir (n = 10) and reference fish maintained in a small impoundments supplied by borehole water (n = 8) and irrigation canal water (n = 8) respectively. Fulton's condition factor was applied as covariate.*

Table 16: *Generalized Linear Model Analysis of Covariance (GLM ANCOVA) of the expression of (A) thyroid receptor α (trα), thyroid receptor β (trβ), type 2 deiodinase (dio2), thyroid stimulating hormone β (tshβ) and (B) glucocorticoid receptor 1 (gr1), glucocorticoid receptor 2 (gr2), mineralocorticoid receptor (mr) and peroxisome-proliferator receptor γ (pparγ) in the (total) brain tissue of O. mossambicus macroscopically diagnosed with pansteatitis in the abdominal fat "pansteatitis" (n = 7) and fish with no visible signs of pansteatitis (n = 3). Fulton's condition factor was applied as covariate.*

Table 17: *The concentrations of selected metals and other elements in water collected from three locations within Lake Loskop during December 2012. The water samples containing these elements were applied in juvenile fish exposures.*

Table 18: *The phytoplankton species composition and relative abundance in water collected from three locations within Lake Loskop during December 2012. The water samples containing algae were applied in juvenile fish exposures.*

LIST OF ABBREVIATIONS

11-KT	11-ketotestosterone
17α-MT	17α-methyltestosterone
5αR	5-alpha reductase
ANOVA	Analysis of Variance
anti-YAS	Yeast Anti-androgen Screen
AR	Androgen Receptor
ar1	*Androgen Receptor 1*
AVMA	American Veterinary and Medical Association
BG	Breeding Gland
C18	Octadecyl
CA	Concentration Addition
CAF	Central Analytical Facility
CDI	Condition Index
CHG	Choriogenin
CYP19	Human Aromatase P450 gene
cyp19a1b	*Aromatase B*
D	Duct
DDE	Dichloro-diphenyl-dichloro-ethylene
DDT	Dichloro-diphenyl-trichloroethane
DES	Di-ethyl-stilbestrol
DHT	Dihydrotestosterone
DHT-EQ	Dihydrotestosterone Equivalent
dio2	*Type 2 deiodinase*
DMSO	Dimethyl Sulphoxide
DNA	Dioxyribonucleic Acid
DPF	Days Post Fertilization
DWAF	Department of Water Affairs and Forestry
E_2	Estradiol
E_2-EQ	Estradiol Equivalent
EC_{50}	Half Maximal Effective Concentration
ED	Endocrine Disruption

EDC	Endocrine Disrupting Contaminant
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EE_2	Ethinyl-estradiol
EEC	Expected Environmental Concentration
EIA	Environmental Impact Assessment
ELISA	Enzyme-Linked Immunosorbent Assay
EMU	Electron Microscopy Unit
EP	Epidermis
ER	Oestrogen Receptor
EU	European Union
FETAX	Frog Embryo Teratogenesis Assay-Xenopus
gr1	*Glucocorticoid Receptor 1*
gr2	*Glucocorticoid Receptor 2*
GSI	Gonadosomatic Index
GWRC	Global Water Research Coalition
hCG	Human Chorionic Gonadotropin
HPG	Hypothalamic Pituitary Gonadal
HPT	Hypothalamic Pituitary Thyroid
HSD	Honest Significant Difference
IA	Independent Action
IC_{50}	Concentration Giving 50% Inhibition
IPCS	International Programme on Chemical Safety
IR	Intermediate Region
KH	Keratinized Hooks
LC_{50}	Lethal Concentration 50%
Lu	Lumen
MCIG	Minimum Concentration Inhibiting Growth
MG	Mucous Gland
Mi	Mitochondria
MOA	Mode of Action
mr	*Mineralocorticoid Receptor*
mRNA	Messenger Ribonucleic Acid
ND	Not Detected

NF	Nieuwkoop and Faber Developmental Stage
NOEL	No Observed Effect Level
NP	Nuptial Pad
OECD	Organisation for Economic Co-operation and Development
PAS	Periodic Acid-Schiff
PBS	Phosphate Buffered Saline
PNEC	Predicted No-effect Concentration
pparγ	*Peroxisome-proliferator Receptor γ*
QSAR	Quantitative Structure-activity Relationship
RER	Rough Endoplasmatic Reticulum
RIE	Relative Induction Efficiency
RO	Reverse Osmosis
RP	Relative Potency
RT-qPCR	Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction
SC	Secretory Epithelial Cells
SPC	Spermatocyte
SPG	Spermatogonia
SPT	Spermatid
SPZ	Spermatozoa
T	Testosterone
T_3	Triiodothyronine
T_4	Thyroxine
TDCs	Tthyroid-disrupting Contaminants
TEDX	The Endocrine Disruptor Exchange
trα	*Thyroid Receptor* α
trβ	*Thyroid Receptor β*
tshβ	*Thyroid Stimulating Hormone β*
USEPA	United States Environmental Protection Agency
VIN	Vinclozolin
VIN-EQ	Vinclozolin Equivalent
VTG	Vitellogenin
WfW	Working for Water
WHO	World Health Organisation

WRC	Water Research Commission
WWTW	Wastewater Treatment Works
XEMA	Xenopus Metamorphosis Assay
YAS	Yeast Androgen Screen
YES	Yeast oestrogen Screen

1. BACKGROUND, INTRODUCTION AND OBJECTIVES

1.1. General: Endocrine Disruption

Evidence to support the concern that there are many environmental chemicals (pollutants) interacting (directly or indirectly) with the endocrine systems of organisms resulting in modulation/disruption of hormonal control are mounting (Myers et al., 2001; Bergman et al., 2013). Pollutants with mechanisms of action interacting in some way with the endocrine system have been referred to as the so-called, endocrine-disrupting contaminants or chemicals (EDCs). The working definition for an EDC would therefore state that it is:

"An exogenous compound that directly interferes with the synthesis, secretion, transport, binding action or elimination of endogenous hormones and neurohormones, resulting in physiological manifestations of the neuroendocrine, reproductive, thyroid or immune systems in an intact organism" (Harris et al., 2000).

Initial concern about the threat posed by environmental contaminants, including pesticides, to human and wildlife health came from the book, *Silent Spring* authored by Rachel Carson in 1962 and a second book, *Our Stolen Future* (Colborn et al., 1996) specifically focussing on the endocrine disruption hypothesis. Although the concept of subtile endocrine disrupting activity was suggested at a much earlier time (Krimsky, 2000; Burkhardt-Holm, 2010), this field of research exploded during the last decade and powerful weight-of-evidence continue to mount showing that all major aquatic wildlife groups are experiencing modulation of the endocrine system in some way or another in a variety of contaminated sites (Hutchinson et al., 2006; Zoeller et al., 2012; Heindel et al., 2013; Kidd et al., 2013). It is not surprising, therefore, to find that aquatic organisms are regarded the most affected, since more than 100 000 chemical are thought to be discharged directly into the freshwater and marine environments (Trudeau and Tyler, 2007). Globally, concern is also mounting for apparent declining of aquatic associated wildlife, fish and amphibian populations, with possible causal links to EDCs (Carey and Bryant, 1995; Davidson et al., 2001; Withgott, 2002; Hayes et al., 2006; Kidd et al., 2007).

Since the initial reports, an exponential increase in the number of published reports, papers, reviews and books on various aspects of endocrine disruption followed and several organizations, including International Union of Pure and Applied Chemistry (IUPAC) (Preziosi, 1998; Miyamoto and Burger, 2003) and the World Health Organization (WHO) (Damstra et al., 2002; Bergman et al., 2013) published extensive review reports assessing the weight of evidence and highlighting the research priorities. However, it was the US Congress, responding to public and scientific concerns that environmental chemicals may modulate endocrine systems, that mandated United States Environmental Protection Agency (USEPA) to design, validate and implement a screening program to investigate these concerns. The

USEPA initiated the Endocrine disrupting Screening Program (EDSP) and in 1992, the USEPA issued a first report including a battery of tests to be implemented at a first tier level followed by second tier (mainly *in vivo*) tests to confirm/disprove initial hypotheses regarding a chemical being an EDC (USEPA, 2009; Juberg et al., 2014). The Organization for Economic Corporation and Development (OECD) (including all the member countries) convened advisory committees to develop and coordinate validation (inter-laboratory research) studies regarding potential screening and testing strategies to evaluate individual chemicals (and environmental samples) for endocrine modulation (disrupting) activity (Holbech et al., 2006; Hecker and Hollert, 20011; Hecker et al., 2011). Others, for example, the Global Water Research Coalition (GWRC) initiated research programmes to comparatively evaluate current screening tests for oestrogenicity testing (Leusch, 2008). Although the initial focus was primarily on human health consequences of EDCs, in particular oestrogenic activity, wildlife health and additional modes of action (androgenicity and thyroid interaction) were added as points of concern.

Multiple potential sources of EDCs in aquatic systems have been reported in the literature, including municipal sewage and domestic waste discharges, industrial effluents, agriculture run-off and groundwater contamination (Burger and Nel, 2008; McKinlay et al., 2008; Frye et al., 2012). Knowledge about potential endocrine disruptors increased dramatically over the past decade and the sources and number of chemicals showing, or predicted, to have some endocrine modulation effects belong to a diverse range of chemical classes (Kidd et al., 2013). Endocrine disruption has been linked to pharmaceuticals (human or veterinary) (including natural and synthetic steroid derivates, antibiotics, analgesics, anti-inflammatory agents, psychotropic agents), personal care products (surfactants, synthetic fragrances (e.g. musk), preservatives), pesticides, metals, metalloids, industrial chemicals and byproducts (Basile et al., 2011; Kidd et al., 2013). Many EDCs are known to be persistent in the environment, may bioaccumulate through food webs and maternally contaminate (through yolk, placenta and breast milk) offspring in wildlife and humans (Kidd et al., 2013). Although some chemicals may have short half-lives, with less bio-accumulative potential, continuous exposure or endocrine disrupting properties of breakdown derivatives they remain a concern. It is recognized that endocrine disruption/modulation through many exposure routes and potential environmental sites is caused by a complex mixture of substances, very often acting at low concentrations in synergy with other compounds (for example, nitrogen compounds) in the mixture (Colborn et al., 1996; Van der Kraak et al., 1998; Porter et al., 1999; Bergman et al., 2013).

Although chemicals are added continuously to the list of potential EDCs (see TEDX list, (TEDX, 2014); >900 compounds), the linking of chemicals to the endocrine disruption (ED) mode of action remains a challenge (Kidd et al., 2007; Phillips et al., 2008). From the endocrine disrupting contaminant (EDC) lists, like the TEDX list, groups of compounds sharing structural features may facilitate QSAR models for

several endocrine receptor related endpoints (Comber et al., 2003; Kidd et al., 2013). However, it seems that the diverse range of "mechanisms of actions" exhibited by many compounds remains a major problem to identify EDC with confidence (Kidd et al., 2013).

1.2. Potential mode of action (MOA) of EDCs

Since the early formulation of the endocrine disruption hypothesis (Krimsky, 2000), the focus of research was mostly on the possible adverse effects on the development and reproductive biology of organisms inhabiting the aquatic environment with potential links to humans as end-user of water resources. Modulation of the reproductive system hormones, in particular oestrogens, female hormones, by far received the most attention (Damstra et al., 2002; Panter et al., 2002; Bergman et al., 2013; Jobling et al., 2013; Van Wyk, 2013). In contrast to the focus on modulation of oestrogenic function or oestrogenic effects in male individuals, relatively few studies addressed the interaction and modulation of the androgenic and related systems by environmental chemicals (Urbatzka et al., 2007a,b). Relatively recently, however, it was recognized that environmental chemicals may also interfere/modulate with the normal functioning of the thyroid endocrine system (Brucker-Davis, 1998; Zoeller, 2007; Crofton, 2008; Kloas et al., 2009; Hecker and Hollert, 2011; Jobling et al., 2013; Van Wyk, 2013).

1.2.1. Oestrogenicity

It is well-known that certain cells are naturally responsive to oestrogens. This means that these cells naturally express/produce a protein(s) when stimulated by oestrogen. The first step in the oestrogen response is the binding of the ligand (oestrogen or an exogenous chemical signal) to oestrogen receptors α or β (ERα or ERβ) (Norris and Carr, 2006). These receptor proteins may, as traditionally recognized, be located in the cytosol or nucleus of the response cell (genomic pathway)(Norris and Carr, 2006) or alternatively be incorporated in the cell membrane (non-genomic pathway) (Cornil et al., 2006). The next step includes the transduction of this signal to an effector mechanism, which is then responsible for the cellular response, for example, changes in enzymatic activity, ion channel kinetics or gene expression (synthesis of a specific protein or so-called oestrogen mediated protein). The basis of most *in vitro* oestrogenicity bioassays include the binding of ligands to the oestrogen nuclear receptors, the transduction of the signal through gene expression and eventually measuring the estrogenic response in the cell. The list of oestrogen mediated proteins, as measurable endpoints, to assess oestrogen activity and/or potency of alleged estrogenic substances is ever-increasing and examples include, several hormone receptor proteins (e.g. progesterone), prolactin, sex hormone binding globulin (plasma protein), the yolk precursor protein, vitellogenin (VTG) (mainly produced in the liver of oviparous species), alkaline phosphatase (ALP) (produced in human endometrial cancer cells), pS2 protein (produced by breast cancer cells) etc.

(Zacharewski, 1997). Important to note that the induction of oestrogenic responses may be cell and/or tissue specific and potentially will occur via either the genomic or non-genomic pathways.

Vitellogenin (VTG) is a high molecular weight phospho-lipo-glycoprotein (250-600 kDa) that is produced and secreted by the liver of oviparous vertebrates as the precursor to several egg yolk proteins to be stored in the oocyte (Arukwe and Goksoyr, 2003). Adult oviparous females synthesizes 17β-estradiol (E_2) in the gonads, and the circulating E_2 is subsequently taken up by hepatocytes where it binds to intra-cellular oestradiol receptors (ER) leading to the transcription of the VTG gene(s). The nuclear produced VTG mRNA leaves the nucleus and the VTG protein complex transcribed in the cytosol and then secreted from the hepatocytes. Vitellogenin enters the blood circulation and is taken up by growing oocyte through receptor-mediated endocytosis (Matozzo et al., 2008). Within the oocyte VTG is degraded to the yolk proteins, lipovitellin 1, lipovitellin 2 and phosphitin (Parks et al., 1999; Brion et al., 2002). These yolk proteins serve as a nutrient store for developing embryos (Matsubara et al., 1999). Vitellogenin is not the only oestrogen-induced protein synthesized in the liver and transported to the ovary. Zona radiata (ZR) or eggshell proteins, so-called choriogenins (CHGs), are synthesized in the liver and transported to the ovary for incorporation in the chorion, specifically the *zona radiata* layer (Arukwe and Goksoyr, 2003). The current status of our understanding of fish VTG and the pathways associated with vitellogenesis has been reviewed extensively (Arukwe and Goksoyr, 2003; Hiramatsu et al., 2006; Matozzo et al., 2008).

Both male and juveniles fish possess hepatic oestrogen receptors, and the potential to produce VTG (Kime et al., 1999; Zhang et al., 2005). Mature female fish will normally be exposed to endogenous oestrogens and therefore produce high levels of VTG in contrast to the low VTG levels normally found in males and juveniles (low endogenous oestrogen levels) (Rose et al., 2002). VTG is therefore not only measurable in the plasma of reproductive active female fish but potentially also in males and immature exposed to exogenous oestrogens or oestrogenic compounds. The fact that VTG expression can be up-regulated and accumulate in the plasma of male fish and juvenile fish (Purdom et al., 1994) has been utilized as *in vivo* bioassays for oestrogenicity screening (Sumpter and Jobling, 1995).

Initially rainbow trout (*Oncorhynchus mykiss*) was used extensively to assess oestrogenic activity of man-made compounds or study oestrogenicity in aquatic environments (Harries et al., 1996; Jobling and Tyler, 2003). However, due to its large body size and relatively late sexual maturation, the rainbow trout has not been included in the OECD's and USEPA's lists of preferred species. The OECD programme includes three small fish species, zebrafish (*Danio rerio*), Japanese medaka (*Oryzius latipes*) and the fathead minnow (*Pimephales promelas*) as preferred species to be used in VTG and other reproductive related bioassays. Since all three species have been used widely in ecotoxicology, several physiological and biochemical biomarkers have been well-studied in these species (Kime, 1998;

Braunbeck et al., 2003). The proposed test species share common features, namely, small body size, robustness, and short life cycles. An important development was the use of juvenile fish in VTG bioassays (Tyler et al., 2000). Although the use of juvenile fish may make it necessary to measure VTG in whole body homogenates, the advantage of excluding mis-identifications of adult sex is important (Pool and Van Wyk, 2007; Slabbert et al., 2007). Recently, researchers also used *vtg* gene expression (VTG mRNA) during the developmental programme of fish as biomarker for oestrogenicity (Esterhuyse et al., 2009). One of the advantages includes the relative short exposure time (Bögi et al., 2002; Helbing et al., 2003).

It was pointed out by Ankley et al. (2009) that sexual development and differentiation during the larval and juvenile stages of development offer a "window" of sensitivity to xenobiotic chemicals since this window corresponds to the period of hormonal control driving sex determination. Therefore, if exposure to EDCs take place inside the sex determination developmental window, information on biased sex ratios and interference with gonadal differentiation can be used can to assess oestrogenicity (Andersen et al., 2003) or inhibition of aromatase (Ankley et al., 2002; Villeneuve et al., 2006). Recently, much attention has also been given to the terminal enzyme, aromatase cytochrome P450 (CYP19) in the oestrogen biosynthetic pathway responsible for catalyzing the irreversible transformation of male hormone, testosterone (androgen) to female hormone 17β-estradiol (E_2; oestrogen) (Hinfray et al., 2006; Esterhuyse et al., 2008; Villeneuve et al., 2009; Hinfray et al., 2010; Massari et al., 2010).

1.2.2. Androgenicity

Research suggests that early life exposure to environmental chemicals (EDCs) may be causative factors to many male human disorders such as testicular dysgenesis syndrome, cryptorchidism, hypospadias, decreased penile length, reduced sperm quality and testicular cancer from sources associated with chlordecone-producing companies (Waring and Harris, 2005), PCBs (Aneck-Hahn et al., 2007) and insecticides such as p,p'-DDE and Endosulfan (Kelce and Gray, 1999; Dalvie et al., 2003; 2004). Cases of cryptorchidism in infants especially have been proposed to be caused by in utero exposure to anti-androgenic compounds found in the environment (Gray et al., 1999; Kortenkamp and Faust, 2010). This led to initial human-related findings of exposure studies with rats and maternal exposures to anti-androgenic compounds such as the androgen receptor (AR) antagonist, Flutamide and several pesticides and fungicides (Kelce et al., 1994; Kelce et al., 1995; Gray et al., 1997; Kelce et al., 1997; Gray et al., 1998; Gray et al., 1999; Kelce and Gray, 1999). Apart from anti-androgenic properties of some contaminants, xenobiotic agonistic, androgenic, action may also be possible. Although limited information regarding environmental chemicals acting as androgens have been published, the growth promoter Trenbolone, used in livestock feedlots and known to contaminate downstream water systems, has been implicated to cause masculinizing effects in

female aquatic organisms (Ankley et al., 2003; Sone et al., 2005; Cripe et al., 2010; Morthorst et al., 2010; Olmstead et al., 2012). Orlando et al., (2007) reported masculinized development and decreased embryo production in mosquitofish collected downstream of a Paper and Pulp Mill in Florida, USA. Furthermore, androgenic or anti-androgenic contaminants might also discharge from wastewater treatment works (WWTWs) into receiving rivers in the same way as found for oestrogenic pollutants (Urbatzka et al., 2007c; Jobling et al., 2009; Metcalfe et al., 2013). Androgenic activity in rivers may also be the result of microbial degradation of phytosterols (Jenkins et al., 2004). Apart from general (anti)-androgenic activity in rivers and lakes, several compounds have been tested to have anti-androgenic activity; including fungicides, insecticides and herbicides, industrial compounds like Bisphenol A (BPA) and Nonylphenol, diesel exhaust particles as well as sunscreen (Urbatzka et al., 2007b).

EDCs can inhibit the normal function of the HPG axis by interfering signalling pathways such as the activation or blocking of androgen or oestrogen hormone receptors (Orton et al., 2012) or by the interference of enzymes (such as CYP19 and/or 5α-reductase) necessary for required steroid biosynthesis and metabolism (Urbatzka et al., 2007a; Langlois et al., 2010; Hecker et al., 2011; Ankley et al., 2012).

The primary steroid hormone testosterone (T) is metabolized in target tissues to a more active androgen, 5α-dihydrotestosterone (5α-DHT) by the enzyme 5α-reductase (5αR) (Urbatzka et al., 2009; Wu et al., 2012; 2013). This conversion of T to DHT may be an important step in the regulation of several secondary sexual traits, for example in male sexual dimorphic traits or other reproductive endpoints responding to DHT rather than T (Urbatzka et al., 2009). The gonads and the prostate in mammals have been shown to be the primary source of 5α-reductase enzymes (Norris and Carr, 2006). Two isoforms of the enzyme 5α-reductase exists namely type 1 (*srd5a1* gene) and type 2 (*srd5a2* gene) and are differentially expressed in different target tissues (Lorenz et al., 2011; Wu et al., 2012). Knowledge about EDCs having effects on the expression and function of 5α-reductase are lacking (Urbatzka et al., 2009).

Clearly, screening for EDCs in the environment that may modulate the reproductive system of aquatic organisms should be extended to include (anti-androgenic) bioassays as well.

1.2.3. Thyroid modulation

Given the importance of thyroid hormone for growth and development and several physiological functions in vertebrates, including humans, it seems reasonable to be concerned that environmental contaminants could influence thyroid functioning and signaling to the extent that populations would be adversely affected (Brucker-Davis, 1998; Hutchinson et al., 2006; Miller et al., 2009). Because of the highly conserved

nature of TH life history (synthesis, transport, regulation, and metabolism) environmental contaminants that affect thyroid function in one vertebrate species may well affect the TH functioning in another, including humans (Cheek et al., 1998; Norris and Carr, 2006). Zoeller and Tan (2007) recognized that while the general functionality of the thyroid system may be the same among vertebrates, there are species-specific differences to be considered; for example, the role of thyroid hormones in controlling amphibian metamorphosis is likely to be different in human development. However, applying vertebrate models to answer the question of whether, or how a contaminant interferes with, or modulates thyroid hormone action or any other aspect of the life history of these hormones, does contribute in initial screening programmes.

Considering the important and wide-spread role of thyroid hormones, and the concern that thyroid disruption may be more than a hypothesis, the development and validation of bioassays to identify potential thyroid-disrupting contaminants (TDCs) received much attention recently (DeVito et al., 1999; Degitz et al., 2005; Fort et al., 2007; Zoeller and Tan, 2007; Crofton, 2008). The challenge of using bioassays to screen for TDCs was to capture the variety of potential points of modulation within the thyroid endocrine system. The one outstanding feature of the thyroid system is the relative large number of regulatory points, which makes it difficult to put together a battery of i*n vitro* screens. It is therefore not surprising that the initial focus in screening for general thyroidal functional effects was on *in vivo*, for example the *Xenopus* metamorphosis assay (XEMA) (Opitz et al., 2005; Fort et al., 2010), rather than *in vitro* bioassays. Assays like XEMA however are labour intensive and may include relatively long exposure periods (Kloas et al., 2003; Degitz et al., 2005; Miyata and Ose, 2012). Molecular endpoints (TH dependent gene expression), for example thyroid hormone receptor (TR) mRNA expression (*trα* and *trβ*) have been quantified following short-term exposure (24 to 48 hours)(Crump et al., 2002; Opitz et al., 2006; Helbing et al., 2011).

Because of the pivotal regulatory role of thyroid hormone (TH) during amphibian metamorphosis and because of extensive research that has been done at several levels of the biological organization, amphibian metamorphosis has been targeted as an *in vivo* model system to screen for thyroid disrupting activity (see Van Wyk, 2013). The USEPA reviewed candidate anuran species and recommended that the African Clawed Frog, *Xenopus laevis* be used in a metamorphosis assay (*Xenopus* metamorphosis assay, XEMA) (Touart, 2002; Fort et al., 2007). The XEMA is basically a morphological assay designed to detect effects of contaminants on metamorphic development, the rationale being that morphological alterations during development reflect effects on TH function. Although the conceptual framework for the XEMA has been developed and the assay recently validated in the USA, Germany, UK and Japan but also here in South Africa (Van Wyk 2013), allowing local scientists, to screen locally used chemicals or water sources for thyroid disruption. It has been recognized that there is a need to include additional endpoints

(morphological, biochemical and genetic) as biomarkers to understand the extent of thyroid disruption (Bögi et al., 2003; Opitz et al., 2005; Van Wyk, 2013), to be implemented alongside XEMA or as short-term preliminary screening tools. For example, additional biochemical markers for specific pathways as well as molecular tools (differential gene expression of TH receptors) have been used as early warning sign-posts for potential thyroid disruption. Although the original XEMA was set-up as a semi-static renewal exposure system, the recommended protocol for XEMA includes a sophisticated flow-through system (Degitz et al., 2005; Tietge et al., 2013). However, in developing countries the semi-static renewal system will be the first step in assessing potential thyroid disrupting activity for a while to come (Van Wyk 2013). More importantly, the time has come to initiate screening and testing of local environmental chemicals and water sources for thyroid disrupting activity in South Africa.

Initial studies using XEMA in South Africa showed that local water source may show either thyroid stimulation or antagonistic activity but also showed the value of using whole body *in vivo* exposures of developing tadpoles to treated water (drinking or waste water) (Van Wyk, 2013). The value of short-term tadpole exposures (96 hours) and assessing differential expression of thyroid hormone receptor mRNAs (Veldhoen and Helbing, 2001; Opitz et al., 2006; Zhang et al., 2006; Opitz and Kloas, 2010) has also confirmed (Van Wyk, 2013) and awaits to be employed locally. Bögi et al., (2002) demonstrated that extending the XEMA protocol to completion of metamorphosis allows for the assessment of organizational effects (Guillette et al., 1995) on reproductive development and expression of sex (sex ratio).

Apart from using amphibians as models for thyroid disruption screening, the use of developing fish has also been recognized (Blanton and Specker, 2007). Van Wyk (2013) suggested that several local fish species have the potential to be used as bioindicator models in EDC studies, for example, *Oreochromis mossambicus*, a species extensively used in the aquaculture industry (Esterhuyse et al., 2008; Esterhuyse et al., 2009; Mlambo et al., 2009; Barnhoorn et al., 2010; Esterhuyse et al., 2010; Swart et al., 2010) and the sharp tooth catfish, *Clarias gariepinus* (Barnhoorn et al., 2004; Marchand et al., 2008; McClusky et al., 2008).

The success of using short-term exposures and quantifying the TRβ mRNA in *Xenopus laevis* tadpoles was the impetus for characterizing similar genes in a local freshwater fish species, *Oreochromis mossambicus* (Van Wyk, 2013). This species has a wide distribution in natural river systems in South Africa and is widely used in ecotoxicological studies (laboratory and field studies). Moreover, it is a well-known aquaculture species and the availability of juvenile and adult fish allows for exposure designs including large numbers of same stage fish in a laboratory exposure set-up. Relative quantifications of gene expression of *O. mossambicus trβ* and *trα* can now be performed and the upregulation of thyroid hormone receptors after a 48 hour exposure of juveniles (20 dpf) to thyroid hormone (T_3) confirmed (Van Wyk, 2013). Similar to *Xenopus laevis* tadpoles, juvenile tilapia fish hold great potential to be

used in short-term exposure studies to indirectly assess effects on the thyroid system by evaluating the differential expression of the TRβ mRNA but at the same time studies focusing on thyroid histomorphology assessments (Grim et al., 2009). *Oreochromis mossambicus* therefore represent a model local fish species to be used to investigate thyroid modulation in wild-populations surviving in polluted water sources or conduct short-term exposure of juvenile fish to water samples in the laboratory and evaluate TR-mRNA expression variation following a short exposure period (Van Wyk, 2013).

In summary, xenobiotic interference with male and female reproduction (HPG axis), including sex determination and differentiation, can occur at several levels; the control pathways of steroid hormone control (positive and negative control), steroidogenesis pathways, transport and metabolism of steroids, uptake into the target cell, binding to steroid receptors, and expression of response proteins. On the other hand, the potential interference in the HPT axis pathways include several additional points of potential modulation, since the production of thyroid hormones require iodine and thyroid hormones are stored as colloid in the thyroid follicles. Although it is generally anticipated that an exogenous EDC can either be an agonist or an antagonist, a concentration effect could be possible (Hogan et al., 2008; Van Wyk, 2013). Therefore, given that effluents are complex mixtures, exposure to effluents could potentially affect all three systems simultaneously, but differentially. Hogan et al. (2008) used the production of VTG and the male protein, spiggin as two endpoints in the stickleback fish to study androgenic and oestrogenic effects in water samples. Although it is not always possible to have biomarkers for more than one endpoint in the same organism, several studies showed the utility of using molecular techniques, for example quantitative PCR to study expression of receptor or other protein mRNAs following short term (12-48 hours) exposure (Veldhoen and Helbing, 2001; Bögi et al., 2002; Villeneuve et al., 2007; Massari et al., 2010). Zoeller and Tan (2007) recognized that while the general functionality of the thyroid system may be the same among vertebrates, there are species-specific differences to be considered; for example, the role of thyroid hormones in controlling amphibian metamorphosis is likely to be different than in human development. However, applying vertebrate models to answer the question of whether, or how a contaminant interferes with, or modulates thyroid hormone action or any other aspect of the life history of these hormones, does contribute in initial screening programmes.

1.3. Mixtures and Mode of Actions

Burger and Nel (2008) identified the need for specific studies related to the variation in chemical use among certain agricultural regions and suggested that the next level of investigation in the South African context should, as a starting point, investigate relevant agrochemical mixtures, specifically selected to represent potential mixture combinations associated with specific intensive and concentrated crop cultivation agricultural practices in the region. The rationale being that selected mixtures would

exclude other non-agricultural environmental chemicals, masking the specific endocrine disruption activity of specific agrochemicals but recognizing the importance of mixtures in potential agrochemical exposures.

This sentiment was also echoed internationally and increasingly scientists recognized that laboratory exposure studies using single chemical exposures to characterize the potential mechanism of action of any given pesticide may have limited value when it comes to understanding complex environmental mixtures as found in rivers and manmade impoundments (Andersen et al., 2002). For example, in pulp and paper mill discharges containing androgenic and oestrogenic compounds may combine with other pollutants from sources such as spray drift or leeching agricultural pesticides or compounds from wastewater treatment plants (WWTPs) (Blake et al., 2010). In the agricultural sector, single or different agricultural crops located near each other are regularly treated with different pesticide types (insecticides, nematocides, herbicides, fungicides) (Bollmohr et al., 2008; Ansara-Ross et al., 2012; Dabrowski and De Klerk, 2013; Dabrowski et al., 2014), potentially treated with chemicals having similar mechanisms of endocrine disrupting effects (therefore additive) modes of action or dissimilar modes of action (therefore independent or antagonistic).

Backhaus and Faust, (2012) pointed out that different chemicals in environmentally relevant aquatic mixtures may not have similar effects on endocrine systems and may therefore interact differently than expected laboratory mixture. However, *in vitro* investigation of a directed endocrine disrupting mechanism of action (such as AR antagonism and steroidogenesis inhibition) will help understand the interaction of contaminants which might end-up in complex mixtures in the environment. Although it is important to confirm the suggested MOA of single compounds (contaminants), it is also important to predict (based on their individual effects) and validate the behaviour of chemicals in mixture. It has been stated in the literature that when investigating the combined effect of EDCs, having the same MOA, it is mostly assumed that a dose addition response will result, so-called additivity null hypothesis (Boone, 2008; Ankley et al., 2010; Ermler et al., 2011; Christiansen et al., 2012; Orton et al., 2013). However, this may not always be the case. Equimolar mixtures of the fungicides epiconazole, tebuconazole and propiconazole, for example, all revealed individual AR antagonistic mechanisms of anti-androgenic action, but in mixture had lower AR antagonistic properties than the predicted additivity model (Kjærstad et al., 2010). A study by Ermler et al. (2011) investigating the AR antagonistic potential of 17 anti-androgens showed that despite varied structural features of the individual compounds, the concentration additive (CA) concept provided a good prediction of the mixture effects having a similar MOA. In terms of the AR antagonistic potential of parabens, their results were in contrast to those reported by Kjærstad et al. (2010) showing deviation (and underestimation) from the CA prediction. It seems that the experimental approach need careful consideration,

since Ermler et al. (2011) suggested that the equimolar approach may not be reliable.

A toxicological mixture interaction which is overlooked in EDC mixture investigations is the occurrence of potentiation. In a study using antimicrobials, such as triclocarban found in the environment may not have an endocrine disrupting action on its own, but in mixture with testosterone induce a greater than expected androgenic response (Blake et al., 2010). On its own, a chemical may therefore show false negatives when screened for endocrine disrupting activity, but in mixture will influence the effect of another ligand and give rise to either an elevated or masked endocrine disrupting effect. It is evident that studies regarding chemical mixtures need more attention, especially in environmental scenarios, and that the additive hypothesis cannot just be an assumption to be used in risk modelling programs.

1.4. Agrichemicals

The pesticide industry is recognized as an important economic sector in the world market (more than 1000 active ingredients), mainly because this sector has grown exponentially along with the growth in industries, for example, agriculture, associated with the use of pesticides to control weeds (herbicides), insects (insecticides), fungi (fungicides) as well as plant and animal diseases that interfere with marketability of produce (Parris, 2011; Plakas and Karabelas, 2012). Pesticides have been used widely for non-agricultural or domestic horticultural purposes though, for example, herbicides are used to eradicate alien species from valuable freshwater catchment areas (DWAF, 2014). Today pesticides are present in most compartments of the environment and detected in ground water as well as surface water, used as source for drinking water (Plakas and Karabelas, 2012). No surprise that authorities around the world are concerned about the potential health effects that pesticides may pose, even at very low concentrations (pg/L - ng/L). The World Health Organization (WHO) report published recently (Bergman et al., 2013) refers to epidemiological evidence linking pesticides to human effects, including malformations, neuro-developmental disorders and effects on the immune system, male reproduction, prostate cancer, thyroid cancer as well as breast cancer. Links to several chronic diseases, genetic damages and epigenetic modifications in humans have also been implicated (Hamid and Eskicioglu, 2012; Bergman et al., 2013; Mostafalou and Abdollahi, 2013).

Freshwater habitats are experiencing rapid biodiversity modifications largely due to agricultural practices (Downing et al., 2008), as agrochemicals have become major sources of water pollution, with devastating health impacts on humans and wildlife (DWAF, 2014). Estimates of freshwater biodiversity loss have been suggesting that the current rate of extinction is the most profound experienced in a long time (Dudgeon et al., 2006; Vaughn, 2010). The concern about the global amphibians decline problem for example (Dinehart et al., 2009; Brühl et al., 2011; Brühl et al., 2013), highlight the need to assess the potential impact of agricultural chemicals on these non-target organisms.

In spite of the increase in laboratory based research suggesting a direct link between certain environmental chemicals (including pesticides), it still remains unclear to what extent environmental relevant concentrations of these compounds does have adverse effects in wildlife individuals and ultimately also populations (Hecker and Hollert, 2009; Hecker and Hollert, 2011). Moreover, the structural diversity found among pesticides make predictions of relationships between chemical structure and endocrine disruption, at whatever level, very difficult.

Internationally regulatory frameworks and best pesticide-use practices programmes have been put forward to limit pesticide presence in drinking water (Karabelas et al., 2009; Bergman et al., 2013). A significant growth in advanced technological to remove pesticides during treatment of drinking water and waste water occurred and continue to do so (Hamid and Eskicioglu, 2012; Sanches et al., 2012; Mostafalou and Abdollahi, 2013; Sadmani et al., 2014), since the initial concerns voiced by Carson (1962).

1.5. Non-specific toxicity threat of agrichemicals (stage dependent toxicity and the Frog Embryonic Teratogenic Assay (FETAX))

Amphibian vulnerability to agrichemicals has been linked to their specific ecological requirements linking them to permanent or temporary water-bodies to complete their life cycle. However, it is these very same water pools and shallow water-bodies or ponds where agrochemicals easily contaminate and accumulate (Mann and Bidwell, 2001). Although it is known that many agrichemicals bind to the sediment the assumption that these chemicals pose relatively little risk to non-target organisms, have been questioned (Relyea, 2009; Brühl et al., 2011) and is it therefore more realistic to assume direct exposure of early life stages in the aquatic environment. Apart from these early stages being sensitive to the chemical component of the environment, early organisational effects may have long term health effects (Guillette et al., 1995).

The global use and presence of the agrichemicals in aquatic systems have been proposed as a contributing factor to the hypothesized declines in amphibian populations (Bishop et al., 1999; Bernabò et al., 2011; Güngördü, 2013; Lajmanovich et al., 2013). Environmental surveys have also reported an association between amphibian population declines and proximity to agricultural lands (Bishop et al., 1999; Mann et al., 2009; Brühl et al., 2013; Edge et al., 2013; Lanctot et al., 2013). But despite the proposed causal links to pesticide exposures, research emanating from developing countries are mostly absent or largely inadequate (Ansara-Ross et al., 2012).

For most pesticides, ecotoxicology studies report LC_{50} concentrations for aquatic organisms, with reference mostly to fish species (aquatic representative), while information regarding toxicity to amphibians are generally scanty (Mann et al., 2009; Relyea and Jones, 2009; Egea-Serrano et al., 2012; Wagner et al., 2013). It has

been shown that amphibians may be just as sensitive to environmental contaminants as fish, due to their permeable skin, therefore advocating the need for studies on potential links between exposure to agricultural chemicals and global amphibian declines.

Variances in acute susceptibility to chemicals and pesticides have been observed among developmental stage of amphibians (Edginton et al., 2003; Greulich and Pflugmacher, 2003). For example, Edginton et al. (2004) in a study on the comparative effects of an herbicide formulation pointed out that *Xenopus laevis* and *Rana pipiens* larvae were 6.8 to 8.9 times more sensitive than their embryos. Although the suggestion of larval stages of amphibians being more sensitive than other developmental stages is now established, variation in sensitivity among stages and species are still high. The use of single developmental stage as opposed to range of developmental stages needs more study and a well-establish laboratory amphibian model, *Xenopus laevis*, is well-suited to clear up the current doubt on the stage sensitivity within a developmental series.

The Frog Embryo Teratogenesis Assay-Xenopus (FETAX) is a standardised 96-hour whole-embryo assay used for the assessment of potential developmental and teratogenic effects in humans (Bantle et al., 1989; Bernardini et al., 1994; Mann and Bidwell, 2000; Leconte and Mouche, 2013; Yu et al., 2013). FETAX has been widely used in aquatic toxicity testing and is well-suited for testing environmental samples or complex mixtures (industrial and wastewater effluents) (Bantle et al., 1999). This assay essentially assesses environmental impacts during embryogenesis. FETAX is a four (4)-day (96-hr) protocol for predicting potential developmental toxicants and teratogens (ASTM, 1998). Endpoints include mortality, malformation and growth inhibition. The use of FETAX as an initial screening test before conducting an amphibian metamorphosis study to investigate disruption of the thyroid seems to be a logical step for predicting pesticide effects on early development. The FETAX assay has been widely used to assess embryotoxicity of various environmental chemicals. Moreover, Bantle et al. (1999) and Leconte and Mouche (2013) reported a predictivity of 75% and 81% respectively for mammals or human teratogenic potential. Leconte and Mouche (2013) correlated FETAX data that span 12 years of screening pharmaceutical compounds and linking results to mammalian embryotoxicity studies. As pointed out by Yu et al. (2013), although the FETAX protocol was designed to test for embryotoxicity, it does have all the potential to generate data useful in ecological risk assessments.

In spite of the wide international application of the FETAX assay, few (if any) studies in South Africa applied this assay on the pesticides used locally and most managers and decision makers must rely on data published elsewhere. It is this lack of baseline toxicity data, especially for locally-used herbicides, that limit studies focusing on non-lethal modulation effects, for example disruption of the endocrine system.

1.6. Agrichemical use in South Africa

South Africa is the highest agri-produce-producing country on the African continent (Bollmohr et al., 2008; Dalvie et al., 2009a; Ansara-Ross et al., 2012; Mensah et al., 2013; Dabrowski et al., 2014). With the high demand in food productivity due to increased population growth and increased food quality, pesticide use in South Africa represents 60% of the pesticide market in Africa (Dabrowski et al., 2014). Therefore, the high demand for local produce for the local and internal markets inevitably impact on South African water sources, higher chance of pollution on the one hand but also the demand for lower residue levels on the other hand.

Approximately 180 different pesticide active ingredients are commercially available in South Africa, with more than 500 registered trade names in use (Pan, 2014). Ansara-Ross et al. (2012) published an extensive review about all pesticide-related research conducted in South Africa since 1970. The summary tables in this report show that relative high levels of pesticides have been detected in various river systems (but still largely understudied) within South Africa (tributaries and major), including most of the main water courses and reservoirs (Ansara-Ross et al., 2012).

In addition, several recent studies attempted to prioritize pesticides or use risk assessment models, including species sensitivity distributions (SSD), to sensitize the producer and user about potential toxicity and health threats (wildlife and human non-targets) posed by pesticides (Ansara-Ross et al., 2008, 2009; Dalvie et al., 2009b; Dalvie and London, 2009; Ansara-Ross et al., 2012; Dabrowski et al., 2014). Data for these reports were mostly generated using acute exposure effects (Dalvie et al., 2009; Dabrowski et al., 2014), pesticide use and toxicity indices (Dabrowski et al., 2014). At most, indirect links have been made to pesticide-use or limited measured concentrations in water sources and published literature, with little or no reference to chronic exposure data, bioassay data, the latter mostly based on *in vitro,* sub-organismal, single chemical exposure studies (Ansara-Ross et al., 2012; Mensah et al., 2013; Dabrowski et al., 2014).

Although it is true that in reality aquatic organisms and humans using untreated water are rarely exposed to single chemicals but rather to complex mixtures (Sun et al., 2009), varying all the time, mimicking such mixtures will be unpractical. Many papers have been published showing combined affects of compounds in mixture, but most include chemicals with the same mode of action (for example oestrogenic). Sun et al. (2009) pointed out that most fish populations showing effects of endocrine disruption are associated with high levels of contamination or occurred in some extreme situation, contaminated with EDCs showing the same MOA, for example exposure to treated sewage effluents. These observations suggest that balancing though exposure to opposing MOAs may occur in nature. Unfortunately, as with most developing countries in the world, MOA information about pesticides acting as endocrine disruption contaminants in South African water systems are rare

(Bornman et al., 2007b; Burger and Nel, 2008; De Jager, 2010; Swart et al., 2011; Van Wyk, 2013) and international studies (where available) not confirmed.

McKinlay et al. (2008) listed a total of 127 pesticides as having an endocrine disruptive mode of action, but giving any specifics regarding mechanism of action. Burger and Nel (2008) listed 15 priority pesticides to consider as EDCs in South Africa. Internationally the list is growing (TEDX, 2014). Several other studies tagged several local-used pesticides as EDCs (Dalvie et al., 2009a; Ansara-Ross et al., 2012b; Dabrowski et al., 2014). Dabrowski et al. (2014) categorized only 22% of 152 active ingredients included, as possible EDCs, but mentioned a few (11) that they felt confident should be regarded as potential EDCs. However, most of these studies rely on database data (Footprint, 2014; PAN, 2014) and given the variation and conflicting results, in the primary literature, these data could be far from reliable or inclusive. Few studies directly link individual or combinations of pesticides to specific endocrine endpoints or mechanisms of action but rather refer to chemicals as being EDCs or in one of three modes of action (Van Wyk et al., 2003; Van Wyk et al., 2005; Van Wyk, 2013).

Specific endocrine (hormonal) activity have been shown to be present in selected water sources, either associated with treated waste water sources, intense farming or industrial areas (Swart and Pool, 2007, 2009a,b). To date, few studies have showed activity in all three endocrine disruption modes of action, oestrogenicity (Hurter et al., 2001; Van Wyk et al., 2005; Esterhuyse et al., 2009; Masgoret et al., 2009), androgenicity (Van Wyk et al., 2003; Aneck-Hahn et al., 2007; McClusky et al., 2007; Marchand et al., 2009; Mlambo et al., 2009) and thyroid modulation (Van Wyk 2013).

With the increasing reports of environmental effects (including endocrine modulating effects) of pesticides upon wildlife and humans raises concern over the legislation regarding pesticide usage in South Africa. The pesticide usage and registration act of South Africa (Act 36/47, Department of Agriculture, 1947; DWAF 1996) does not consider the environmental consequences or pollution of water from agricultural pesticides (London et al., 2000; London and Rother, 2000; Mensah et al., 2013).

1.7. Aquatic Vertebrate Models for EDC Studies in South Africa

Amphibians are generally regarded as good models to understand the mechanism of action for pesticides (including insecticides, fungicides and herbicides) when affecting physiological functioning (Lajmanovich et al., 2013; Attademo et al., 2014). But in general, relatively few studies used amphibians as model organisms (Mann et al., 2009; Brühl et al., 2013). The African clawed frog, *Xenopus laevi*s, a local aquatic species with a widespread distribution in Southern Africa (Tinsley and Knobel, 1996; Du Preez and Carruthers, 2009) is now a well-recognized international animal model used in most countries of the world for medical research, toxicological and endocrine disruption research (Kloas and Lutz, 2006) and listed as the preferred

species to use in several validated international assays (FETAX) (Bantle et al., 1989), recently validated as the preferred species to use when screening for thyroid disruption (XEMA test, USEPA; Grim et al., 2009). Despite the fact this species is widely (internationally) used in endocrine disruption screening programmes, it has been used on rather limited bases in South Africa, with only few studies including field studies (Hurter et al., 2002; Van Wyk et al., 2003; Du Preez et al., 2005; Jooste et al., 2005; Van Wyk et al., 2005; Du Preez et al., 2008; Van Wyk, 2013) included this species as model. The XEMA protocol for thyroid disruption has only been used in one laboratory, and only using a semi-static exposure approach (Van Wyk, 2013).

A local freshwater fish, *Oreochromis mossambicus* (Mozambique tilapia) has been widely used in South Africa as model species in ecotoxicological research (Nussey et al., 1995; Van Dyk et al., 2007). In terms of endocrine disruption, in the laboratory juvenile and adult fish were used to study relative gene expression, mainly mRNA of steroid hormone receptors (oestrogen receptor, ER) (Esterhuyse et al., 2010), enzymes (aromatase) (Esterhuyse et al., 2008) and proteins (vitellogenin, VTG) (Esterhuyse et al., 2009). The expression of Tilapia TH-mRNA was also recently included in the battery of mRNA transcripts (Van Wyk, 2013). Exposures of juvenile Tilapia fish were also used assess oestrogenicity (VTG as biomarker) (Swart et al., 2011). The disruption of other pathways (androgenic, adrenal) has not been studied locally before. Tilapia fish were also used for histopatological studies of the reproductive organs (Mlambo et al., 2009) in laboratory exposure research as well as in wild caught fish (Barnhoorn et al., 2009; 2010).

Although endocrine endpoints needs more research in *Oreochromis mossambicus*, this local species is well-suited for a freshwater species model to study endocrine disruption in water bodies as well as for laboratory exposure studies (especially using juvenile fish). In the northern (highly industrialized as well as intensively farmed) parts of South Africa (Shelton, 2001) this species occurs in most rivers and therefore exposed to a diverse mixture of manmade chemicals.

1.8. EDC Research Progress and Needs in South Africa (Pesticides)

South Africa is fast growing into a developed country, showing all the characteristics and human activities associated with a modern developed country, in the developing content of Africa. Together with the largest agricultural sector in Africa comes the increased use of pesticides and several compounds being categorized as potential endocrine disruptors, posing the risk of adversely modulating the endocrine systems of wildlife and humans, similar to what has been reported for developed countries in Europe and the Americas. However endocrine disruptor research activity remains limited with a low priority status, compared to developed countries. Initially, the focus was on studying the endocrine disrupting activity (mostly oestrogenic activity, feminization) of internationally well-known contaminants, like nonylphenol (De Jager et al., 1999; McClusky et al., 2007) and DDT (Dalvie et al., 2004; Bornman et al.,

2007a; De Jager et al., 2009; Sereda et al., 2009; Bornman et al., 2010; Van Dyk et al., 2010; Gyalpo et al., 2012; Bouwman et al., 2013). Since the realization that EDCs in South Africa may be a real environmental and human health concern, focus shifted to bioassay development and validation, which culminated in the establishment of a toolbox programme (De Jager et al., 2010). Initially, *in vivo* models were used to assess oestrogenicity (Hurter et al., 2002; Van Wyk et al., 2005; Esterhuyse et al., 2010; Swart et al., 2011) and anti-androgenicity (De Jager et al., 1999; Van Wyk et al., 2003; Aneck-Hahn et al., 2007; Kilian et al., 2007), the shift to *in vitro* bioassays followed (Swart and Pool, 2009b, a; De Jager et al., 2010; Swart et al., 2011). *In vitro* screening at a first tier level has been implemented by the USEPA in the endocrine disruptor screening programme (EDSTAC; Juberg et al., 2014). In South Africa several *in vitro* bioassays are used to screen chemicals or environmental water samples, E-screen (MCF7 cells; ER binding) (Swart and Pool, 2009b), *Xenopus laevis* liver slice bioassay (VTG production)(Hurter et al., 2002), recombinant yeast screens (ER and AR binding)(Aneck-Hahn et al., 2005; Slabbert et al., 2007; Verhoog et al., 2007a, b; Aneck-Hahn et al., 2008; Mahomed et al., 2008; Mfenyana et al., 2008; Masgoret et al., 2009; De Jager et al., 2010), Kbluc mammalian cell reporter gene assay (ER binding) (De Jager et al., 2011). In conjunction with *in vitro* studies, measuring human hormones in water sources, in particular where treated waste water effluents are discharged in rivers, have gained popularity (Swart and Pool, 2007; Swart et al., 2011; Faul et al., 2014).

In contrast to the use of the range of *in vitro* bioassays, few studies reported on *in vivo* laboratory exposures (Van Wyk et al., 2003; Mlambo et al., 2009) or endocrine endpoints (reproduction or thyroid) in aquatic vertebrates (fish and amphibians) collected from water bodies (Barnhoorn et al., 2004; Van Wyk et al., 2005; Marchand et al., 2009; Barnhoorn et al., 2010; Bornman et al., 2010; Van Wyk, 2013). Although in recent years few studies directly investigated EDC activity in aquatic systems most of these were focused on reproductive disruption (Van Wyk et al., 2005; Bornman et al., 2007b; Barnhoorn et al., 2009; Barnhoorn et al., 2010; Kruger et al., 2013).

In terms of thyroid disruption, the *Xenopus* metamorphosis assay (XEMA) to screen for thyroid disruption has recently been implemented and is now in use in South Africa (Van Wyk, 2013). In addition, the expression of Thyroid Hormone (TH) receptor mRNAs has been used in short-term tadpole and fish exposures (Van Wyk, 2013; Truter et al., 2014).

The fact that South Africa is a major user of pesticides in Africa and reports of the presence of pesticides and their metabolites in local water bodies makes it surprising that so few studies report on *in vitro* and *in vivo* exposures and/or collection of sentinel species from natural water bodies. Prioritizing locally used pesticides, using physical properties and published toxicity data, whether the chemical compound may be regarded as an EDC or not, is an important management exercise. However, the importance of considering the specific mode of action, especially if mixtures are to be considered, needs to be addressed when considering potential endocrine disruptive

activities. On the other hand, in South Africa, health assessment of aquatic vertebrates needs more attention. Information about the pesticides in use, together with potential modes of actions and behaviour of pesticides when in mixture, allows only for making predictions about real exposures in the field situation. Local populations in, specifically in potentially affected ecosystems, need to be studied from an endocrine disruption perspective (reproductive and thyroid modulation).

The objective of this study was to begin to evaluate the potential endocrine disruption potential of selected pesticides (individual and binary mixtures) representing the three different classes (insecticides, herbicides and fungicides) and to collect animals (fish and amphibians) from selected environmental sites (suspected to be polluted) to assess the possibility of endocrine disruption caused by pollutants (including pesticides).

2. Laboratory Studies: Summary of results

2.1. Pesticides: Oestrogenic activity: *In vitro* screening and mixture studies

Researcher: E Jansen van Rensburg, PhD, Stellenbosch University

2.1.1. Aims

The aim of this study was to assess potential oestrogenic activity of four insecticides (used in the Western Cape agricultural region) as well as the potential interaction of the selected pesticides when applied in a mixture.

2.1.2. Methods

Pesticide selection

Based on use and environmental detection, four insecticides were selected. Literature references were used to predict the potential oestrogenic activity (Table 1).

Table 1: *Four insecticides selected for oestrogenic assessment as individual chemicals or as binary mixtures.*

Active Ingredient	Oestrogenicity	Reference
Azinphos-methyl	Suspected	Croplife, 2009 Pesticide Action Network (PAN, 2014)
Carbaryl	Oestrogenic	Cocco, 2002
Chlorpyrifos	Weakly oestrogenic	Croplife, 2009 Viswanath et al., 2010
Endosulfan	Oestrogenic	Andersen et al., 2002; Bulayeva and Watson, 2004; Lemaire et al., 2004

T47D-kBluc assay (oestrogenicity and anti-oestrogenicity)

In vitro methods were developed to provide a tool to screen for endocrine disrupting activity of compounds and/or water sources. In order to assess the oestrogenicity of water samples, the USEPA developed a stably transfected cell line using the T47D human breast cancer cell line. This cell line contains both endogenous oestrogen receptors, ER-α and ER-β, and was transfected with an oestrogen responsive element (ERE) luciferase reporter gene construct (De Jager et al., 2011).

All chemicals were obtained from Sigma Chemical Company. The cells were obtained from the American Type Culture Collection (ATCC, catalog no CRL-2865), Manassas, USA, through the University of Pretoria's Ecophysiology Laboratory. The assay was performed as described in Wilson et al. (2004), including modifications as

described in De Jager et al. (2011). In brief, the cells were cultured in RPMI medium in the incubator for a week, before being placed in starvation medium in preparation for the assay. The assay medium was mixed with RPMI medium to prepare medium containing 5% dextran/charcoal filtered FBS. The cells were then seeded at 5×10^4 cells per well in 96-well luminometer plates and left to attach overnight. The next day the cells are exposed to the test chemicals which are prepared in the medium and once again left to incubate overnight. The next day the cells are lysed using the reaction buffer and placed in the freezer. Once the plates have been in the freezer long enough for the medium to freeze, the plates were removed since the lysis buffer was activated by a freeze-thaw cycle. The luciferase activity was determined using a luminometer. With the T47D-kBluc assay, oestrogen (*17β-oestradiol,* E_2) was used as the positive control (causing a dose-dependent luciferase activity) that can be inhibited by the anti-oestrogen ICI.

The single chemicals and the mixtures were evaluated using two parameters, the Relative potency (RP) and the Relative induction efficiency (RIE) (Fang et al., 2000; Versonnen et al., 2003). The RP is the ratio of the E_2-EC_{50} to the EC_{50} of the tested chemical, while the RIE is the ratio of the maximum absorbance of the test chemical to the maximum absorbance of the E_2.

The assay was performed at the EDC laboratory of the University of Pretoria (Dr. N. Aneck-Hahn). All calculations were performed using Graphpad Prism 6.0 and Microsoft Excel.

2.1.3. Results

Single Chemicals

Azinphos-methyl did not show significant interaction with the ERs and therefore not presented in single chemical response graphs. Carbaryl showed some interaction with the ERs (Fig. 1A) and displayed a relatively low RP and a moderate RIE compared to E_2 (Table 2). This result correspond with the report of Cocco (2002), reporting that Carbaryl has been shown to be weakly oestrogenic in *in vitro* studies and may have negative impacts on fertility in animals. Chlorpyrifos showed interaction with the ERs (Fig. 1B), but also displaying a very low RP and a RIE of less than 30% (Table 2). According to Juberg et al. (2013), Chlorpyrifos is not interactive with ERs when tested using a rat uterine cytosol oestrogen receptor binding assay. However, when using an oestrogen receptor transactivation assay with the stably transfected human hERa-HeLa-9903 cell-line, a slight elevation of oestrogen receptor mediated response at the highest concentrations was reported. In the current study, the T47D kBluc reporter gene assay showed that even at the highest point of induction Chlorpyrifos did not reach 40% of the maximum induction by E_2. This confirms that Chlorpyrifos as a single chemical can be regarded as a weak oestrogen. Endosulfan interacted with the ERs, but as with the other chemicals showed cytotoxicity at higher concentrations (Fig. 1C). Using the T47D kBluc assay,

endosulfan induced its maximal response at 10^{-6} M. Endosulfan had by far the highest RP of all the chemicals tested and a calculated RIE of 41% (Table 2). Andersen et al. (2002) also showed that Endosulfan will increase cell proliferation and ER transactivation in MCF7 cells.

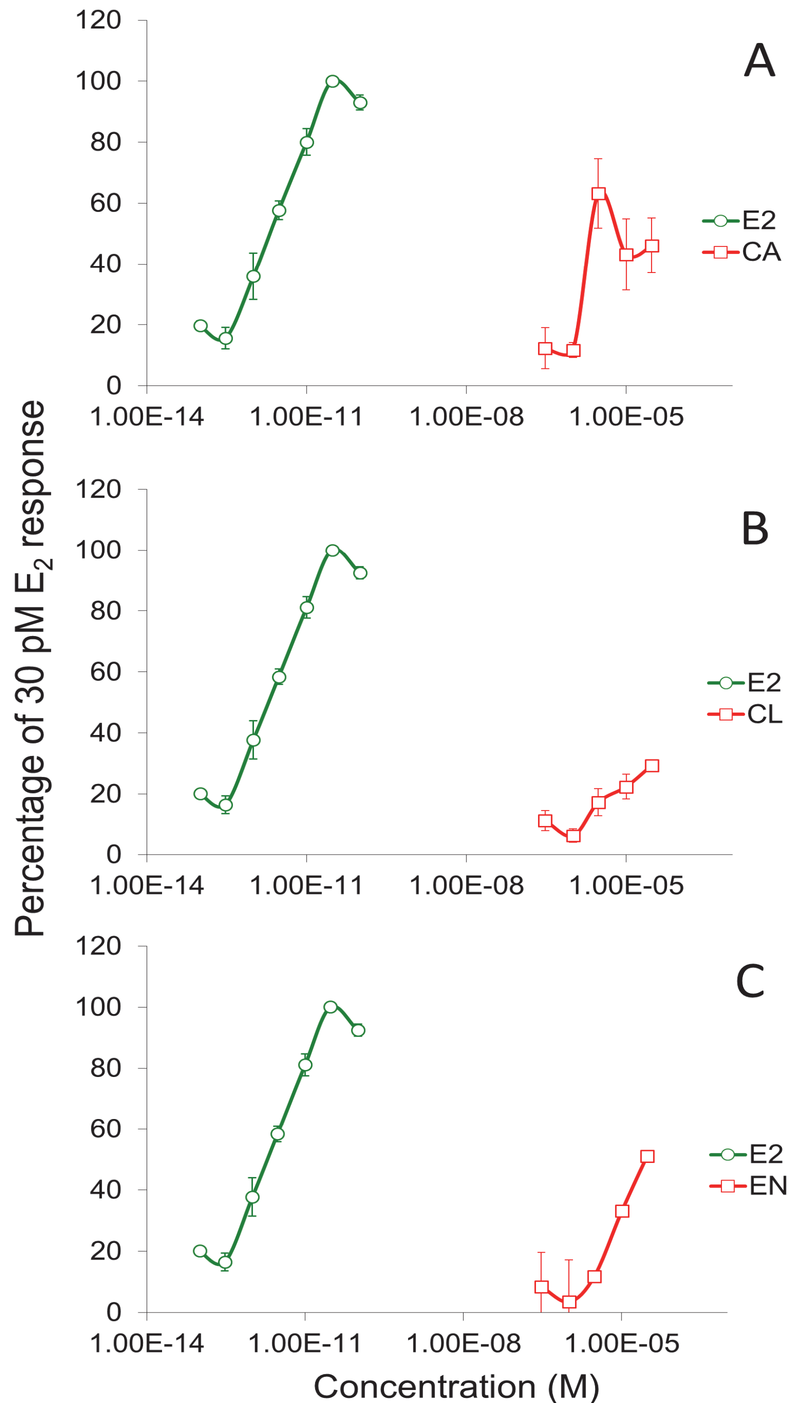


Figure 1: *Concentration-response curves for the positive control (17β-estradiol, E_2) and the pesticides, Carbaryl (CA;* ***A****), Chlorpyifos (CL;* ***B****) and Endosulfan (EN;* ***C****), inducing oestrogen receptor (ER) transactivation in T47D human breast cancer cells. Data points represent the mean of duplicate samples.*

Table 2: *Calculated oestradiol (E_2) EC_{50} values, Chemical EC_{50} values, Relative Potency (RP) and Relative Induction Efficiency (RIE) for the selected insecticides.*

Chemicals	EC_{50} E_2	EC_{50} Chemical	RP (%)	RIE (%)
Carbaryl	$2.29E^{-12}$	$7.13E^{-07}$	0.000346	56
Chlorpyrifos	$2.29E^{-12}$	$2.28E^{-06}$	0.000101	28
Endosulfan	$2.29E^{-12}$	$8.36E^{-07}$	0.001245	41
Carbaryl & Chlorpyrifos	$1.13E^{-12}$	$3.55E^{-06}$	0.000123	19
Carbaryl & Endosulfan	$1.59E^{-12}$	$2.25E^{-06}$	0.000309	36
Chlorpyrifos & Azinphos-methyl	$1.13E^{-12}$	$3.67E^{-07}$	0.000316	76
Chlorpyrifos & Endosulfan	$1.59E^{-12}$	$1.34E^{-05}$	$1.21E^{-05}$	33
Endosulfan & Azinphos-methyl	$1.59E^{-12}$	$3.55E^{-06}$	0.000263	30

Mixtures

Carbaryl and Chlorpyrifos as an equimolar mixture has a higher EC_{50} value than either of the individual chemicals (Fig. 2A). The RP for this mixture was lower than for their individual chemicals (Table 2). However, the RIE of this mixture was only slightly higher than calculated for Chlorpyrifos alone, even-though Carbaryl has a much higher RIE (Table 2). In the case of the equimolar mixture of Chlorpyrifos and Azinphos-methyl (Fig. 2B), the mixture showed a higher RP and RIE than Chlorpyrifos alone (Table 2), and higher than Azinphos-methyl. Azinphos-methyl showed almost no interaction with the ERs when considering individually during the initial screening.

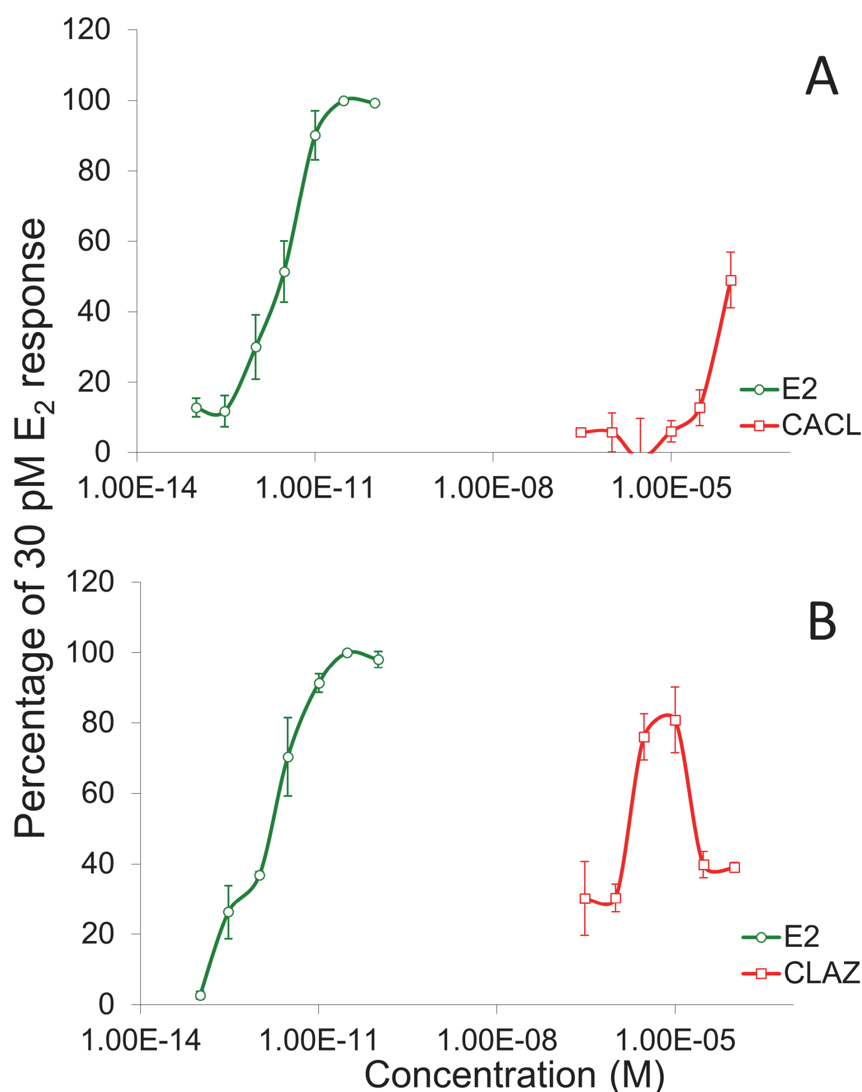


Figure 2: *Concentration-response curves for the positive control (17β-estradiol, E_2) and binary pesticide mixtures, Carbaryl-Chlorpyifos (CACL; **A**), Chlorpyifos-Azinphos-methyl (CLAZ; **B**), inducing the oestrogen receptor (ER) transactivation in T47D human breast cancer cells. Data points represent the mean of duplicate samples.*

The equimolar mixture of Endosulfan and Azinphos-methyl did not show anything unexpected (Fig. 3A). Both the RP and the RIE of this mixture fall between the values for Endosulfan and Azinphos-methyl alone (Table 2). The equimolar mixture of Endosulfan and Chlorpyrifos (Fig. 3B) showed a far lower RP than that of the separate chemicals, while the RIE was closer to the mean (Table 2). Although Chlorpyrifos showed some interaction with the ER (Fig. 1B), Azinphos-methyl, showed almost no interaction at all during the screening process. However, when these two insecticides were mixed they showed the highest induction efficiency of all the test chemicals or mixtures assessed (Table 2). The equimolar mixture of Carbaryl and Endosulfan displayed a higher EC_{50} value than either one of the single chemicals (Fig. 3B). The RP for this mixture and for Carbaryl alone is very similar,

while the RIE of this mixture is lower than either of the single chemicals (Table 2). In a study by Presibella et al. (2005), female rats were exposed to mixtures of Endosulfan and Deltamethrin daily, but did not show any adverse effect. This lead to the conclusion that either Endosulfan and/or Deltamethrin did not show the same effects in *in vivo* and *in vitro* studies, or that these chemicals were not delivered to the target organs. The suggestion is that perhaps there were interactions between the chemicals when exposed together in a mixture, which may mask some of the possible effects resulting from exposure to Endosulfan alone.

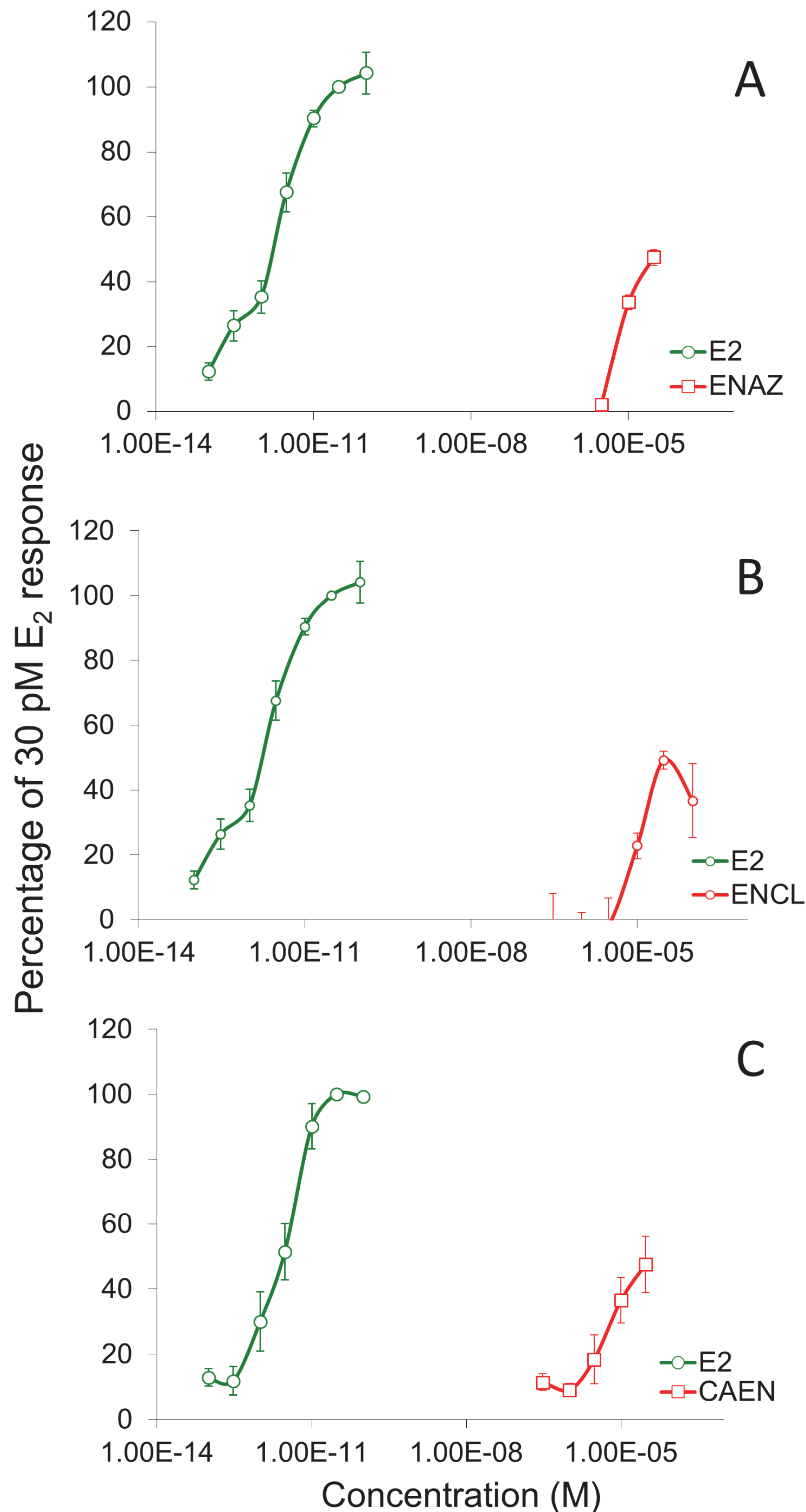


Figure 3: *Concentration-response curves for the positive control (17β-estradiol, E_2) and binary mixtures pesticides, Endosulfan-Carbaryl (ENCA;* ***A****), Endosulfan-Chlorpyifos (ENCL;* ***B****) and Carbaryl-Endosulfan (CAEN;* ***C****), inducing the oestrogen receptor (ER) transactivation in T47D human breast cancer cells. Data points represent the mean of duplicate samples.*

2.1.4. Conclusions

Three of the four pesticides selected showed interaction with the ER, albeit low and therefore considered weak oestrogenic EDCs for this particular assay. Relative potency is the potency of the chemical tested in comparison to the positive control where the positive control = 100%. If the RP of a test chemical is 10% it means that a 10 times greater concentration would be needed to obtain an EC_{50} of the test chemical compared to the EC_{50} of the positive control (De Jager et al., 2011). For all the chemicals tested, the relative potency was extremely low. During the screening process, higher concentrations of the test chemicals (above 0.2 M) all showed cytotoxicity, therefore higher concentrations will not lead to greater effects, but merely lead to cell death.

Although additivity was suggested in some of the mixture combinations of the present study, in other combinations, suggestions of antagonism or independent action were observed. However, this represents a preliminary study and more research is needed to understand the interaction of commonly-used insecticides with ERs, as well as interaction when in mixture. Additional mechanism of action pathways, for example modulation of the aromatase enzyme, catalyzing the androgen-oestrogen transformation in the steroidogenesis pathway, should be investigated.

2.1.5. Location of the full version:

Jansen van Rensburg, E. 2014. *Endocrine disruption effects of agrichemical mixtures in the aquatic environment: Bio-responses and linkage to population-level impacts*. Chapter in PhD Dissertation, Stellenbosch University, Stellenbosch University.

2.2. Herbicides as biological toxicants and endocrine disruptors

Researcher: O.O. Babalola, PhD, Stellenbosch University.

2.2.1. Specific aims of this project

- To assess comparative toxicity and Identification of most sensitive developmental stage of *Xenopus laevis*.
- To assess lethal and teratogenic Impacts using Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX)
- To assess potential thyroidal disruption by Glyphosate, Imazapyr, Diquat dibromide and Glufosinate ammonium formulations using the *Xenopus* Metamorphosis Assay (XEMA).
- To assess the potential developmental and reproductive impacts in post-metamorphic juveniles.
- To assess reproductive and thyroid endocrine impacts following exposure of adult male *Xenopus laevis* frogs to Diquat dibromide and Imazapyr formulations.

2.2.2. Materials and Methods

Herbicides

The herbicide-formulations, Roundup (360 g/L, Monsato, USA), Enviro Glyphosate (360g/L, Enviro Industries Ltd, RSA), Kilo Max (700 g/kg, Glyphosate, Volcano Agroscience Ltd, RSA), Midstream (373 g/L, Diquat dibromium, Syngenta Ltd, USA), Basta (200 g/L, Glufosinate ammonium, Bayer Crop Science AG Ltd, DE) and Arsenal (250 g/L, Imazapyr, BASF, DE).

Xenopus laevis as model

The African clawed frog, *Xenopus laevis* was selected as test-species. Adult male and female frogs were obtained from a commercial dealer and housed in separate aquaria as breeding stock. Housing and maintenance was according to the SANS guidelines (SANS, 2008) as well as guidelines presented in the American Society for Testing and Materials (ASTM, 1998) and XEMA guidelines (Degitz et al., 2005; Opitz et al., 2005; OECD, 2009).

Xenopus laevis care and breeding of tadpoles

Breeding induction was performed according to ASTM (1998) protocol. All the eggs and tadpoles staging were done according to the Nieuwkoop and Faber (1956) (referred to as NF-stages) normal table. All breeding and general maintenance procedures were approved by the Animal Ethics Committee of the Stellenbosch University (Approval no- SU-ACUM 12-00013).

2.2.3. Exposure procedures

Acute toxicity testing

Three *Xenopus laevis* developmental (tadpole) stages (according to Nieuwkoop and Faber, 1956), NF-stage 8-11 (embryo, pre-metamorphic phase), NF-stage 48 (larval, pro-metamorphic phase) and NF-stage 60 (larval, metamorphic phase) was assessed. The exposures followed the ASTM (1997), 96-hour acute toxicity protocol. Twenty tadpoles per developmental stages were exposed against each of the six herbicide formulations. Mortality/survival records were recorded every six hours. At 96 hours, the assay was terminated and the final dataset used to determine the 96-hour lethal concentrations (LC_{50}) for each of the test substances using Probit Software 3.3 (USEPA, 2014).

FETAX Testing

The Frog Embryo Teratogenesis Assay of Xenopus (FETAX) was performed following the guidelines as described by American Society for Testing and Materials (ASTM, 1998). In other to confirm the experimental concentrations, four random concentrations per formulation were sampled from the exposure tanks. The sampled concentrations were independently (blind) analyzed at Synexa Analytical laboratory in Cape Town, in South Africa. The laboratory used a liquid chromatography tandem mass spectrometry (LC/MSMS) method. The analytical results showed very low variation compared to the predicted nominal concentrations (data not shown). Each exposure vessel (500 mL) contained 20 selected embryos, replicated for each concentration. Exposure tanks were set-up in a controlled climate room for the duration of the experiment – under the physical conditions protocol (OECD, 2009).

The following endpoints were determined: Mortality incidence (measured every six hours and added together at 96-hour mark), body growth inhibition, incidence of developmental malformations (defined according to Atlas of Abnormalities; Bantle et al., 1998), facial as well as axial malformations (tail, and notochord abnormalities), and the calculated teratogenic index (TI). Mortality and malformation incidence data obtained over the range of concentrations were used to calculate the 96-hour lethal concentration of 50% of the animals (LC_{50}) and effective concentration for malformations in 50% of the animals (EC_{50}) in each exposure treatment. The LC_{50} and EC_{50} were used to calculate the teratogenic index (TI), calculated as $TI = LC_{50}/EC_{50}$. A substance is considered teratogenic when $TI \geq 1.5$ (ASTM, 1998; Mann and Bidwell, 2000; Leconte and Mouche, 2013).

The Xenopus Metamorphosis Assay (XEMA)

The exposures were carried out following a standardised XEMA protocol (Degtz et al., 2005; Opitz et al., 2005; OECD, 2009). In brief, pre-metamorphic tadpoles (NF stage 51; n = 20 per exposure tank) were obtained using in-house breeding.

Tadpoles were randomly selected from the holding tanks and transferred to 10 L exposure tanks. Individual exposure tanks were replicated at each of the selected concentrations (a control plus three concentrations per chemical). The selected exposure concentrations were centered on 15, 30 and 45% of the 96-hour LC_{50} results for NF stage 48 tadpoles. Test chemicals included Arsenal (0, 0.5, 2.0, and 3.5 mg/L), Basta (0, 0.05, 0.15 and 0.25 mg/L), Midstream (0, 0.05, 0.11 and 0.14 mg/L), Roundup (0, 0.2, 0.4, and 0.6 mg/L), Enviro Glyphosate (0, 9, 19, and 28 mg/L), and Kilo Max (0, 90, 190 and 280 mg/L) herbicide formulations.

The exposure was done under controlled climatic conditions according to the XEMA experimental protocol. The following physical conditions were applied: Water temperature of 23 ± 1ºC, pH ranging between 7.5 and 8.5, dissolved oxygen of >3.5 mg/L and a 12 hours light and dark photoperiod (L12:D12). The tadpoles were fed with 65 mg/L of algae powder (Sera Micron, Sera Germany) three times daily.

At the end of the 21-day exposure period, the tadpoles were carefully collected and euthanased in buffered MS-222 (200 mg/L, buffered with 5 nM sodium bicarbonate). They were blotted dry and individually weighed (to the nearest 0.01g), and snout-vent-length (SVL) (to the nearest 0.1 mm) recorded and fixed in Davidson's solution for 72 hours before preserving the animals in 10% neutral buffered formalin (Bancroft and Stevens, 1977).

The tadpoles were individually sampled and developmental stages (according to Nieuwkoop and Faber, 1956) were determined. The hind-limb lengths and fore-limb lengths were measured to the nearest 0.1 mm using a Leica EZ4D stereo-microscope (Leica, Wetzlar GmbH). The heads of tadpoles, containing the lower jaws and the thyroid glands, were carefully decapitated just behind the eye, using a sharp blade and preserved in buffered formalin for routine histological studies (see Van Wyk, 2013 and Grim et al., 2009 for details).

Photometric assessments of the slides were carried out using a Leica DMLB bright light-microscope equipped with digital camera (Leica Microscope Ltd, DE). Epithelial cell height (measure from the base to the apical edge of the cell), follicular cross-sectional area (follicle lumen area) as well as thyroid cross sectional area were measured using image analysis software (SigmaScan, SPSS Science; Leica EZ software). A sample size of five tadpoles per exposure group (n = 5) were used for the measurements. Ten follicles were randomly measured for each thyroid gland. A mean value was then calculated per individual and used with other individual group members to calculate a group mean.

Juveniles (post-metamorphic)

In addition to the *Xenopus laevis* tadpoles exposed in the XEMA, tadpoles were continued to be exposed to the selected herbicide formulations until the completion of metamorphosis. Growth disruption was determined by measuring body mass (BM)

at prometamorphic stages and at completion of metamorphosis. Gonadal sex was determined macroscopically and a subsample was validated using routine histological procedures. Gonadal sex ratio was compared between the control and the herbicides graded concentrations. Gonadal morphological malformations, categorized according to Lutz et al. (2008), were recorded by macroscopic inspection of the gonads. The diversity of malformations was noted and the incidence included in a malformation index (Lutz et al., 2008).

The identification of the malformed testes and ovaries included the following morphological characteristics (see Lutz et al., 2008 for descriptions):

(a) Adhension, (b) Aplasia (agenesis), (c) Segmented aplasia, (d) Bifurcation, (e) Angular deformity, (f) Displaced gonads, (g) Fused, (h) Hypertrophy, (i) Segmented hypertrophy, (j) Hypoplasia (k) Segmented hypoplasia, (l) Intersex, (m) Mixed sex, (n) Translucent, (o) Segmental translucent.

Adult male exposures

Adult male *Xenopus laevis* frogs were obtained from a local commercial dealer. Adult male frogs (n = 5 per exposure group) were exposed to a subset of two herbicide formulations, Midstream (Diquat dibromide) (0, 0.25, 0.45 and 0.65 mg/L) and Arsenal (Imazapyr) (0, 2, 4 and 8 mg/L) for 28 days. Exposure water was statically renewed three times a week for all exposure groups. The exposure study was performed under the following physical conditions: pH between 6.5 and 8, dissolved oxygen of > 3.5 mg/L and 12 hours of light and 12 hours dark (12L:12D) photoperiod cycle. All the procedures were performed according to the ethical protocol and principles as approved by the central Ethical Committee of the Stellenbosch University (Approved no SU-ACUM13-00017). No mortalities were recorded during the 28-day exposure period. Following the exposure, male frogs were decapitated and pithed (Brown, 1970). Blood samples were collected directly from the heart and organs (gonad and liver) dissected and weighed on an electronic balance (to the nearest 0.01 g) and fixed in 10% buffered formalin (Bancroft and Stevens, 1977).

The following endpoints were assessed: (a) body mass (BM), testes mass to calculate a gonadosomatic index (GSI; gonad mass/BM x 100), (b) hepatosomatic index (HSI; liver mass/BM x 100), (c) qualitative testes histopathology, (d) plasma vitellogenin (VTG) concentrations, (e) plasma testosterone concentrations, (f) plasma thyroid hormone (T_4) concentrations. The Xenopus VTG ELISA is based on a VTG antibody developed and validated using an in-house protocol (Hurter et al., 2002). Commercial ELISA kits were purchased to determine testosterone and Thyroxine concentrations in the plasma (DRG International, USA).

2.2.4. Statistical analyses

Normality and homogeneity of variance was assessed using the Shapiro-Wilk’s W and Levene’s tests respectively. Non-parametric data was analysed using Kruskal-

Wallis ANOVA and Dunn's test for multiple comparisons. Parametric data was analysed using 1-ANOVA and Tukey's HSD post hoc test. Significant differences between treatments were taken at $p < 0.05$.

2.2.5. Results and Discussion

Acute toxicity of X. laevis tadpoles

The results of this study showed a stage-dependent differential toxicity response across all the six herbicide formulations (Table 3). In each of the formulation, the stage specific response pattern in terms of 96-hour LC_{50} showed that NF stage 48 was the most vulnerable tadpole stage within the life cycle of the *X. laevis* (Fig. 4).

Table 3: *Xenopus laevis tadpoles NF-stages, exposure concentrations and calculated 96-hr LC_{50} values for the selected herbicide formulations. The LC_{50} values are in bold and the upper and lower ranges are in brackets.*

Herbicide	NF Stage	Exposure Conc. (mg/L)	96hr LC_{50} (mg/L) (95% CI)
Roundup	8-11	0.3, 0.5, 0.7, 0.9, 1.1, 1.3	**1.05** (0.91-1.32)
	48	0.8, 0.85, 0.9, 0.95, 1.0, 1.2	**0.89** (0.85-0.91)
	60	1, 3, 4, 5, 6	**2.75** (2.1-3.2)
Kilo Max	8-11	130, 160, 190, 220, 250, 280	**207** (197-218)
	48	20, 40, 60, 80, 100, 120	**58.1** (44.8-73.0)
	60	400, 450, 500, 550, 600	**455** (435-473)
Enviro Glyphosate	8-11	320, 360, 400, 440, 480, 520, 540	**466** (434-512)
	48	100, 120, 140, 160, 180	**134.6** (127-142)
	60	4800, 5000, 5200, 5400, 5600	**5257** (5098-5466)
Midstream	8-11	0.5, 1, 1.5, 2, 2.5, 3	**0.83** (0.431-1.14)
	48	0.09, 0.1, 0.2, 0.3 & 0.4	**0.2** (0.16-0.26)
	60	6, 8, 12, 16, 20	**11.8** (10.6-13)
Basta	8-11	1.0, 1.2, 1.4, 1.6, 2, 2.5, 3	**2.24** (1.97-2.71)
	48	0.1, 0.5, 1.0, 1.5, 2 & 2.5	**0.59** (0.36-0.85)
	60	15, 20, 25, 30, 35	**24.9** (22.7-27.1)
Arsenal	8-11	20, 25, 30, 35, 40, 45	**36** (33.3-39.5)
	48	30, 33, 36, 39, 42	**32.8** (31.7-33.8)
	60	150, 160, 170, 180, 190	**173.5** (169-178.5)

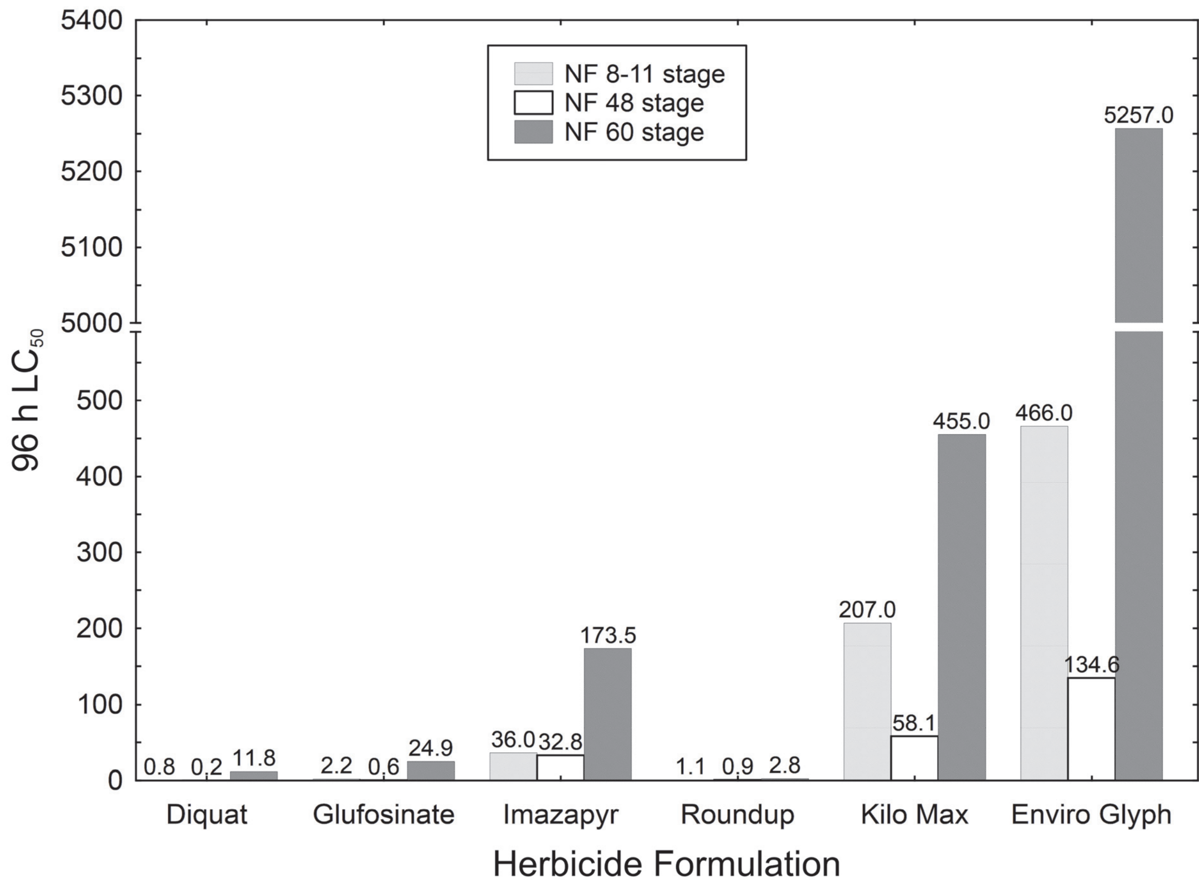


***Figure 4**: The 96-hour LC_{50} indexes determined for three different X. laevis developmental stages exposed to six herbicides formulations. The 96-hour LC_{50} comparison suggests marked variation among exposed chemicals as well as among developmental stage. In general, NF-stage 48 tadpoles exposed to the six herbicide formulations, proved to be the most sensitive stage of the three developmental stages in the X. laevis developmental life cycle*.

This result was consistent with the findings of several studies including Dutta and Mohanty-Hejmadi (1978), Herkovit and Perez-Coll (1993), Anguiano et al. (1994), Berrill et al. (1998), Bridges et al. (2000), Edington et al. (2004) and Ortiz-Santaliestra (2006) who all showed that the pre-limb tadpole stages (premetamorphic) of various species of amphibians were more susceptible to various environmental chemicals formulation than their eggs and cleaving embryos or the older prometamorphic larvae at metamorphic climax. However, the results from this study and many others mentioned above are contrary to the findings of Ezenmoye and Tongo (2009) who noted that using graded concentrations of Atrazine on tadpoles of *Ptyychadena bibroni* between one to four weeks post-hatch developmental stages for 96-hours, the percentage mortality decreased with increasing maturity.

With the susceptibility of pre-metamorphic larvae, it is clear that the protection of one developmental stage will be ecological inadequate if another developmental stage is seriously vulnerable to the same chemical. The fact that similar trends have also

been identified in different anuran species suggest that pre-metamorphic tadpoles represent a vulnerable life stage that could be an important factors in understanding the global amphibian declines. This study therefore confirmed the importance of using a selection of developmental stages, but including pre-metamorphic tadpoles (for example NF-stage 48) in exposure studies to evaluate the potential threat that pesticides formulations, directly or indirectly, may hold for non-target amphibian species in a polluted aquatic environment.

Non-lethal and teratogenic impacts

The present study examined the lethal effects along with non-lethal, developmental effects including body size effects, teratogenic (dysmorphogenic) potential and malformation-type, following the exposure to a concentration series for six selected herbicide formulations including Midstream, Basta, and Arsenal as well as Roundup glyphosate formulation, together with two new glyphosate formulations including Kilo Max and Enviro Glyphosate.

Embryotoxicity/Mortality

The 96-hr LC_{50} for Midstream, Arsenal, Basta, Roundup, Kilo Max and Environ Glyphosate were 0.24, 28.13, 2.01, 1.05, 207.25 and 465.95 mg/L respectively (Table 4).

Table 4: *The exposure concentrations (mg/L), LC_{50}, EC_{50}, Teratogenic Index (TI), and minimum concentration that inhibit growth (MCIG) for the herbicide formulations (95 % CI in brackets).*

Treatment	Exposure Concentrations	LC_{50} (mg/L) (95%CI)	EC_{50} (mg/L) (95%CI)	TI	MCIG
Roundup	0, 0.5, 0.7, 0.9, 1.1, 1.3.	**1.1** (0.9-1.3)	0.8 (-)	1.4	0.9
Kilo Max	0, 130, 160, 190, 220, 250, 280	**207.2** (196.6-217.6)	**150.8** (139-160)	1.4	190
Enviro Glyphosate	0, 320, 360, 400, 440, 480, 520	**466.0** (434-51)	**287** (142.7-32)	1.6	440
Midstream	0, 0.5, 1.0, 2.0, 2.5, 3.0	**0.833** (0.4-1.1)	0.24 (-)	3.5	1.0
Arsenal	0, 20, 25, 30, 35, 40, 45	**36** (33.3-39.5)	**28.1** (26.2-30.0)	1.3	30
Basta	0, 1.6, 2.0, 2.5, 3.0	**2.240** (2.0-2.7)	**2.01** (1.8-2.3)	1.1	2.0

Growth effects

The results from this study revealed a significant reduction in length of tadpoles exposed to Roundup, Kilo Max, Midstream, Arsenal and Basta formulation compared to the Control tadpoles in several of the exposure concentrations ($p < 0.05$).

Malformations and teratogenic Index (TI)

The 96-hour EC_{50} malformation index obtained for these herbicides were 0.241, 28.13, 2.01, 0.76, 150.76 and 287 mg/L for Midstream, Arsenal, Basta, Roundup, Kilo Max, and Enviro Glyphosate respectively (Table 4). The TIs (LC/EC) obtained were 3.5, 1.3, 1.1 1.4, 1.4, and 1.6 for Midstream, Arsenal, Basta, Roundup formulation, Kilo Max and Enviro Glyphosate respectively (Table 4).

The observed malformations appeared closely related between the three formulations of glyphosate while it varies from one formulation to another between Midstream, Arsenal and Basta formulations. The various malformations generally included gut abnormalities, generalised edema, cardiac edema, abdominal edema, blistering, eye, head, wavy tail and curved tail (Fig. 5).

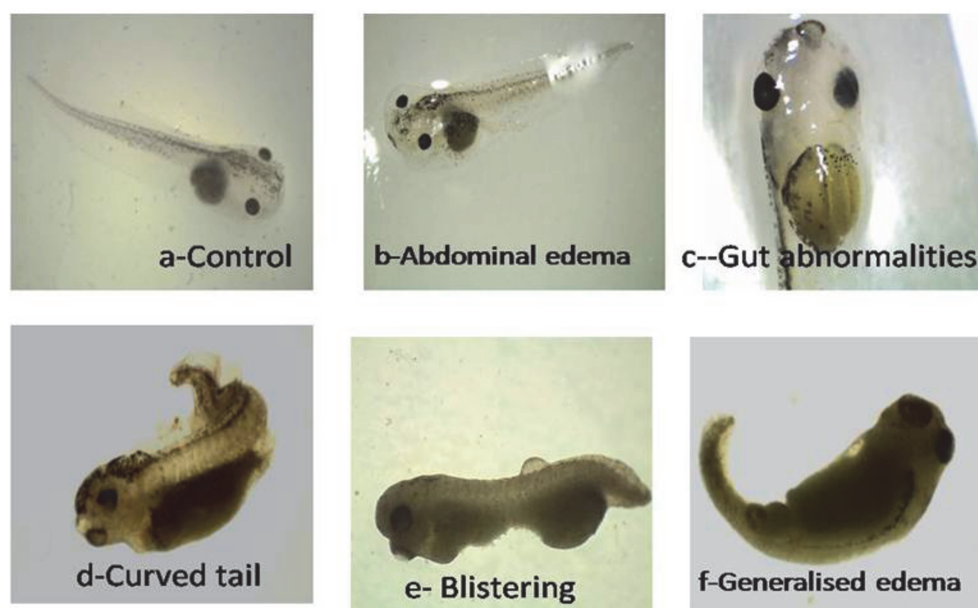


***Figure 5**: Examples of malformations associated with the various formulations used.*

The present study confirmed that Roundup (Glyphosate formulation), Midstream and Basta formulation had differential toxic effects but also differentially affected early growth of *Xenopus laevis* embryos. The incidence of malformations revealed a range of different malformation-types in all exposure groups. Teratogenic potential for the selected herbicides were mostly positive (>1), two (Midstream and Enviro Glyphosate formulations) of the selected six herbicides exceeding TI values of 1.5

(Bantle et al., 1999) but only one (Basta formulation) had a TI below 1.2. Leconte and Mouche, (2013) used 1.2 as threshold value.

Of the three glyphosate formulations, only Roundup showed a very high toxicity at 96 hour LC_{50} of 1.052 mg/L. This 96-hour LC_{50} is below the expected environmental concentration (EEC) of 1.43 mg/L of this formulation at the recommended aquatic application rate (Govandarajulu, 2008). This supports the claims of Lanctot et al. (2013) and Güngordü (2013) that Roundup formulation is not suitable for use in aquatic habitats, especially where amphibians and other sensitive biota are found. Midstream formulation also showed a high toxicity with 96-hour LC_{50} of 0.83 mg/L. This supports the report of Anderson and Prahlad (1976), who reported that Diquat formulation at concentration of 0.001-0.002 mg/L, was highly embryotoxic to *Xenopus laevis*. This 96-hour LC_{50} is very close to the EEC of 0.733 mg/L of this herbicide at the recommended application rate of 0.1-2.0 mg/L (Dial and Dial, 1987; Peterson et al., 1994). Therefore, at the EEC concentration of 0.733 mg/L, more than 30% mortality is expected. The results hence indicate that Diquat is not appropriate for the aquatic environment, where amphibians and other equally sensitive aquatic organisms reside.

In the case of the Basta formulation (Glufosinate ammonium), with 96-hour LC_{50} of 2.24 mg/L, the embyotoxicity of this herbicide to *X. laevis* and compared to the EEC of 1.0 mg/L (Dinehart et al., 2010). This Basta formulation, if applied correctly in aquatic habitat does not guarantee toxicity safety at 96-hour LC_{50} of 2.24 mg/L, as concentrations spikes just after application may exceed the LC_{50} concentration. This therefore calls for concern on the application of this formulation on the safety of the sensitive embryos of aquatic organisms like amphibians. The Arsenal formulation was found to be relatively less toxic, with 96-hour LC_{50} of 36 mg/L, which when compared to the EEC of 0.083 mg/L is higher than the EEC and toxicity is unlikely to reach that level under normal application rates.

For the MCIG (minimum concentration inhibiting growth), the results showed that of the three glyphosate-based formulations, only Kilo Max and Roundup showed a significant concentration dependent growth inhibition. The Midstream formulation, on the other hand showed strong inhibition of growth at all exposure concentrations. The Arsenal and Basta formulations revealed a concentrated dependent significant growth reduction, which showed the inhibiting potential of these formulations. For Basta formulation, the fact that this herbicide has an expected environmental concentration of 1.0 mg/L (Dinehart et al., 2010) and resulted in significant growth inhibition from 2 mg/L concentration, makes it a marginal case.

The teratogenic potential has been widely used as one of the FETAX endpoints (Bantle et al., 1999; Leconte and Mouche, 2013). Bantle et al. (1999) suggested that a teratogenic index value larger than 1.5 would indicate positive teratogenic potential. On the other hand, Leconte and Mouche (2013) regarded TI values > 1.2 as positive dysmophogenic. In the present study, of the three glyphosate-based

formulations, only exposure to Enviro Glyphosate (TI of 1.6) suggested positive teratogenic potential. In terms of the Leconte and Mouche (2013) threshold TI value of 1.2, Roundup and Kilo Max could also be regarded as teratogenic at TI of 1.4 each. The teratogenic index (TI) for Midstream formulation (Diquat) was 3.5, which would be considered relatively high (TI > 1.5; Bantle et al., 1999). Midstream formulation also exhibited widespread occurrence of malformations with severe generalised edema, as the most common abnormalities. For Basta formulation, the TI was 1.1, which is lower than the 1.5 by Bantle et al. (1999) and the Laconte and Mouche (2013) TI index. Lastly, the Arsenal formulation, resulted in a TI of 1.3, which is below the 1.5, Bantle et al. (1999) threshold but above the Leconte and Mouche (2013) TI value of 1.2, indicating a positive dymorphogenic potential. The main abnormalities observed in this herbicide included gut malformation that showed slightly improper gut coiling as well as complex improper gut formation. Severe generalised edema was also frequently observed and may be linked to disruption in osmoregulation cause by cell membrane lipid layer disruption (Osano et al., 2002).

In conclusion, this present study showed that Roundup, Midstream and Basta formulations are embryotoxic to *X. laevis,* while Arsenal, Enviro Glyphosate and Kilo Max showed relative low toxicity to the *X. laevis* embryos. Kilo max, Roundup, Basta, and Arsenal formulations revealed significant growth disruption. In terms of teratogenicity, Midstream formulation showed a strong teratogenic potential, while Enviro Glyphosate showed positive of teratogenic potential at relative high exposure concentrations. These results confirm that the alleged contribution of pesticides/chemicals contamination to the global declines of amphibian cannot be ruled out, as many of these formulations exhibited toxicity, growth inhibition and teratogenicity to the challenges facing amphibians, particularly at the sensitive embryonic stages.

Xenopus Metamorphosis Assay (XEMA)

<u>Morphometric endpoints</u>

The results of the XEMA study revealed that all six herbicide formulations affected the final outcome of development following a 21-day semi-static exposure of NF stage 48 tadpoles to the selected herbicide formulations. In terms of growth (body size), tadpole growth was significantly compromised at higher-end of the exposure concentrations in Arsenal (3.5 mg/L), Roundup (0.4 and 0.6 mg/L), Kilo Max (90, 190 and 290 mg/L), Enviro Glyphosate (28 mg/L), but for the Basta and Midsteam formulations not significant variation was observed (Kruskal-Wallis, $p > 0.05$; Table 5).

Table 5: *Summary of all measured endpoints with the standard deviations for the six herbicide formulation after 21 day exposure.*

Herbicides	Conc. (mg/L)	Whole wet Weight (g)	Whole Body Length (mm)	Hind Limb Length (mm)	Front Limb Length (mm)	Snout-Vent Length (mm)
Midstream	0	0.84 ± 0.1	61.3 ± 5.6	5.10 ± 2.4	2.54 ± 0.7	21.2 ± 2.94
	0.05	0.89 ± 0.1	61.3 ± 6.0	6.56 ± 2.6	2.04 ± 0.8	21.4 ± 2.23
	0.11	0.78 ± 0.2	59.5 ± 7.8	4.89 ± 3.0*	2.00 ± 0.8*	21.0 ± 2.54
	0.14	0.74 ± 0.1	58.6 ± 9.3	5.10 ± 3.2*	2.10 ± 1.0*	21.0 ± 3.27
Arsenal	0	0.80 ± 0.3	51.3 ± 25.7	8.51 ± 3.9	3.38 ± 1.5	20.7 ± 3.11
	0.5	0.9 ± 0.2	60.8 ± 10.6	7.70 ± 2.6	2.89 ± 1.2	20.2 ± 3.56
	2.0	0.97 ± 0.1	62.3 ± 7.2	6.96 ± 3.2*	2.65 ± 1.1*	21.1 ± 2.58
	3.5	0.91 ± 0.2*	59.1 ± 15.2	7.40 ± 4.4*	2.91 ± 1.7*	21.3 ± 4.03
Basta	0	0.99 ± 0.2	59.5 ± 17.0	6.75 ± 4.7	2.66 ± 1.6	20.3 ± 3.55
	0.05	1.04 ± 0.2	62.2 ± 8.4	5.47 ± 3.4	2.30 ± 1.1	20.5 ± 1.09
	0.15	1.09 ± 0.3	62.1 ± 13.6	6.10 ± 4.6	2.51 ± 1.6	20.8 ± 1.72
	0.25	1.09 ± 0.2	61.5 ± 11.6	6.23 ± 4.2	2.59 ± 1.3	20.7 ± 1.71
Roundup	0	0.96 ± 0.2	59.4 ± 3.6	6.01 ± 1.8	2.13 ± 0.6	20.2 ±1.54
	0.2	0.94 ± 0.2	63.5 ± 5.0*	6.58 ± 1.3	2.56 ± 0.5	21.0 ± 1.94
	0.4	0.78 ± 0.2*	57.9 ± 6.7	6.68 ± 1.9	2.70 ± 0.8*	19.7 ± 2.22
	0.6	0.83 ± 0.2*	58.9 ± 7.1	6.80 ± 2.0	2.58 ± 0.9*	20.4 ± 2.12
Enviro. glyphosate	0	0.89 ± 0.1	60.8 ± 3.5	6.5 ± 1.5	2.41 ± 0.6	20.9 ± 0.81
	9	0.93 ± 0.1	61.1 ± 3.4	6.34 ± 1.5	2.36 ± 0.6	21.3 ± 1.45
	19	0.80 ± 0.2	58.0 ± 5.2	6.68 ± 2.0	2.58 ± 0.8	20.2 ± 2.2
	28	0.68 ± 0.2*	54.0 ± 5.1*	5.69 ± 1.5	2.24 ± 0.5	18.7 ± 1.88*
Kilo Max	0	0.96 ± 0.2	59.5 ± 3.7	6.05 ± 1.8	2.13 ± 0.6	20.2 ± 1.54
	90	0.72 ± 0.1*	56.9 ± 5.6	6.75 ± 1.5	2.73 ± 0.7 *	19.3 ± 1.9
	190	0.62 ± 0.1*	55.0 ± 4.2*	4.97 ± 1.8	2.0 ± 0.5	19.4 ± 2.89*
	280	0.57 ± 0.2*	53.0 ± 6.2*	4.59 ± 2.1*	1.87 ± 0.7	18.7 ± 1.84*

Hindlimb development was significantly decreased in three of the herbicides tested, Arsenal (2 and 3.5 mg/L), Midstream (0.11 and 0.16 mg/L) and Kilo Max (280 mg/L), but for Roundup, Basta and Enviro Glyphosate no significant variation in hindlimb development was recorded (Dunn's test, $p > 0.05$; Table 5). Frontlimb development also varied among concentrations and groups in developing tadpoles. Frontlimb development was decreased significantly in response to Arsenal (2 and 3.5 mg/L)

and Midstream (0.11 mg/L and 0.14 mg/L; Table 5). In the Roundup group, frontlimb development was significantly increased (0.4 mg/L) relative to the control tadpoles (Dunn's test, $p > 0.05$, Table 5). In the Kilo Max exposure frontlimb development was significant increased in a lower exposure group (90 mg/L), but not in the higher concentrations (190 mg/L and 280 mg/L). For Basta and Enviro Glyphosate formulations no significant variation in front leg development occurred (Table 5).

<u>Developmental stage endpoint</u>

Although it is standard practice to present the frequency distribution of tadpoles developmental stages following exposure to a suspected thyroid disruptor, here we only present the mean developmental stage achieved after a 21-day exposure to the different concentrations of the selected herbicides.

Mean developmental stage at 21 days, varied significantly only in three formulations (Kruskal-Wallis, $p < 0.05$), showing decreased development in Arsenal (at 3.5 mg/L) (Fig. 5), Midstream (at 0.11 and 0.14 mg/L) (Fig. 6), and Kilo Max (at 190 and 280 mg/L) (Fig. 7) compared to the controls (Dunn's test: $p < 0.05$). Tadpoles exposed to Roundup and Enviro Glyphosate did not show variation among concentration groups and the Control, and the same was concluded for the Basta formulation (although there was a trend of decreased stages in all groups) (Fig. 8).

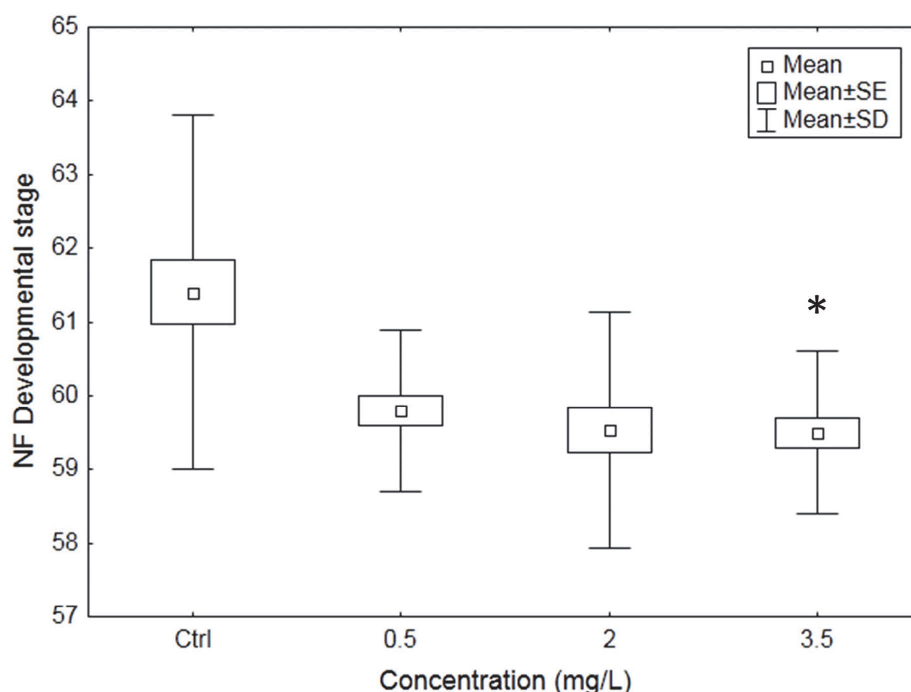


Figure 6: *Stage differentiation of X. laevis exposed for 21 days to a range of different concentrations of the Arsenal formulation compared to the control (Ctrl). Asterisks indicate significant difference from the control. The developmental stage was reduced across all the three exposure concentrations, but significantly different at 3.5 mg/L (Dunn's test, p < 0.05) compared to the control.*

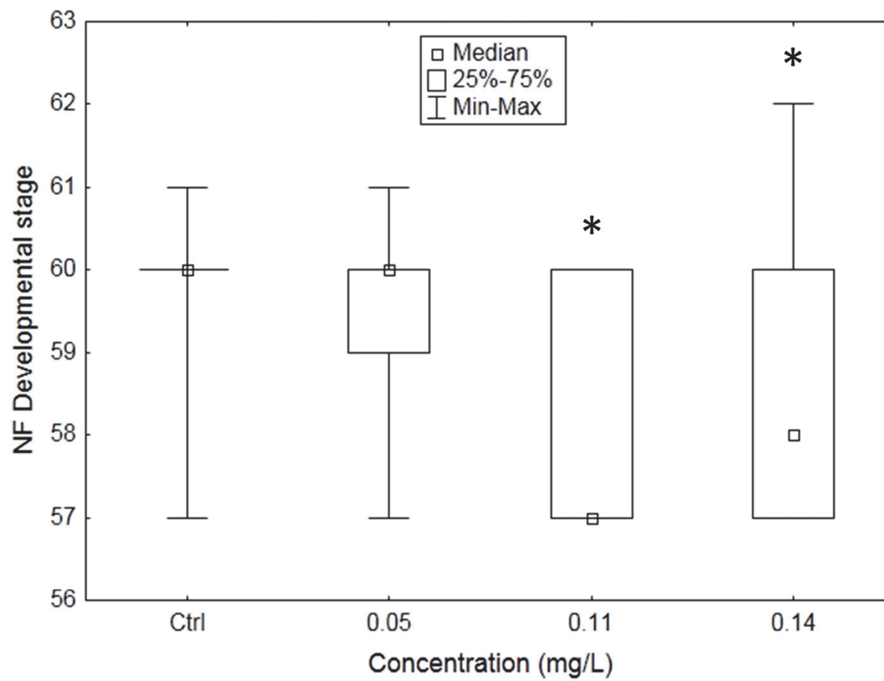


Figure 7: *Stage differentiation following exposure (21 days) to different concentrations of the Midstream formulation compared to the control (Ctrl). Asterisks indicate significant difference from the control. The mean developmental stage was significantly reduced at concentrations of 0.11 and 0.14 mg/L (Dunn's test, p < 0.05) compared to the control.*

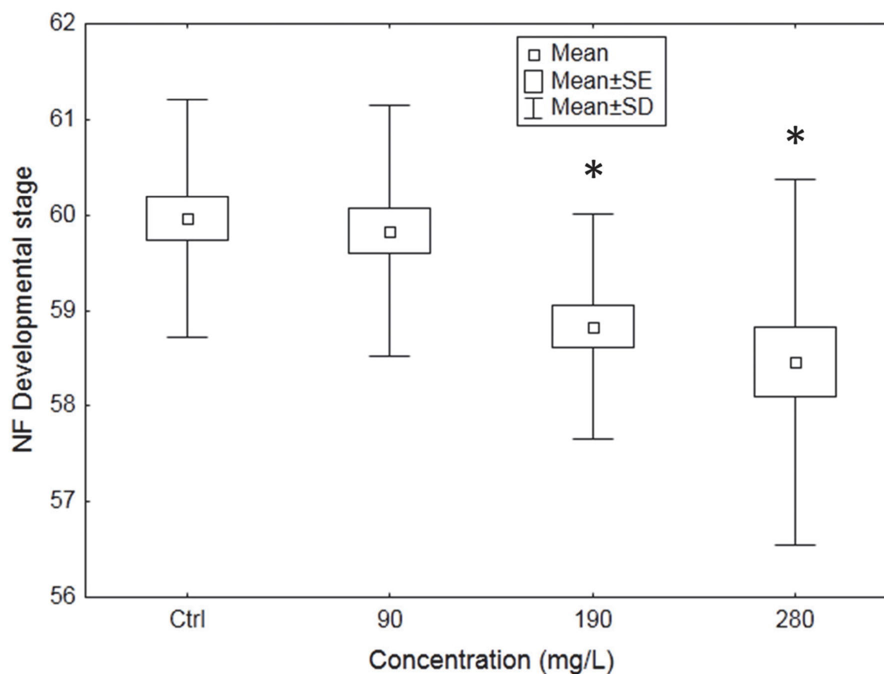


Figure 8: *Stage differentiation following 21 days of exposure to different concentrations of the Kilo Max formulation compared to the control. Asterisks indicate significant difference from the control. The tadpole developmental stages were reduced in dose dependent manner across the exposure concentrations. The stages were significant differences at concentrations of 190 mg/L and 280 mg/L (Dunn's test, p < 0.05) compared to the control.*

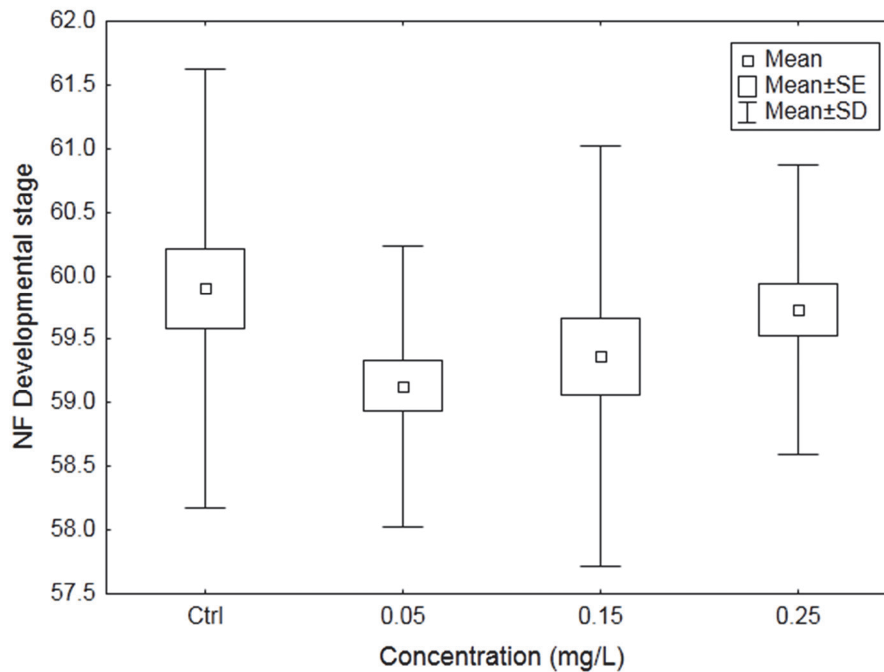


Figure 9: *Stage differentiation following exposure for 21 days to different concentrations of the Basta formulation. Developmental stages of tadpoles exposed to graded concentrations of Basta formulations were reduced generally across the exposure concentrations but did not prove to be significantly different from the mean developmental stage of the control (Dunn's test, p > 0.05).*

Thyroid histopathology endpoints

The thyroid histology confirmed this result of effects on developmental stage reduction for three of the herbicide formulations including Arsenal (at 0.2 mg/L), Basta (at 0.15 and 0.25 mg/L) and Roundup (at 0.6 mg/L) compared to the control (Table 6). In the Arsenal exposure, the coloidal area was significantly reduced ($p < 0.05$) as well as the overall gland area, showing glandular atrophy. The mean follicular epithelium height in the Arsenal exposure was significantly increased ($p < 0.05$), showing cellular hypertrophy. Tadpoles from the Basta formulation exposure exhibited increased mean follicular height (cellular hypertrophy), but also increased glandular hypertrophy, although the colloidal area did not vary significantly compared to the control. For the Midstream formulation, the colloidal (luminal) area was significantly reduced at 0.14 mg/L ($p < 0.05$) compared to the control. The follicle epithelium was hypertrophied at only the lowest exposure concentration of 0.05 mg/L Midstream formulation compared to the control ($p < 0.05$). Overall gland area was not significantly different for the Midstream formulation at all concentrations compared to the control. The gland area and colloidal (luminal) area of the thyroid gland in the tadpoles exposed to 0.6 mg/L Roundup formulation was significantly increased compared to the control ($p < 0.05$). Folliclule cell hypertrophy was also found at all Roundup formulation concentrations compared to the control (Table 6). In both the Kilo Max and Enviro Glyphosate exposures were the colloidal areas reduced ($p < 0.05$) and the mean follicular epithelium height increased ($p < 0.05$),

showing signs of glandular hypertrophy compared to the thyroid glands in the control tadpoles.

Table 6: *Summary data of histopathology morphometrics of the six herbicide formulation used in a 21 day XEMA exposure assessment. Bold values with asterisk indicate significantly different values compared to control (0 mg/L) exposed tadpoles (Dunn's test, p < 0.05).*

Herbicide formulation	Treatment (mg/L)	Follicle Epithelium (µm; mean ± SD)	Colloidal Area (μm^2; mean ± SD)	Gland Area (μm^2; mean ± SD)
Roundup	0	7.48 ± 1.02	2968.4 ± 2455.5	68737.3 ± 15494.6
	0.2	9.81 ± 1.65*	2508.9 ± 1755.7	79514.1 ± 26146.0
	0.4	8.23 ± 1.33*	3016.8 ± 1704.8	78167.2 ± 21408. 0
	0.6	11.31 ± 1.77*	8501.6 ± 4354.3*	100907.3 ± 24470.8*
Kilo Max	0	7.48 ± 1.02	2968.4 ± 2455.5	68737.3 ±15494.6
	90	10.15 ± 1.77*	3499.8 ± 2387.8	76658.1 ± 26164.1
	190	9.46 ± 2.42*	2179.3 ± 1795.7	56645.6 ± 21408.0
	280	9.21 ± 2.25*	2830.8 ± 2183.0	47363.3 ± 24470.8
Env glyph	0	8.44 ± 1.43	4925.2 ± 2626.2	92515.9 ± 31110.2
	9	8.69 ± 1.26	3026.5 ± 1622.6*	89325.4 ± 35154.5
	19	9.25 ± 1.21*	3264.5 ± 1742.7*	104426.8 ± 19727.6
	28	11.80 ± 0.78*	4666.8 ± 3915.5	81688.8 ± 16599.9
Midstream	0	7.82 ± 1.65	3410.6 ± 2299.2	53903.8 ± 27630.7
	0.05	8.92 ± 1.68*	3146.0 ± 1793.6	69585.7 ± 12328.7
	0.11	8.27 ± 1.22	2413.7 ± 1195.1	59964.9 ± 15335.3
	0.14	8.28 ± 1.33	1832.8 ± 999.8*	39340.3 ± 10961.2
Arsenal	0	7.85 ± 1.48	4798.4 ± 3012.1	119157.5 ± 33814.9
	0.05	10.93 ± 1.52*	3040.0 ± 2122.6*	92922.8 ± 10403.5
	2.0	11.34 ± 1.50*	3189.1 ± 1783.1*	83186.2 ± 25250.6*
	3.5	11.15 ± 1.46*	3588.1 ± 1964.8	93683.0 ± 21768.9
Basta	0	7.30 ± 1.08	1910.0 ± 1055.5	47014.7 ± 6771.1
	0.05	8.05 ± 1.11*	1711.8 ± 1013.1	43919.0 ± 8161.9
	0.15	8.02 ± 1.18*	1916.9 ± 1078.6	86800.9 ±17907.1*
	0.25	10.02 ± 1.86*	1843.4 ± 793.4	78414.8 ±16871.5*

Juvenile Results

Percentage sex ratios

Two of the six herbicide formulations (Midstream and Kilo Max) significantly altered the percentage of sex ratios in the metamorphic froglets (Table 7). Midstream significantly altered the percentage of sex ratios to a female bias at concentrations of 0.14 mg/L to 60:40 compared to 50:50 at the control ($p < 0.05$; Table 7). Kilo Max significantly altered the sex ratio to female bias at concentration of 280 mg/L to 68:32 compared to 50:50 ($p < 0.05$; Table 7).

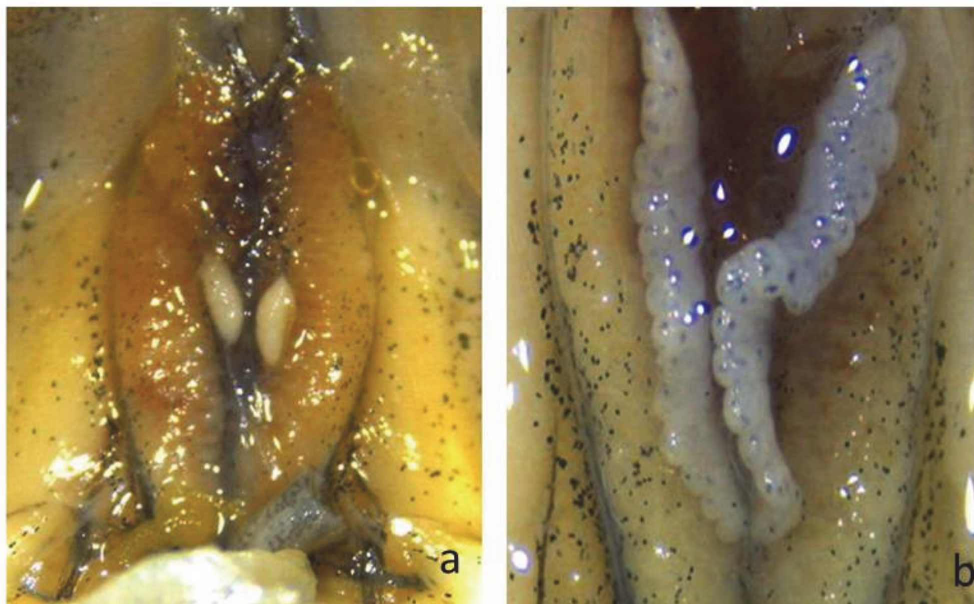
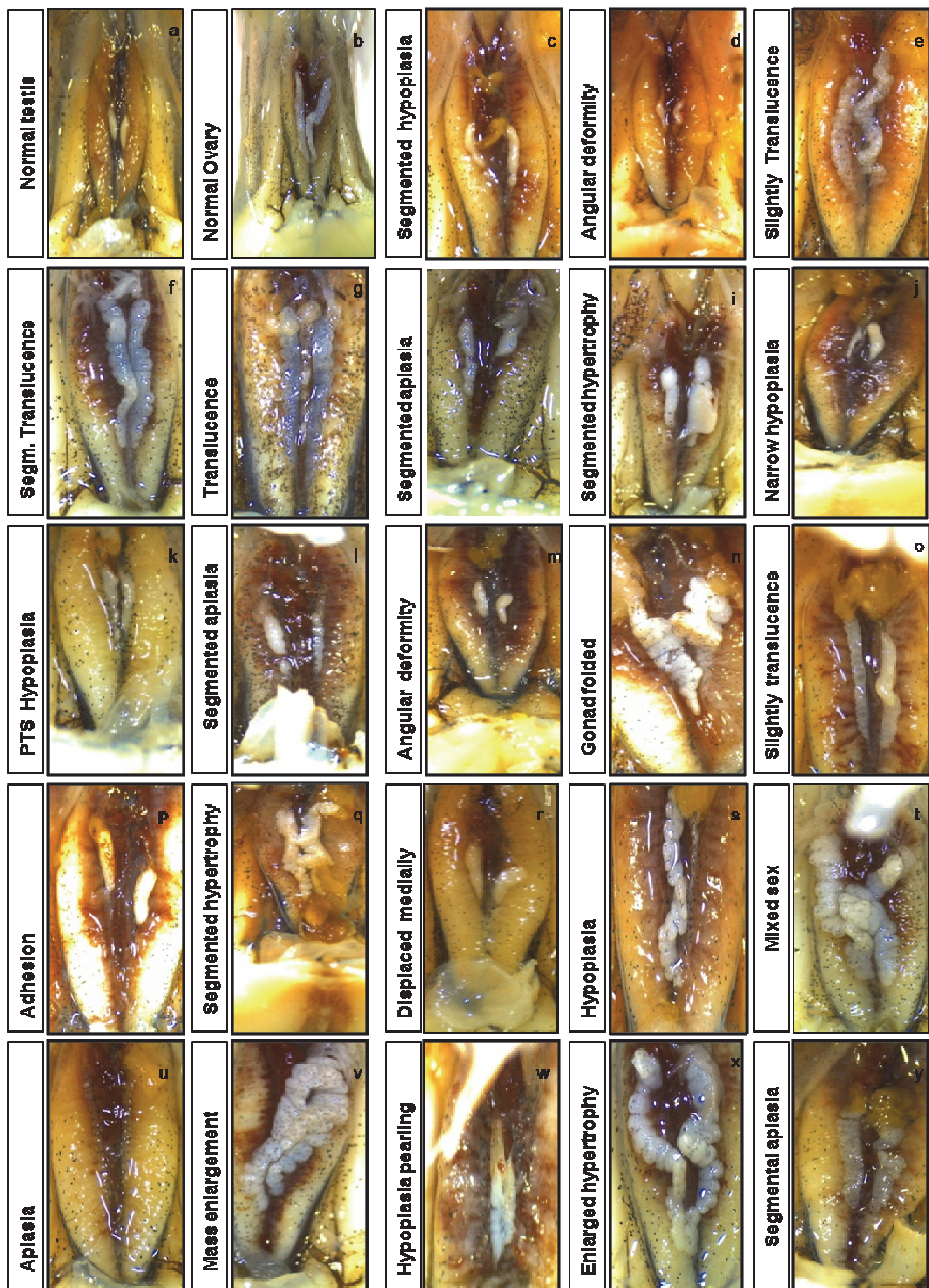


Figure 10: *Macroscopic view of normal male (a) and female (b) gonads of juvenile, post-metamorphic, X. laevis frogs.*

The potential of these herbicide formulations to alter the sex ratios of amphibians at concentrations below their expected environmental concentrations (EEC) deserve attention. Midstream formulation for example, seriously feminized developing frogs at concentrations well below its EEC of 0.73 mg/L. This feminisation potential could negatively impact the population of exposed aquatic organisms. Kilo Max formulation also feminized the developing frogs, although at a higher concentration than would be regarded as an environmental relevant concentration. However, the feminisation potential at relevant environmental concentration still needs further study. For Basta, Arsenal, Enviro Glyphosate and Roundup formulations, the potential to disrupt the sex ratios below their various EEC also deserve more study.

Gonadal Malformations

The normal macroscopic views of male and female gonad development in post metamorphic *Xenopus laevis* juveniles are shown in Figure 9. Examples of gonadal malformations (after Lutz et al., 1999) encountered are presented in Figure 10.



*PST: Partly thinned segmented hypoplasia

Figure 11: *Examples of the morphological malformations recorded in reproductive gonads of X. laevis frogs, following the exposure to the selected herbicides.*

Midstream formulation

Several gonad malformation-types were recorded following exposure to the Midstream formulation (Table 7). Gonad malformations including *segmented hypoplasia*, *segmented aplasia*, *enlarged hypertrophy*, *translucence*, *gonad folded*, *mixed sex, segmented hypoplasia* and partly *narrow hypoplasia were observed* (Table 7). The abnormality index ranged from 32.5-60% through a concentration range of 0.05-0.14 mg/L compared to a natural malformation index of 7.5% recorded in the control group (Table 7).

Basta formulation

The observed malformation-types recorded following exposure to Basta include *gonad folded*, *adhesion*, *hypoplasia*, *slightly translucence* and *angular deformity* (Table 7). The rate of malformations following exposure to this formulation increased in a dose dependent manner, varying from 15-27.5% through a concentration range of 0.05-0.25 mg/L compared to a natural occurring abnormality index of 12.5% recorded in the control group (Table 7).

Arsenal formulation

Exposure to the Arsenal formulation resulted in several gonad malformations (Table 7). The observed malformations included *tissue separation*, *segmented aplasia*, *aplasia*, *hypoplasia* and *angular deformity*, *folded gonads* and *narrow hypoplasia* (Table 7). The malformation index (%) ranged from 17.5-35% through a concentration range of 0.5-3.5 mg/L compared natural occurring abnormality index of 7.5 % recorded in the control group (Table 7).

Kilo Max formulation

Numerous gonad abnormalities, of which the incidences were concentration dependent, were observed following exposure to this formulation (Table 7). The abnormalities included, *slightly translucence*, *aplasia*, *segmented aplasia*, *gonad folded*, *tissue separation*, *protuberance*, *translucence*, *segmented hypoplasia*, *segmented bifurcation* and *segmented aplasia* (Table 7). The malformation index range from 22.5-43 % through a concentration range of 90-280 mg/L compared to an abnormality index of 10 % recorded in the control group (Table 7).

Roundup formulation

Exposure to Roundup formulation also resulted in several gonadal abnormalities (Table 7). The observed abnormalities included, *segmented aplasia*, *narrow hypoplasia*, *partly narrow hypoplasia, segmented hypoplasia*, *gonad folded*, *translucence*, *aplasia*, *adhesion*, *angular deformity* and *displaced* (Table 7). The abnormality index ranged from 20-30% through a concentration range of 0.2-0.6 mg/L compared to natural abnormality index of 7.5% recorded in the control group (Table 7).

Enviro Glyphosate

The Enviro Glyphosate formulation resulted in numerous gonad abnormalities (Table 7). The observed abnormalities included, *slightly translucence*, *hypoplasia*, s*egmented aplasia*, *narrow hypoplasia*, *aplasia* and *segmented hypoplasia* (Table 7). The abnormality index ranged from 17.5-37.5% through a concentration range of 9-28 mg/L compared to a natural abnormality index of 7.5 % recorded in the control group (Table 7).

Table 7: *Numbers and percentage of morphological abnormalities and sex ratios at various concentrations of the herbicide formulations.*

Herbicide Formulation	Conc. (mg/L)	No. of Morphological malformations	% Occurrence	% Sex ratio (F:M)
Diquat	0	Narrow hypoplasia (2) Slightly translucence (1)	8	50:50
	0.05	Segmented hypoplasia (8) Slightly translucence (2) Segmental aplasia (3)	33	50:50
	0.11	Segmented hypertrophy (2) slightly translucence (3) mixed sex (1) translucence (2) enlarged hypertrophy (5) gonad folded (2)	37.5	55:45
	0.14	Segmented hypertrophy (2) gonad folded (9) segmental aplasia (3) slightly translucence (4) enlarge hypertrophy (4) segmented hypoplasia (1)	57.5	60:40
Kilo max	0	segmented hypoplasia (1) Narrow hypoplasia (3) hypoplasia (1)	10	50:50
	90	Aplasia (2) segmented aplasia (2) segmented hypoplasia (1) slightly translucence (4)	23	47.5:52.5
	190	Tissue separation (2) folded gonadal (2) protuberances (2) translucence (2) segmented aplasia (1) segmented hypoplasia (1)	25	52.5:47.5

Herbicide Formulation	Conc. (mg/L)	No. of Morphological malformations	% Occurrence	% Sex ratio (F:M)
Glufosinate	280	Aplasia (4) segmented bifurcation(2) segmented hypertrophy(2) translucence(8) segmental aplasia (1)	40	68:32
	0	Hypoplasia (4) folded gonad (1)	12	50:50
Roundup	0.05	Gonad folded (1) adhesion (2) angular deformity (2)	13	50:50
	0.15	Gonad folded (4) enlarged hypertrophy (1) segmental aplasia (1)	15	52.5:47.5
	0.25	Gonad folded (6) slightly translucence (2) angular deformity (1) hypoplasia (2)	28	55:45
	0	Aplasia (2) Hypoplasia (1)	8	50:50
Environ	0.2	Aplasia (1) segmented aplasia (1) narrow hypoplasia (1) partly narrow hypoplasia (1) segmented hypoplasia (1) folded gonadal (1) angular deformity (1) displaced (1)	23	45:55
	0.4	Aplasia (1) segmented aplasia (1) narrow hypoplasia (2) segmented hypoplasia (1) folded gonadal (1) angular deformity (2)	25	45:55
	0.6	Translucence (4) segmented hypoplasia (2) narrow hypoplasia (1) aplasia (1) adhesion (1) segmented aplasia (1) hypoplasia (1)	28	47.5:52.5
	0	Hypoplasia (3)	8	52.5: 47.5

Herbicide Formulation	Conc. (mg/L)	No. of Morphological malformations	% Occurrence	% Sex ratio (F:M)
Glyphosate	9	Slightly translucence (2) segmental aplasia (2) segmented hypoplasia (1) aplasia (2)	18	52.5:47.5
	19	Slightly translucence (2) hypoplasia (1) segmental aplasia (2) aplasia (4)	23	52.5:47.5
	28	Folded gonadal (6) segmented hypoplasia (1) aplasia (4) segmental aplasia (2) narrow hypoplasia (2)	38	55:45
Imazapyr (Arsenal)	0	Segmented aplasia (1) narrow hypoplasia (1) slightly translucence (1)	8	47.5:52.5
	0.5	Aplasia (1) angular deformity (1) segmental aplasia (2) tissue separation (2) partly narrow segmented (1)	18	47.5:52.5
	2.0	Aplasia (2) angular deformity (1) segmental aplasia (1) tissue separation (3) hypoplasia (2) slightly translucence (1)	25	47.5:52.5
	3.5	Folded gonadal (6) segmented hypoplasia (2) aplasia (4) segmental aplasia (1) narrow hypoplasia (1)	35	45:55

The diverse morphological abnormalities observed in all the six formulations confirmed the potential of these formulations to cause subtle reproductive abnormalities. Most of the formulations initially tested showed relative high abnormality indexes, including Midstream (32.5-60%), Basta (15-27.5%), Arsenal 17.5-35%), Kilo Max (22.5-43%), Roundup (20-30%) and Enviro Glyphosate (17.5-37.5%), incidence mostly being much higher than the natural occurrence of these abnormalities recorded for tadpoles in the buffered RO water control group.

The functional significance of these abnormalities, in particular regarding decreased reproductive success, still needs further study.

Adult Male exposure

No endpoint measured showed significant variation following the 28-day exposure to the range of herbicide concentrations (ANOVA, $p > 0.05$) although some trends were observed. The Midstream formulation reduced the body mass of the exposed frogs, while frogs exposed to the Arsenal formulation showed an increased in body mass as compared to the control frogs (Table 8). Both formulations resulted in an increase the length dimension of the testes compared to the males in the clean water control group. Midstream reduced the testes width, while the Arsenal formulation increased the width except at the highest exposure concentration. The hepato-somatic index and gonado-somatic index showed slight increases in the herbicide exposed male frogs.

Plasma Vitellogenin (VTG) as a biomarker for oestrogenic activity in males did not vary significantly ($p > 0.05$) among treatment groups (Table 8). Plasma thyroxine (T_4) hormone concentrations varied in males exposed to the two herbicide formulations, increased trend following exposure to the Midstream formulation, but no real trend following the exposure to the Arsenal formulation as compared to the control frogs. Plasma testosterone did not vary significantly among treatment groups (Table 8). Exposure to Arsenal showed slight reductions in mean plasma testosterone when compared to the males from the control group.

Table 8: *Summary of the exposure results (mean ±SD) following a 28-day exposure to different concentrations of the two selected herbicides,* ***A:*** *Midstream and* ***B:*** *Arsenal. Group means differing significantly from the Control (0) are marked with asterisk (Dunn's test, $p < 0.05$).*

A)

Endpoint (mean ± SD)	Midstream concentration			
	0 mg/L	0.25 mg/L	0.45 mg/L	0.65 mg/L
Body Mass (g)	42.8 ± 7.4	32 ± 6.8	41.6 ± 8.2	41.6 ± 8.9
Testes Length (mm)	6.6 ± 0.9	7.1 ± 0.8	6.89 ± 0.9	6.85 ± 0.9
Testes Width (mm)	3.09 ± 0.3	2.8 ± 0.5	2.96 ± 0.5	2.77 ± 0.3
GSI	0.21 ± 0.1	0.27 ± 0.1	0.26 ± 0.1	0.23 ± 0.1
HSI	5.51 ± 2	5.05 ± 1.7	5.29 ± 1.9	5.91 ± 1.5
VTG (ng/mL)	1 ± 0.6	1.88 ± 1.3	1.6 ± 0.4	2.53 ± 1.6
T_4 (ng/mL)	20.3 ± 5.8	26.8 ± 7.2	24.2 ± 5	21.2 ± 2.9
Testo (ng/mL)	42.4 ± 8.6	43.5 ± 7.8	42.3 ± 6.3	44.2 ± 13.1

B)

Endpoint (mean ± SD)	Arsenal concentration			
	0 mg/L	2 mg/L	4 mg/L	8 mg/L
Body Mass (g)	42.8 ± 7.4	53.3 ± 8	49.9 ± 8.3	44.1 ± 9.5
Testes Length (mm)	6.6 ± 0.9	7.9 ± 0.9	7 ± 1	7.4 ± 1.1
Testes Width (mm)	3.09 ± 0.3	3.27 ± 0.5	3.15 ± 0.4	2.79 ± 0.4
GSI	0.21 ± 0.1	0.2 ± 0.1	0.22 ± 0.03	0.23 ± 0.1
HSI	5.51 ± 2	5.6 ± 1.1	5.6 ± 1	5.51 ± 1.51
VTG (ng/mL)	1 ± 0.6	1.39 ± 1	3 ± 2.6	1.23 ± 0.5
T_4 (ng/mL)	20.3 ± 5.8	22.1 ± 7.6	18.4 ± 5.4	22 ± 6.2
Testo (ng/mL)	42.4 ± 8.6	43.6 ± 7.7	41.9 ± 6.3	40.8 ± 4.8

The histopatological assessment revealed that the Midstream formulation increased percentages of both spermatogonia and spermatocytes, while reducing both spermatids and spermatozoa in the testis. Arsenal formulation exposure led to a decrease in the percentage of spermatogonia, spermatocytes and spermatids compared to the control treatment.

2.2.6. Conclusions

Acute Toxicity (96-hour LC_{50} test)

The result of this study revealed the high sensitivity of premetamorphic NF-stage 48 of *X. laevis* to all the tested herbicides formulation relative to early embryos (NF-stage 8-11) and tadpoles undergoing metamorphosis (NF-stage 60). The study showed differential response to different glyphosate formulations (real world scenario) affected survival differentially, and with Enviro Glyphosate having the lowest LC_{50} concentration among the glyphosates. The 96-hour LC_{50}s of the premetamorphic larva (NF-stage 48) for Roundup, Midstream and Basta formulations were found to be lower than the expected environmental concentration (EEC) of those formulations, which can therefore potentially affect survival rates and long-term amphibian population dynamics. Although the LC_{50} concentrations of the embryos were generally higher, these pre-hatch stages may not be adequately protected. With the susceptibility of premetamorphic larva, it is clear that the protection of one developmental stage will be ecological inadequate if another developmental stage is seriously vulnerable to the same chemical. The fact that similar trends have also been identified in several anuran species, suggest that premetamorphic tadpoles represent a vulnerable life stage that could be an important factor in understanding the global amphibian declines.

This study therefore confirmed the importance of using premetamorphic tadpoles (for example NF stage 48) in exposure studies to evaluate the potential threat that pesticides formulation reaching the aquatic environment, directly or indirectly, may hold for non-target amphibian species. This may prove to be an important consideration for aquatic alien plant eradication programmes using chemical means, since the mobility of glyphosate for example is known to be slow in the soil but may pose a greater risk in aquatic system when applied directly

FETAX (Embryotoxicity test)

The present study showed that Roundup, Midstream and Basta formulations may be embryotoxic to the *X. laevis*, while Arsenal, Enviro Glyphosate and Kilo Max showed relatively low toxicity to the *X. laevis* embryo. Kilo max, Roundup, Basta, and Arsenal formulation revealed significant growth disruption. In terms of teratogenicity, Midstream formulation showed a strong teratogenic potential, while Enviro Glyphosate showed positive of teratogenic potential at relative high exposure concentrations.

These results confirm the potential contribution of pesticides/chemicals contamination as a causal factor associated with global declines of amphibian populations, as many of these formulations revealed toxicity, growth inhibition and teratogenicity particularly at the sensitive embryonic stages. The importance of embryotoxicity assessment at the first tier stage of testing must not be underestimated, especially screening the formulation as they are applied in practice, and not just the active ingredient. This information will be valuable when planning investigations including extended endpoints, for example screening for endocrine disrupting activity. Although the link between developmental affects and endocrine disruptors is still under investigation, thyroid disruption has been implicated in certain cases, for example Atrazine and Roundup.

In South Africa, herbicide formulation selection for application in agriculture and non-agriculture fields, for example directly to aquatic environments for alien plant control (WfW programme), could benefit from embryotoxicity and malformation incidence data using amphibians as models.

Xenopus Metamorphosis Assay (XEMA) – Thyroid disruption

Herbicides formulations like Midstream, Basta, Arsenal, Roundup and Kilo Max differentially inhibited the developmental stages of *X. laevis*, and therefore considered to be thyroid active. It was also evident that Arsenal, Roundup, Basta and Midstream formulations, at relevant environmental concentrations, have the potential to disrupt the developmental growth the functioning of the thyroid axis. Given the antecedent of Atrazine herbicides and the fact that more than 60% of herbicides are alleged to be thyroid disrupting chemicals, more attention should be directed towards understanding the thyroidal-herbicide interaction (including all the

different herbicide groups). Characterization mechanism of action regarding the interaction with the thyroid systems needs more study and will be helpful in herbicide selection as well as ecological risk assessment models. In disrupted thyroids, histopathology mostly showed severe follicular cell hypertrophy but with variation regarding thyroid gland area ranging from severe atrophic (Arsenal) to hypertrophic with varying amount of colloidal area. Important to note that several of the formulations did not significantly affect the mean development stage after the 21-day exposure but histopathology showed significant variation when compared to the control tadpoles.

Post-metamorphic juvenile exposure (sexual development)

The results of this study showed the potential capacities of Midstream (Diquat dibromide), Basta (Glufosinate ammonium), Arsenal (Imazapy) and Glyphosate formulations (Roundup, Kilo Max and Environ Glyphosate) to negatively impact the reproductive fitness of amphibians. This is either through the reduced growth rate as demonstrated by Midstream and Kilo Max, Roundup, and Enviro Glyphosate formulations on the growth rate of *X. laevis*. Developing frogs showed Th sexual bias towards the female phenotype when exposed to Midstream, Basta and Kilo Max or to male bias when exposed to the Arsenal formulation. Gonadal development and differentiation phenotype were also effected following exposure to the six herbicide formulations. Even though the physiological and reproductive impact of gonadal malformations are still largely unknown, in particularly as it may have effects on reproductive success within the larger population. Clearly, more attention is therefore required to understand the implications of induced malformations on reproduction in amphibians. Attention should also be extended to other herbicides formulations, as they may have more reproductive toxicity potential than currently thought. As noted by several authors, gonadal malformations, as found in this study, may lead directly to reproductive dysfunction and are therefore likely to reduce the reproductive success of exposed aquatic individuals (Howe et al., 2004).

Adult male exposure (reproductive endocrine disruption)

The 28-day exposure of adult male *Xenopus laevis* frogs to two herbicide formulations, Midstream and Arsenal, had very little effect on the reproductive and thyroid endpoints measured. Large individual variation was recorded in many of the endpoint responses and more individuals should be included in exposure groups. Endpoints should be increased to include secondary sexual characters and in light of some evidence of oestrogenic activity, separate adult female exposures conducted.

2.2.7. *Location of the full version:*

Babalola, O.O. 2014. Ecotoxicological and potential endocrine effects of selected aquatic herbicides on life stages of the African clawed frog, *Xenopus laevis*. Chapter in PhD Thesis, Stellenbosch University, Stellenbosch.

2.3. Fungicides as endocrine disruptors

Researcher: E. Archer, MSc, Stellenbosch University

2.3.1. *Specific aims of this project*

- To describe and validate male breeding glands in *Xenopus laevis* as biomarkers for androgenic activity.
- To conduct *in vitro* screening of fungicides used in the Western Cape for (anti)androgenic activity.
- To assess the exposure effects of regularly-used fungicides (individual and in mixture) in adult male African clawed frogs (*Xenopus laevis*).

2.3.2. *Materials and Methods*

Breeding gland description and validation

Adult male frogs were exposed for 21 days to human chorionic gonadotropin (hCG) (100 i.u.), the AR antagonistic pharmaceutical Flutamide (100 µg/g body mass), the 5αR inhibitor Dutasteride (5 µg or 50 µg/g body mass) or to the dithiocarbamate fungicide Mancozeb (50 µg/g body mass). Test chemicals were injected once a week into the dorsal lymph sac, as illustrated in Brown (1970). Dimethyl-sulfoxide (DMSO) was used as solvent for the Flutamide, Dutasteride and Mancozeb stock solutions. Six frogs were used for each exposure and kept in 10 L activated charcoal filtered tap water at a temperature of 24 ± 2°C and photoperiod of 14 hours light and 10 hours dark cycle. All frogs were injected with hCG for the exposure period and frogs in the Flutamide, Dutasteride and Mancozeb treatment were additionally injected with the test chemicals following the hCG injection. Six frogs were also injected with only hCG during the 21-day exposure, which served as a control group. At the onset of the 21-day exposure, all frogs were euthanized according to the American Veterinary and Medical Association (AVMA, 2007). Blood was drawn directly from the heart using a heparinized needle for plasma T and DHT steroid hormone analysis of control, Flutamide and Dutasteride-exposed frogs, using Enzyme-Linked Immunosorbert Assay (ELISA) kits directed for each steroid hormone. Testis and skin samples (containing breeding glands) were removed and taken for basic histology using Heamatoxilin and Eosin (H&E) staining (Bancroft and Stevens, 1977). Skin samples of frogs from the control and Flutamide treatment were taken for ultrastructural investigation using transmission electron microscopy (TEM) and was done at the University of Cape Town (EMU unit, RW James Building,

UCT upper campus, Cape Town, South Africa). Skin samples from the control, Flutamide and Mancozeb treatment were also used for immunohistochemistry investigation of AR localization using the PG21 primary anti-body. Staining procedures were done according to the anti-body manufacturer (Upstate Cell Signalling Solutions, Millipore) and analyses were performed at the Central Analytical Facility Imaging Unit of Stellenbosch University (SU-CAF).

Tadpoles and juvenile *X. laevis* frogs were reared in the lab using an in-house artificial breeding protocol and according to literature (Brown, 1970; Opitz *et al*., 2005). Staging of the tadpoles throughout metamorphosis was done by following the anatomical description of developing *X. laevis* tadpoles (Nieuwkoop and Faber, 1956). Upon reaching NF stage 47/48, forty tadpoles were randomly selected and transferred to 10 L glass tanks (Opitz *et al*., 2005) and cultured to developmental stage NF 56-59 (pre-metamorphic stage), when the forearms are starting to develop (Nieuwkoop and Faber, 1956). Remaining tadpoles from the in-house breeding program were cultured through metamorphosis until a post-metamorphic juvenile stage was achieved (Nieuwkoop and Faber, 1956), which were then used for the juvenile exposure experiments. Forty pre-metamorphic *X. laevis* tadpoles (NF stage 56-59) with well-developed front limbs were exposed to the synthetic androgen 17α-Methyltestosterone (17α-MT). Tadpoles were exposed to nominal concentrations of 0, 30, 100 and 300 μg/L 17α-MT with ten tadpoles in each group. The stock solutions of 17α-MT were dissolved in DMSO at the three varying concentrations and then added to the exposure tanks to be dissolved in the water. Twenty juvenile *X. laevis* frogs (5 days post-metamorphic) were used to assess the development of breeding glands in normal frogs, as well as frogs exposed to 17α-MT. Two exposure groups were used with ten frogs in each group, either exposed to 0 or 600 μg 17α-MT per 10 g food. The stock solution of 17α-MT was dissolved in absolute ethanol and added to trout pellets (AquaNutro). The wet food mixture was then dried under a heat source to allow the alcohol to vaporize. Frogs were then fed with the 17α-MT-spiked trout pellets twice a week, estimating five pellets per frog in each exposure tank. Upon completion of the 21-day exposure, both tadpoles and juveniles were euthanized with an overdose of Benzocaine (250 mg/L; AVMA, 2007). Skin samples taken from the front extremities in both tadpoles and juvenile froglets were taken and subsequently subjected to routine Heamatoxilin and Eosin (H&E) histology (Bancroft and Stevens, 1977). The glandular composition of the integuments was compared between exposure groups as well as with the integument containing breeding glands in adult male *X. laevis* frogs.

In vitro screening of fungicides

We followed the basic protocol as described in Sohoni and Sumpter (1998) for the recombinant yeast anti-androgen screen (anti-YAS), but with modifications as described in later studies (Urbatzka *et al*., 2007; Kolle *et al*., 2011). The fungicides Vinclozolin, Folpet, Procymidone, Dimethomorph, Flusilazole, Fenarimol and

Mancozeb as well as the insecticide Chlorpyrifos were selected according to their regular use in agriculture, especially on vineyards (Table 9).

Table 9: *Test chemicals and their suggested mechanisms of endocrine disrupting action. Test chemicals were used in the current study to evaluate anti-androgenic (inhibition of AR binding) activity of binary mixtures as well. Selected compounds were diluted to obtain 12 different concentrations, before added to the yeast cultures along with 6.25 nM DHT. Vinclozolin and DHT served as positive controls for the anti-YAS and YAS respectively. Pesticide active ingredients indicated with an asterisk (*) are anti-fungal pesticides.*

Pesticide active ingredient	Chemical class	Anti-androgenic mechanism of action	Reference
Vinclozolin*	Dicarboximide	AR binding antagonist	Kolle et al., 2011
Fenarimol*	Pyrimidinyl carbinol	AR binding antagonist	Vinggaard et al., 2005
Mancozeb*	Dithiocarbamate	AR binding antagonist	Viswanath et al., 2010
Folpet*	Dicarboximide	Expected AR binding antagonist (same chemical group as vinclozolin and procymidone)	n/a
Dimethomorph*	Cinnamic acid	AR binding antagonist	Orton et al., 2011
Procymidone*	Dicarboximide	AR binding antagonist	Ostby et al., 1999
Flusilazole*	Triazole	Decrease androgen biosynthesis	Trösken et al., 2004
Chlorpyrifos	Organophosphate	Decreased testosterone biosynthesis, AR binding antagonist	Viswanath et al., 2010

Test chemicals were evaluated in the presence of 5α-androstan-17ß-ol-3-one (DHT) at a concentration of 6.25 nM (EC_{50} from a generated DHT standard curve in the assay). All pesticides were tested for their ability to inhibit the agonistic action of the androgen spike. The fungicide Vinclozolin was used as the negative control in the assay due to its similar dose-response than that of the model AR antagonistic compound Flutamide. Stock solutions of test chemicals were made up at a concentration of 20 mM in absolute ethanol and serially diluted in a sterile 96-well flat bottom plate to obtain 12 concentration ranges of each test chemical (Greiner Bio-one, DE). Each diluted concentration was transferred to a sterile 96-well flat bottom assay plate with a low evaporation lid (Corning Costar, USA) at 10 µL/well along with the DHT (EC_{50}) spike. Assay medium was added (200 µL/well) to give a final 1/20 dilution of the test chemicals in the assay. The pesticide dilutions were tested in duplicate in two separate plates and in two separate experiments. A colour change of the assay medium was observed after 48 hours of incubation (31°C) and

absorbance was measured at 570 nm for colour change and 620 nm for turbidity using a plate reader (Thermo multiscan, type 355, Ascent, version 2.6, Thermo Labsystems). These measurements were used to calculate the final absorbance (corrected value) of each dose-response effect from each test compound. The absorbance of the test chemical dilutions was also used to calculate the IC_{50} (inhibition concentration at 50% relative to the control respectively) for each chemical as well as the relative potency (RP) for each pesticide in the assay. The RP indicates the efficiency (%) of the chemical to antagonize the DHT-AR binding relative to the model AR antagonistic fungicide Vinclozolin.

Following the individual chemical exposures, the pesticides were screened in the anti-YAS for simple mixture effects. Pesticides used for binary mixtures were added at equimolar concentrations (starting concentration, 1 mM) and tested in the same manner as with individual pesticides. Mancozeb was the only pesticide that was included in mixture at starting concentration of 1.95 µM due to its high individual potency in the assay. The IC_{50} for each mixture was calculated and used to determine the isobole coefficient of the mixture as described by Birkhøj *et al.* (2004).

Adult male frog exposure to fungicides

Twenty four adult male *X. laevis* frogs were used for the exposure experiment. Six frogs were grouped into for treatment groups consisting of a control (no treatment), Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) and a Manzozeb/Folpet mixture (0.25 + 0.05 mg/L respectively). Frogs were kept in a climate controlled room under 25ºC temperature and photoperiod of 14 hours light and 10 hours darkness. Treatment of test chemicals was done by keeping frogs in 15 L glass aquaria containing 10 L of reverse-osmosis (RO) water supplemented with 2.5 g of iodized sea salt (Seepo, 0.004 g/100 g NaCl). Test chemicals were dissolved in DMSO and exposed directly in the water, according to the exposure concentrations shown above. Control-treated frogs also received the same amount of DMSO in their water. The concentration of DMSO in the exposure tanks was below 0.01% relative to the volume of tank water (< 1 mL). Water was replaced three times a week following new test chemical exposure. Exposure lasted for 21 days. At completion of the 21-day exposure, the body mass (BM) of each frog was measured to the nearest 0.01g and snout-to-vent length (SVL) to the nearest 0.01 cm, to calculate a condition index (CDIndex; Janssen *et al*., 1995).

After the morphometric measurements, frogs were sacrificed by decapitation and pitching (according to AVMA, 2007) and blood samples were taken directly from the heart using a heparinized syringe and needle. Blood was centrifuged at 8000 rpm for 2 min; plasma extracted and stored at -80ºC for plasma steroid hormone (testosterone, T; dihydrotestosterone, DHT; oestradiol, E_2) and Vitellogenin (VTG) analysis. The gonads and liver were removed and weighed to the nearest 0.01 g to calculate a gonadosomatic index (GSI) and hepatosomatic index (HSI) for each frog. Gonads were then placed in Bouin's fixative (Bancroft and Stevens, 1977) for

histological analyses. Skin samples (5 x 10 mm) including the NP integument, situated on the forearms of the frogs (location of breeding glands), were also removed and fixated in 10% buffered formalin (Bancroft and Stevens, 1977) for histological analyses.

Circulating hormone and vitellogenin (VTG) determinations

Plasma testosterone (T), dihydrotestosterone (DHT) and oestradiol (E_2) levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (DRG Instruments GmbH). For T analysis, unextracted plasma samples were diluted 40 fold with PBS containing 0.1% human serum albumin (HSA) and put through the steps provided by the ELISA kit in duplicate. Standard curves were calculated using standards provided in the ELISA kits. Validation of the T assay is explained elsewhere (Van Wyk *et al*., 2003) and is shown to have a sensitivity of 0.083 ng/mL with low cross-reactivity for other hormones. For the DHT analysis, unextracted plasma samples were diluted 200 fold with PBS containing 0.1% HSA and put through the steps provided by the ELISA kit in duplicates. The DHT ELISA kit has a sensitivity of 0.019 ng/mL and also a low cross-reactivity for other hormones. For plasma E_2 analysis, unextracted plasma samples were diluted 10 fold with PBS containing 0.1% HSA and put through the steps provided by the ELISA kit in duplicates. The E_2 ELISA kit has a sensitivity of 0.0097 ng/mL and also a low cross-reactivity for other hormones. Data generated for plasma T, DHT and E_2 concentrations were also used to calculate the ratio of DHT relative to T (plasma DHT/T ratio) and E_2 relative to T (plasma E_2/T ratio) of each frog to compare androgen turnover between frogs within the different treatments. Blood plasma samples were also used for the determination of the circulating protein VTG produced in the liver, which serves as a biomarker to indicate estrogenicity in male frogs (Van Wyk *et al*., 2003). Plasma VTG were measured using an in-house ELISA protocol as described elsewhere (Van Wyk *et al*., 2003). Plasma VTG levels was detected and compared between treatments and expressed as relative fold change from the levels obtained from the control treatment.

RNA isolation and cDNA synthesis

Liver tissue was collected from adult male frogs and stored in RNAlater (Sigma, DE). The tissues were subsequently homogenized in Trizol (Invitrogen, US) using an ultrasound sonicator (Omni-ruptor 400, Omni International Inc., USA) and total RNA isolated according to the manufacturer's instructions with slight modifications. Isopropanol and 0.8 M sodium citrate were applied to precipitate RNA, followed by two washes with ice cold 70% ethanol. RNA integrity was assessed spectrophotometrically using a Nanodrop (Thermo Scientific, USA) and through agarose gel electrophoresis. The RNA of suitable integrity was subsequently DNase I (Zymo Research, USA), treated. Complementary DNA (cDNA) was synthesized using RevertAid reverse transcriptase kits (Thermo Scientific, US) (2 µg RNA per 20 µL reaction volume) using a combination of Oligo (dT)18 and random hexamer primers.

RT-qPCR

The expression of *thyroid receptor-β b* (*trβ-b*), *type 2 deiodinase* (*dio2*) was evaluated using real-time RT-qPCR with *ribosomal protein l8* (*rpl8*) as housekeeping gene. The PCRs were performed as 15 µL reactions containing 2 µL cDNA (10 ng/reaction), 7.5 µL Jumpstart® SYBRgreen mix (Sigma, DE), 0.33 µM of each primer and nuclease free water. The PCR programs for al primer pairs included an enzyme activation step at 95°C (9 minutes), followed by 40 cycles of denaturing at 95°C (15 seconds), annealing at 55°C (*trβ* and *rpl8*), or 63°C (*dio2*) (30 seconds) and elongation at 72°C (45 seconds). Each PCR plate contained an internal non-template control (no cDNA) as well as a six point two-fold serial dilution. All samples and controls were run in triplicate and dissociation curves were applied to confirm single-fragment amplification. Gene expression was quantified using the Pfaffl method (Pfaffl, 2001). Amplification efficiencies were determined for each primer pair per PCR programme.

The primers sequences and sources were as follows: *rpl8* sense 5' AGAGCCCATGTAAAGCAC 3', *rpl8* antisense 5' CCTGTAAGGGTCACGGAA 3' (Esterhuyse *et al.*, 2009); *dio2* sense 5' AGGCTGAGTGTGGACTTG 3', *dio2* antisense TGACCTGCTTGTAGGCATCCA 3' (Searcy *et al.*, 2012); *trβ-b* sense 5' AAAGTGCCAGGAAGGTTTCCT 3', *trβ-b* antisense 5' GGTCGGTGACTTTCATCAGCA 3' designed using Premier Primer (Version 5, Premier Biosoft International, USA) (Genbank: NM_001087781.1).

2.3.3. Statistical Procedures

All statistical analyses were performed using Statistica 10.0 (StatSoft Inc., USA). All datasets were tested for normality (Shapiro Wilk-W) and parametric or non-parametric analyses were selected accordingly. Parametric analyses were done using a breakdown and one-way analysis of variance (ANOVA). When statistical significance was obtained, ANOVA was followed by Tukey's honest significant difference (HSD) post-hoc test to test for significant differences between treatments. Statistical significance for all analyses was reported at p-values < 0.05.

2.3.4. Results

Breeding glands of Xenopus laevis males

Adult male *X. laevis* exposures

Histological observations of breeding glands in the hCG-induced control group showed a typical stimulated state of breeding glands in male *X. laevis* frogs, which is characterized by a large cross-sectional gland area with densely packed columnar secretory cell epithelium (Fig. 12A). The stimulated state of the breeding glands in these frogs was also accompanied by the development of well-defined epidermal keratinized hooks (KHs; Fig. 12A). Frogs from a treatment with the AR antagonist

(Flutamide) show decreased secretory cell heights, with cells being more cuboidal in shape (Figs. 12A & B). Few or no KHs were seen at the surface of the epidermis as compared to control-treatment frogs (Fig. 12B).

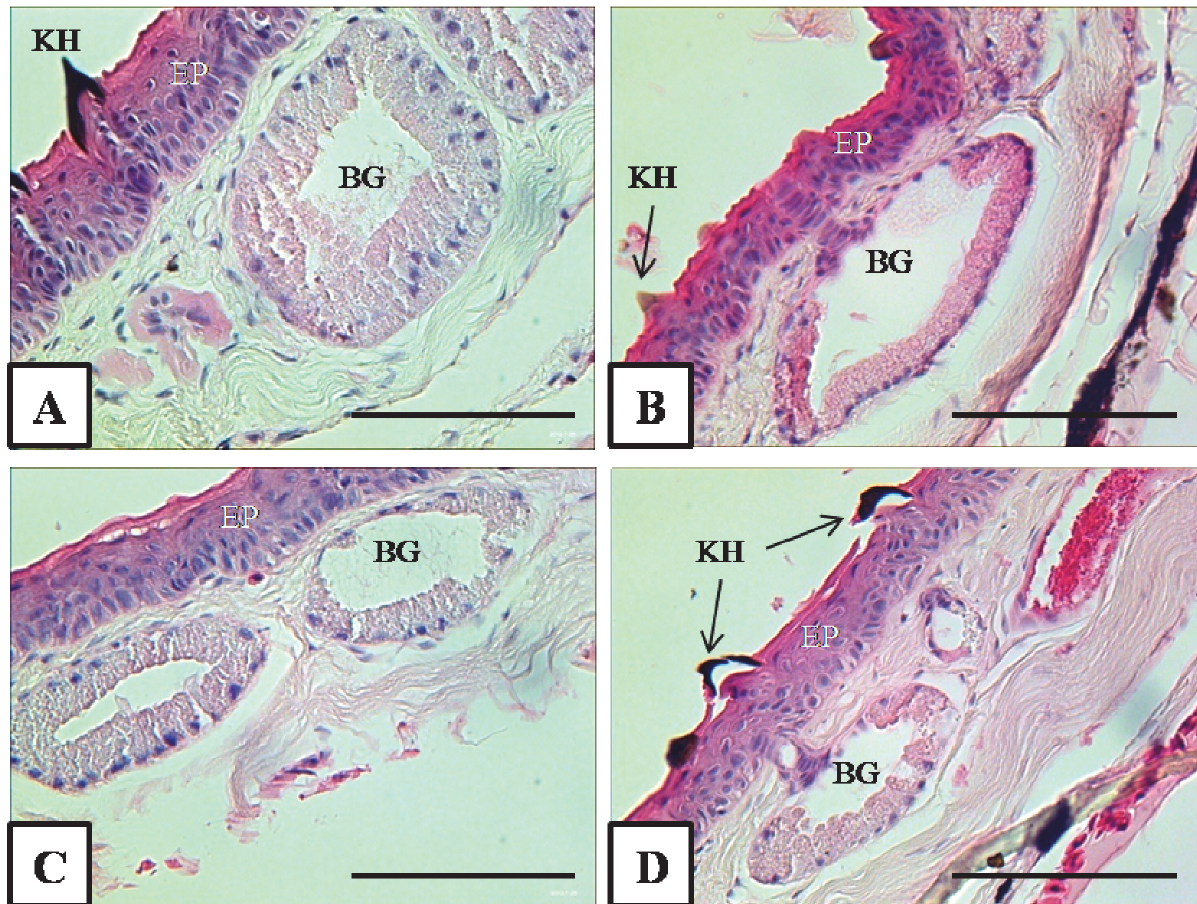


***Figure 12**: Cross sections (400x) of the NP area on the ventral forearm of male X. laevis frogs containing breeding glands. Treatment groups: (**A**) 100 i.u. hCG (control); (**B**) 100 ug/g Flutamide + 100 i.u. hCG, (**C**) 5 µg/g Dutasteride + 100 i.u. hCG and (**D**) 50 µg/g Dutasteride + 100 i.u. hCG. Labels: KH, keratinized hooks; EP, epidermis; BG, breeding gland. Scale bar: 100µm.*

All frogs within the different exposure groups showed significant variation in breeding gland morphometrics when compared to the control group (Fig. 12). Male frogs administered with the anti-androgens Flutamide and Dutasteride, along with an hCG injection of 100 i.u. per week, showed reduced development of breeding glands compared to hCG-treated frogs (Fig. 13). Flutamide and Dutasteride treatment significantly reduced breeding gland cross-sectional areas (Figs. 13A & B; ANOVA, $F_{3,26} = 7.54$; Tukey's HSD, $p < 0.05$) as well as gland secretory cell epithelium height compared to frogs injected with only hCG (control treatment) (Figs. 12 & 13B; ANOVA, $F_{3,26} = 20.45$; Tukey's HSD, $p < 0.05$). No significant differences were recorded in either cross-sectional breeding gland areas or secretory cell epithelium height between the different anti-androgen treatments (Tukey's HSD, $p > 0.05$). The epidermis of the NP skin area also did not show significant hypertrophy (variation in

thickness) when comparing frogs in all treatments with the frogs from the hCG-induced control group (Fig. 13; ANOVA, $F_{3,26} = 5.47$, $p > 0.05$). In the anti-androgen-treated frogs (Flutamide and Dutasteride), breeding gland cross-sectional areas were shown to be decreased in size, with secretory cell epithelium being more cuboidal in shape, but still showing densely packed secretory cells containing eosinophilic granules. The outer epidermis of the Flutamide and Dutasteride exposed males also showed fewer or no keratinized hooks compared to the hCG-control treatment frogs (Fig. 12).

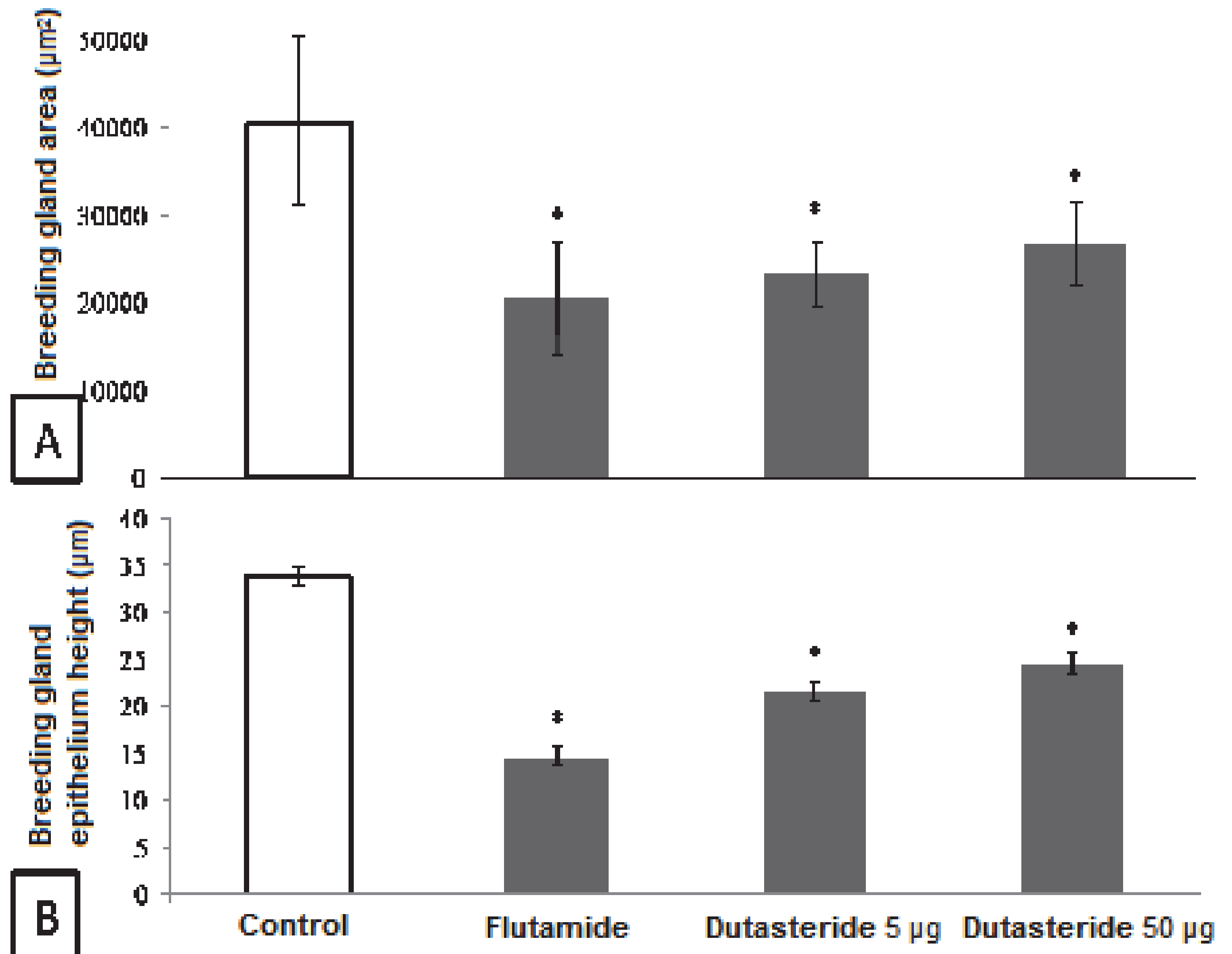
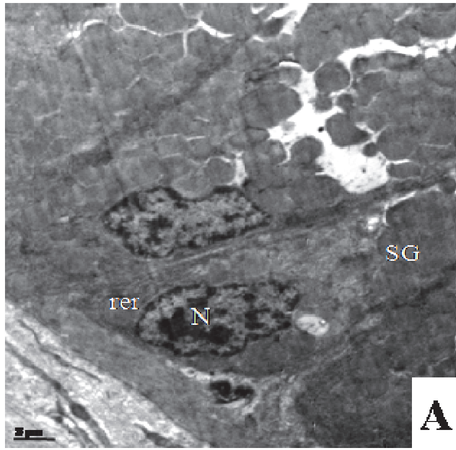


Figure 13: *Variation in the overall size (area) of breeding glands (**A**; μm^2 ± SD) and breeding gland epithelium height (**B**; μm, ±SD) among male X. laevis frogs exposed to hCG (control), Flutamide (AR antagonist) and two concentrations of Dutasteride (5αR antagonist). Six breeding glands and the length of ten epithelial cells within each breeding gland were measured per frog (n = 6) in each exposure group. The asterisk indicates a significant difference of treatment groups compared to control frogs (ANOVA; Tukey's HSD post hoc test: p < 0.05). Treatment groups: hCG control = 100 i.u. hCG; Flutamide = 100 μg/g body mass + 100 i.u. hCG; Dutasteride 5 μg = 5 μg/g body mass + 100 i.u. hCG; Dutasteride 50 μg = 50 μg/g body mass + 100 i.u. hCG.*

The cytoplasm of the SCs contained secretory granules (SGs), which were irregular in shape and filled the SC up to the apical portion of the cell (Fig. 14A). The nucleus located at the basal portion of BG SCs exhibited prevalent diffuse chromatin (euchromatin) content (Figs. 14A & B). The density of the chromatin differed between adjacent cell nuclei; indicating asynchronous cell secretion activity (Fig. 14A). The perinuclear cytoplasm contained clearly visible rough endoplasmatic reticulum (rer) and mitochondria (mi) organelles reaching towards the inner part of the secretory cell cytoplasm (Figs. 14B & C). The apical region of the secretory cells showed large, irregular-shaped SGs and microvilli (mv) on the surface of the cell membrane, directing towards the lumen of the BG (Fig. 14D). The release of secretory product at the apical portion of the cell appeared to be merocrine, in which the membrane of secretory granules fuses with the apical plasma membrane and secretory product is released into the lumen via exocytosis (Fig. 14E).

The ultrastructure of the breeding gland secretory cells in Flutamide-treated frogs displayed less-densely packed SGs occupying the cell cytoplasm, as well as a less-differentiated cell membrane dividing adjacent cells (Fig. 14F). The basal portion of these cells showed less visible rough endoplasmatic reticulum and mitochondria surrounding the nucleus (Fig. 14G). The nucleus appeared pyknotic in shape with more prevalent heterochromatin content and less visible nuclear pores (Figs. 14F & G). The apical region of the secretory cells showed smaller secretory granules as compared to a stimulated breeding gland as well as the presence of a different type of secretory granules and less electron-dense secretory product (Fig. 14H).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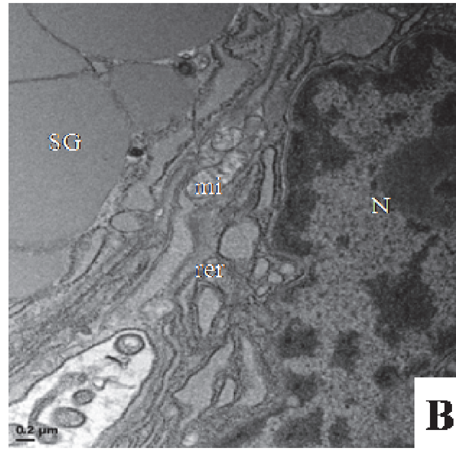
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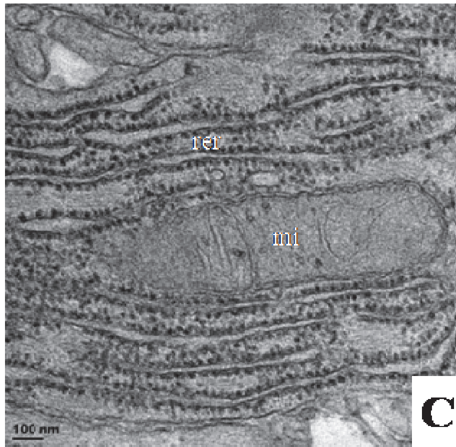
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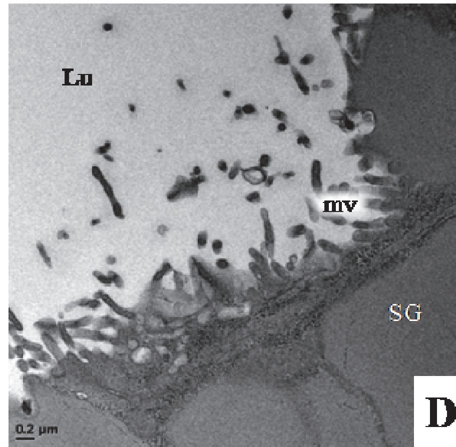
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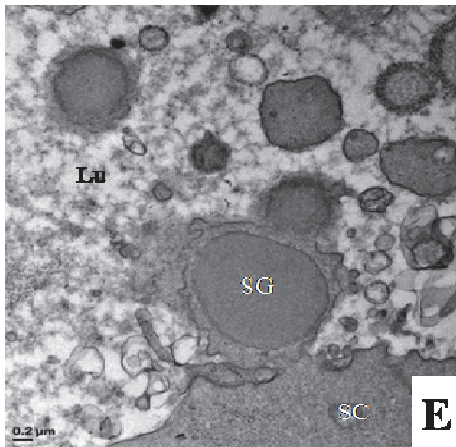
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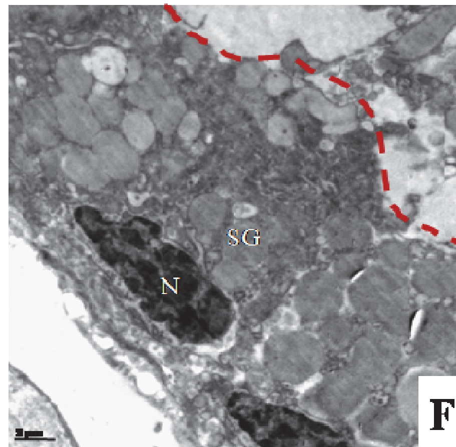
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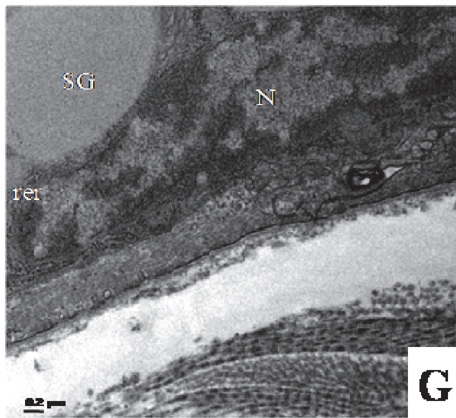
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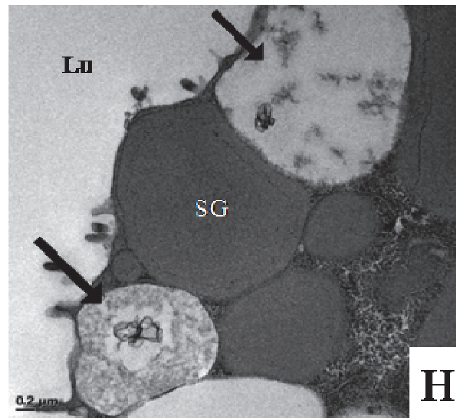
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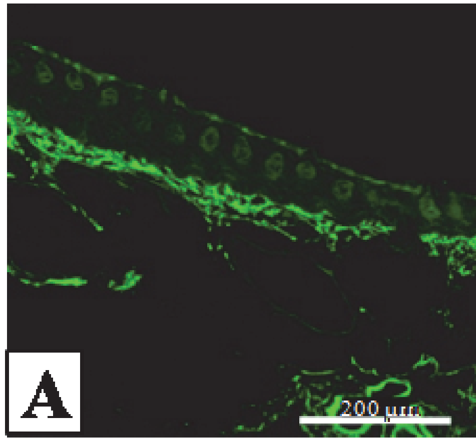
Figure 14 (on previous page): *Transmission electron micrographs of regions of a stimulated (hCG-induced; A, B, C, D, E) and inhibited (Flutamide-exposed; F, G, H) breeding gland situated on the front extremities of male X. laevis frogs.* ***A:*** *Columnar secretory cell (SC) epithelium of a stimulated gland, showing irregular-shaped secretory granules (SG) and a nucleus situated at the basal portion of the cell.* ***B & C:*** *Clearly visible rough endoplasmatic reticulum (rer) and mitochondria (mi) situated in the perinuclear portion of the SC.* ***D:*** *Apical portion of the SC showing microvilli (mv) located on the membrane of the cells leading towards the gland lumen (Lu).* ***E:*** *Merocrine secretion of a SG being released into the Lu.* ***F & G:*** *SCs of an inhibited gland (Flutamide-exposure) showing less developed SGS and a nucleus which is pyknotic in shape.* ***H:*** *Apical region of an inhibited gland SC showing SGs which are smaller in size and some SGs which differ in secretory content.*

Adult male frogs exposed to hCG showed high levels of AR immunopositive nuclei in the epidermis, keratin hooks KHs and breeding gland epithelium (Figs. 15A & B). Skin sections which were treated with PBA lacking the primary PG-21 antibody showed to generate little or no AR-positive immunostaining (Figs. 15C & D). Skin sections of the NP epidermis exposed to either the AR antagonist Flutamide (Figs. 15E & F) or the dithiocarbamate fungicide Mancozeb (Figs. 15G & H) showed low expression of AR-immunolocalization at the epidermis, KHs and breeding gland epithelium compared to the hCG-treatment (Figs. 15A & B).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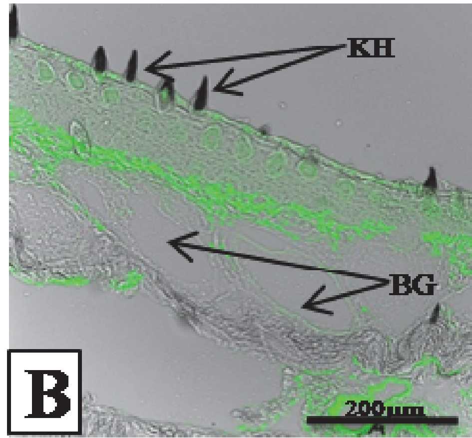
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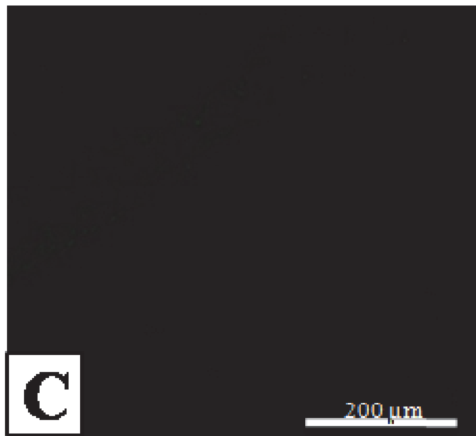
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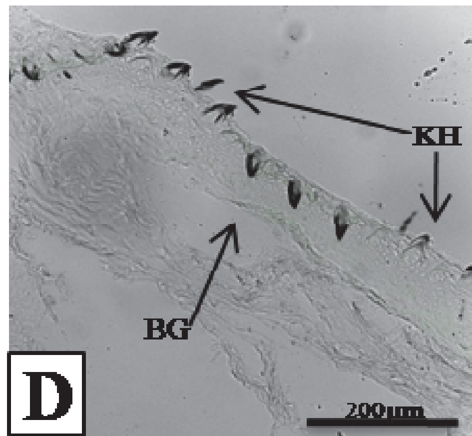
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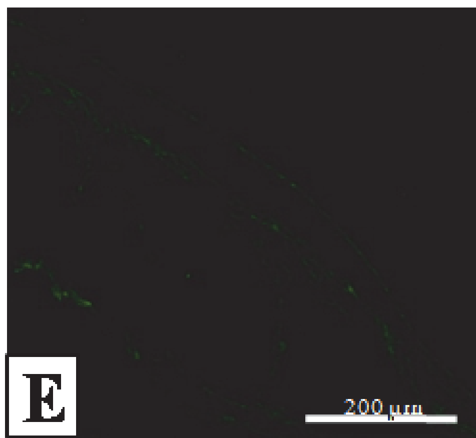
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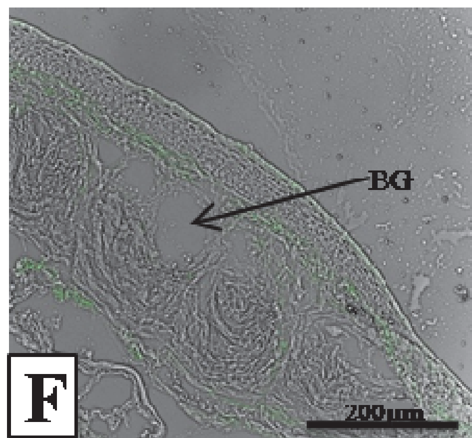
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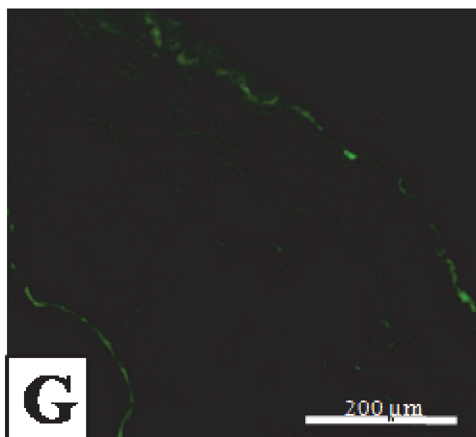
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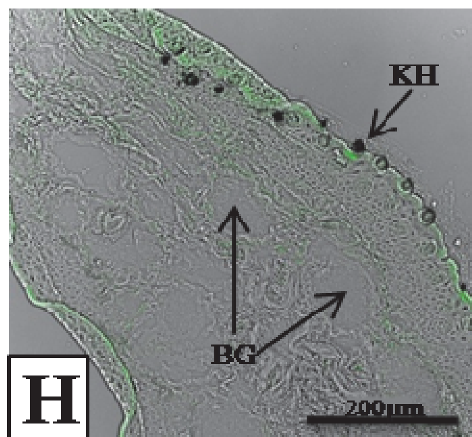
KH
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H
200µm

Figure 15 (on previous page): *Immunohistochemical visualization of the distribution of AR in the NP area of adult male X. laevis frogs exposed to an hCG control (**A** & **B**), negative stained control (**C** & **D**), Flutamide pharmaceutical (**E** & **F**) and mancozeb fungicide (**G** & **H**). The negative stained control (**C** & **D**) represents skin sections stained with PBA only in the absence of the PG-21 primary antibody. Less immunolocalization of AR can be observed in the Flutamide and Mancozeb-exposed frogs (**E, F, G** & **H**) compared to the hCG control (**A** & **B**). Labels: BG, breeding gland; KH, keratinised hooks. Scale bar: 200 µm.*

Plasma hormone analyses

Flutamide treatment did not cause significant modulation of plasma T or DHT concentrations relative to the control (Fig. 16; ANOVA, $F_{3,16} = 8.187$; $p > 0.05$). In contrast, plasma T levels were significantly elevated in the Dutasteride treatments (Fig. 16; ANOVA, $F_{3,16} = 100.70$; Tukey's HSD, $p < 0.05$). Exposure to the highest concentration of Dutasteride (50 µg/g BM) caused a significant decrease in plasma DHT levels relative to control frogs (Fig. 16; ANOVA, $F_{3,16} = 8.19$; Tukey's HSD, $p < 0.05$). There was a significant difference in the plasma DHT/T ratio of both the Dutasteride treatments compared to control frogs (Fig. 17; ANOVA, $F_{3,16} = 48.34$; Tukey's HSD, $p < 0.05$).

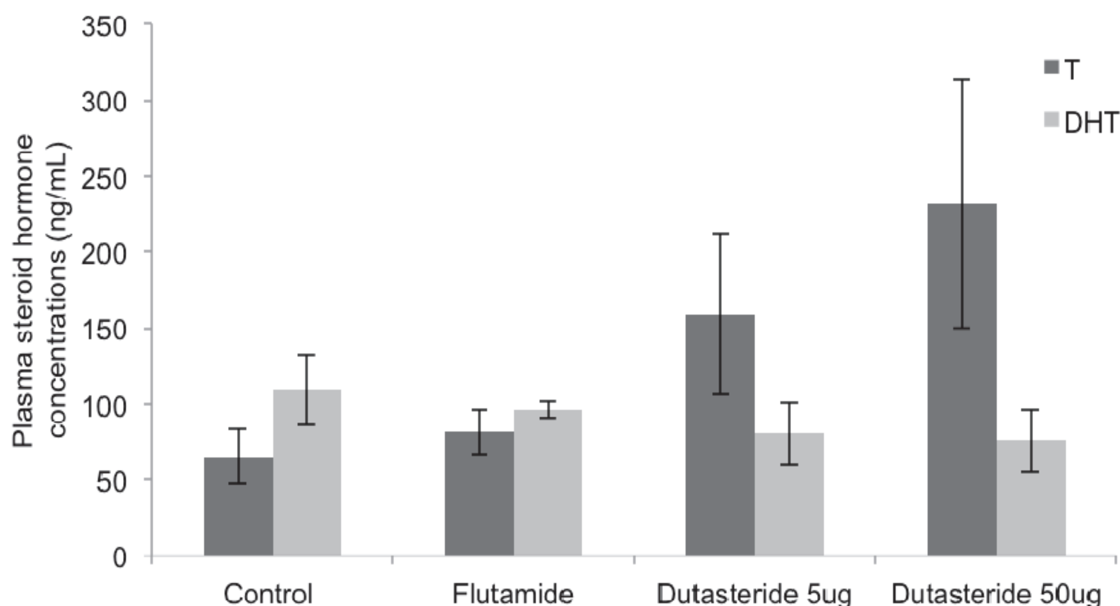

Figure 16: *Variation in plasma testosterone (T) and dihydrotestosterone (DHT) concentrations (ng/ml ± SD) among male X. laevis frogs (N = 6) exposed to hCG (control), Flutamide and two concentrations of Dutasteride. The asterisk indicates a significant difference of treatment groups compared to control frogs (ANOVA: $F_{3,16} = 100.7$; Tukey's HSD post hoc test: $p < 0.05$). Treatment groups: Control = 100 i.u. hCG; Flutamide = 100µg/g body mass + 100 i.u. hCG; Dutasteride 5 µg = 5 µg /g body mass + 100 i.u. hCG; Dutasteride 50 µg = 50 µg/g body mass + 100 i.u. hCG.*

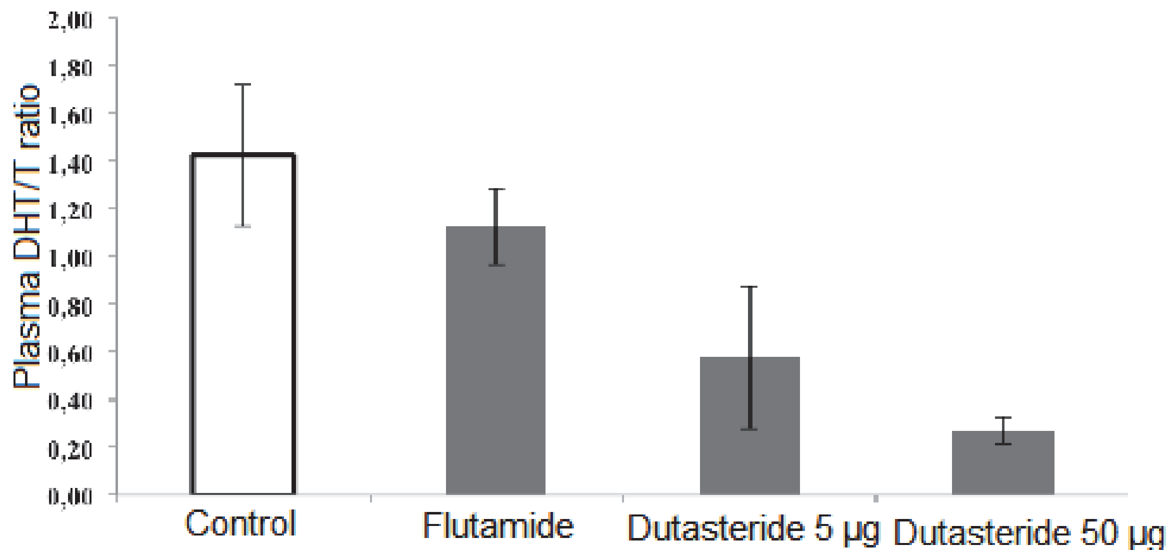

Figure 17*: Variation in the ratio of plasma DHT/T among male X. laevis frogs exposed to hCG (control), Flutamide and two concentrations of Dutasteride. The asterisk indicates a significant difference of treatment groups compared to control frogs (ANOVA: $F_{3,16} = 48.34$; Tukey's HSD post hoc test: $p < 0.05$). Treatment groups: Control = 100 i.u. hCG; Flutamide = 100 µg/g body mass + 100 i.u. hCG; Dutasteride 5 µg = 5 µg /g body mass + 100 i.u. hCG; Dutasteride 50 µg = 50 µg/g body mass + 100 i.u. hCG.*

Spermatogenesis

A differential occurrence of specific germ cell stages in the seminiferous tubules were observed in frogs of all treatment groups, indicating active spermatogenesis for all these animals (Fig. 18). Qualitative data revealed that both Dutasteride treatment concentrations caused a significant increase in percentage of secondary spermatogonial germ cells relative to the hCG control treatment (Fig. 18; ANOVA, $F_{3,26} = 3.83$; Tukey's HSD, $p < 0.05$). In contrast, Flutamide treatment caused a significant reduction in spermatocyte germ cell percentage (Fig. 18; ANOVA, $F_{3,26} = 8.55$; Tukey's HSD, $p < 0.05$) as well as a reduced percentage of spermatozoa compared to the hCG control treatment group (Fig. 18; ANOVA, $F_{3,26} = 5.95$; Tukey's HSD, $p < 0.05$).

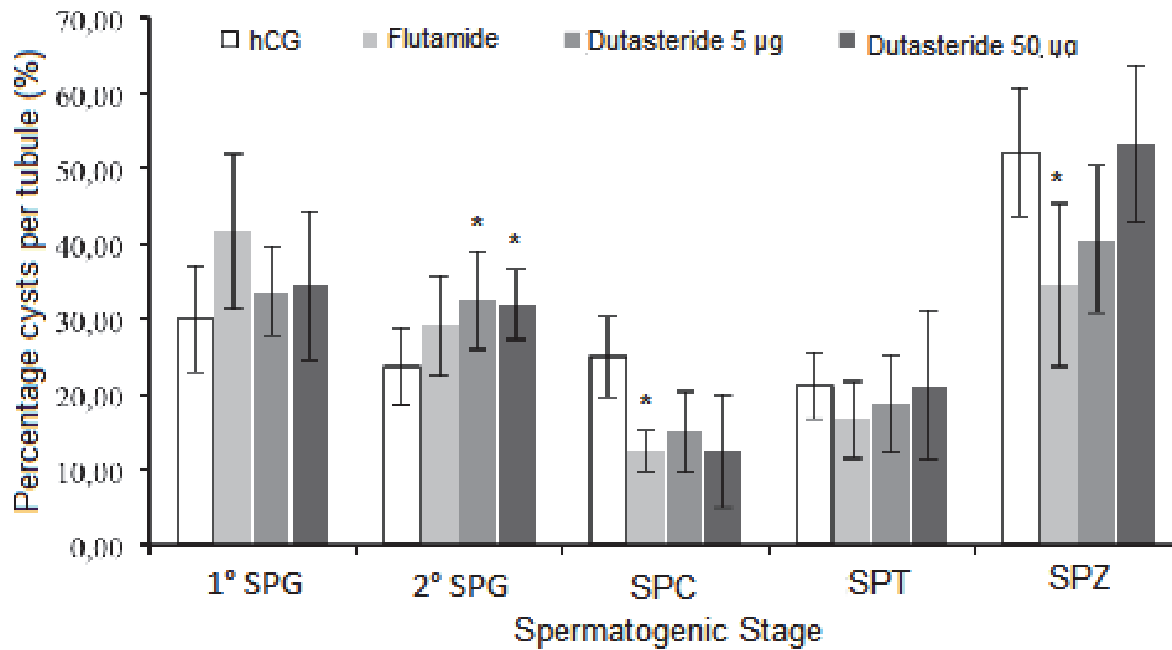

***Figure 18**: Variation in the average percentage (% ±SD) of germ cell cysts and spermatozoa per seminiferous tubules in male X. laevis testes exposed to hCG (control), Flutamide and two concentrations of the 5αR inhibitor Dutasteride. The asterisk indicates a significant difference from the hCG treated control (ANOVA, $p < 0.05$; Tukey's HSD post hoc test, $p < 0.05$).1° SPG, primary spermatogonia; 2° SPG, secondary spermatogonia; SPC, spermatocyte; SPT, spermatid; SPZ, spermatozoa.*

Pre-metamorph tadpole breeding gland development response

The nuptial pad (NP) region in control pre-metamorphic tadpoles (NF 59-62) showed the exclusive presence of dermal mucous glands (Fig. 19A). These glands were characterized as small, simple alveolar glands containing basophilic cuboidal epithelium cells lining the lumen of the gland. The epidermis of control tadpoles did not show any indications of keratin hook (KH) development (Fig. 19A). Skin sections of the NP integument surface of tadpoles treated with 30 µg/L 17α-MT reveal the prevalence of KHs with underlying glands resembling a mucous gland-type (Fig. 19B). Tadpoles exposed to 100 and 300 µg/L 17α-MT exhibited a higher frequency of KHs on the skin surface as well as the presence of both mucous and breeding gland types (Figs. 19C & D). Some of the sub-epidermal glands in tadpoles from the 300 µg/L 17α-MT treatment also showed a mixed-type of gland, which included secretory cell epithelium resembling both mucous and breeding gland-types (Figs. 19D & E, arrows). The presence of breeding glands in the 300 µg/L treatment of 17α-MT was also confirmed by showing breeding gland secretory cell epithelium stained highly positive for PAS (Fig. 19F). Categorical data indicated a significant correlation between the prevalence of KHs and breeding glands with increasing Testosterone treatment (Fisher's exact test, $p < 0.05$) with the highest density of breeding glands and KHs per mm NP skin surface area observed in the 300 µg/L treatment group (Figs. 20A & B).

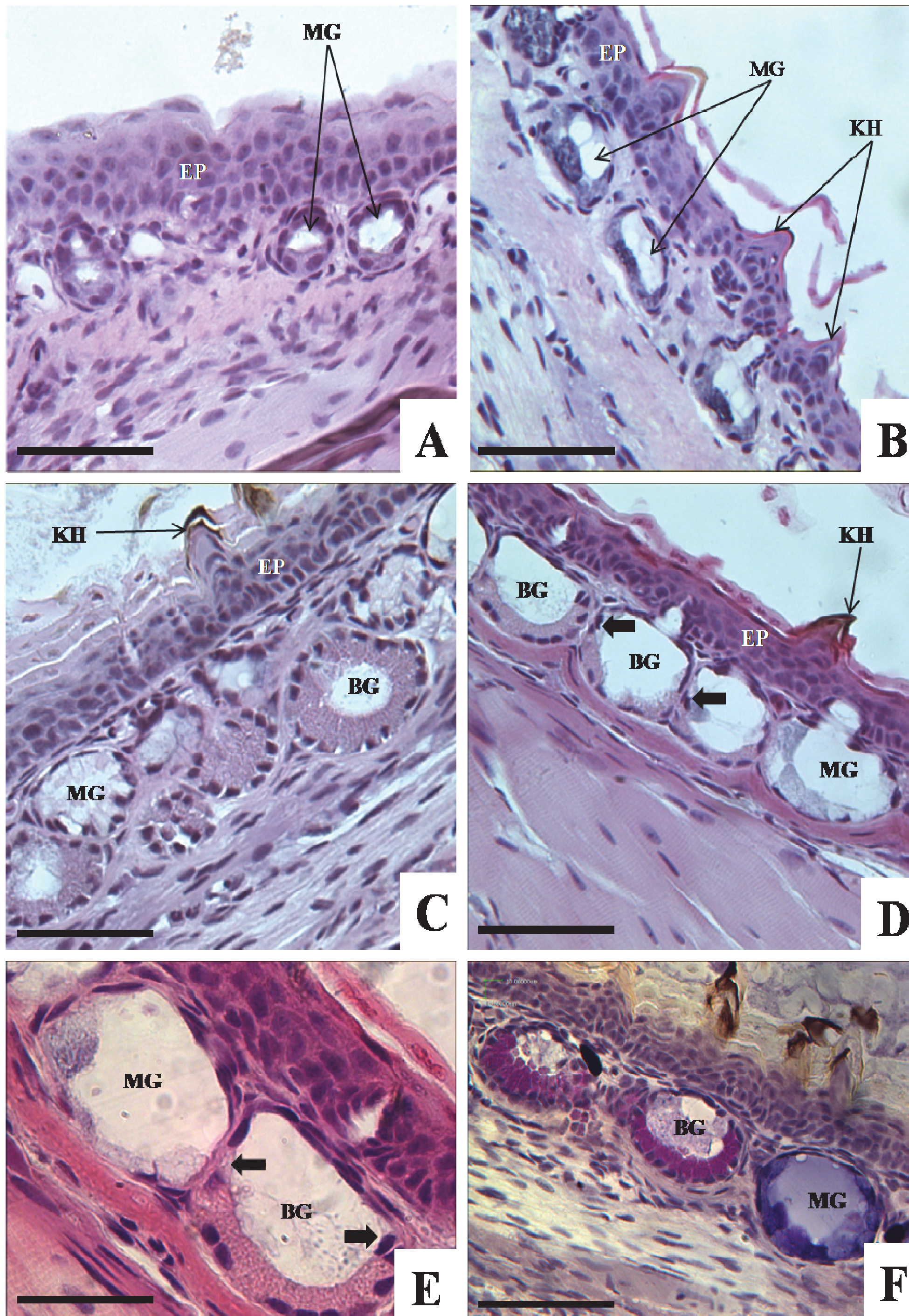

Figure 19: *Development of BGs and epidermal KHs in pre-metamorphic X. laevis tadpoles (NF 59-62) given either a DMSO supplement (**A**) or a 17α-MT supplement of 30 µg/L (**B**), 100 µg/L (**C**) or 300 µg/L (**D, E & F**). Some of the sub-epidermal glands in tadpoles from the 300 µg/L 17α-MT treatment showed a mixed-type of gland (**D** & **E**, arrows). Staining of skin sections with PAS clearly differentiate between breeding- and mucous glands in the 300 µg/L treatment (**F**). Labels: MG, mucous gland; BG, breeding gland; KH, keratin hooks; EP, epidermis. Scale bar: A, B, C, D, F (100 µm); E (40 µm).*

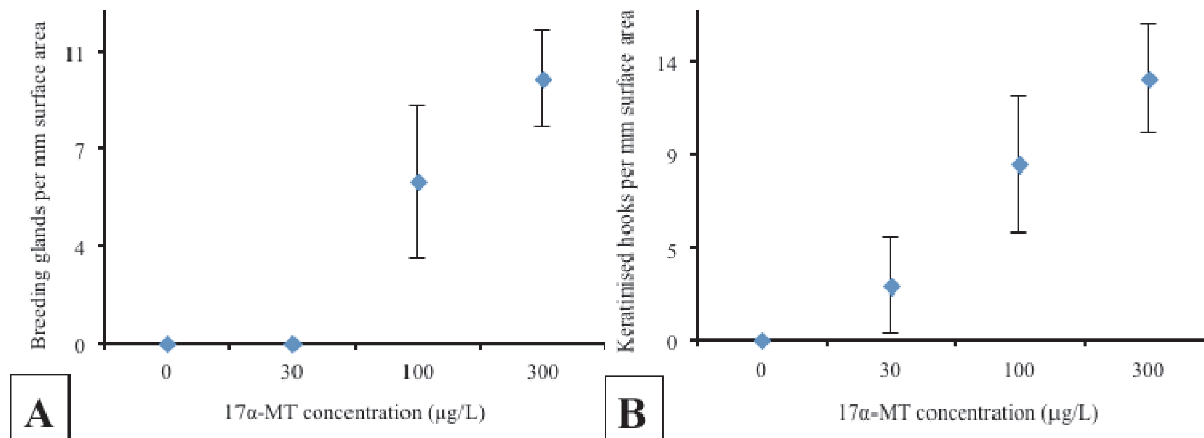

Figure 20: *Occurrence of the breeding gland density per mm NP surface area (**A**) and epidermal KH density (**B**) per mm NP surface area in pre-metamorphic tadpoles (NF stage 56-59; n = 10) exposed to different concentration ranges of 17α-MT. Values are expressed as mean (± SD).*

Juvenile (froglet) Xenopus laevis breeding gland development response

The NP skin sections of control juveniles (5-days post-metamorphic froglets) did not show any epidermal KHs on the skin surface, with only glands resembling a mucous gland type situated underneath the epidermis (Fig. 21A & B). Serous and/or other specialized gland types were absent in the skin sections of the control froglets (Fig. 21A). These mucous glands correlate with mucous glands found in adult frogs, which are characterized by the basophilic cuboidal epithelium cells lining the inner aspect of the gland (Fig. 21B). Juveniles subjected to 17α-MT treatment developed epidermal KHs in the NP skin surface (Fig. 21C). Underlying glands only resembled a breeding gland type, along with the absence of mucous and serous gland types (Fig. 21D). The lining of the luminal area of the gland characteristically show eosinophilic columnar epithelial cells containing secretion granules in the apical region and nuclei located in the basal region of the cells (Fig. 21D). These glandular characteristics resemble breeding glands such as found in the NP area of sexually mature adult male *X. laevis* frogs.

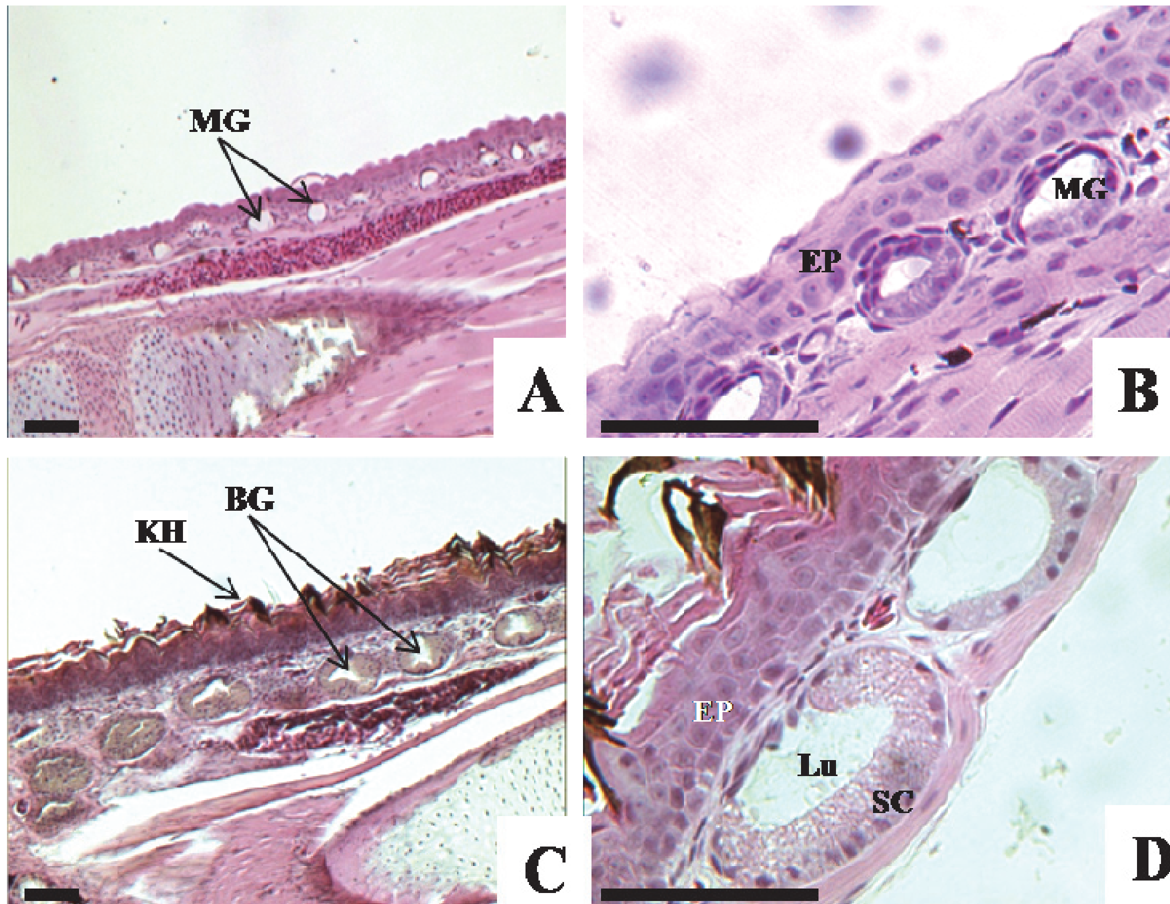

Figure 21: *Skin sections of the NP area in juvenile X. laevis frogs (five days post-metamorphosis) given a daily androgen supplement of 0 ug/g food (**A** & **B**) or 600 ug/g food (**C** & **D**) for 21 days. Labels: MG, mucous gland; BG, breeding gland; KH, keratin hooks; EP, epidermis; Lu, lumen; SC, secretory cell. Scale bar: 100 µm.*

In Vitro screening of regular-used fungicides

A clear dose-response of all test pesticides upon inhibiting the binding of DHT to AR (AR antagonism) was observed between 0.3 µM and 3 mM with the exception of Mancozeb, which had a dose-response concentration range between 0.03 nM and 32 µM (Fig. 22). Mancozeb was shown to be the most potent AR antagonist in the anti-YAS (RP, Table 10; Fig. 22) with the IC_{50} of Mancozeb being $2.5e^{-5}$ mM in the assay (7 µg/L; Table 10).

Table 10: The IC_{50} of test compounds and their isobole coefficients in the mixtures as well as the relative potency (RP) of the pesticides to the control fungicide, Vinclozolin. Isobole coefficients for the mixtures with a value > 1 indicates antagonistic activity, value < 1 indicates synergism and a value = 1 indicates additivity. Predicted IC_{50} concentrations for the binary pesticide mixtures assuming concentration addition (CA) or independent action (IA) is also estimated.

Pesticides	IC_{50} Conc. (mM)	RP (%)			
Vinclozolin	$4.94e^{-03}$	100			
Procymidone	$1.53e^{-01}$	3			
Fenarimol	$4.72E^{-02}$	11			
Chlorpyrifos	$1.18e^{-02}$	42			
Folpet	$5.50e^{-03}$	90			
Mancozeb	$2.54e^{-05}$	19449			
Dimethomorph	$3.80e^{-01}$	1			
Flusilazole	$4.34e^{-02}$	11			
			Isobole Coefficient (IC_{50})	Predicted concentration addition (CA; IC_{50})	Predicted independent action (IA; IC_{50})
Mancozeb + Dimethomorph	$4.65e^{-05}$	10624	0.92	$5.07e^{-05}$	$3.80e^{-01}$
Dimethomorph + Folpet	$2.49e^{-02}$	20	2.29	$1.08e^{-02}$	$3.83e^{-01}$
Vinclozolin + Folpet	$2.98e^{-02}$	17	5.72	$5.21e^{-03}$	$1.04e^{-02}$
Vinclozolin + Mancozeb	$7.70e^{-03}$	64	152.55	$5.05e^{-05}$	$4.97e^{-03}$
Vinclozolin + Fenarimol	$2.36e^{-02}$	21	2.63	$8.95e^{-03}$	$5.19e^{-02}$
Vinclozolin + Chlorpyrifos	$1.23e^{-01}$	4	17.59	$6.96e^{-03}$	$1.67e^{-02}$

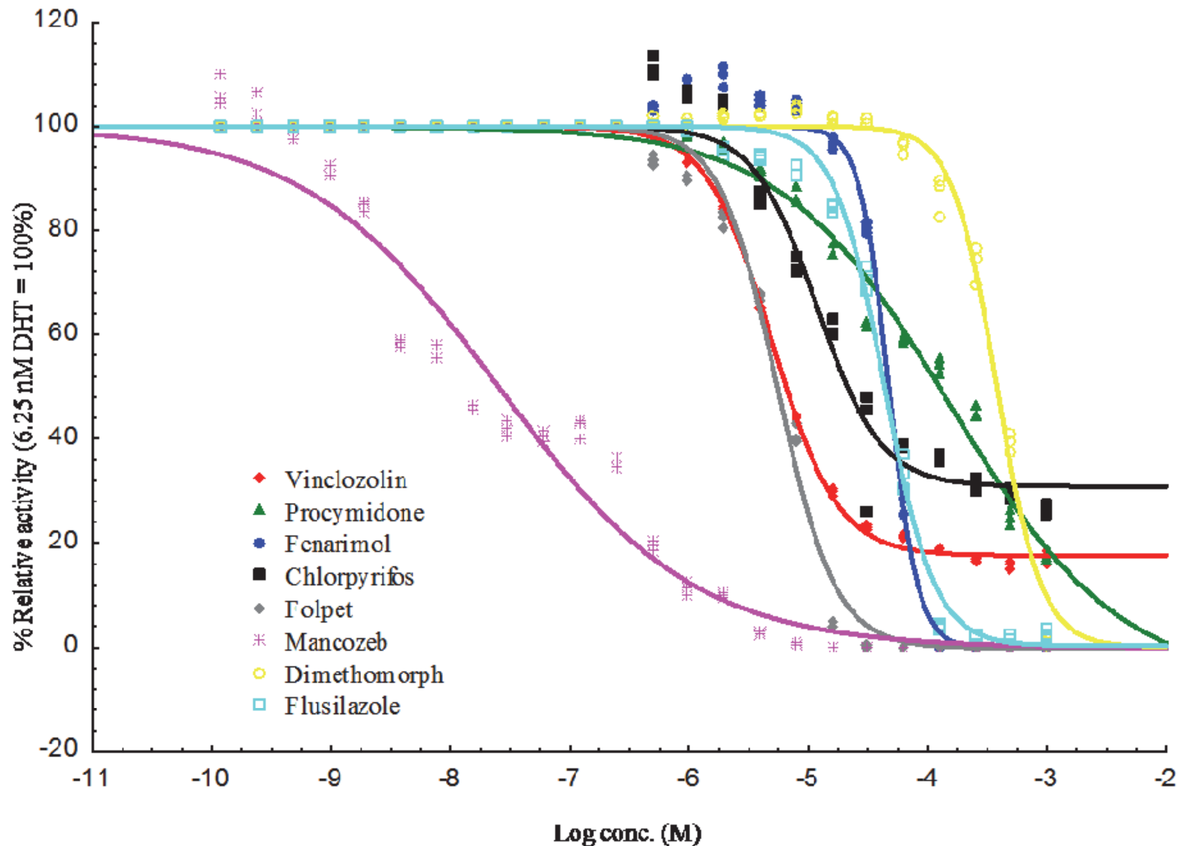

Figure 22: *AR antagonistic dose-response of the test pesticides regularly used in agriculture in the Western Cape. Values are expressed as a percentage relative to the model AR antagonistic fungicide Vinclozolin.*

A dose-response concentration range between binary, equimolar mixtures of selected test pesticides was generated between 1 µM and 1 mM with the exception of a binary mixture between Mancozeb and Dimethomorph, which ranged between 0.1 nM and 32 µM in the assay (Fig. 23). The binary mixtures did not follow the expected additive mixture response at IC_{50} concentrations (isobole coefficient > 1, Table 10; Fig. 23) with the exception of a mixture between Mancozeb and Dimethomorph which generated an additive mixture response at IC_{50} concentrations (isobole coefficient ≈ 1, Table 10; Fig. 23).

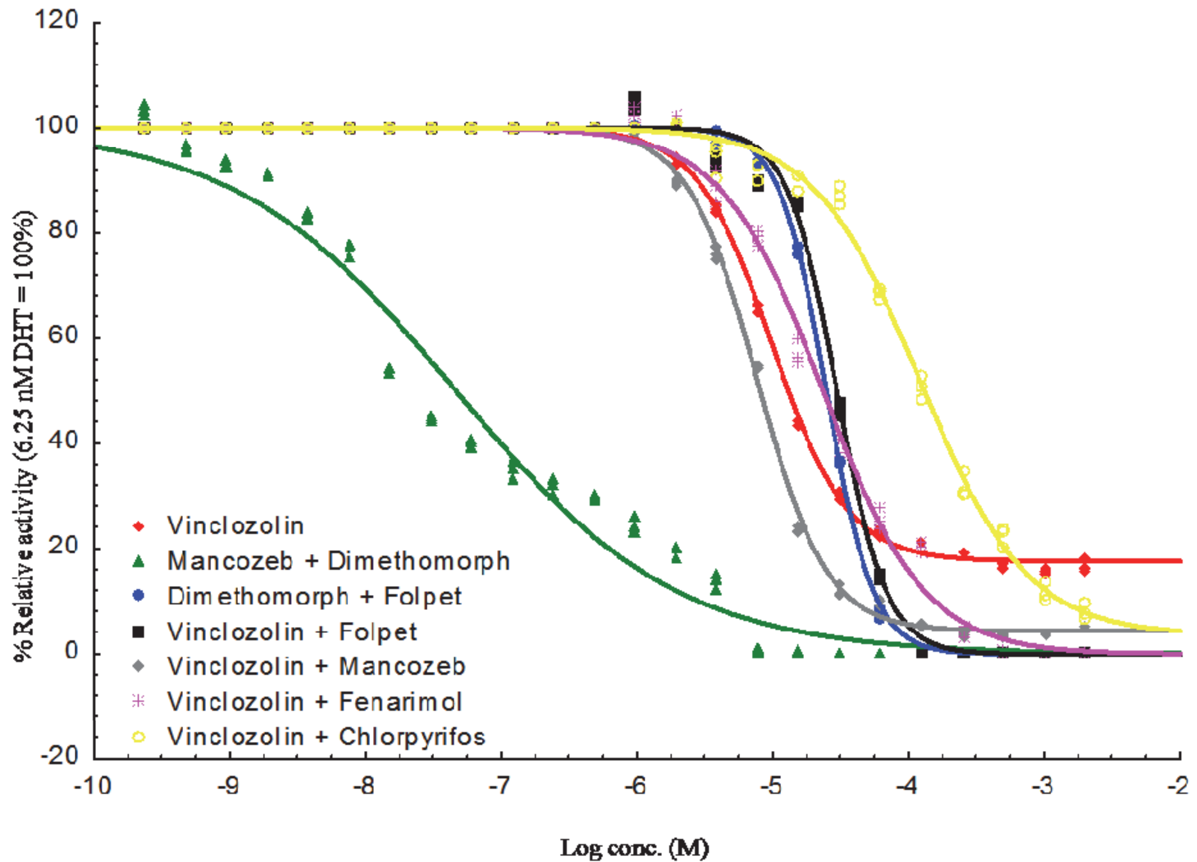

Figure 23: *AR antagonistic dose-response of equimolar binary mixtures of the test pesticides. Values are expressed as a percentage relative to the model AR antagonistic control fungicide, Vinclozolin.*

Adult male frog exposure to fungicides

Plasma hormone analysis

A significant variance was found in plasma Testosterone concentrations between frogs in the control and mixture groups (Fig. 24; ANOVA, $F_{3,16} = 7.98$; Tukey, $p < 0.05$). No significant variance was found in plasma Dihydrotestosterone concentrations between frogs in the treatment groups (Fig. 25; ANOVA, $F_{3,16} = 0.94$; Tukey, $p > 0.05$). Concentrations of plasma VTG expression between treatment groups did not show any significant variance compared to the control treatment frogs (Fig. 27; ANOVA, $F_{3,16} = 8.43$; Tukey, $p > 0.05$). A significant variance was found in the ratio of plasma Dihydrotestosterone and Testosterone concentrations, in which frogs in the Mancozeb treatment showed lower DHT/T ratios compared to the control treatment and the mixture treatment showed higher DHT/T ratios compared to the control treatment (Fig. 28; ANOVA, $F_{3,16} = 7.88$; Tukey, $p < 0.05$). Although no significant variance was found in plasma E_2 concentrations between treatment groups (Fig. 26; ANOVA, $F_{3,16} = 3.52$; Tukey, $p > 0.05$), a significant variance was found in the ratio of plasma E_2 and T concentrations in which frogs in the mixture

treatment showed higher E_2/T ratios compared to the control treatment (Fig. 29; ANOVA, $F_{3,16} = 0.39$; Tukey, $p < 0.05$).

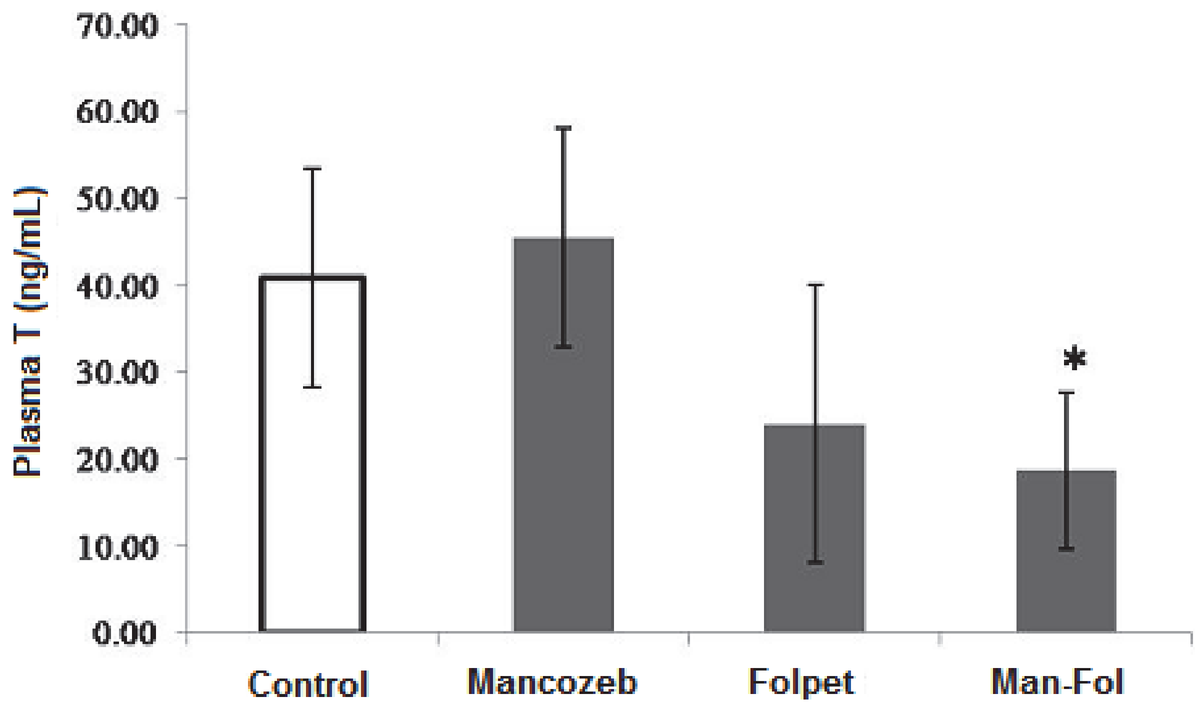

Figure 24: *Concentrations of plasma testosterone (T) of adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Test chemicals were exposed in the water using dimethylsulfoxide (DMSO) as solvent. The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, p < 0.05) from frogs in the control exposure.*

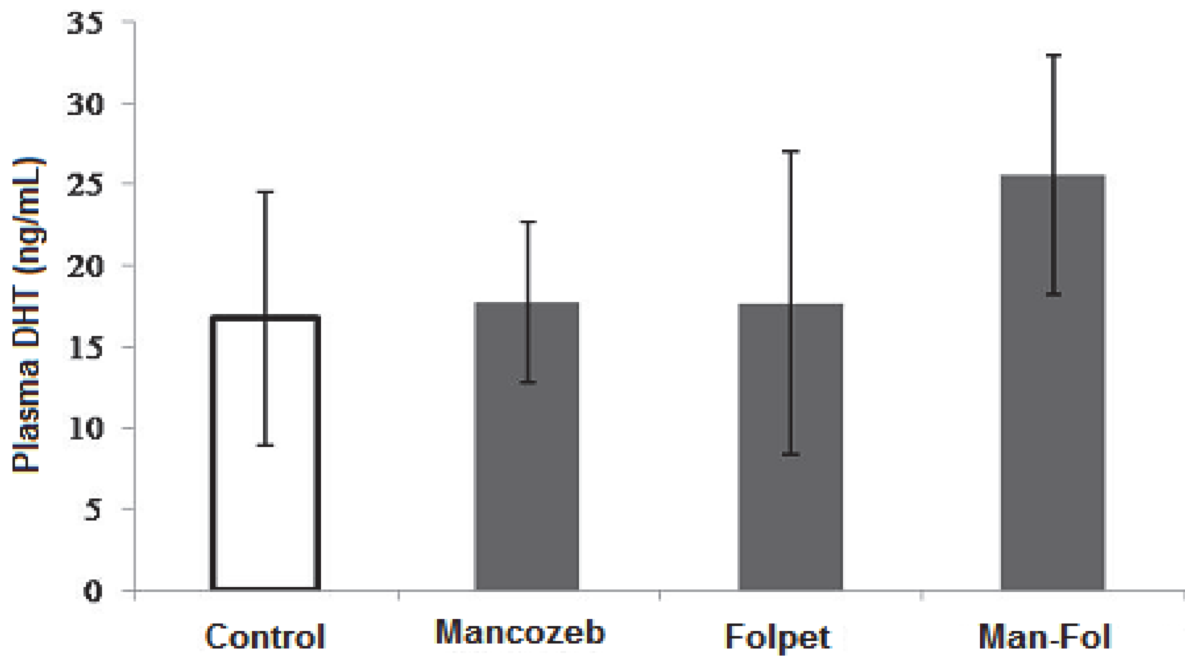

Figure 25: *Concentrations of plasma dihydrotestosterone (DHT) of adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Test chemicals were exposed in the water using dimethylsulfoxide (DMSO) as solvent. The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, p < 0.05) from frogs in the control exposure.*

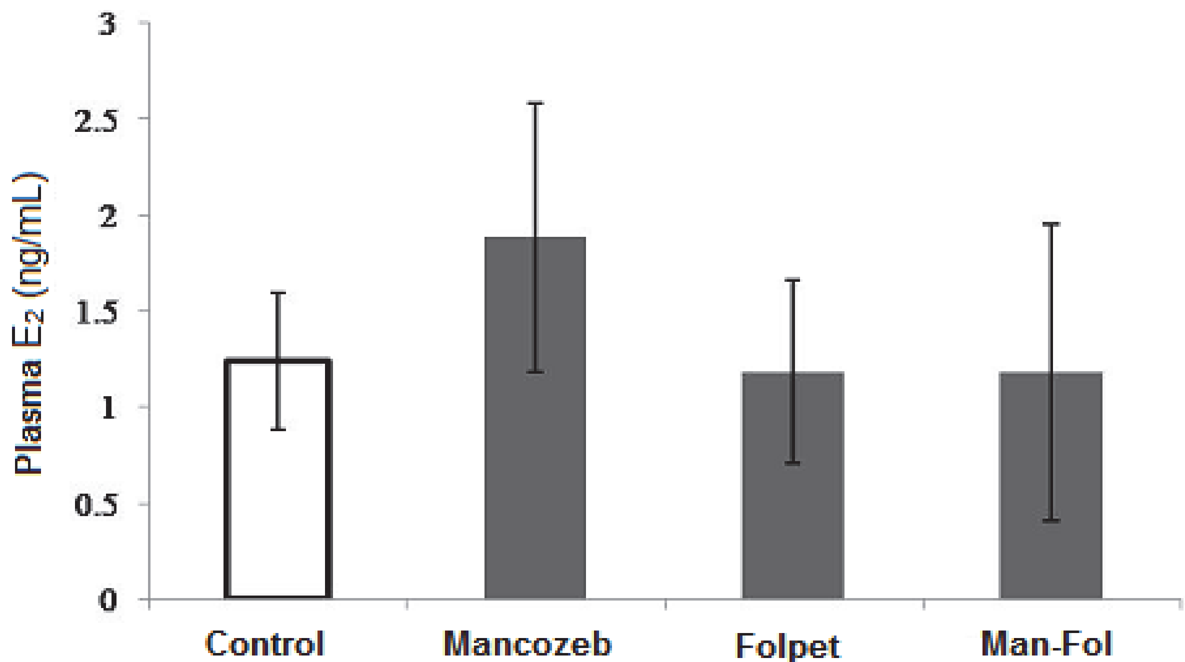

Figure 26: *Concentrations of plasma oestradiol (E_2) of adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides mancozeb and folpet (0.25 + 0.05 mg/L respectively). Test chemicals were exposed in the water using dimethylsulfoxide (DMSO) as solvent. The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, p < 0.05) from frogs in the control exposure.*

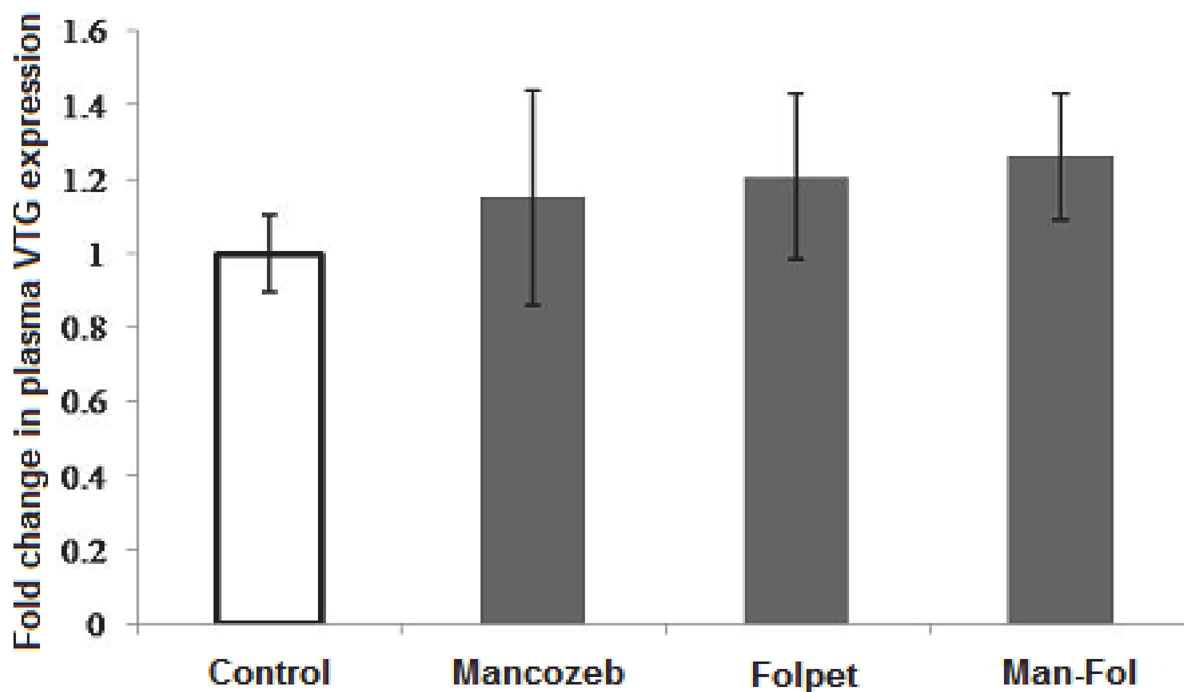

Figure 27: *Relative fold change in plasma VTG concentrations of adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Test chemicals were exposed in the water using dimethylsulfoxide (DMSO) as solvent. The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, $p < 0.05$) from frogs in the control exposure.*

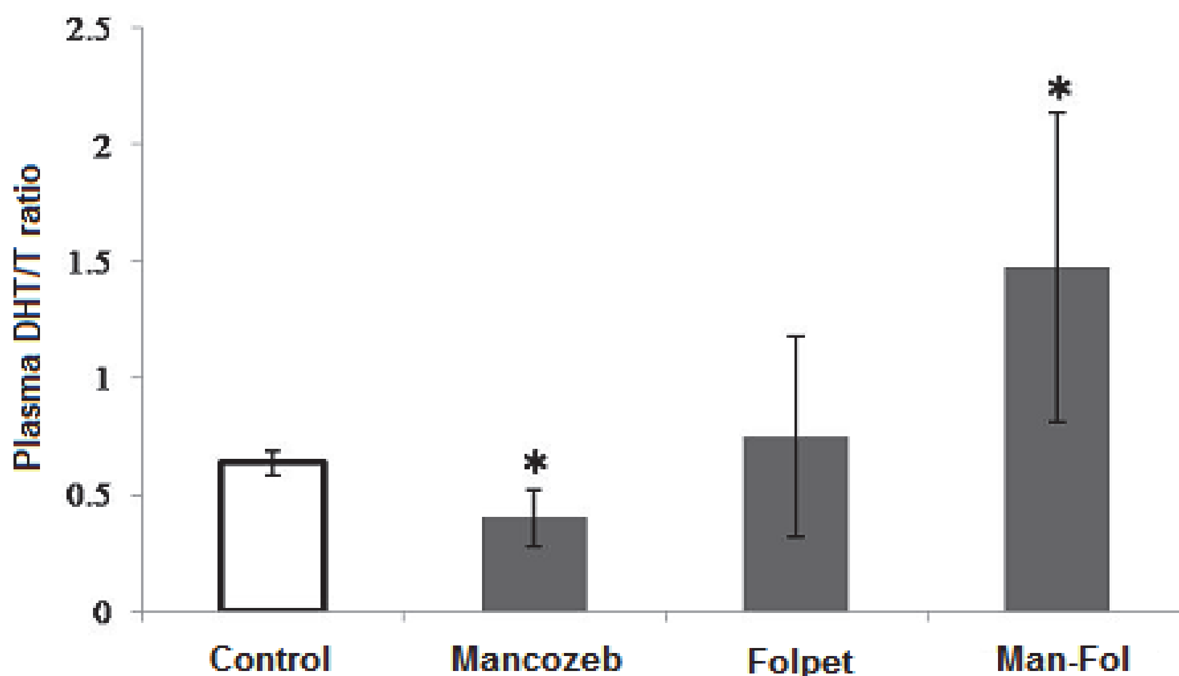

Figure 28: *Ratios of plasma dihydrotestosterone (DHT) relative to plasma testosterone (T) of adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Test chemicals were exposed in the water using dimethylsulfoxide (DMSO) as solvent. The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, $p < 0.05$) from frogs in the control exposure.*

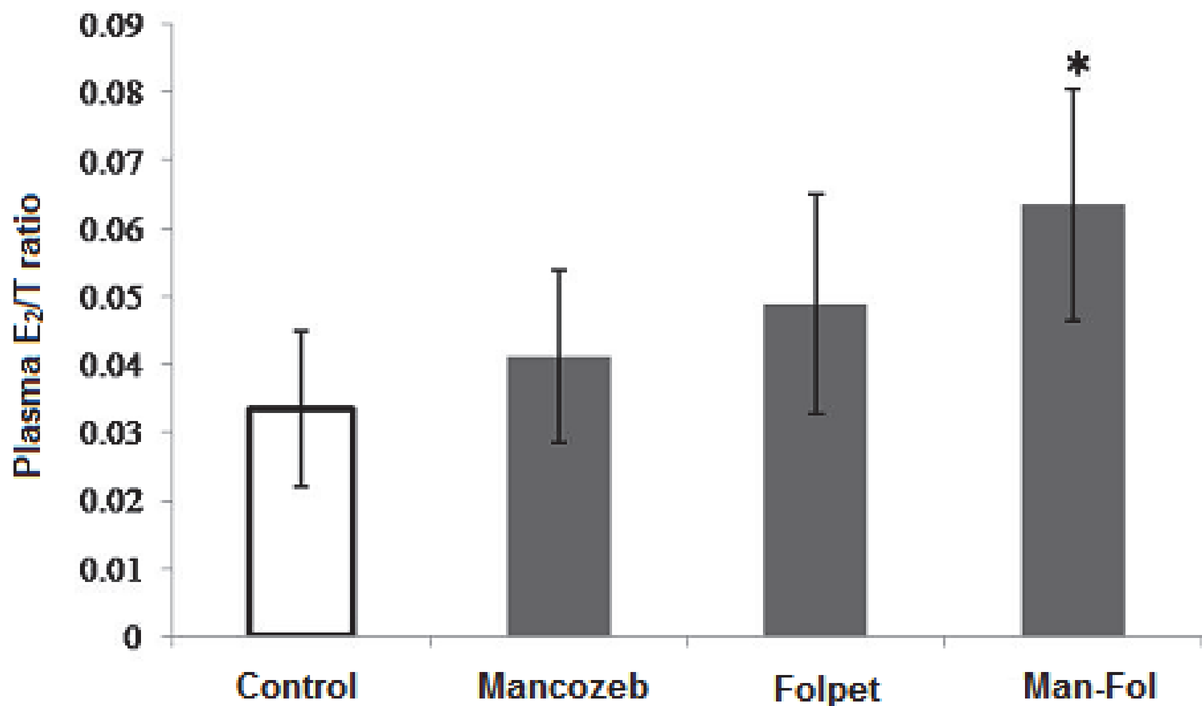

Figure 29: *Ratios of plasma oestradiol (E_2) relative to plasma testosterone (T) of adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Test chemicals were exposed in the water using dimethylsulfoxide (DMSO) as solvent. The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, $p < 0.05$) from frogs in the control exposure.*

Breeding gland morphology

A significant variance in breeding gland area was shown between frogs from the control treatment compared to the fungicide treatments, as well as the mixture treatment group (Fig. 30; ANOVA, $F_{3,140} = 18.15$; Tukey, $p < 0.05$). However, no significant variance was found in breeding gland area between the different fungicide treatments (Fig. 30; ANOVA, $F_{3,140} = 7.98$, $p > 0.05$).

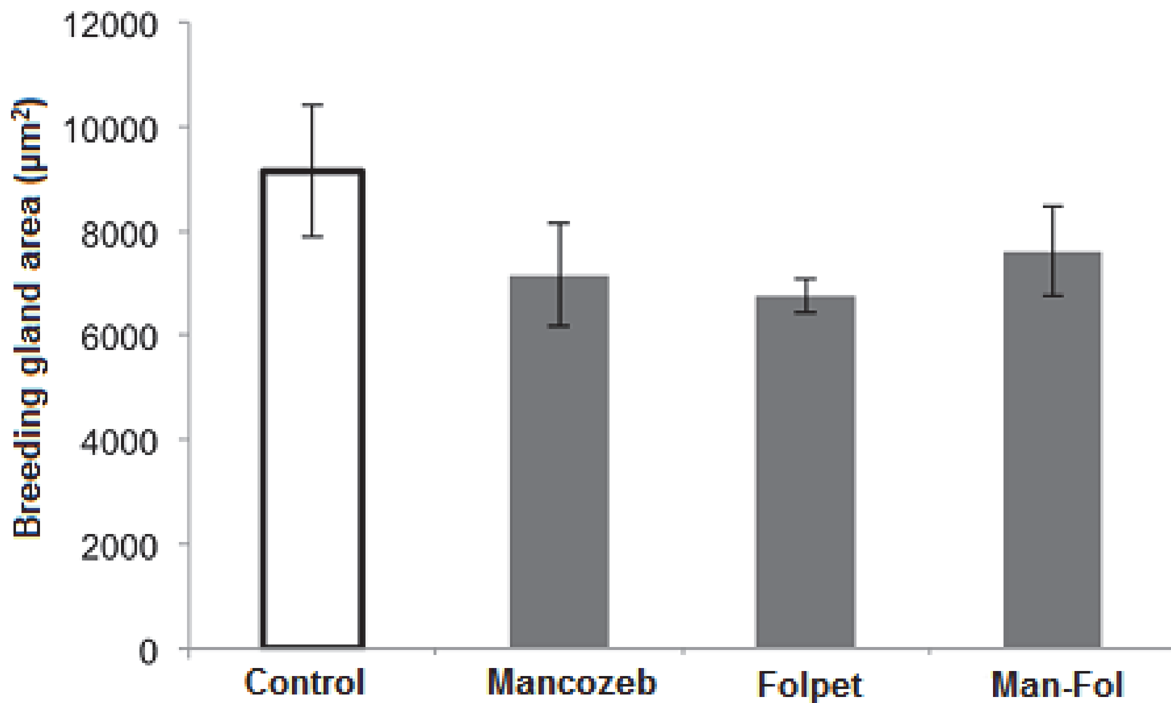

Figure 30: *Variation in the overall size (area) of breeding glands (µm^2 ± SD) among adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Six breeding glands and the length of ten epithelial cells within each breeding gland were measured per frog (n = 6) in each exposure group. The asterisk indicates a significant difference of treatment groups compared to control frogs (ANOVA; Tukey's HSD post hoc test: p < 0.05).*

A significant variance in breeding gland secretory cell epithelium height was shown between frogs from the control treatment compared to the fungicide treatments as well as the mixture treatment group (Fig. 31; ANOVA, $F_{3,860} = 139.55$; Tukey, $p < 0.05$). Furthermore, a significant variation was found in breeding gland secretory cell epithelium heights between the Mancozeb and Folpet treatment as well as between Folpet and the mixture treatment group (Fig. 31; ANOVA, $F_{3,860} = 139.55$; Tukey, $p < 0.05$).

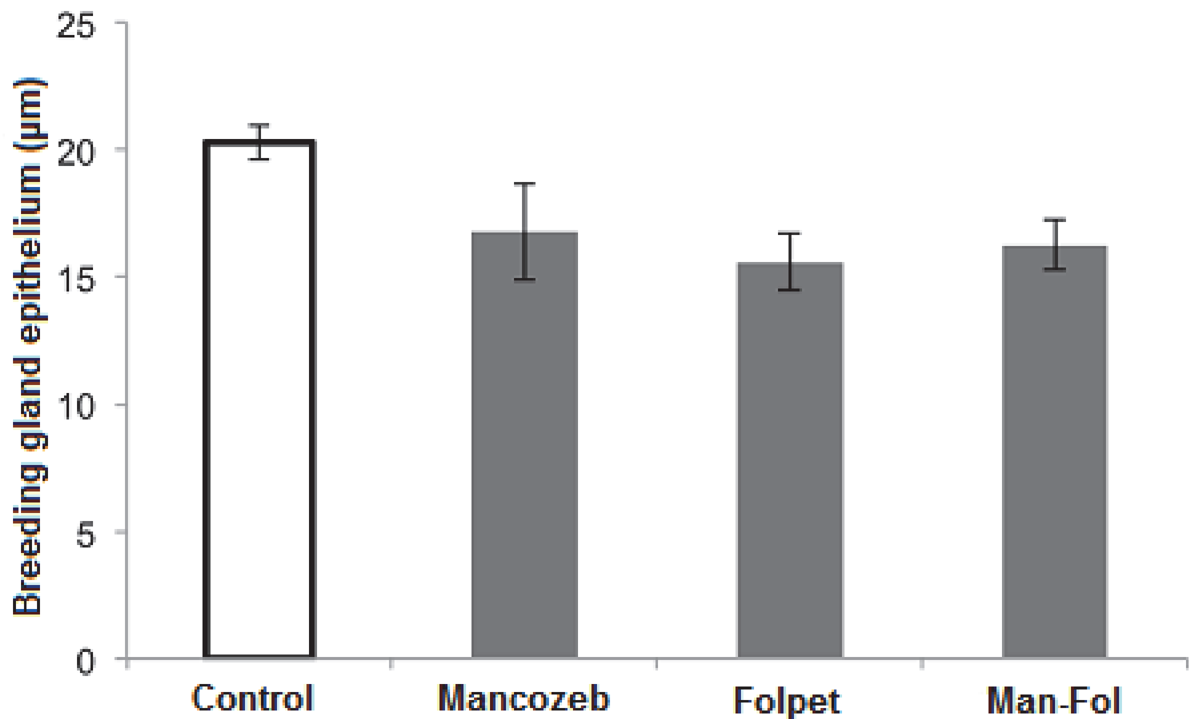

Figure 31*: Variation in overall breeding gland epithelium height (µm, ±SD) among adult male X. laevis frogs (n = 6) exposed to the fungicide Mancozeb (0.5 mg/L), Folpet (0.1 mg/L) or a mixture between the fungicides Mancozeb and Folpet (0.25 + 0.05 mg/L respectively). Six breeding glands and the length of ten epithelial cells within each breeding gland were measured per frog (n = 6) in each exposure group. The asterisk indicates a significant difference of treatment groups compared to control frogs (ANOVA; Tukey's HSD post hoc test: $p < 0.05$).*

RT-qPCR analyses

There was no significant difference in the expression of *trβ-b* and *dio2* among individuals exposed to Folpet, Mancozeb or a mixture of Mancozeb and Folpet relative to control frogs maintained in buffered RO water (Fig. 32).

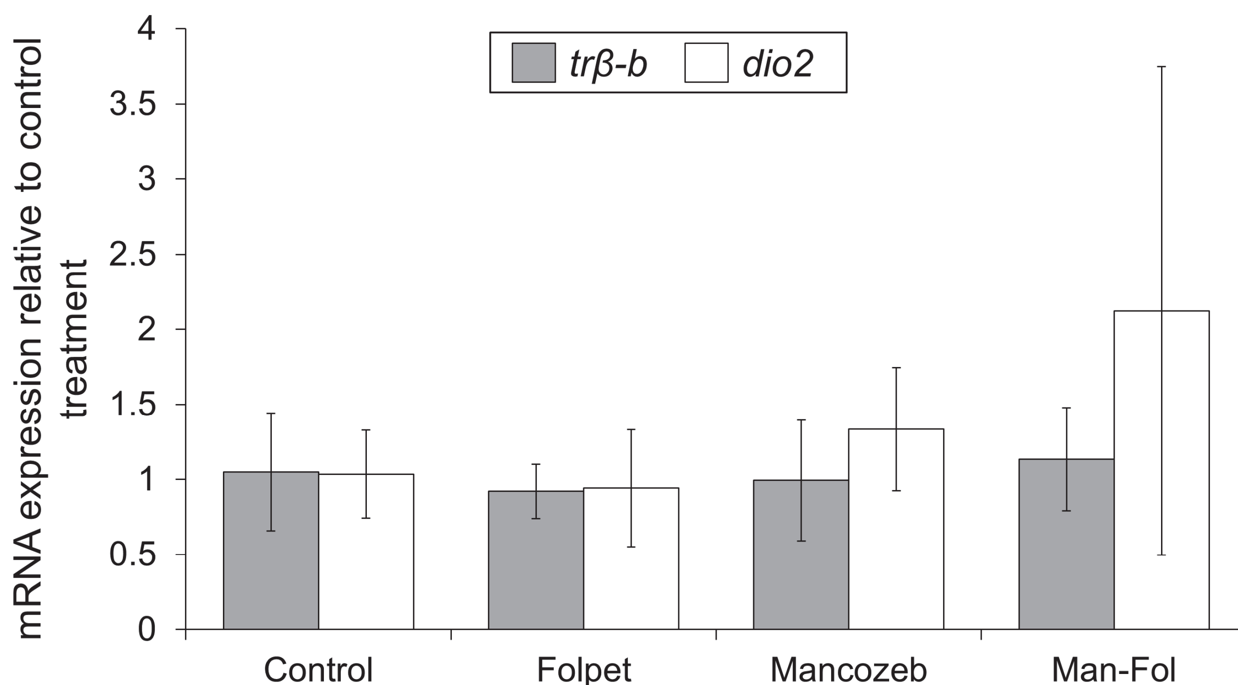

Figure 32: *The mRNA expression (mean ± SD) of thyroid receptor β b (trβ-b) and type 2 deiodinase (dio2) in the liver tissue of adult X. laevis exposed to Folpet (0.1 mg/L), Mancozeb (0.5 mg/L or a Mancozeb-Folpet mixture (0.25 mg/L Mancozeb; 0.05 mg/L Folpet) (n = 5 to 7 frogs per treatment).*

2.3.5. Conclusions

The morphology and androgen dependency for breeding gland development in adult male *X. laevis* frogs have been confirmed and corresponds to previous studies (Fujikura *et al*., 1988; Thomas *et al*., 1993; Emerson *et al*., 1999; Van Wyk *et al*., 2003). Androgen-dependent breeding glands in male *X. laevis* frogs represent a sensitive secondary sexual trait that can be used in EDC bioassays or in wild caught male frogs to assess (anti)androgenic endocrine disrupting activity. These glands were shown to be modulated by compounds having different mechanisms of anti-androgenic action, either by AR antagonism (Flutamide and Mancozeb) or by 5αR inhibition (Dutasteride). This suggests that the activity of these glands is regulated by various androgen endocrine pathways. Furthermore, the study has also shown, for the first time, that the expression of these androgen-regulated breeding glands can be prematurely induced in pre-metamorphic tadpoles and juvenile frogs, which allows for breeding glands in *X. laevis* to be used as a biomarker of (anti)androgenic endocrine disruption during various life stages of the frog.

The present study confirmed the value of a recombinant yeast bioassay to screen for the specific mechanism of anti-androgenic action in agrichemicals (fungicides and insecticides) on a first-tier basis. Furthermore, we confirmed the AR antagonistic activity of commonly-used pesticides in South African agricultural practices and investigated the interaction of binary chemical mixtures. We showed that additivity

predictions of EDC mixtures having a similar mechanism of anti-androgenic action may not always be accurate. The basis of the non-additivity of these pesticide mixtures needs further study in order to eventually apply predictive risk assessment models to complex environmental mixtures.

Following the initial screening of locally-used fungicides, *in vivo* exposure of adult male frogs to two selected fungicides and a binary mixture of these confirmed that in mixtures, additive responses in specific androgen dependent biomarkers may occur in male *X. laevis* frogs. Although individual chemicals did not result in clear anti-androgenic responses, mixture of the two fungicides did modulate hormones and secondary sexual endpoints. However, our data suggests no significant effects of Folpet, Mancozeb or a mixture of the said chemicals on the signaling of two key genes, *trβ-b* and *dio2*, within the thyroid cascade in *X. laevis* after 28 days of exposure. Nevertheless, these outcomes demonstrated that more research is needed to include pesticides, especially fungicides, in *in vivo* mixture experiments to aid in the extrapolation of endocrine disruption results obtained from laboratory studies to biomarker outcomes found in field experiments. Future research should consider interactions of complex pesticide mixtures upon biomarker outcomes of indigenous aquatic species, such as *X. laevis* frogs, to show endocrine disruption activity in environmental water systems.

3. Field studies

3.1. Agriculture dams in Stellenbosch area, Western Cape: Using *in vitro* and *in vivo* biomarkers (African clawed frog, *Xenopus laevis*).

Researcher: E. Archer, MSc, Stellenbosch University

3.1.1. Aims

The aim of the current study was to screen for oestrogenic, androgenic or anti-androgenic endocrine disrupting activity in impoundments located within intensive farmed areas, mostly surrounded by vines and orchards. In light of the relative high use of pesticides, especially fungicides, associated with the local farming practices (Dabrowski et al., 2014.) and the anti-androgenic activity of several of these regularly used pesticides, water samples were collected and screened for endocrine disruption activity. Secondly, adult male *X. laevis* frogs were collected from a subset of sites to investigate whether long-term exposure to agrichemicals may have compromised male reproduction, by using primary and secondary sexual endpoints to assess the reproductive and thyroid state of these local frogs.

3.1.2. Materials and Methods

The area of study consisted of water impoundments located in the Stellenbosch wine district, which are mostly situated at the bottom of converging slopes (Fig. 33). Nine impoundments were selected to be situated close to agricultural practices and receiving water runoff from converging slopes (Sites 2-10; Fig. 33) whereas a control impoundment was selected to be surrounded by natural vegetation, not receiving runoff from converging slopes and fully isolated from any agricultural practices (Site 1; Fig. 33). Sampling was done twice during the year of 2013 at two different seasons; during the pre-harvest period (summer, late February) and early winter (end of May). Water was extracted in the laboratory using a standard solid phase extraction (SPE) protocol (Swart and Pool, 2007).

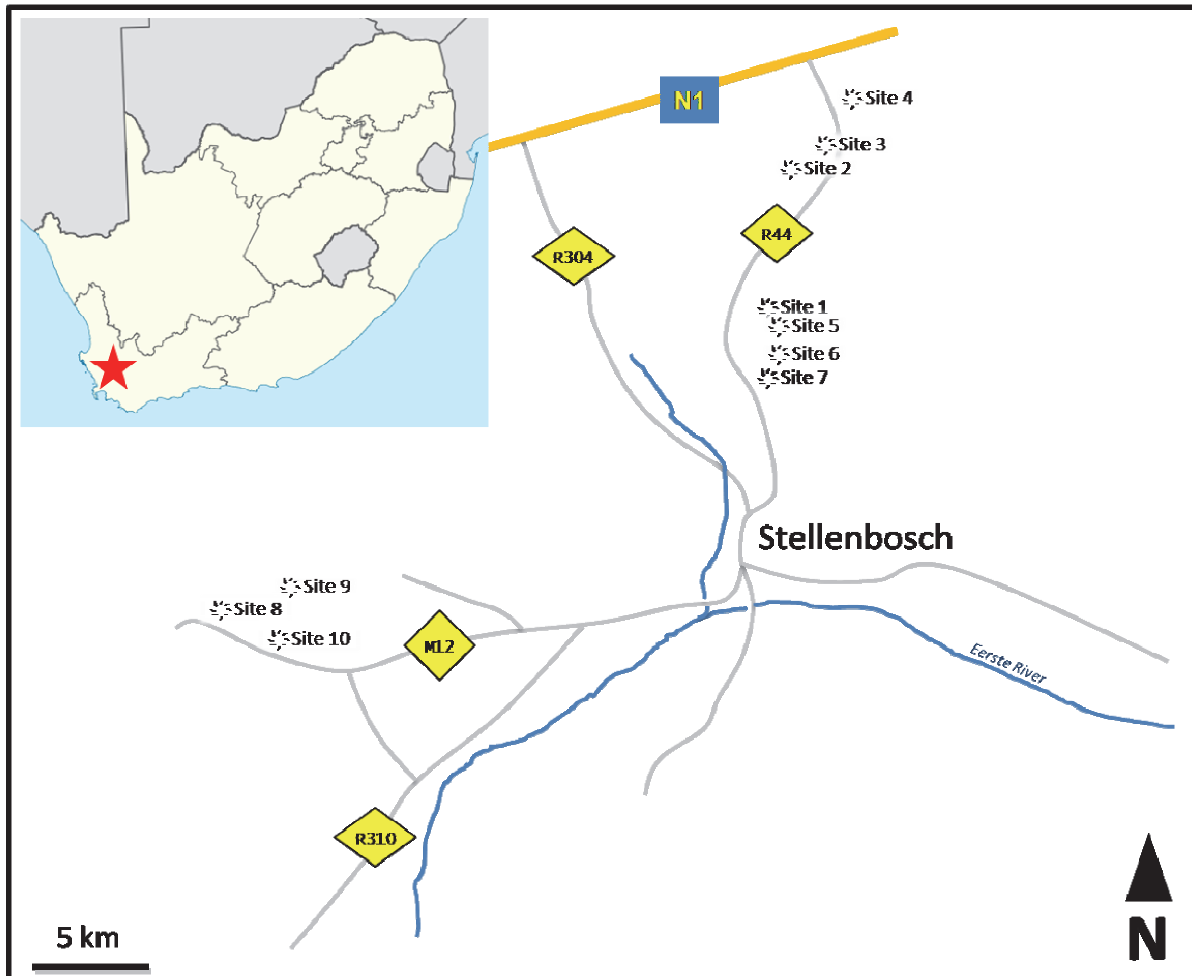

Figure 33: *Localities of water samples taken from water impoundments located close to agricultural practices. Site 1 was selected as relatively secluded from agricultural practices, surrounded by natural vegetation and not receiving runoff from converging slopes whereas the other sites (Site 1-3 and 5-10) were located at the bottom of slopes converging to a specific impoundment (dam) within intensive agricultural activity, mostly vineyards. Sampling was done during late summer and early winter periods. Ten adult male X. laevis frogs (n = 10) were also captured at sites 1, 2, 3 and 4 for biomarker investigations.*

To assess the presence of pollutants modulating androgen and oestrogen receptor binding, a recombinant yeast oestrogen, androgen and anti-androgen screen (YES, YAS/anti-YAS) were used. The protocol followed was according to Sohoni and Sumpter (1998) and De Jager et al. (2011) with some slight modifications from other literature (Urbatzka et al., 2007; Kolle et al., 2011). To assess whether ligands in the water samples may modulate steroid biosynthesis, a minced testis bioassay was performed as described by Ebrahim and Pool (2010). The effects of the water sample extracts on cell viability were also determined using a XTT cell viability assay.

Forty adult male *X. laevis* frogs were collected from a subset (n = 10 per sample site) of the water impoundments selected for the present study (Site 1, 2, 3 and 4). Frogs

were sacrificed by decapitation and pitching (according to AVMA, 2007) and blood samples were taken directly from the heart using a heparinized syringe and needle. Blood was centrifuged at 8000 rpm for 2 min, plasma extracted and stored at -80ºC for plasma hormone and VTG analysis. The testis and skin samples including the NP integument (location of breeding glands) were removed from six frogs per impoundment site and fixated in 10% buffered formalin (Bancroft and Stevens, 1977) for histological analyses using hematoxylin and eosin (H&E) staining (Bancroft and Stevens, 1977). Histology of the cystic-type testis in *X. laevis* was assessed according to literature (Rastogi et al., 1986; Kaptan and Murathanoğlu, 2008). Plasma Testosterone (T), Dihydrotestosterone (DHT) and oestradiol (E_2) levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (DRG Instruments GmbH, Germany). Blood plasma samples were also used for the determination detection of the circulating protein, VTG, produced in the liver which serves as a biomarker to indicate oestrogenic activity in male frogs (Van Wyk et al., 2003). Plasma VTG were measured using an in-house ELISA protocol as described elsewhere (Van Wyk et al., 2003).

Liver tissue was collected from adult male frogs and stored in RNAlater (Sigma, DE). The tissues were subsequently homogenized in Trizol (Invitrogen, US) using an ultrasound sonicator (Omni-ruptor 400, Omni International Inc., USA) and total RNA isolated according to the manufacturer's instructions with slight modifications. Isopropanol and 0.8 M sodium citrate were applied to precipitate RNA, followed by two washes with ice cold 70% ethanol. RNA integrity was assessed spectrophotometrically using a Nanodrop (Thermo Scientific, USA) and through agarose gel electrophoresis. The RNA of suitable integrity was subsequently DNase I (Zymo Research, USA), treated. Complementary DNA (cDNA) was synthesized using RevertAid reverse transcriptase kits (Thermo Scientific, US) (2 µg RNA per 20 µℓ reaction volume) using a combination of Oligo (dT) 18 and random hexamer primers.

The expression of *thyroid hormone receptor-β b* (*trβ-b*), *type 2 deiodinase* (*dio2*) was evaluated using real-time RT-qPCR with ribosomal protein l8 (rpl8) as housekeeping gene. The PCRs were performed as 15 µL reactions containing 2 µL cDNA (10 ng/reaction), 7.5 µL Jumpstart® SYBRgreen mix (Sigma, DE), 0.33 µM of each primer and nuclease free water. The PCR programs for al primer pairs included an enzyme activation step at 95°C (9 minutes), followed by 40 cycles of denaturing at 95°C (15 seconds), annealing at 55°C (*trβ* and *rpl8*), or 63°C (*dio2*) (30 seconds) and elongation at 72°C (45 seconds). Each PCR plate contained an internal non-template control (no cDNA) as well as a six point two-fold serial dilution. All samples and controls were run in triplicate and dissociation curves were applied to confirm single-fragment amplification. Gene expression was quantified using the Pfaffl method (Pfaffl, 2001). Amplification efficiencies were determined for each primer pair per PCR programme.

The primers sequences and sources were as follows: rpl8 sense 5' AGAGCCCATGTAAAGCAC 3', rpl8 antisense 5' CCTGTAAGGGTCACGGAA 3' (Esterhuyse et al., 2009); dio2 sense 5' AGGCTGAGTGTGGACTTG 3', dio2 antisense TGACCTGCTTGTAGGCATCCA 3' (Searcy et al., 2012); trβ-b sense 5' AAAGTGCCAGGAAGGTTTCCT 3', trβ-b antisense 5' GGTCGGTGACTTTCATCAGCA 3' designed using Premier Primer (Version 5, Premier Biosoft International, USA) (Genbank: NM_001087781.1).

3.1.3. Statistical procedures

All statistical analyses were performed using Statistica 10.0 (StatSoft Inc., USA). All datasets were tested for normality (Shapiro Wilk-W) and parametric or non-parametric analyses were selected accordingly. For incidence percentages data were arcsine transformed. Parametric analyses were done using a breakdown and one-way analysis of variance (ANOVA). When statistical significance was obtained, ANOVA was followed by Tukey's honest significant difference (HSD) *post-hoc* test to test for significant differences between treatments. For non-parametric measurements, data was analyzed using Kruskal-Wallis ANOVA. Statistical significance for all analyses was reported at p-values < 0.05.

3.1.4. Results and Conclusions

Recombinant yeast androgen- and oestrogen receptor screens of impoundments

Three impoundment localities (Sites 2, 5 and 6; Fig. 33) in the summer period showed elevated levels of ligand-induced AR binding compared to blank (untreated) yeast cells (ANOVA, $F_{9,39}$ = 3.48; Tukey, p < 0.05, Fig. 34; Table 11). Water collected from five impoundments (Sites 1, 2, 4, 5 and 7; Fig. 33) significantly induced AR antagonistic (anti-androgenic) effects in the anti-YAS (ANOVA, $F_{9,39}$ = 0.44; Tukey, p < 0.05, Fig. 35; Table 11). Most AR antagonistic activity in the water samples were observed during the summer period (dry season) of sampling with little or no detection (nd) of AR antagonistic activity during the first rainfall period of the winter (Fig. 35; Table 11). The YES assay showed oestrogenic activity in water samples collected from two sampling localities compared in the summer (ANOVA, $F_{9,39}$ = 2.29; Tukey, p < 0.05; Site 3 > Site 10; Fig. 36; Table 11), which corresponded to E_2 equivalent concentrations ranging between 0.082 and 0.029 ug/L.

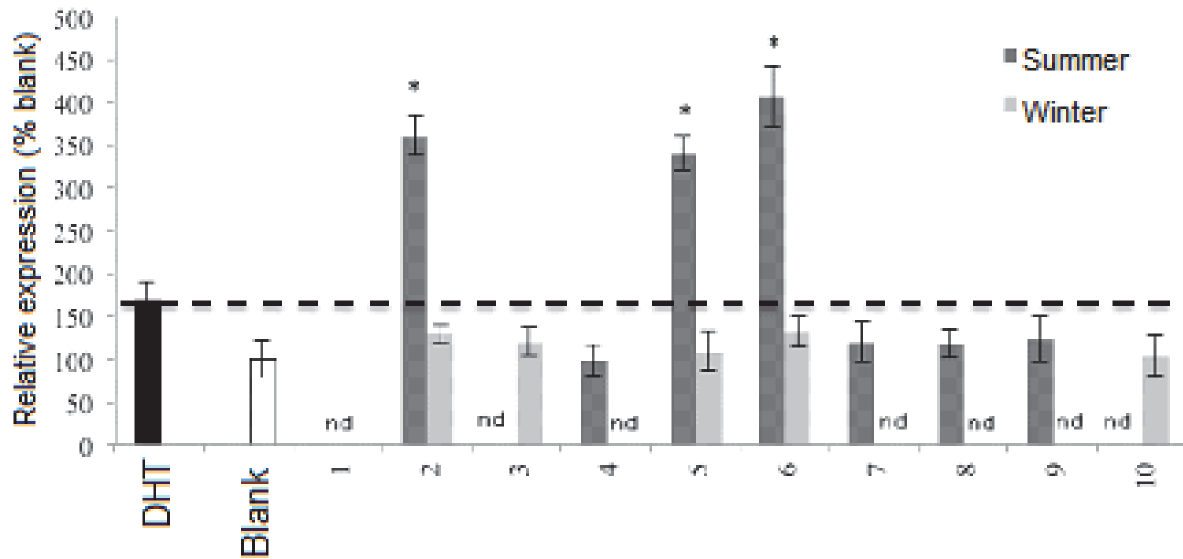

Figure 34: *Androgenic activity in water samples collected from impoundments situated close to agricultural practices (Sites 2-10) and an impoundment isolated from agricultural practices (Site 1) in the YAS. Samples were taken during summer and winter seasons and expressed relative to untreated (blank) cells in the assay. The concentration shown for DHT represents the EC_{50} concentration of DHT in the standard curve (dashed line, 6.25 nM). Expression of water samples under the detection limit of the assay were shown as not detected (nd). The asterisk indicates significant difference from the blank (untreated) cells.*

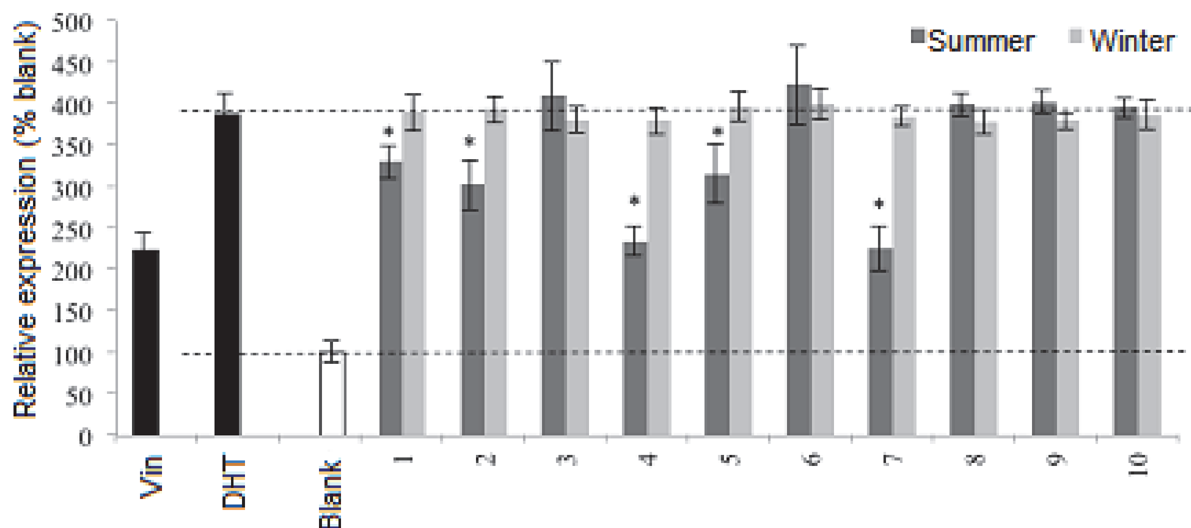

Figure 35: *Anti-androgenic activity in water samples collected from impoundments situated close to agricultural practices (Sites 2-10) and an impoundment isolated from agricultural practices (Site 1) in the anti-YAS. Samples were taken during summer and winter seasons and expressed relative to untreated (blank) cells in the assay (bottom dashed line). The concentration of Vinclozolin (Vin) represents the generated IC_{50} in the assay (4.94 nM). Expression of water samples under the detection limit of the assay were shown as not detected (nd). The asterisk indicates significant difference from the EC_{50} of the DHT spike (top dashed line).*

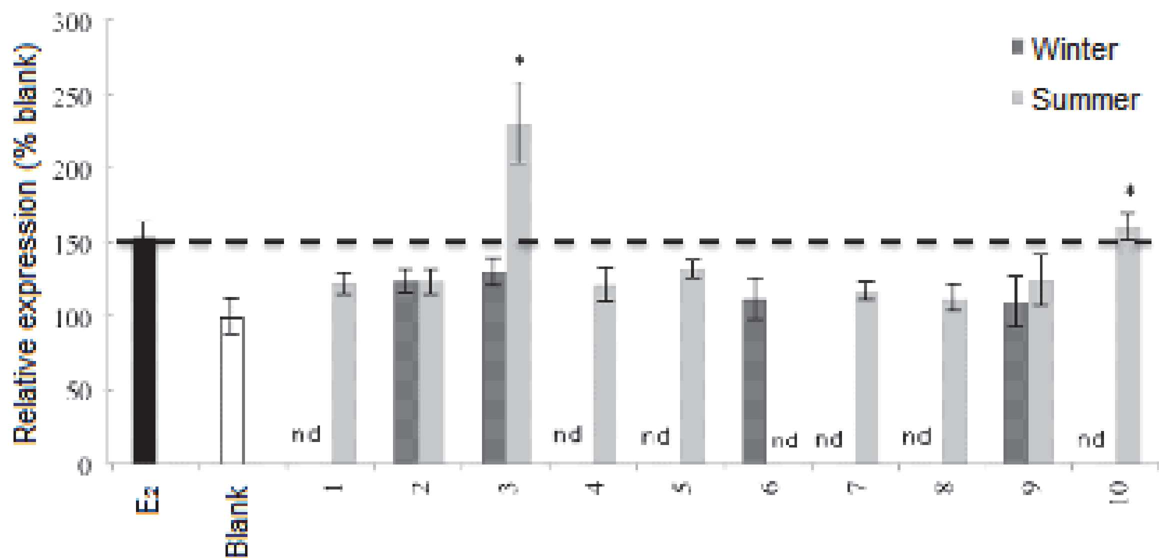

Figure 36: *Oestrogenic activity in water samples collected from impoundments situated close to agricultural practices (Sites 2-10) and an impoundment isolated from agricultural practices (Site 1) in the YES. Samples were taken during summer and winter seasons and expressed relative to untreated (blank) cells in the assay. The concentration shown for E_2 represents the EC_{50} concentration of E_2 in the assay (5.39 nM). Expression of water samples under the detection limit of the assay were shown as not detected (nd). The asterisk indicates significant difference from the blank (untreated) cells.*

Cell viability and minced testis assay

Only one of the water impoundments (Site 8) had a significant effect on cell viability during the summer season of sampling (t-test, $t = 0.79$, $p < 0.05$; Fig. 37). Water samples collected from two impoundments (Sites 6 & 7; Fig. 33) stimulated testosterone production significantly in the *ex vivo* rat minced testes culture ranging from 7.58 to 15.72 ng/mL as compared to untreated (blank) cells (Table 11; Fig. 38; ANOVA, $F_{9,39} = 5.59$; Tukey, $p < 0.05$).

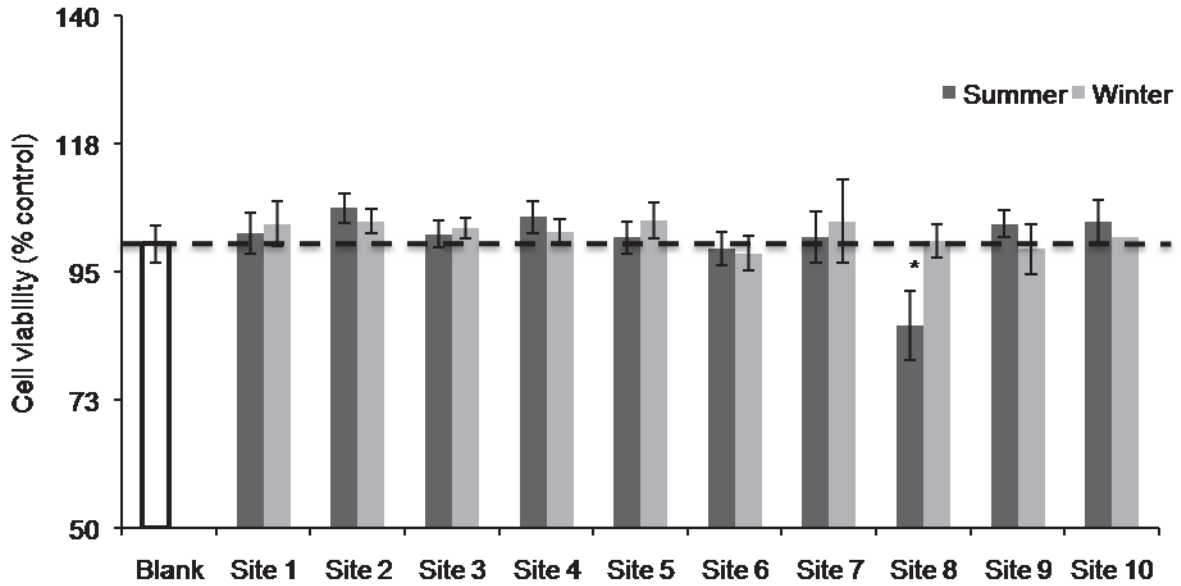

Figure 37: *Cell viability of the minced testis cell suspension treated with the water catchment samples taken during summer and winter periods. Values are expressed as the measured formazan formation in the XTT assay in percentage of absorbance (% ± SD) relative to control treated (blank) cells. The asterisk indicates significant difference from the blank cells (ANOVA; Tukey's HSD test, p < 0.05.).*

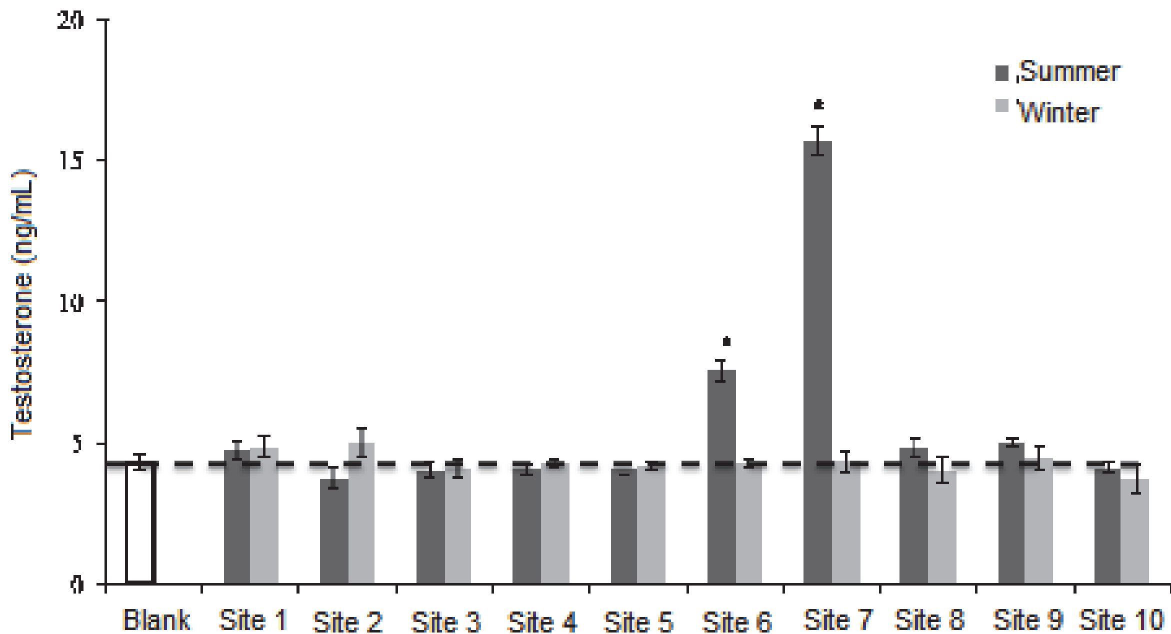

Figure 38: *Effect of water samples at the catchment sites during summer and winter sampling on testosterone secretion (ng/ml ± SD) in the rat minced testis culture. Cells were exposed to the water samples obtained from the various impoundments. Samples were screened in duplicates and values are expressed as average testosterone concentration in the culture medium. The asterisk indicates significant difference from untreated (blank) cells (ANOVA; Tukey's HSD test, p < 0.05.).*

***Table 11**: Summary incidences of significant interaction with the steroid receptors (hAR and hER) and testosterone production using a rat minced testis assay. S: Summer, W: Winter. The + sign indicate significant variance from untreated (blank) cells used in the respective assays (ANOVA, p < 0.05).*

Impoundment Sites	**YAS** (androgenicity)		**anti-YAS** (anti-androgenicity)		**YES** (oestrogenicity)		**Mince testis** (androgenicity/steroidogenic)	
	S	**W**	**S**	**W**	**S**	**W**	**S**	**W**
1	-	-	+	-	-	-	-	-
2	+	-	+	-	-	-	-	-
3	-	-	-	-	+	-	-	-
4	-	-	+	-	-	-	-	-
5	+	-	+	-	-	-	-	-
6	+	-	-	-	-	-	-	-
7	-	-	+	-	-	-	+	-
8	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-
10	-	-	-	-	+	-	-	-

Breeding gland morphology of male X. laevis frogs and comparison between impoundment sites

The NP skin sections of male frogs (n = 6) sampled from the different water impoundments (Sites 1, 2, 3 & 4; Fig. 33) did not show significant variation in glandular area (Fig. 39A & 40; ANOVA, $F_{3,23}$ = 12.34, p > 0.05) or in breeding gland secretory cell epithelium height (Fig. 39B & 40; ANOVA, $F_{3,23}$ = 15.72, p > 0.05) between localities.

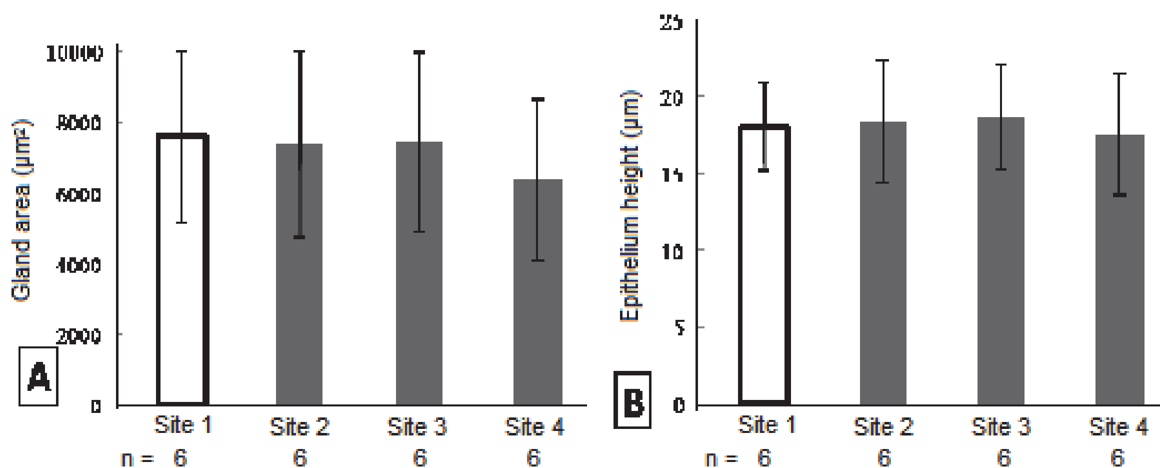

***Figure 39**: Morphological variation in breeding gland area (**A**; µm^2 ± SD) and breeding gland secretory cell epithelium height (**B**; µm ± SD) in adult male X. laevis frogs from the different water impoundments. Male frogs (n = 6) from a water impoundment isolated from agricultural practice (Site 1) was compared to frogs from water impoundments situated in close proximity of agricultural practices (Sites 2, 3 & 4).*

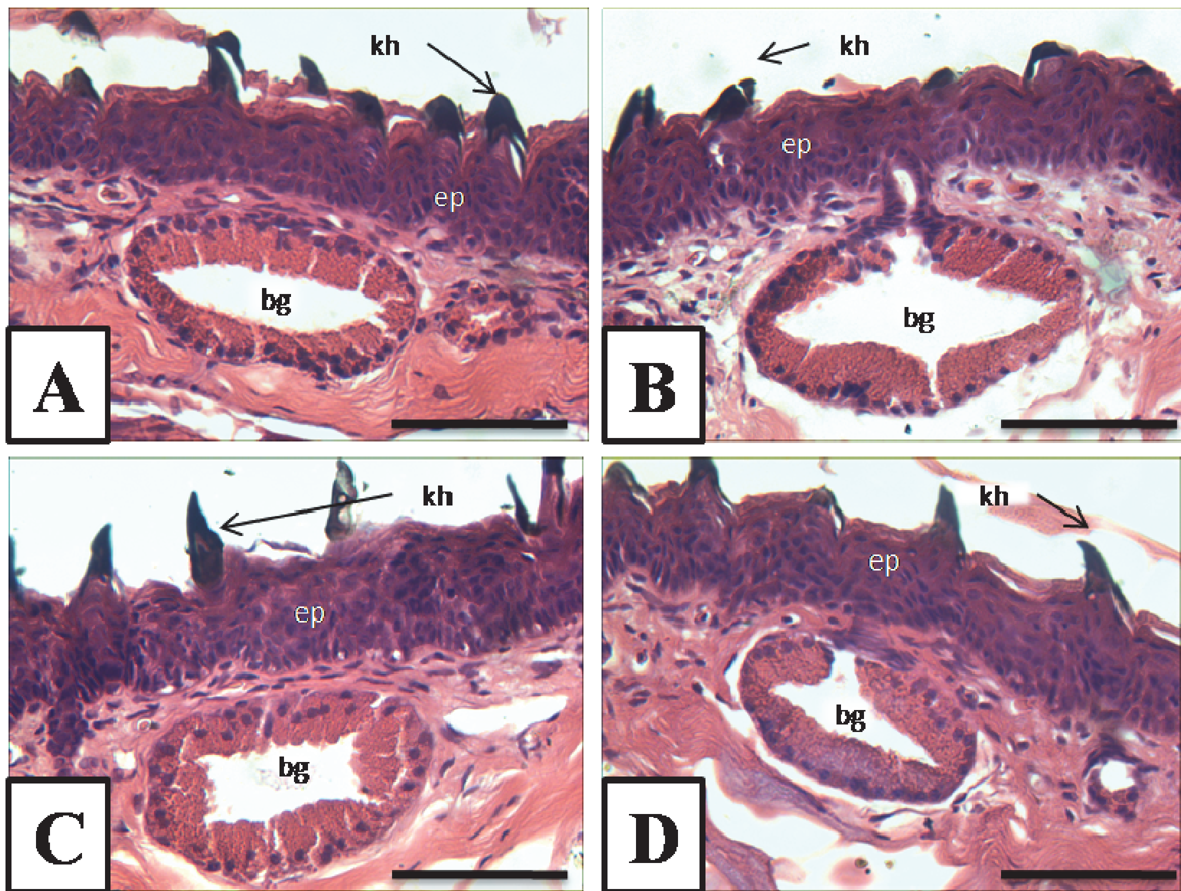

Figure 40: *Breeding gland morphology (400x magnification) of adult male X. laevis frogs (n = 6) captured from a water impoundment surrounded by natural vegetation and not receiving runoff from converging slopes with the impoundment isolated from agricultural practice (**A**; Site 1, control) and water impoundments receiving water from catchments in close proximity to agricultural crops (**B**, Site 2; **C** Site 3; **D**, Site 4). Labels: kh, keratinised hooks; ep, epidermis; bg, breeding gland. Scale bar: 50 µm.*

Spermatogenesis comparison of male X. laevis frogs between impoundment sites

Frogs collected from impoundment Site 3 showed an increase in the percentage of SPG germ cell stages (Fig. 41; ANOVA, $F_{3,23} = 0.46$; Tukey's HSD, $p < 0.05$) and a significant reduction in SPC germ cell stages (Fig. 41; ANOVA, $F_{3,23} = 2.35$; Tukey's HSD, $p < 0.05$) compared to frogs from water impoundment Site 1. Frogs sampled from water impoundment Site 3 also showed a significantly reduced percentage of SPZ in each SN tubule of these frogs compared to frogs from water impoundment Site 1 (Figs. 41 & 42E; ANOVA, $F_{3,23} = 2.55$; Tukey's HSD, $p < 0.05$).

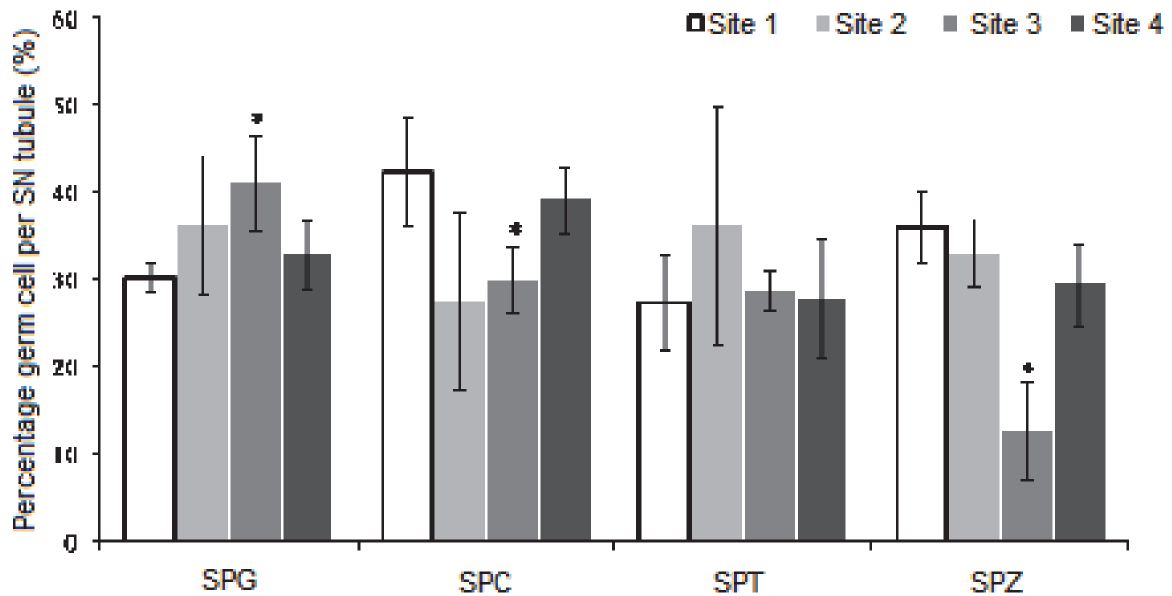

Figure 41: *Variation in the average percentage (% ± SD) of germ cell cysts and spermatozoa per seminiferous tubules in male X. laevis testes (n = 6) obtained from water impoundments isolated from agricultural practice (Site 1, Fig. 28) and close to agricultural practices (Sites 2, 3 & 4, Fig. 28). The asterisk indicates a significant difference from frogs in water impoundments isolated from agricultural practice (Site 1, Fig. 5.1) (ANOVA; Tukey's HSD post hoc test, p < 0.05). SPG, spermatogonia; SPC, spermatocyte; SPT, spermatid; SPZ, spermatozoa.*

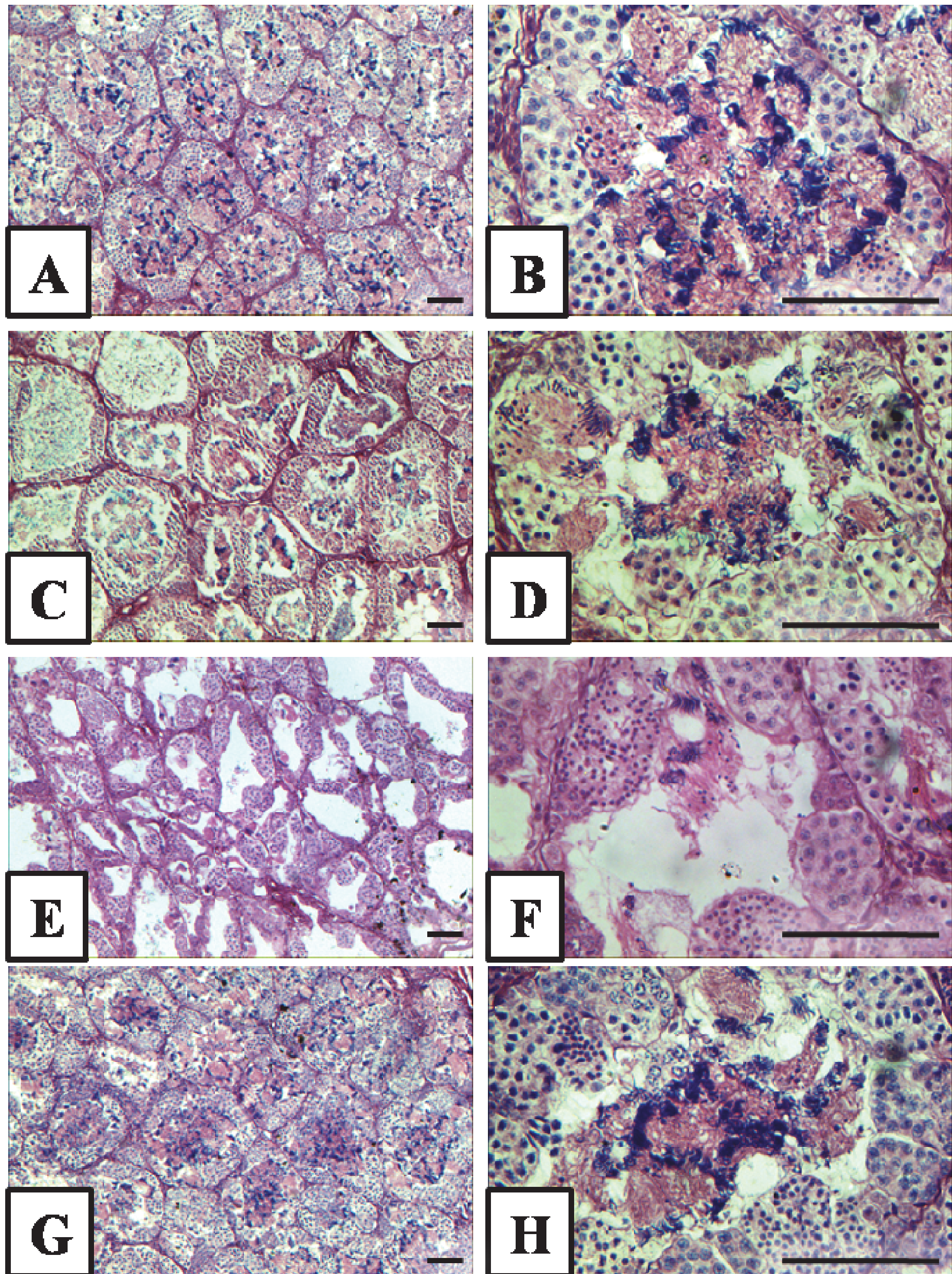

Figure 42: *Variation in spermatogenic germ cell types between adult male X. laevis frogs captured from a water impoundments isolated from agricultural practice (**A** & **B**; Site 1) and water impoundments situated close to agricultural practices (**C** & **D**, **E** & **F**, **G** & **H**; Sites 2, 3 & 4 respectively). Magnification: 100x (A, C, E, G); 400x (B, D, F, H). Scale bar: 100 µm.*

Plasma steroid hormone levels of male X. laevis frogs between impoundment sites

A significant difference was found in plasma T concentrations in frogs (n = 6 per site) collected from Site 3 compared to frogs from control Site 1 (ANOVA, $F_{3,23} = 0.94$, $p <$

0.05; Fig. 43A). Significant differences was shown in plasma DHT concentrations in frogs (n = 6 per site) collected from Site 3 compared to frogs from control Site 1 (ANOVA, $F_{3,23} = 0.37$, $p < 0.05$; Fig. 43B). Frogs from both impoundment Sites 2 and 3 (n = 6 per site) showed a significantly lower ratio of plasma DHT relative to plasma T concentrations compared to frogs from control Site 1 (ANOVA, $F_{3,23} = 0.82$, $p < 0.05$; Fig. 43C). Frogs collected from impoundment Sites 3 and 4 (n = 6 per site) showed a significant difference in plasma E_2 concentrations relative to the control Site 1 (ANOVA, $F_{3,23} = 4.23$, $p < 0.05$; Fig. 43D). Expression of plasma VTG was significantly increased in frogs (n = 6 per site) from impoundment site 3 relative to plasma VTG concentrations in frogs from the control Site 1 (ANOVA, $F_{3,16} = 2.89$, $p < 0.05$; Fig. 43E). The ratios of plasma E_2 relative to T concentrations in frogs from the different impoundment sites (n = 6 per site) did not differ significantly (ANOVA, $F_{3,23} = 0.94$, $p > 2.48$; Fig. 43C).

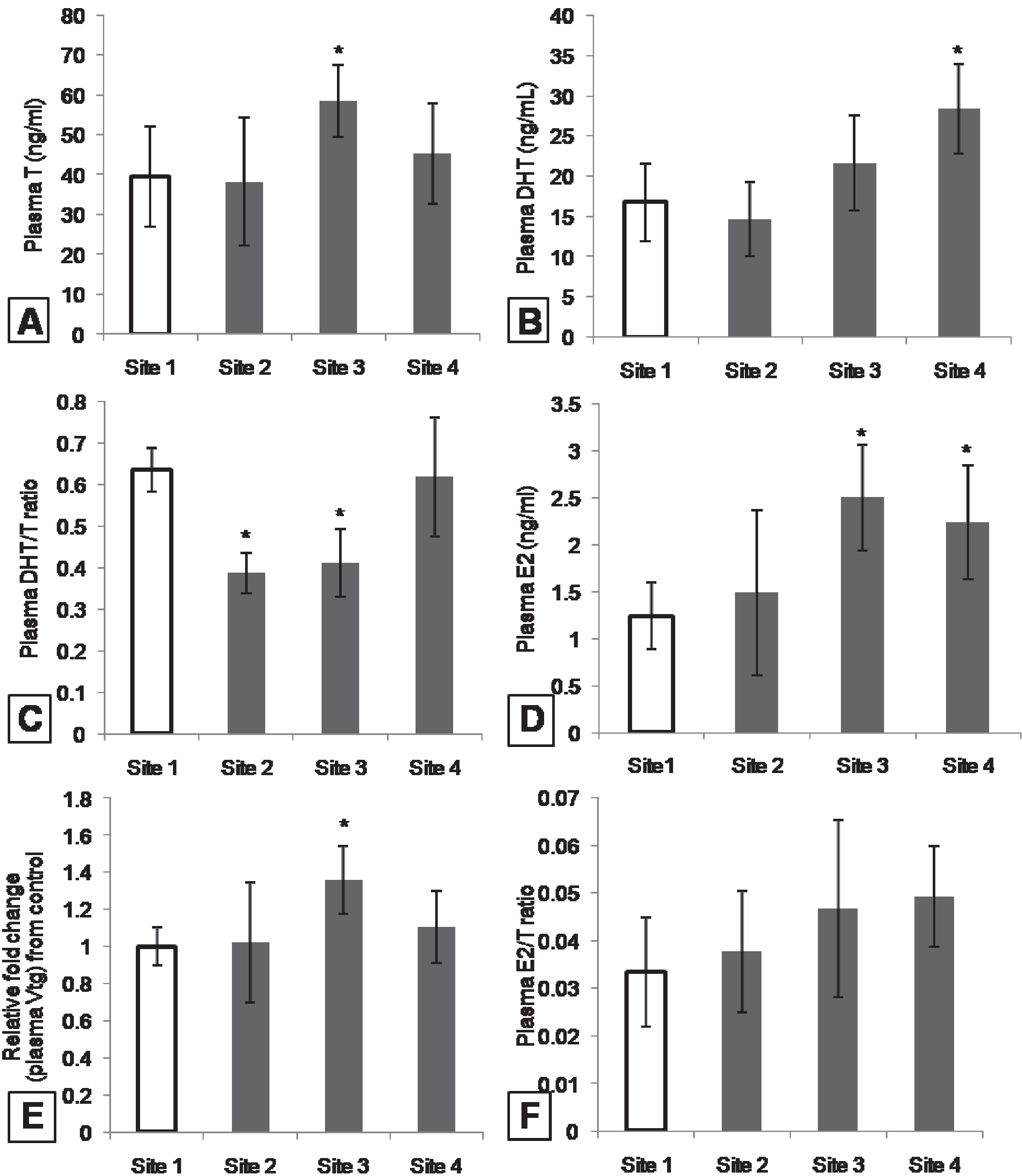

Figure 43: *Concentrations of plasma T (**A**), DHT (**B**), DHT:T ratio (**C**), E_2 (**D**), fold change in plasma VTG expression (**E**) and E_2:T ratios (**F**) of adult male X. laevis frogs (n = 6 per impoundment site). Frogs were captured from a water impoundment not receiving runoff from converging slopes, surrounded by natural vegetation and isolated from agricultural practices (Site 1, control) and water impoundments receiving water from catchments in close proximity to agricultural practices (Sites 2, 3 & 4). The asterisk indicates a significant difference (ANOVA; Tukey's HSD test, $p < 0.05$) from frogs in the control water impoundment (Site 1).*

Thyroid disruption of male X. laevis frogs between impoundment sites

Thyroid receptor β b expression was significantly lower in liver tissues of frogs captured from Site 4 compared to frogs collected from Site 4 ($p < 0.05$) and the control frogs. Expression was also lower in frogs collected from Site 2, but the latter comparison proved not to be significantly ($p > 0.05$) different from other groups (Fig. 44).

Type 2 deiodinase (*dio2*) expression varied significantly ($p < 0.05$) between male frogs collected from three impoundments (Figure 2). Significantly higher *dio2* mRNA levels were expressed in frogs collected from Site 2 relative to frogs collected from Site 3 but statistically not different from the control frogs (Fig. 44).

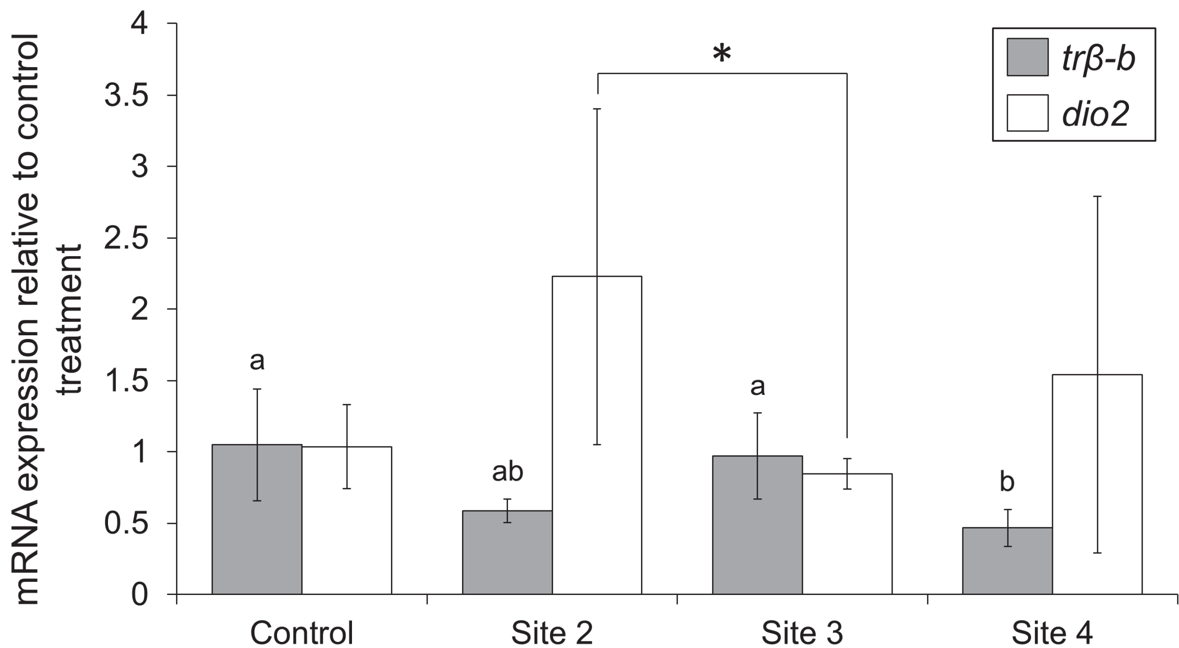

Figure 44: *The mRNA expression (mean ± SD) of thyroid receptor β b (trβ-b) and of type 2 deiodinase (dio2) in the liver tissue of adult X. laevis individuals captured from three impoundments (dams) in the Stellenbosch agricultural area (N = 5 to 7 frogs per treatment). Dissimilar characters indicate statistically significant differences (Tukey's HSD post hoc test, α = 0.05). Significant differences are indicated by asterisks (Student's t-test).*

3.1.5. Conclusions

In vitro screening of androgenic, anti-androgenic and oestrogenic activity within water impoundments situated around agricultural areas in the Western Cape Province of South Africa was confirmed using recombinant yeast screens and a rat minced testis assay. These results raised the importance of screening environmental water sources for anti-androgenic endocrine disruption activity to the same extend as screened for oestrogenic endocrine disruption activity. These results confirmed the value of using a recombinant *in vitro* yeast assay to detect gonadal endocrine

disruption from impoundments among vineyard and orchard plantations. Screening for gonadal endocrine disruption activity in impoundments among agricultural activities has shown that several modes of endocrine disruption activities can be detected in environmental surface waters, possibly contaminated by agricultural pesticides. However, the present study showed that predicted gonadal endocrine disruption activity, especially (anti)androgenic activity, did not always correlate with biomarkers displaying (anti)androgen activity in wild caught male frogs. The decrease in spermatozoa in male testes in frogs collected from the Site 3 impoundment together with the decreased expression of diodinase 2 (*dio2*) enzyme mRNA (relative to Site 2) calls for further study to understand the thyroid-reproductive link. The decreased *mRNA expression of thyroid receptor β b (trβ-b)* in males collected from Site 4 corresponded to high plasma DHT levels and an increased DHT/T ratio. Whether this variation in expression represents endocrine disruption, linked to pesticides, is not known. Studying wild-caught frogs collected from local impoundments, confirmed the importance of including both *in vitro* screening and *in vivo* endpoint assessment associated with a local indicator species in environmental assessments regarding EDC activity. By only using *in vitro* screening may give a false reflection of the real state of affairs regarding endocrine disruption activity in the environment.

3.1.6. Location of full version

Archer, E. 2014. Androgen-controlled secondary sexual characters in the male African clawed frog, *Xenopus laevis*, as potential biomarkers for endocrine disruptor contaminants (with special reference to fungicides) in aquatic systems. Chapter in MSc thesis, Stellenbosch University, Stellenbosch.

3.2. The application of *in vitro* and *in vivo* techniques to evaluate endocrine disruption activity in the Olifants River, a major South African river.

Researcher: C. Truter, PhD, Stellenbosch University

3.2.1. Aims

The objective of this study was to use *in vitro* and short-term in vivo exposures to evaluate the endocrine disrupting potential in a river catchment receiving multiple man-made effluents. The aims included: (1) the determination of human steroid oestrogen concentrations in the upper Olifants River during summer, autumn, winter and spring; (2) the assessment of (anti)oestrogenic and (anti)androgenic activity of organic compounds extracted from water collected from the river during summer; (3) the expose of juvenile Mozambique tilapia *Oreochromis mossambicus* to organic

compounds extracted from Olifants River surface water, followed by an assessment of variation (response) regarding endocrine-linked gene expression.

3.2.2. Materials and Methods

Six study sites were selected based on their representation of different land use areas (Fig. 45). Surface water was collected during April, July, September and November 2011. Organic compounds were extracted from the water through solid phase extraction (SPE) using 12 mL 2 g C-18 columns (Supelco, Sigma-Aldrich, DE) and reconstituted in DMSO (Sigma, DE) to a 1000x concentrated state.

The land cover of Site 1 (near the town Davel) and Site 2 catchments are principally occupied by dry-land and irrigated agriculture as well as livestock farming. In contrast, Site 3 is downstream of urbanised areas hence being subject to potential contamination through runoff and deliberate releases such as the effluent of the Kriel WWTP (Fig. 45). Similarly, Site 4 is adjacent to the town of Emalahleni and downstream of the Riverview WWTP, which receives industrial and domestic wastewater. Site 5 is approximately 40 km downstream of Site 4, being located in a region chiefly covered by natural vegetation and the contaminants/impacts expected at this locality are predominantly introduced via the Olifants River itself (Fig. 45). Similar to Site 5, Site 6 is surrounded by natural vegetation, but is located downstream of the confluence of the Klein Olifants and Olifants rivers, therefore potentially receiving contaminants from both the greater Emalahleni and Middelburg regions as well, although both Middelburg and Emalahleni are both at least 50 km upstream of this site. Furthermore, dry-land agricultural practices are located within a 10 km radius of Site 6 (Fig. 45).

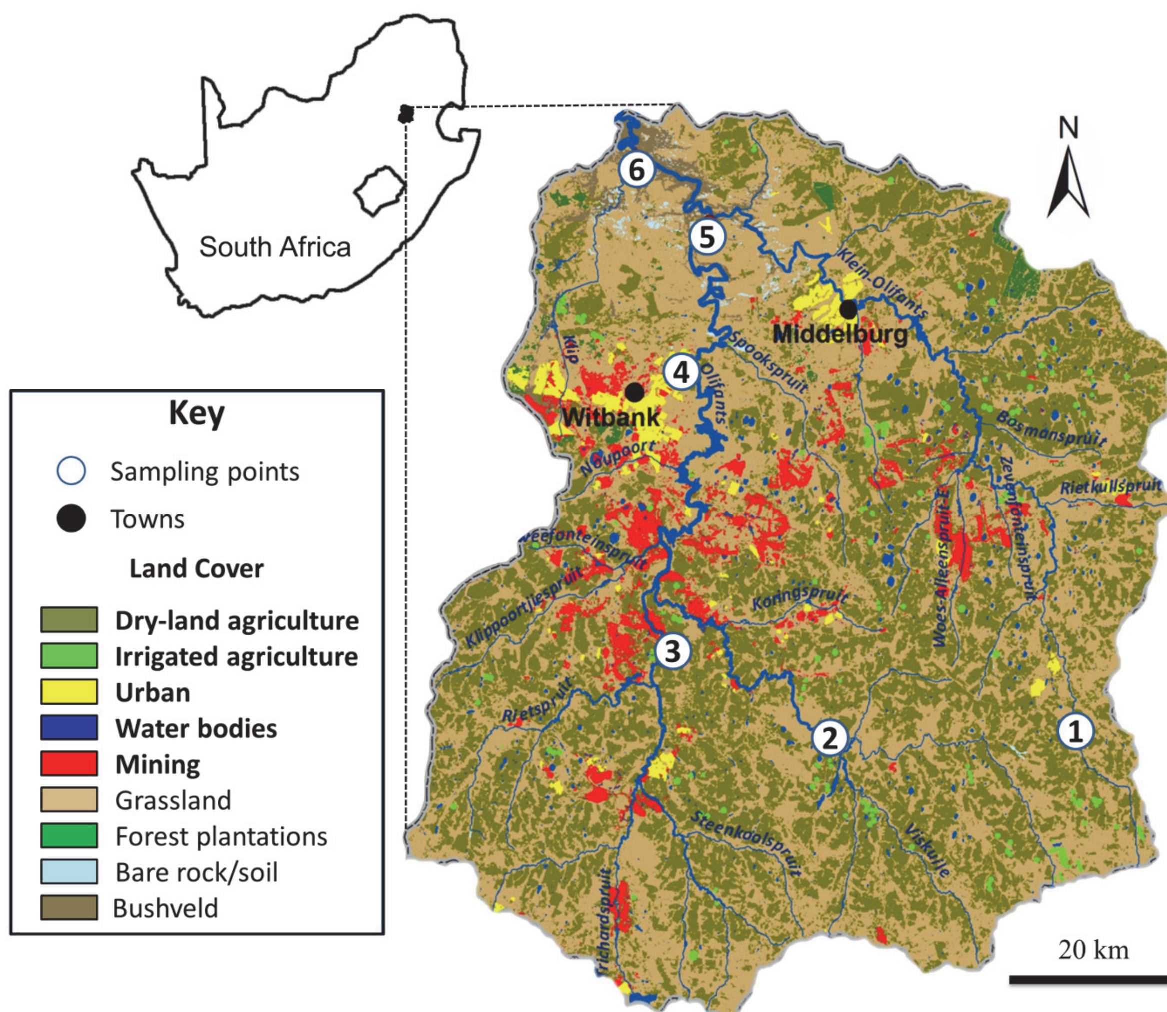

Figure 45: *Map of the upper Olifants River catchments with land use (Dabrowski and De Klerk, 2013) and the current sampling locations indicated.*

Enzyme-linked Immunosorbent Assays (ELISAs)

17-β-estradiol (E_2) and 17α-ethinylestradiol (EE_2) levels were measured in the C18 SPE extracts of water collected from the six localities across four seasons using commercially available ELISA kits (E_2: DRG International Inc., USA; EE_2: R-Biopharm, GE).

In vitro recombinant yeast assay

The Yeast Oestrogen Screen (YES), Yeast Anti-oestrogen Screen (anti-YES), Yeast Androgen Screen (YAS) and Yeast Anti-androgen Screen (anti-YAS) recombinant yeast bioassays were applied to evaluate oestrogenic, anti-oestrogenic, androgenic and anti-androgenic activity in the C18 SPE extracts of water collected from the six localities during summer. The recombinant yeast was kindly provided by J.P. Sumpter, Brunel University, Uxbridge, United Kingdom). The receptor transactivation

screens were applied as described by Sohoni and Sumpter (1998) using tamoxifen and flutamide as anti-oestrogenic and anti-androgenic standards respectively.

In vivo fish exposures

Oreochromis mossambicus individuals at the 17 days-post-fertilization (dpf) stage were obtained from a single breeding pair at the Welgevallen Experimental Farm, Stellenbosch University. The fish were exposed at 22 dpf to the C18 solid phase extraction extracts of water collected during the summer of 2011. Twenty fish were assigned per treatment in duplicate 1-litre capacity glass tanks. The C18 extracts representing the six localities were reconstituted in 600 mℓ of buffered iodated RO water to the concentrated state as in nature (0.001% DMSO). A negative as well as DMSO solvent control exposure group were also included. The fish were exposed without food for 48 hours under constant aeration at 28 ± 1°C with a 14:10 light:dark cycle, pH 7.

Whole body homogenates of juvenile fish were prepared in TriReagent using an ultrasound sonicator (Omni-ruptor 400, Omni International Inc., US). Total RNA was isolated according to the TriReagent technical bulletin. The RNA was subsequently DNase I (Sigma, DE) treated, and complementary DNA (cDNA) was prepared from 2 µg of total RNA in 20 µℓ-, or 1 µg in 10 µℓ reaction volumes using Enhanced Avian HS RT-PCR kits (Sigma, DE) according to manufacturer’s instructions. Messenger RNA expression of *cyp19a1b*, *ar1, gr1, mr, trα* and *trβ* with *β-actin* as reference gene was evaluated using real-time RT-qPCR as described in Truter et al. (2014). Outliers were identified per treatment group through the Grubbs’ test (Burns et al., 2005) and removed.

3.2.3. Statistical analysis

Normality and homogeneity of variance of the data was assessed using the respective Shapiro-Wilks and Levene’s tests. Data that were still non-parametric after log transformation were analysed using Kruskall-Wallis ANOVA followed by Multiple Comparisons of Mean Ranks to assess pair-wise differences, whereas One-way ANOVA followed by Fisher LSD Multiple Comparisons were applied on parametric data. Seasonal variation in steroid oestrogens were assessed using generalized linear mixed models (PROC GLIMMIX) in SAS (V 9.3, SAS Institute Inc., USA), with season as the repeated measure, and locality as the subject identifier. The variance components covariance structure was applied based on the Akaike Information Criterion. Probability values of ≤ 0.05 were deemed significant. A Redundancy Analysis (RDA) triplot was used to describe the among-site gene expression signatures with the basic water quality and element concentrations overlaid. The data applied in the RDA were log transformed. Ordination triplots were created using CanoDraw 4.12 (Ter Braak and Smilauer, 2002).

Statistical analyses were performed using Statistica 11 (Statsoft Inc., USA), SAS 9.3 (SAS Institute, USA) and CANOCO version 4.5 (Ter Braak and Smilauer, 2002). Ordination biplots and triplots were created using CanoDraw 4.12 (Ter Braak and Smilauer, 2002).

3.2.4. Results

Steroid hormone analyses

17β-Estradiol (E_2) and 17α-ethinylestradiol (EE_2) was detected at each of the six locations sampled in the Upper Olifants River catchment at concentrations ranging between 0.72 to 30.8 ng/L and 0.45 to 10.83 ng/L, respectively (Figs. 46 and 47). The geographic variation in E_2 and EE_2 is as expected, concentrations being higher at the sites 3 and 4 both located downstream of WWTPs (Figs. 48 and 49). EE_2 was detected during a single sampling event at site 2 and site 5 and at low concentrations, whereas sites 1 and 6 had EE_2 within the current detectable range on two of the four sampling events.

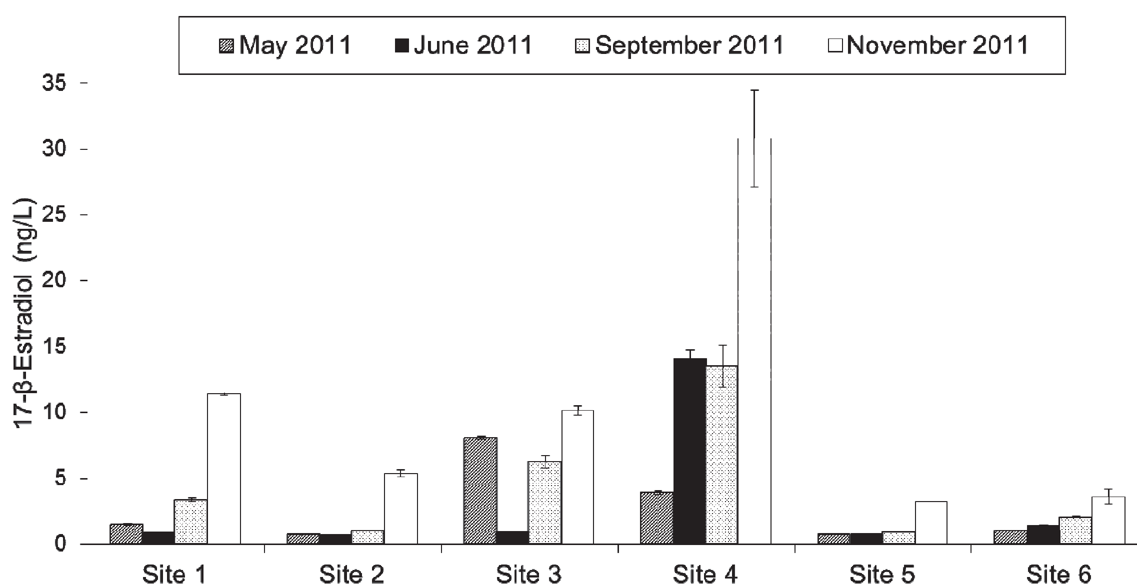

Figure 46: *Mean 17β-estradiol (E_2) concentrations (ng/L) measured in water collected from a selection of localities within the upper Olifants River catchment during May, June, September and November 2011. Error bars denote the standard deviation (SD) of technical repeats within immunoassays.*

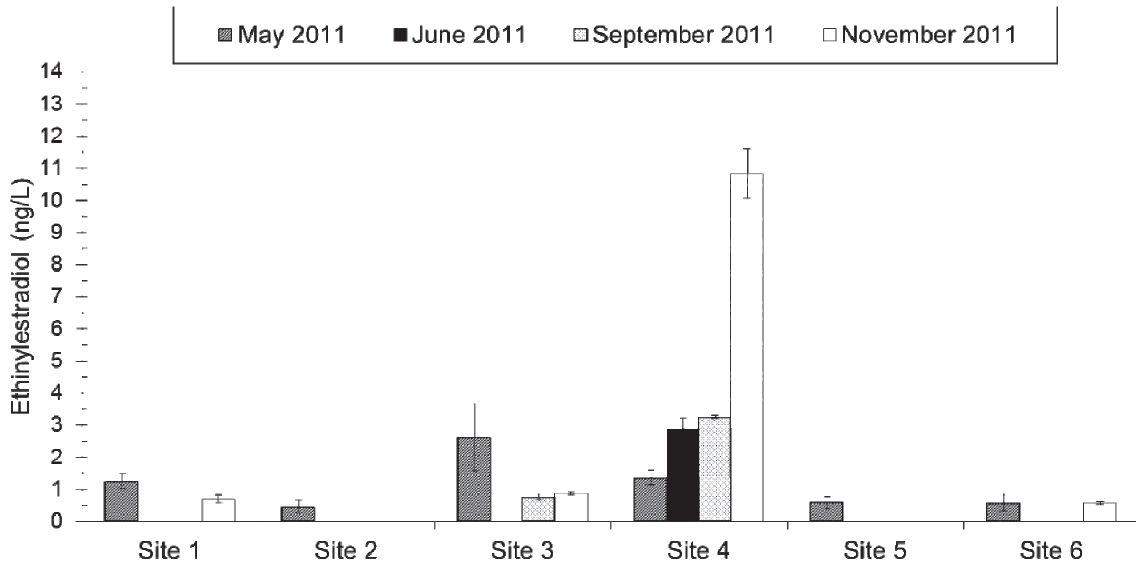

Figure 47: *Mean 17α-ethinylestradiol (EE_2) levels (ng/L) measured in water collected from six localities within the upper Olifants River catchment during May, June, September and November 2011. Error bars denote the standard deviation (SD) of technical repeats within immunoassays.*

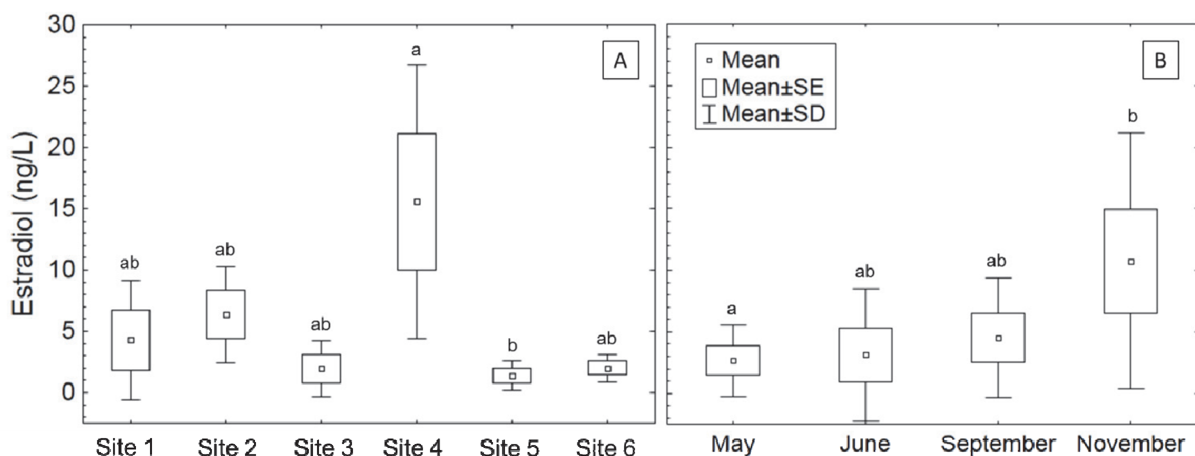

Figure 48: *17β-Estradiol levels ng/L (**A**) across seasons (i.e., summer, autumn, winter and spring) (**B**) among six localities within the upper Olifants River catchment. Significant differences are indicated by dissimilar characters above figure bars. Locality (within the river) was a significant source of variation in E_2 concentrations measured across seasons ($H_{5,24}$ = 12.3, p = 0.03).*

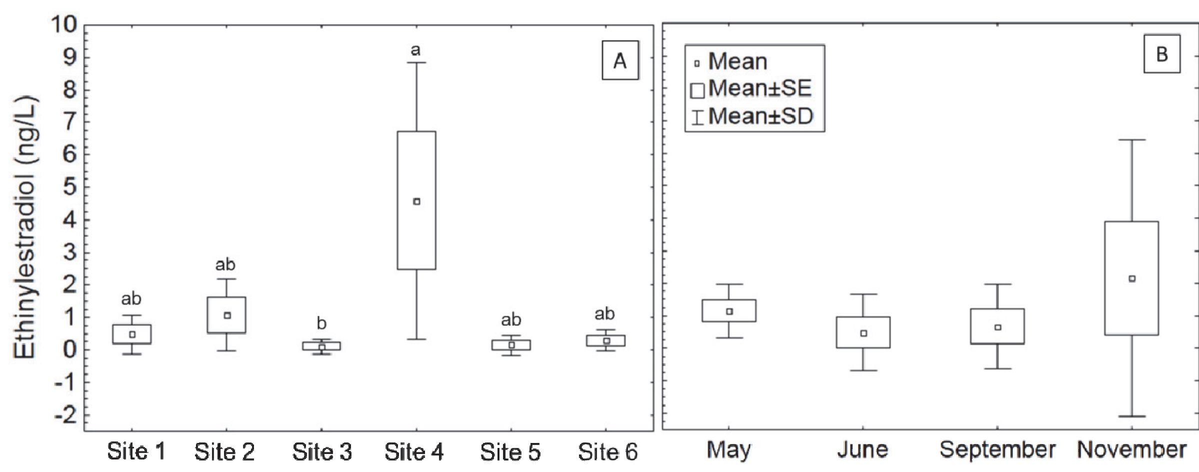

Figure 49: *17α-Ethinylestradiol levels ng/L (**A**) across seasons (i.e., summer, autumn, winter and spring) (**B**) among six localities within the upper Olifants River catchment. Significant differences are indicated by dissimilar characters above figure bars. Locality (within the river) was a significant source of variation in EE2 ($H_{5,24}$ = 13.73, p = 0.01, Kruskal-Wallis ANOVA).*

17β-Estradiol was present at all six locations during all four seasons sampled, and when the detected concentrations were pooled among localities, a stepwise seasonal increase from May to November was evident (Fig. 48b), although the only significant pair-wise difference was among May and November (p = 0.05, PROC GLIMMIX) (Figs. 46 and 48). Similarly EE_2 was detected in the upper Olifants catchment throughout the year, although only the Site 4 had detectable concentrations during all four seasons sampled (Figs. 47 and 49). The seasonal variation in EE_2 concentrations followed a similar trend to E_2 at Site 4, being highest in November (Figure 47).

Yeast Reporter Gene Assays

Oestrogenic activity was limited to site 4, whereas anti-androgenic activity was observed in water collected from sites 1 and 2 (Fig. 50 & 51). Neither anti-oestrogenic nor androgenic activity was observed in any of the surface water samples.

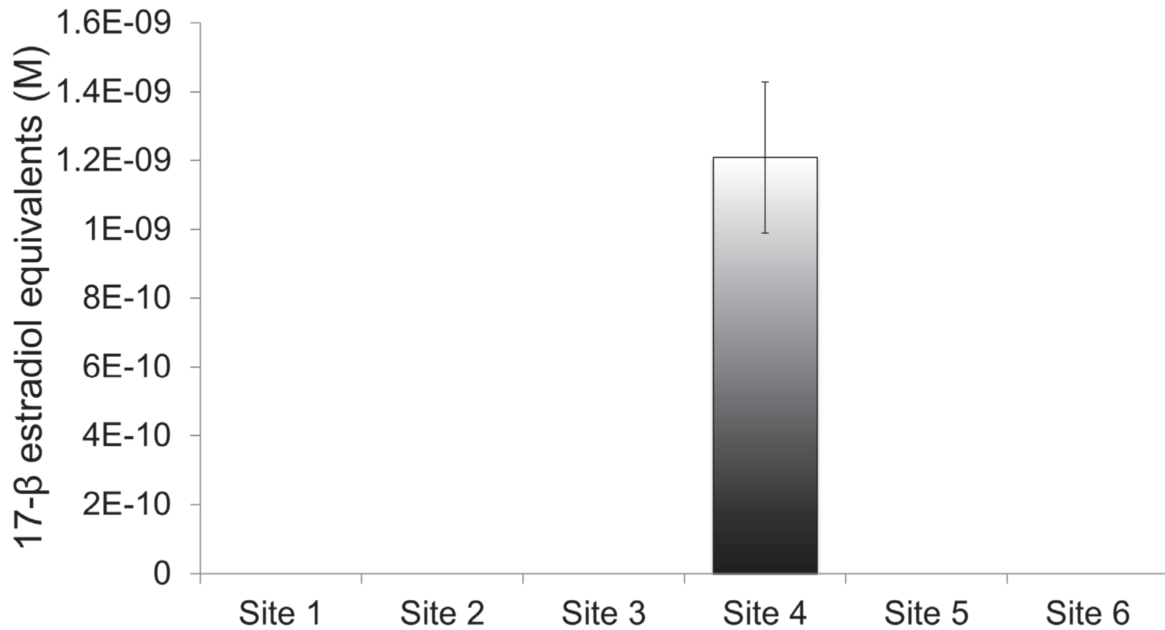

Figure 50: *Oestrogenic activity (expressed as 17β-estradiol equivalents) observed in surface water collected from six localities within the upper Olifants River catchment during summer. The error bar indicates inter-assay variability as standard deviation.*

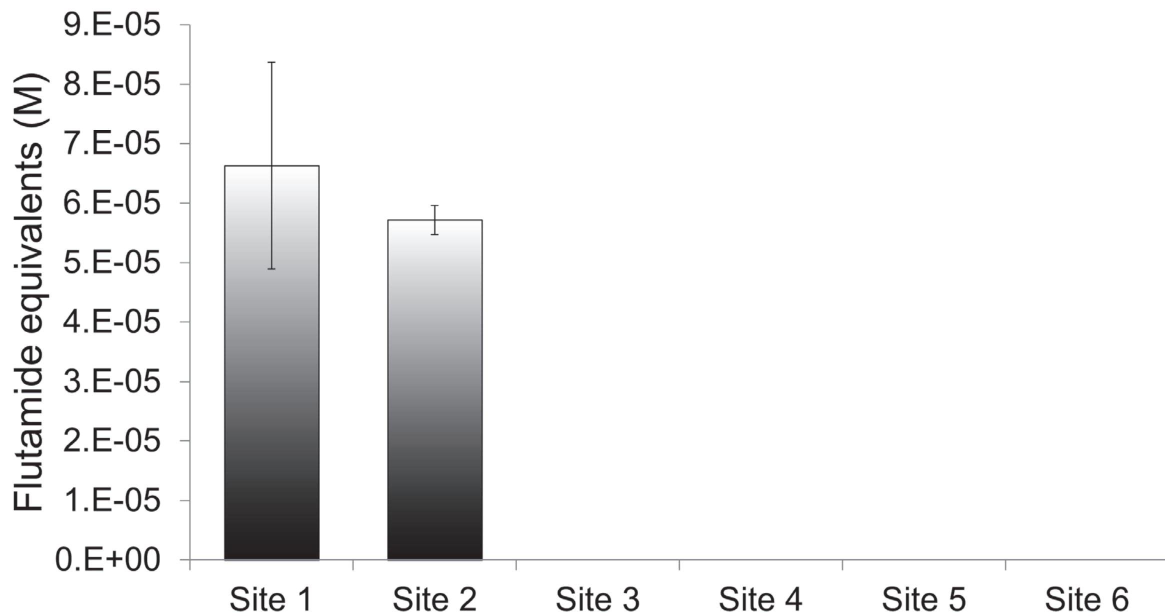

Figure 51: *Anti-androgenic activity (expressed as Flutamide equivalents) observed in surface water collected from six localities within the upper Olifants River catchment during summer. Error bars indicate inter-assay variability as standard deviation.*

Gene expression

No significant differences were observed among any of the treatment groups for any of the genes under investigation (Fig. 52 & 53).

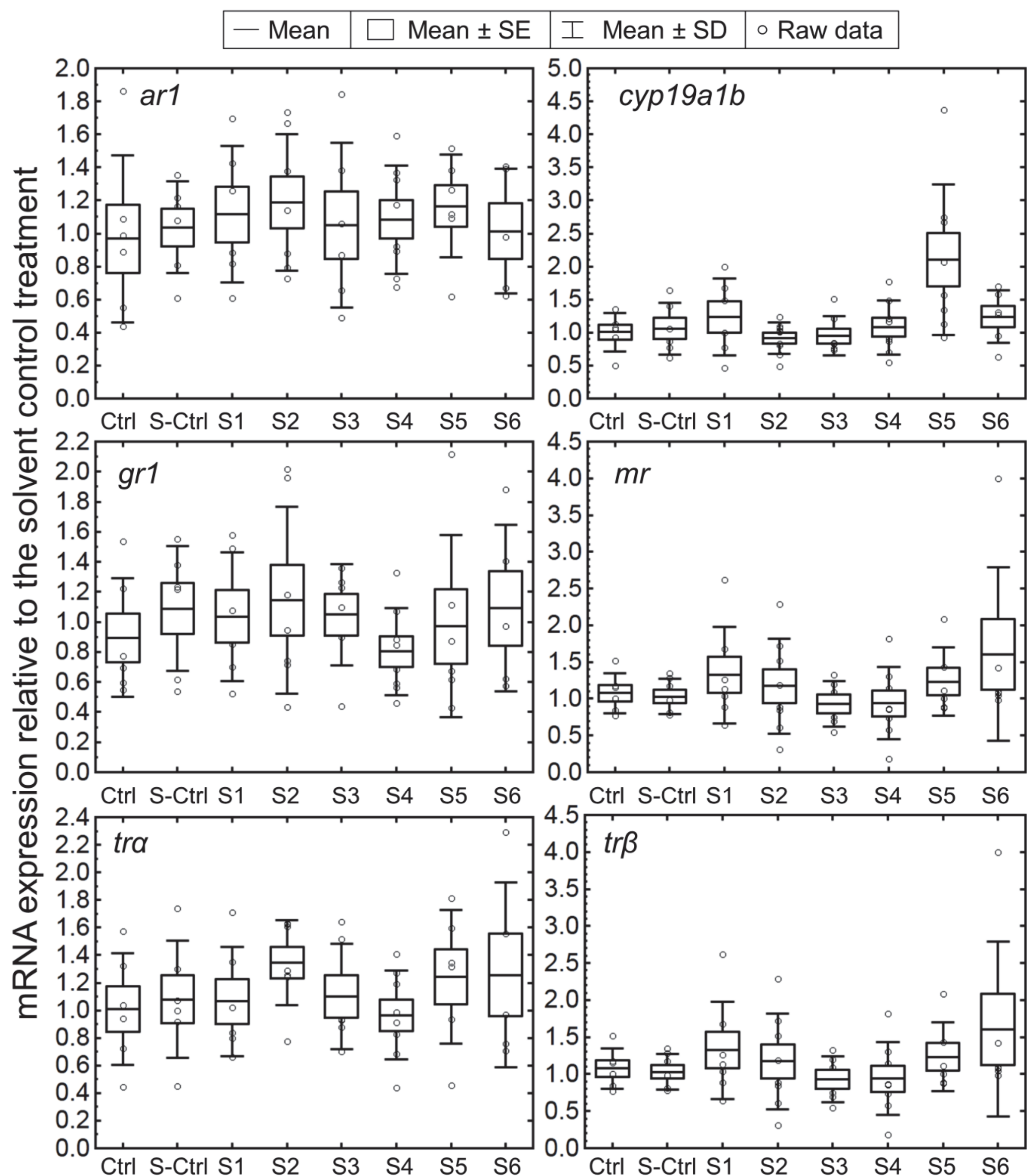

Figure 52: *Expression of androgen receptor-1 (ar1), aromatase (cyp19a1b), glucocorticoid receptor-1 (gr1), mineralocorticoid receptor (mr), thyroid receptor-α (trα) and trβ in juvenile Oreochromis mossambicus (22 dpf) exposed for 48-hours to C18 solid phase extracts of water collected from six localities within the upper Olifants River catchment, a buffered RO water control and solvent (DMSO) control. Expression was determined using RT-qPCR. Expression is expressed relative to the solvent control exposure group and was quantified using the Pfaffl method (Pfaffl, 2001) with β-actin as reference gene.*

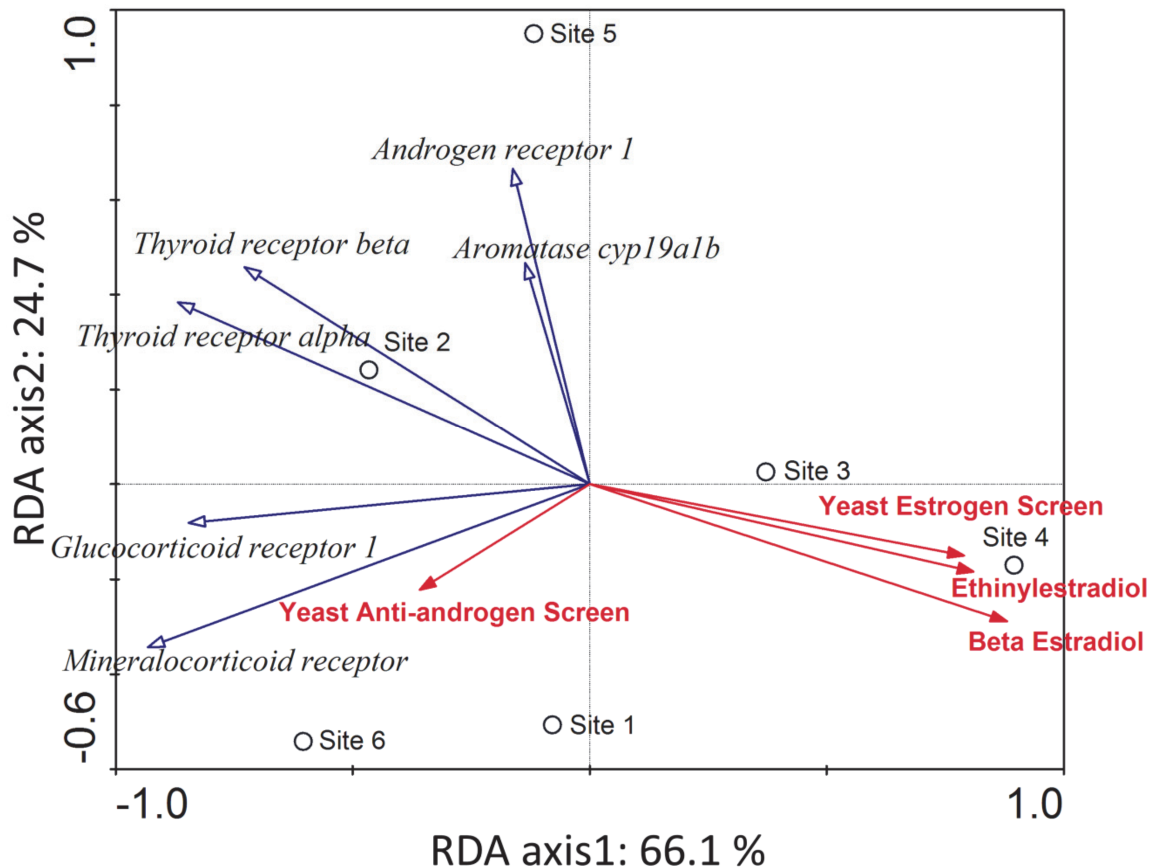

Figure 53: *A Redundancy Analysis triplot indicating the (dis)similarities among six sampling locations within the upper Olifants River catchment with regard to the expression of O. mossambicus trα, trβ, ar1, gr1, mr and cyp19a1b expression in combination with 17β-oestradiol, ethinylestradiol concentrations and oestrogenicity and androgenicity as indicated by the yeast oestrogen- and anti-androgen screens respectively. The mean of gene expression per treatment group was applied in the analysis.*

3.2.5. Conclusions

The current study provides evidence of steroid oestrogen contamination in the upper Olifants River catchment, further indicating a primary association of these contaminants with WWTPs, although agricultural practices were shown to be a further vector. The E_2 concentrations were high at sites 3 and 4 relative to previous reports for South African surface water (Pool and Swart, 2007; Manickum et al., 2010), in fact exceeding the levels observed in waste water treatment plant effluents in the Western Cape (Swart and Pool, 2007). *In vitro* recombinant yeast screens indicate oestrogenic activity at a single location downstream of a waste water treatment works, and anti-androgenic activity at two locations within an agricultural region. The anti-androgenic activity is likely associated with pesticides, which are known anti androgens (Orton et al., 2011). Limited effects were observed in the expression of a number of endocrine-linked genes in response to organic contaminants in surface water collected from the Olifants River catchment. The lack

of responses in reproductive linked genes is surprising seeing that oestrogen concentrations exceeding the reproduction predicted no-effect concentration (PNEC) for fish were measured at all six the localities sampled (Caldwell et al., 2012). Our results therefore suggest that *O. mossambicus* juveniles are insensitive to exogenous oestrogen exposure. A redundancy analysis triplot evaluating gene expression signatures in light of oestrogenic and anti-androgenic activity together with E_2 and EE_2 concentrations indicates a grouping of the waste-water treatment works impacted sites 3 and 4, and close association with oestrogenicity and oestrogen loads. No grouping in the agriculture impacted or downstream sites were evident suggesting no clear links in the responses of the genes under investigation and land-cover regions when anti-androgenicity, oestrogenicity and oestrogen loads are accounted for.

3.2.6. Location of full version

Truter, J.C. 2014. Testing for endocrine disruptors in South African waters: a comparative study to evaluate in vitro and *in vivo* testing approaches in laboratory and field situations. Chapter in PhD thesis, Stellenbosch University.

3.3. Potential disruption of the Thyroid and Adrenal endocrine axes in the Mozambique tilapia (*Oreochromis mossambicus)* population Loskop Dam: I. Thyroid health in wild caught fish from Loskop Dam and Flag Boshielo Dam

Researcher: J Dabrowski (PhD, Pretoria University & CSIR)

3.3.1. Aims

The aims of this study included the assessment of thyroid function in fish collected from the two sites. Thyroid function was assessed from data, including variation in total plasma concentrations of the two main thyroid hormones T_3 (3,3',5-triiodo-L-thyronine) and T_4 (thyroxine) using radioimmunoassay (RIA), and a corresponding thyroid histopathology study.

3.3.2. Materials and Methods

Study Site

Fish were collected from Loskop Reservoir (LR) in Mpumalanga Province, and Flag Boshielo Reservoir (FBR) in Limpopo Province, on the Olifants River (Fig. 54). Water quality in LR is heavily impacted by various land uses including coal mining, agriculture, industry and effluent originating from wastewater treatment works that are not functioning optimally. These impacts have resulted in increased eutrophication and frequent blooms of *Microcystis aeruginosa* and *Ceratium*

hirundinella. In contrast, the main land use in the catchment of FBR is agriculture, and the reservoir is oligotrophic.

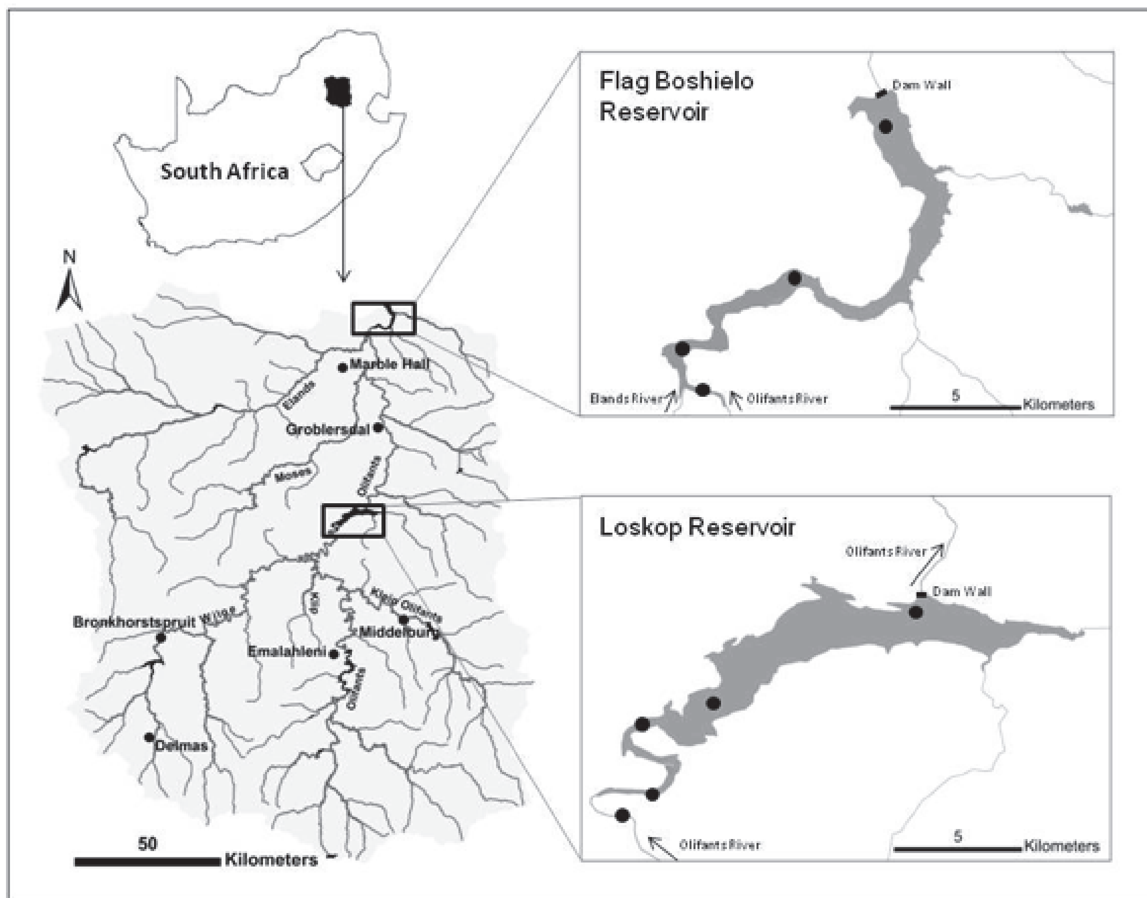

Figure 54: *Map showing the location of Loskop Reservoir (LR) and Flag Boshielo Reservoir (FBR) on the Olifants River. Black circles indicate the location of established water quality monitoring points at both reservoirs (Dabrowski et al., 2013; 2014).*

Fish Collection

At both reservoirs, approximately 20 adult fish were collected per season using gill nets during April (autumn), June (winter), October (spring) and December (summer) in 2011 to incorporate variation related to feeding and reproductive cycles. Nets were set during daylight hours using three 25 m panels with 70, 90 and 130 mm multi-filament stretched-mesh. An approximately even sex ratio and consistent size class (> 200 mm total length) was collected from each reservoir. Condition factor (K) was calculated as; K = 100 W L-3 where W is body weight (g) and L is length (cm). After collection of blood samples, fish were killed by severing the spinal cord. Collection methods and animal handling followed approved university guidelines and relevant permits were obtained.

Plasma Thyroid Hormones

While fish were alive a sample of blood was collected from the caudal vein. After centrifugation of whole blood, 0.5 mL of plasma was collected and frozen for analysis. A sample of 0.5 ml of serum was collected and frozen at -80°C for analysis of total T_3 and total T_4 concentrations using Siemens Coat-A-Count® commercial kits by radioimmunoassay (RIA) according to manufacturer's guidelines. Sensitivity of the assay was defined by standards which ranged from 0.31 to 9.22 nmol/L for T_3, and 6.4 to 193 nmol/L for T_4. All samples were assayed in duplicate for each hormone in a single assay and the intra-assay coefficient of variation (% CV) was 3.26% for the T_3 assay and 13.89% for the T_4 assay. Thyroid hormone levels were expressed as nmol/L. As an indicator of peripheral conversion of T_4 to T_3 (5'-deiodination), the ratios of T_3/T_4 were calculated (Brar et al., 2010).

Histomorphology of Thyroid Follicles

The subpharyngeal tissue of a sub-sample of 30 fish from each reservoir was dissected and preserved in buffered formaldehyde solution for histological analysis of thyroid follicles. Approximately even sex ratios were evaluated with 14 females and 16 males from LR, and 17 females and 13 males from FBR. Thyroid tissue was not collected during winter; therefore this season was excluded from the analysis. All fish included in the analysis had corresponding values for plasma T_3 and T_4. Tissue sections were processed and stained with Haematoxylin and Eosin (H & E) following standard methods (Bancroft and Stevens, 1977).

The perimeter (μm) and area (μm^2) of ten thyroid follicles per fish was measured. Thyroid follicle activity was determined by counting the numbers of vacuoles along the inner perimeter of each follicle (vacuoles / μm), and measuring the epithelial cell height of three thyrocytes per follicle (both indicators of thyroid activity related to TSH stimulation). The appearance of colloid storage of each follicle was subjectively scored numerically along a gradient with lower values indicating more dilute storage, and higher values indicating more concentrated storage: 0 = empty; 1 = granular appearance; 2 = light eosinophilic stain; 3 = intense eosinophilic stain. The mean of these values for ten follicles per fish was presented as the average colloid concentration.

Tissue sections were analysed with an Olympus BX43 light microscope fitted with an AxioCam ICc 3 Zeiss microscope camera, and all parameters were measured using AxioVision 4 software.

3.3.3. Statistical procedures

Thyroid hormone data violated assumptions of normality and homogeneity of variance, despite data transformation efforts, and were therefore analyzed using a non-parametric Kruskal-Wallis analysis of variance (ANOVA) by ranks, with site,

season and gender as grouping variables. Thyroid follicle histomorphology was analysed using a multi-factor analysis of variance (MANOVA) with site, season, and gender as factors. Results from ten thyroid follicles per fish were used to calculate mean values that were used in the analysis, and variables were transformed to meet assumptions where necessary. Relationships between measured parameters were evaluated using Spearman’s rank correlation analysis. All statistical analyses were completed using Statistica Version 12 (StatSoft, Inc. Tulsa OK) and significance level (α) was set at 0.05.

3.3.4. Results

Fish Summary

Fish from LR had abundant mesenteric fat which contained typical pansteatitis lesions of yellow, orange and brown spots (ceroid pigment) that varied in intensity and hardness. Fish from FBR had little to no fat, which when present, was white in colour. The mean body weight of fish from LR (1458 g) was significantly heavier (T-test, $p < 0.0001$), and approximately double the mean weight of fish from FBR (730 g). The mean TL of fish from LR (398 mm) was also significantly longer than fish from FBR (325 mm; T-test, $p < 0.0001$).

Table 12: *Summarised catch statistics for Oreochromis mossambicus sampled from Loskop (LR) and Flag Boshielo (FBR) Reservoirs in 2011 including the sex ratio (M:F), weight and total length (TL; Mean ± SD).*

Site	Season	*n*	M : F	Weight (g)	TL (mm)	*K*
LR	Autumn	31	17 : 14	1207 ± 597	372 ± 6.5	2.2 ± 0.2
	Winter	20	7 : 13	1725 ± 468	421 ± 3.9	2.2 ± 0.2
	Spring	20	11 : 9	1683 ± 337	420 ± 3.4	2.8 ± 0.2
	Summer	20	6 : 14	1353 ± 448	395 ± 4.8	2.1 ± 0.2
	Total	**91**	**41 : 50**	**1458 ± 532**	**398 ± 5.4**	**2.2** ± 0.2
FBR	Autumn	21	16 : 5	876 ± 404	365 ± 6.3	2.0 ± 0.2

Site	Season	*n*	M : F	Weight (g)	TL (mm)	*K*
	Winter	20	15 : 5	906 ± 312	353 ± 4.8	2.0 ± 0.2
	Spring	20	7 : 13	662 ± 252	321 ± 5.2	1.9 ± 0.2
	Summer	20	4 : 16	474 ± 223	283 ± 3.8	1.9 ± 0.1
	Total	**81**	**42 : 39**	**730 ± 348**	**325 ± 5.7**	**1.99** ± 0.2

Plasma Thyroid Hormones

Thyroid hormone analysis showed significantly ($p < 0.0001$) elevated T_3 plasma concentrations in fish from LR in all seasons and for both genders compared to FBR (Fig. 55B). The interaction between site and season was significant with an increasing trend over the study period observed in fish from LR, while levels were seasonally consistent in fish from FBR. Female fish had significantly higher T_3 levels than males at both sites. There was no significant difference in plasma T_4 concentrations between reservoirs (Fig. 55B). There was a significant effect of both season and gender on circulating T_4 in fish from both reservoirs. Concentrations increased from autumn to summer over the sampling period, and were slightly higher in females than in males.

Within season T_3 levels were consistently higher than T_4 levels in LR fish, while the opposite trend was observed in fish from FBR.

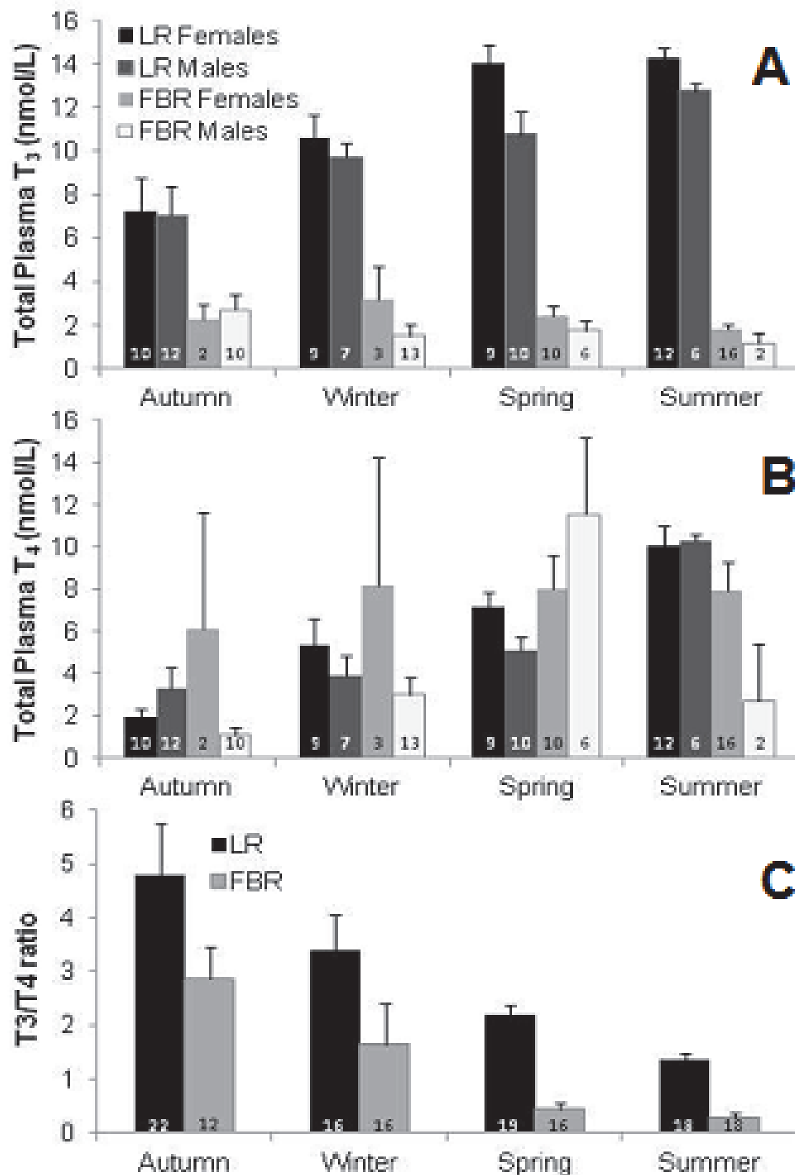

Figure 55: *Plasma T_3 (A) and T_4 (B) concentrations, and T_3/T_4 ratio (C) measured in Oreochromis mossambicus sampled from Loskop Reservoir (LR) and Flag Boshielo Reservoir (FBR). Bars represent mean values ± S.E., with the number of individuals per mean shown within bars.*

Histomorphology of Thyroid Follicles

In fish from LR, thyroid follicles were frequently enlarged (Fig. 56A) with hypertrophy and hyperplasia of the follicular epithelium (Fig. 56B) and increased numbers of vacuoles along the epithelial-colloid interface (Fig. 56C). Follicles in fish from FBR showed a large degree of variation in size and colloid staining intensity (Fig. 56D).

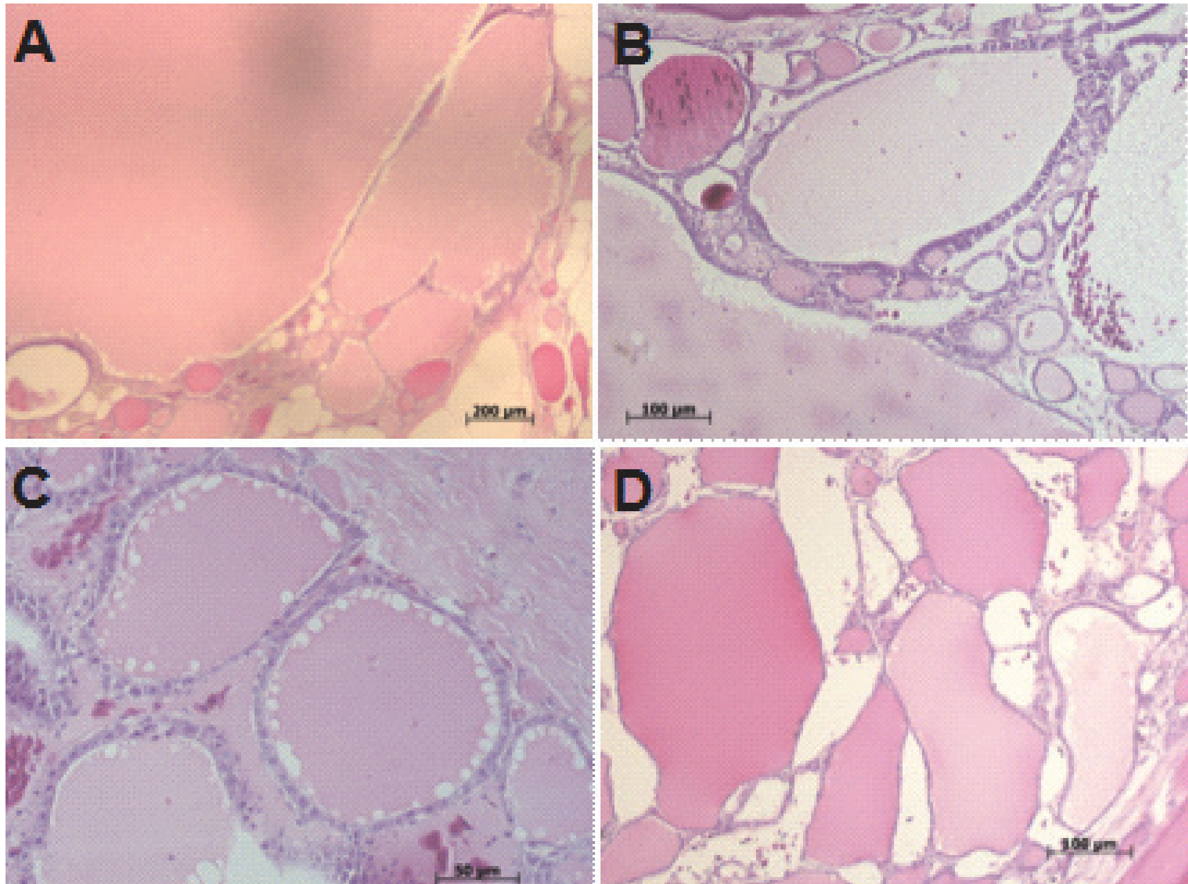

Figure 56: Photomicrographs (H&E) of thyroid follicle sections collected from Oreochromis mossambicus. **A**. follicles in fish from Loskop Reservoir were abnormally enlarged with greater surface area (40x magnification); **B**. hypertrophy and hyperplasia of the follicular epithelium in fish from LR (100x magnification); **C**. increased number of epithelial-colloid interface follicular vacuoles in fish from LR (200x magnification); **D**. typical variation in size, colloid staining intensity, resting epithelium and low vacuolar activity of follicles in fish from FBR (100x magnification).

Fish from LR had thyroid follicles with a significantly larger area, greater epithelial cell height, a higher number of vacuoles / µm and lower colloid storage than fish from FBR (Table 13).

Table 13: Mean (±SE) histopathological and morphometric descriptor values for thyroid follicles evaluated in Oreochromis mossambicus from Loskop Reservoir (n = 30) and Flag Boshielo Reservoir (n = 30).

Thyroid follicle descriptors	Loskop Reservoir	Flag Boshielo Reservoir
Area (μm^2) [L]	64,005 ± 9562 *	25,639 ± 4118 *
Epithelial Cell Height (µm)	4.5 ± 0.09 ***	3.5 ± 0.06 ***
Colloid droplets / µm [S]	0.04 ± 0.00***	0.02 ± 0.00***
Average colloid storage	2.02 ± 0.07**	2.3 ± 0.05**

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. Statistics based on MANOVA

[L] Log transformed; [S] Square root transformed for statistical analysis.

There was a weak but very similar positive correlation between condition factor and T_3/T_4 ratios in fish from both reservoirs. Thyrocyte height was positively correlated with T_3 levels and negatively correlated with T_4 levels in fish from FBR, but showed no relationship to hormone levels in fish from LR. Perpheral colloid vacuoles density was positively correlated with T_4 in fish from LR.

Table 14: *Spearmans rank correlation coefficients (R) and p-values of relationships between nutritional and thyroid parameters measured in Oreochromis mossambicus from Loskop Reservoir and Flag Boshielo Reservoir. Bold font indicates that the correlation coefficient is significant (p < 0.05).*

Parameters	Loskop Reservoir						Flag Boshielo Reservoir					
	T_3		T_4		T_3/T_4		T_3		T_4		T_3/T_4	
	R	p	R	p	R	p	R	p	R	p	R	p
T_3	-	-	0.50	***	0.05	NS	-	-	0.20	NS	0.41	**
T_4	0.50	***	-	-	-0.76	***	0.20	NS	-	-	-0.77	***
T_3/T_4	0.05	NS	-0.76	***	-	-	0.41	**	-0.77	***	-	-
Condition factor	0.05	NS	-0.15	NS	0.29	*	0.30	*	-0.10	NS	0.27	*
Thyroid follicle area	-0.25	NS	-0.14	NS	0.07	NS	0.12	NS	0.24	NS	-0.08	NS
Thyrocyte height	-0.18	NS	-0.17	NS	0.01	NS	0.39	*	-0.41	*	0.62	**
Colloid droplets	0.32	NS	0.40	*	-0.12	NS	0.16	NS	0.03	NS	-0.09	NS
Colloid storage	-0.09	NS	-0.07	NS	-0.00	NS	0.14	NS	0.15	NS	-0.08	NS

3.3.5. Conclusions

Overall, the fish were significantly larger (weight and length) in Loskop Dam than Flag Boshielo Dam. Every aspect of the thyroid cascade examined in *O. mossambicus* from LR indicated very high levels of activity in comparison to fish from FBR. Elevated epithelial cell height, decreased colloid staining, and a greater number of colloid droplets within thyroid follicles are all indirect but reliable indicators that TSH was being actively released by the pituitary gland (Eales and Brown, 1993).

These results also indicated that T_4 was being actively synthesized and secreted, and no disruption was detected at this level of the thyroid cascade. This was further confirmed by the similar T_4 concentrations observed in fish from both reservoirs, which emphasized the homeostatic capacity of the thyroid system at the central level (Eales et al., 1999). In hyperthyroidism, colloid stains less intensively; epithelial cell height increases and follicles are smaller (Eales, 1999). The latter is not consistent with our observations in fish from LR, as their follicles were significantly larger than those from FBR. The T_3 and T_4 levels of *O. mossambicus* from FBR, and T_4 levels in fish from LR were comparable to control fish used in experimental studies, but T_3 levels in LR fish were higher than published values (Peter, 2009; Peter and Peter, 2009). We did not directly measure deiodination in peripheral tissues, but there was no evidence of inhibition of extra-thyroidal conversion of T_4 to T_3 because elevated T_3 levels and the T_3/T_4 ratio indicated high rates of deiodination in fish from LR (Brar et al., 2010).

Additional work is underway, investigating the possible influence of nutritional status on the thyroid system, as the elevated lipids and large body size of fish from Loskop Dam could be a confounding factor in this regard.

3.3.6. Location of full version

Dabrowski, J. 2014. Thyroid and nutritional status of *Oreochromis mossambicus* from two sub-tropical African reservoirs. Chapter in PhD thesis, University of Pretoria, Pretoria

3.4. Potential disruption of the Thyroid and Adrenal endocrine axes in the Mozambique tilapia (*Oreochromis mossambicus*) population Loskop Dam: II. Molecular markers associated with the thyroid, adrenal glands and lipids storage systems

Researcher: C. Truter, PhD, Stellenbosch University

3.4.1. Aims

The aims of the current investigation was, firstly, to identify potential links between altered thyroid, corticosteroid and PPARγ signalling, pansteatitis and obesity in a wild fish population, by evaluating the expression of selected mRNA genes (i.e. *thyroid receptor α* [*trα*], *trβ*, *iodothyronine type 2 deiodinase enzyme* [*dio2*], *thyroid stimulating hormone β* [*tshβ*], *glucocorticoid receptor-1* [*gr1*], *gr2*, *mineralocorticoid receptor* [*mr*] and *peroxisome-proliferator receptor gamma* [*pparγ*] (nuclear receptor associated with lipid uptake by fat cells) in total brain tissue of wild-caught *Oreochromis mossambicus* fish. Secondly, to evaluate potential contaminant-induced (48 hour exposure) thyroid modulation and altered PPARγ signalling of

water collected from the Loskop reservoir by quantifying the response expression of *trα, trβ, dio2* and *pparγ* in juvenile *O. mossambicus* as biomarkers.

3.4.2. Materials and Methods

Adult fish collection

Live adult *O. mossambicus* were obtained from the Loskop reservoir (Fig. 57) during an annual angling event as well as from an alternate population in the North West Province using drag nets. Pansteatitis lesions were identified macroscopically in the mesenteric fat of each individual. The body mass and standard length (snout to fork) of each fish was recorded and applied to calculate Fulton's Condition Factor (CF = W/L3) (Ricker, 1975).

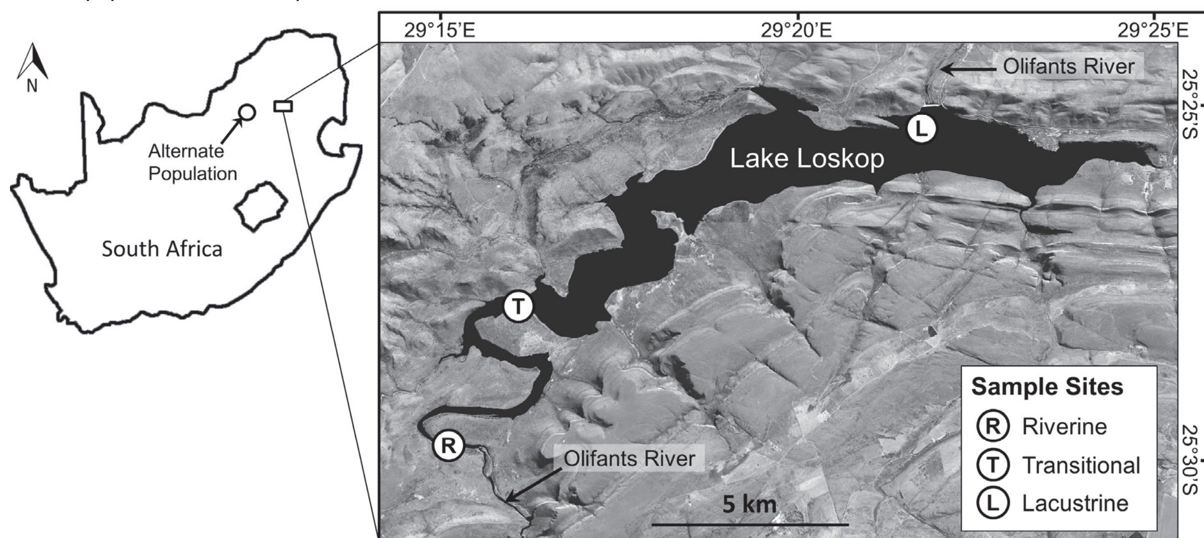

Figure 57: *Lake Loskop and the lake's position within South Africa, as well as the location of a further O. mossambicus population in the North West Province from which fish were obtained. Three collection points within Lake Loskop from where water applied in fish exposures and chemical analysis was collected are indicated. Map credits: Google Inc.*

Juvenile fish exposures

Water was collected from the lacustrine, transitional and riverine zones of Lake Loskop (1.5 m integrated samples) (Fig. 51). Each locality was represented by three exposure groups namely: (1) water as collected from the lake (containing algae and other microorganisms); (2) filtered water (1.2 µm glass fibre MGC filters [Munktell, DE]); (3) filtered water containing 50 µg/L triiodothyronine (Sigma, DE). 32 days post fertilization *O. mossambicus,* obtained from a single breeding pair (Rivendell Hatchery, Grahamstown, RSA) were exposed to 800 mℓ of liquid in 1 L glass containers for 48 hours, without food (28 ± 1°C, 14h:10h light:dark cycle). The juvenile fish were euthanized in 0.1% benzocaine at exposure termination.

Whole body homogenates of juvenile fish and brain specimens were prepared in TriReagent using an ultrasound sonicator (Omni-ruptor 400, Omni International Inc.,

US). Total RNA was isolated according to the TriReagent technical bulletin. The RNA was subsequently DNase I (Sigma, DE) treated, and complementary DNA (cDNA) was prepared from 2 µg of total RNA in 20 µL-, or 1 µg in 10 µL reaction volumes using Enhanced Avian HS RT-PCR kits (Sigma, DE) for brain tissue and Maxima H-minus cDNA synthesis kits (Thermo Scientific, USA) for juvenile whole body homogenates according to the manufacturers' instructions. Messenger RNA expression of *trα, trβ, dio2, tshβ, mr, gr1, gr2* and *pparγ* with *β-actin* as reference gene was evaluated using real-time RT-qPCR. The PCR programs and primer sequences of *trα, trβ, dio2, mr, gr1* and *gr2* are described in Truter et al., (2014). The primer sequences of *tshβ* were: sense, 5' AGGGACAGCAACATGAGGGA 3'; antisense, 5' GGACAGCCAGGCAGAATAGC 3' (Genbank: XM003453648.1), and *pparγ*: sense, 5' TGCGAGGGCTGTAAGGGTTT 3' and antisense 5' ACTTGTTGCGGGACTTCTTGTG' 3 (Genbank: AY590304.1), designed using PRIMER 3 (Rozen and Skaletsky, 2000). Outliers were identified per treatment group through the Grubbs' test (Burns et al., 2005) and removed.

Chemical analyses

The concentrations of a selection of inorganic elements were determined in the water samples collected from Lake Loskop (applied in the juvenile fish exposures), as well as water collected from the North West province *O. mossambicus* population. In particular, the concentrations of Ca, K, Mg, Na, P, Rb and Si were measured using a Thermo ICAP 6300 ICP-AES (Thermo Scientific, USA), and Al, As, Ba, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Mo, Ni, Pb, Sb, Se, Sn, Sr, Ti, V, Zn using an Agilent 7700x ICP-MS (Agilent Technologies, USA).

Phytoplankton identification

The phytoplankton was identified in each of the samples collected from Lake Loskop in order to evaluate the potential influence of the phytoplankton assemblage and abundance on *trα*, *trβ*, *dio2* and *pparγ* expression in the juvenile fish exposed to the "total" unfiltered water samples. Aliquots (50 mL) of the water collected from Lake Loskop were applied for phytoplankton identification using a light microscope at 1250x magnification (Van Vuuren et al., 2006). The samples were analysed using the strip-count method, after being sedimented in an algae chamber.

3.4.3. Statistical analysis

Normality and homogeneity of variance was assessed in the data using the respective Shapiro-Wilk's W test, normal probability plots and Levene's test. The Student's t-test and Man-Whitney U-test was applied for pairwise comparisons in gene specific mRNA abundance. The Variance Estimation and Precision (VEPAC) module was applied for Generalized Linear Model Analysis of Covariance (GLM ANCOVA) in combination with the LSD post hoc test (Statistica 11, Statsoft, USA). Probability values (p) of less than 0.05 were deemed significant.

3.4.4. Results

Adult fish

The effect of condition factor, gender, locality and the gender*locality interaction on the expression of *trα, trβ, dio2, tshβ, mr, gr1, gr2* and *pparγ* observed in the fish from Loskop reservoir and other impoundments were evaluated using GLM ANCOVA. No significant association between condition factor and the expression of any of the genes investigated were observed. Gender was however a significant source of variation in the expression of *trβ* and *gr2*, in both case being higher in males than females (Table 15a). In addition, *trα* varied significantly among localities (Table 15). In particular, *trα* expression was significantly higher in the Loskop reservoir fish than both the borehole ($p = 0.02$) and irrigation canal fish ($p = 0.01$; Fig. 58). Although there was a clear trend for higher *dio2* expression in both the Loskop and canal water fish relative those collected from borehole water supplied lakes (Figure 58), the GLM ANCOVA indicated no significant effect by CF, gender, locality and the gender-locality interaction (Table 15a). However, when the variation of *dio2* expression was re-analysed using a one way ANOVA, location was indicated as a significant source of variation and *dio2* expression was significantly lower in fish captured from lakes supplied by borehole water than both the canal water ($p = 0.02$) and Loskop reservoir fish ($p = 0.01$; Fig. 58).

Table 15: Generalized Linear Model Analysis of Covariance (GLM ANCOVA) of the expression of: (**A**) thyroid receptor α (trα), thyroid receptor β (trβ) and type 2 deiodinase (dio2); (**B**) peroxisome-proliferator receptor γ (pparγ) and thyroid stimulating hormone β (tshβ); (**C**) glucocorticoid receptor 1 (gr1), glucocorticoid receptor 2 (gr2) and mineralocorticoid receptor (mr) in the (total) brain tissue of adult O. mossambicus captured from Loskop reservoir (n = 10) and reference fish maintained in a small impoundments supplied by borehole water (n = 8) and irrigation canal water (n = 8) respectively. Fulton's condition factor was applied as covariate.

A)

Source of Variance	*df*	*trα*		*trβ*		df	*dio2*	
		F	*P*	*F*	*P*		*F*	*P*
Condition Factor	1,19	1.24	0.28	0.00	0.95	1,18	0.18	0.68
Locality	2,19	4.80	0.02	0.62	0.55	2,18	2.01	0.16
Gender	1,19	0.04	0.85	4.92	0.04	1,18	0.16	0.69
Locality*Gender	2,19	2.02	0.16	1.98	0.17	2,18	0.25	0.78

B)

Source of Variance	*df*	*pparγ*		df	*tshβ*	
		F	*P*		*F*	*P*
Condition Factor	1,19	0.59	0.45	1,17	0.18	0.68
Locality	2,19	0.17	0.84	2,17	0.98	0.40
Gender	1,19	2.46	0.13	1,17	0.86	0.37
Locality*Gender	2,19	0.12	0.89	2,17	0.27	0.77

C)

Source of Variance	*df*	*gr1*		*gr2*		*mr*	
		F	*P*	*F*	*P*	*F*	*P*
Condition Factor	1,19	1.46	0.24	0.07	0.80	0.58	0.45
Locality	2,19	0.54	0.59	0.11	0.90	0.92	0.42
Gender	1,19	0.56	0.46	7.94	0.01	0.49	0.49
Locality*Gender	2,19	1.61	0.23	0.58	0.57	1.27	0.30

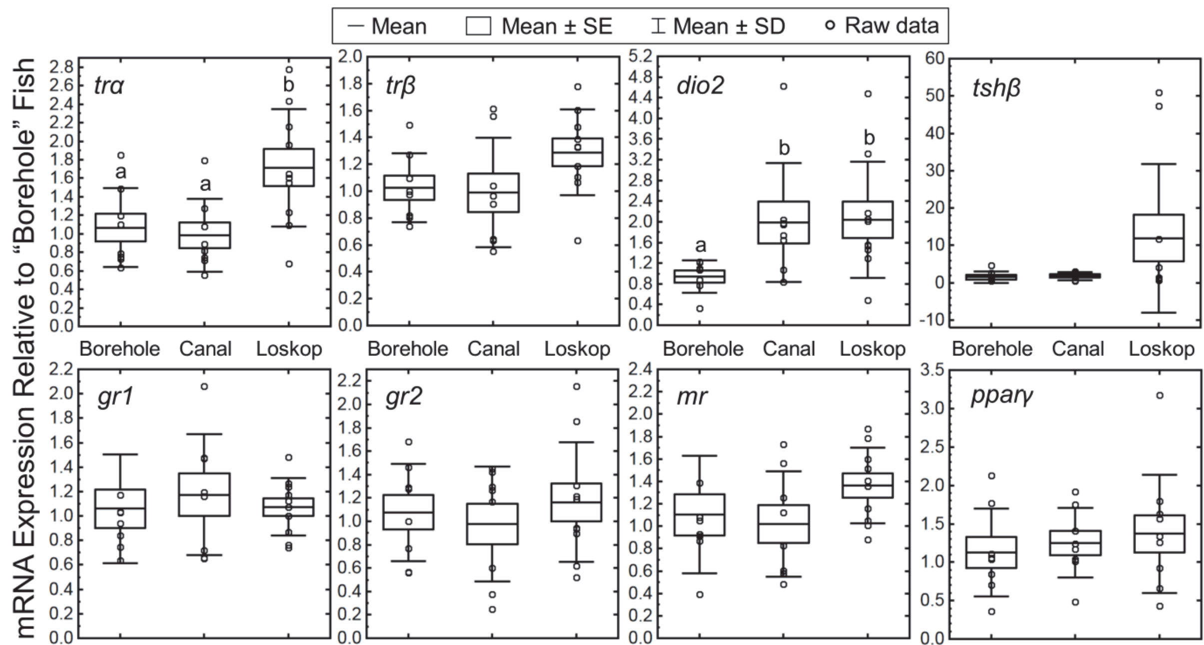

Figure 58: *The mRNA expression of thyroid receptor α (trα), thryroid receptor β (trβ), type 2 deiodinase (dio2), thyroid stimulating hormone β (tshβ), glucocorticoid receptor 1 (gr1), glucocorticoid receptor 2 (gr2), mineralocorticoid receptor (mr) and peroxisome-proliferator receptor γ (pparγ) in the (total) brain tissue of adult O. mossambicus captured from Loskop reservoir (n = 10) and reference fish maintained in a small impoundments supplied by borehole water (n = 8) and irrigation canal water (n = 8) respectively. β-actin was applied as normalizer. Dissimilar characters indicate statistically significant differences (Fisher's LSD post hoc test, α = 0.05).*

Condition factor (CF) varied significantly among the localities ($F_{2,20} = 13.15$; $p < 0.01$), and was significantly higher in the Loskop reservoir fish (0.038 ± 0.0047 g/cm^3, mean ± SD) than both the borehole (0.031 ± 0.0024 g/cm^3) ($p < 0.01$) and canal water (0.030 ± 0.0057 g/cm^3) ($p < 0.01$) reference fish. Gender was a further significant source of variation in CF ($F_{1,20} = 6.32$; $p = 0.02$), being significantly higher in females than males.

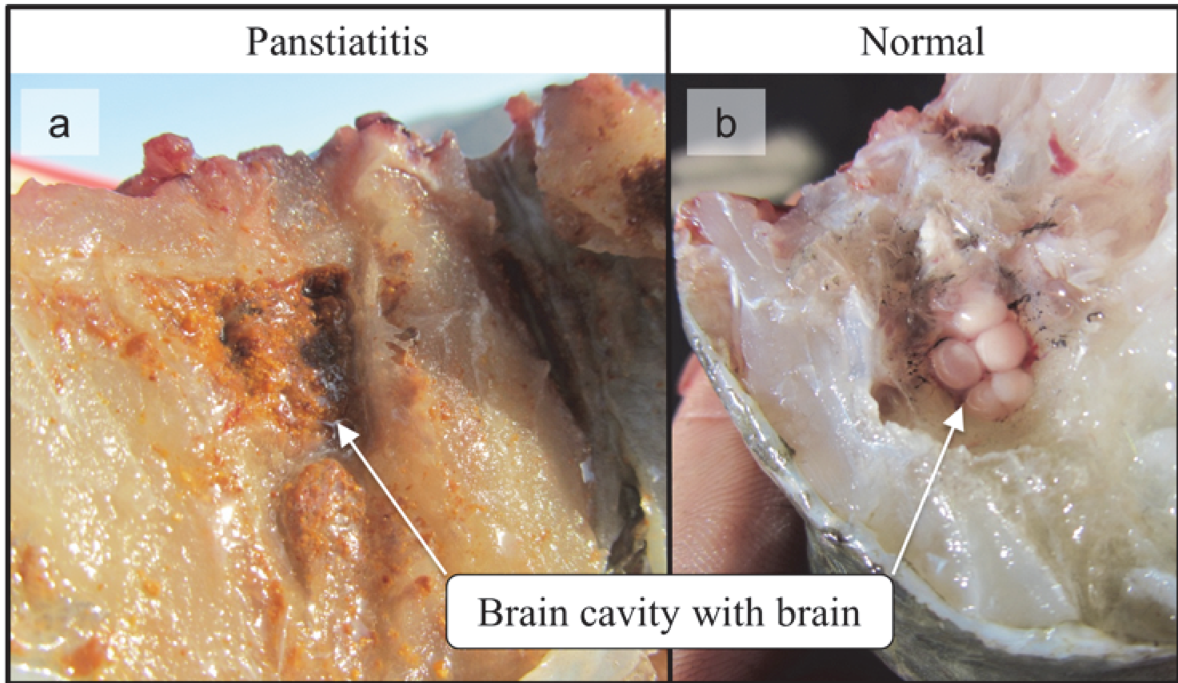

Figure 59: *Examples of the brain cavities of pansteatitis suffering O. mossambicus captured from Loskop reservoir (**a**) and pansteatitis free fish from an alternative populations (**b**).*

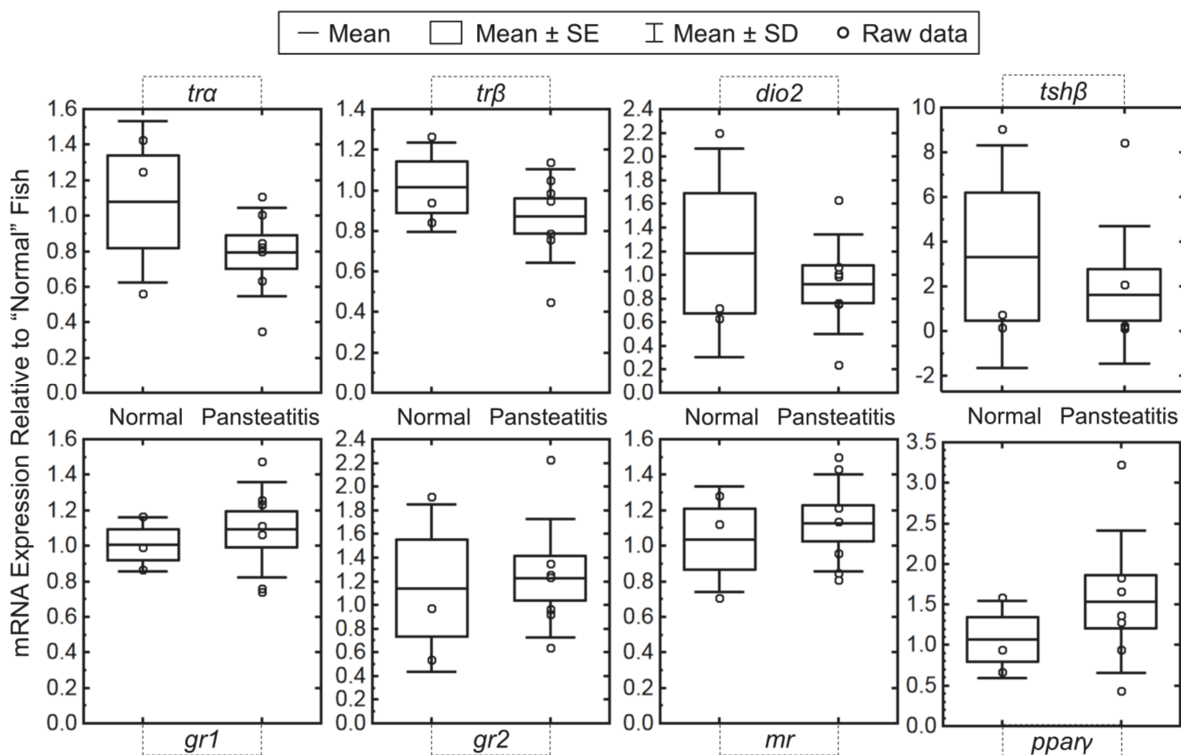

Figure 60: *The expression of thyroid receptor α (trα), thryroid receptor β (trβ), type 2 deiodinase (dio2), thyroid stimulating hormone β (tshβ), glucocorticoid receptor 1 (gr1), glucocorticoid receptor 2 (gr2), mineralocorticoid receptor (mr) and peroxisome-proliferator receptor γ (pparγ) in the (total) brain tissue of adult O. mossambicus captured from Loskop reservoir. The fish diagnosed with pansteatitis in the abdominal fat "pansteatitis" (n = 7) were compared to those with no visible signs of pansteatitis "normal" (n = 3). β-actin was applied as normalizer. No significant differences were observed among the "normal" and "pansteatitis" groups (Student's t-test, α = 0.05).*

The association between the prevalence of pansteatitis and the expression of *trα, trβ, dio2, gr1, gr2* and *mr* was evaluated using GLM ANCOVA using pansteatitis and gender as fixed effects and condition factor as covariate. The presence of pansteatitis was not associated with significant variation in the expression profile of any of the genes investigated. Gender was however a significant source of variation in the expression of *gr2* (Table 16; Fig. 60). Moreover, condition factor (CF) had a significant effect on *trβ* expression (Table 16; Fig. 60). When the association between CF and *trβ* expression within the Lake Loskop population was however evaluated with linear regression analysis, no significant association between *trβ* expression and CF was observed ($R^2 = 0.21$, p = 0.18).

Table 16: *Generalized Linear Model Analysis of Covariance (GLM ANCOVA) of the expression of (**A**) thyroid receptor α (trα), thyroid receptor β (trβ), type 2 deiodinase (dio2), thyroid stimulating hormone β (tshβ) and (**B**) glucocorticoid receptor 1 (gr1), glucocorticoid receptor 2 (gr2), mineralocorticoid receptor (mr) and peroxisome-proliferator receptor γ (pparγ) in the (total) brain tissue of O. mossambicus macroscopically diagnosed with pansteatitis in the abdominal fat "pansteatitis" (n = 7) and fish with no visible signs of pansteatitis (n = 3). Fulton's condition factor was applied as covariate.*

A)

Source of Variance	*df*	*trα*		*trβ*		*dio2*		*tshβ*	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Condition Factor	1,5	0.38	0.57	7.09	0.04	1.06	0.35	1.06	0.35
Pansteatitis	1,5	1.00	0.36	3.89	0.11	1.72	0.25	1.72	0.25
Gender	1,5	1.07	0.35	0.90	0.39	0.14	0.72	0.14	0.72
Panst*Gender	1,5	0.08	0.79	2.67	0.16	1.24	0.32	1.24	0.32

B)

Source of Variance	*df*	*gr1*		*gr2*		*mr*		*pparγ*	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Condition Factor	1,5	0.22	0.66	6.04	0.06	0.84	0.40	1.06	0.35
Pansteatitis	1,5	1.83	0.23	12.43	0.02	3.51	0.12	1.72	0.25
Gender	1,5	0.09	0.78	0.05	0.83	0.39	0.56	0.14	0.72
Panst*Gender	1,5	0.00	0.99	1.43	0.29	1.06	0.35	1.24	0.32

Juvenile exposures

The expression of trα in the juvenile fish did not vary significantly among the control and environmental water treatments (Fig. 61A).

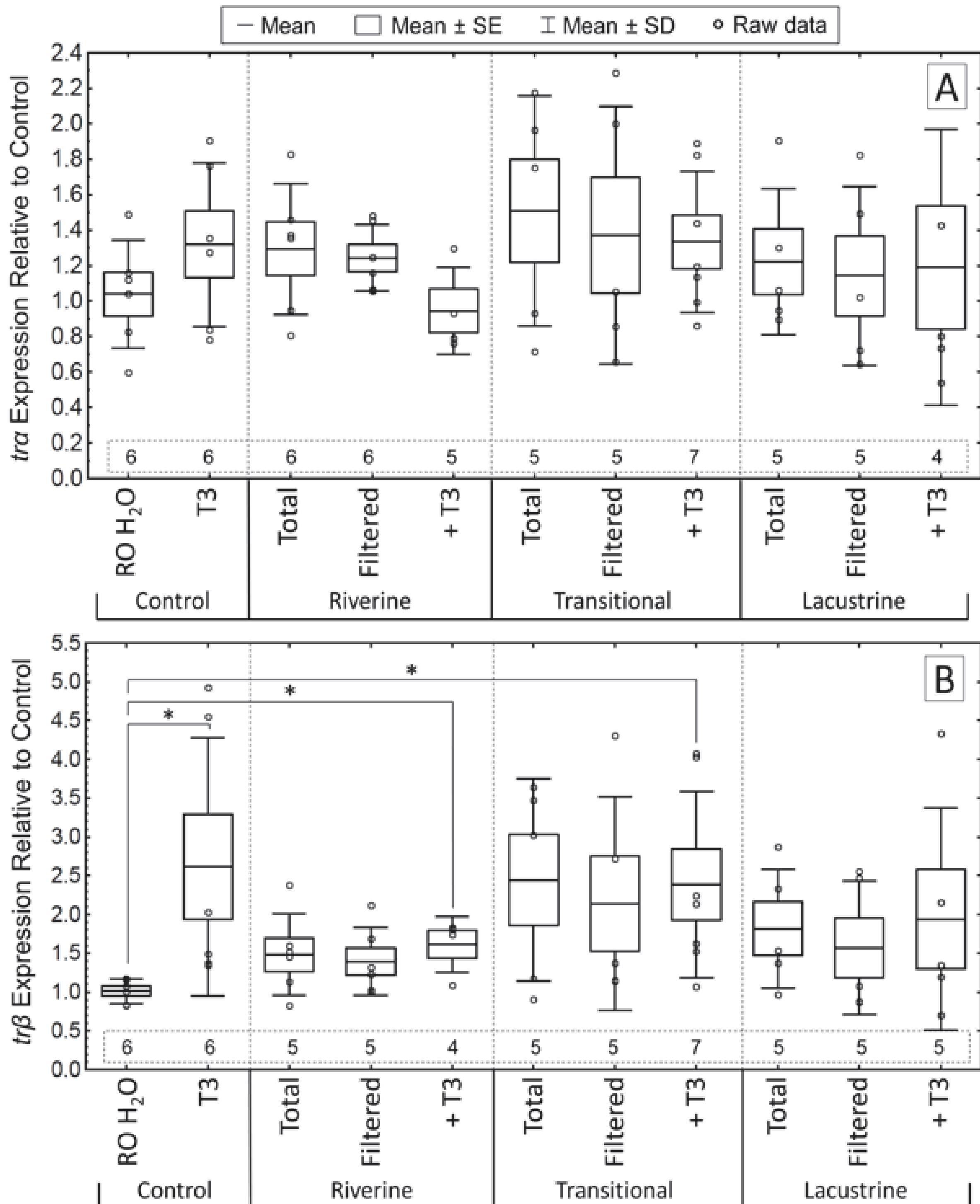

Figure 61: *Expression of (**A**) thyroid receptor α (trα) and (**B**) thyroid receptor β (trβ) in juvenile Oreochromis mossambicus (32 dpf) (whole body homogenates) exposed for 48h to water from the lacustrine, transitional and riverine sections of Lake Loskop. Significant differences are indicated by asterisks (Student's t-test or Man-Whitney U-test). The numbers within the horizontal boxes indicate the number of fish representing each treatment.*

The expression of trβ did no vary significantly among the riverine, transitional and lacustrine treatment groups and both the negative and positive (T_3) control treatments (Fig. 61B). The only significant differences in trβ expression was between the negative control and the 5 μg/L T_3 control ($p < 0.01$), riverine supplemented with

T_3 (p = 0.04) and transitional supplemented with T_3 (p = 0.05) (Fig. 61B). There was however a trend for *trβ* to be higher in the total and filtered transitional treatments than the control (Fig. 61B) (total: p = 0.08; filtered: p = 0.06).

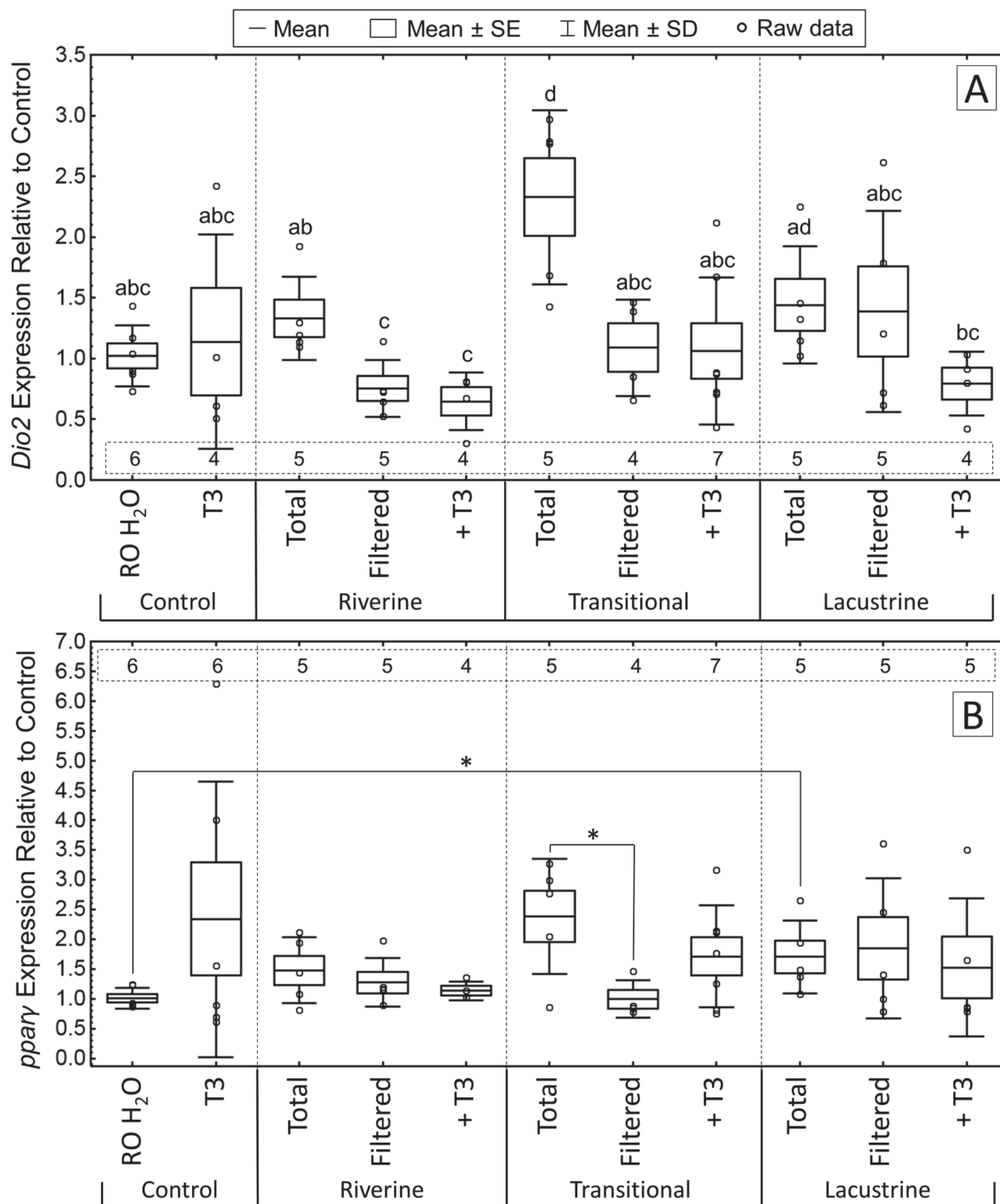

Figure 62: *Expression of (**A**) type 2 deiodinase (dio2) and (**B**) peroxisome- proliferator receptor γ (pparγ) in juvenile Oreochromis mossambicus (32 dpf) (whole body homogenates) exposed for 48h to water from the lacustrine, transitional and riverine sections of the Loskop reservoir. Significant differences are indicated by asterisks (Student's t-test or Man-Whitney U-test). The numbers within the horizontal boxes indicate the number of fish representing each treatment.*

Type 2 deiodinase (*dio2*) expression varied significantly among the control, T_3 supplemented, riverine, transitional and lacustrine treatment groups ($F_{10,43}$ = 3.23,

$p < 0.01$; Fig. 62A). In particular, *dio2* was significantly higher in the transitional (microorganism containing) treatment than the control treatment ($p < 0.01$; Fig. 62A). Moreover, *dio2* expression was significantly higher in the transitional unfiltered treatment than both the filtered and T_3 supplemented (filtered) transitional treatments ($p = 0.01$ and $p < 0.01$ respectively). Similarly, *dio2* expression was significantly higher in the transitional unfiltered treatment than the lacustrine filtered ($p = 0.03$) and T_3 supplemented ($p < 0.001$), riverine filtered ($p < 0.001$) and T_3 supplemented ($p < 0.001$), and the 5 µg/L T_3 control treatments ($p < 0.01$) (Fig. 62A).

Similar to the trend observed in the transitional treatments, *dio2* expression was significantly higher in the riverine unfiltered treatment than both the filtered ($p = 0.04$) and T_3 supplemented ($p < 0.01$) groups. Furthermore, the dio2 expression was significantly higher in the unfiltered lacustrine treatment than the T_3 supplemented treatment ($p = 0.04$; Fig. 62a). Finally, *dio2* expression was significantly higher in the lacustrine unfiltered treatment than both the riverine filtered ($P = 0.02$) and riverine T_3 supplemented ($p < 0.01$) treatments (Fig. 62). Peroxisome-proliferator receptor γ (pparγ) expression differed significantly among the control- and lacustrine unfiltered treatment ($p < 0.01$), as well as among the transitional filtered and unfiltered treatments ($p = 0.03$; Fig. 62B).

Metal analysis

Moderate to low concentrations (relative to the South African Water Affairs Guideline; DWAF, 1996) of metals were detected in the water collected from the Loskop reservoir (Table 17). Iron was the only element that exceeded the South African Water Affairs Guideline for aquaculture (10 µg/L) (DWAF, 1996) in water collected from the riverine (19.48 µg/L) and transitional (12.96 µg/L) localities. There was a general trend for increased concentrations of metals at the riverine locality (Table 17).

Table 17: *The concentrations of selected metals and other elements in water collected from three locations within Lake Loskop during December 2012. The water samples containing these elements were applied in juvenile fish exposures.*

Element	Locality (µg/L)		
	Lacustrine	Transitional	Riverine
Al	3.51	6.67	11.17
As	0.54	0.55	0.33
B	28.60	31.71	28.11
Ba	51.77	58.55	29.12
Ca	25040	21460	20010
Cd	0.03	0.007	0.001
Co	0.11	0.34	0.57
Cr	0.10	0.12	0.31
Cu	0.81	1.03	1.16
Fe	5.38	12.96	19.48
Hg	<L.O.D.	<L.O.D.	<L.O.D.
K	4610	3707	3317
Li	11.82	13.55	11.14
Mg	17577.5	15177.5	15177.5
Mn	0.86	2.79	4.47
Mo	0.31	0.15	0.15
Na	24110	22290	21160
Ni	2.09	3.05	4.59
P	<L.O.D.	8.50	14.10
Pb	0.01	0.01	0.004
Rb	0.3	1.73	0.03
Sb	<L.O.D.	<L.O.D.	<L.O.D.
Se	0.06	0.10	0.02
Si	300	1725.10	1946.10
Sn	0.07	0.06	0.06
Sr	145.99	119.97	113.68
Ti	0.05	0.05	0.01
V	0.52	1.68	1.53
Zn	4.11	4.56	8.63

Limit of detection (L.O.D.) = 0. 1 ng/L

Phytoplankton classification

The lacustrine zone had the highest phytoplankton diversity, and eight species could be identified, yet at low numbers (Table 18). The transitional zone phytoplankton community was dominated by the dinoflagelate *Ceratium hirundininella* (57 cells/mL). A low number of the cyanobacterium, *Microcystis aeruginosa* (9 cells/mL) was present in the sample collected from the transitional zone, whereas higher

concentration of this species was present in the riverine zone sample (14 cells/mL) (Table 18).

Table 18: *The phytoplankton species composition and relative abundance in water collected from three locations within Lake Loskop during December 2012. The water samples containing algae were applied in juvenile fish exposures.*

Algal group or genus and species	Locality (Cells/mL)		
	Lacustrine	Transitional	Riverine
Bacillariophyceae			
Melosira varians	-	-	23
Fragililaria crotonensis	21	13	-
Aulacoseira granulata	-	3	6
Diatoma vulgaris	-	-	11
Pinnularia viridus	-	-	4
Chlorophyceae			
Coelastrum reticulatum	2	-	-
Pandorina morum	8	-	-
Straurastrum anatium	3	-	-
Cyanophyceae			
Microcystis aeruginosa	3	9	14
Dinophyceae			
Ceratium hirundininella	5	57	-
Peridium bipes	-	23	-
Euglenophyceae			
Trachelomonas volvocina	7	-	4
Cryptophyceae			
Cryptomonas ovata	9	-	-

3.4.5. Conclusions

The expression of trα and dio2 was significantly higher in the Loskop reservoir *O. mossambicus* than fish obtained from a population in the North West Province suggesting a degree of thyroid disruption. The exact cause of the altered gene expression however remains unclear, although it may likely be associated with obesity (pansteatitis) through leptin signaling (Araujo and Carvalho, 2011; Pearce, 2012). The lack of significant association between condition factor (CF) and the expression of the genes investigated provide evidence that the obese condition prevalent in the Loskop reservoir fish may not be a direct response of thyroid, corticosteroid disruption or altered PPARγ signalling. The expression of dio2 was significantly higher in juvenile *O. mossambicus* exposed to water collected from both the riverine and transitional zones of the Loskop reservoir, relative to those exposed to 1.2 µm filtered samples obtained from the same localities. Phytoplankton

assemblage data in light of previous studies linking feeding with dio2 suggests that dio2 expression was likely decreased in the filtered treatments due to fasting (Van der Geyten et al., 1998). There was no significant association between the expression of any of the genes investigated and pansteatitis, suggesting no direct influence of pansteatitis on the thyroid and interrenal systems or PPARγ signalling. Transcript signatures will however likely differ in adipose tissue itself and further investigation is required.

3.4.6. Location of full version

Truter, J.C. 2014. Testing for endocrine disruptors in South African waters: a comparative study to evaluate *in vitro* and *in vivo* testing approaches in laboratory and field situations. Chapter in PhD thesis, Stellenbosch University.

4. PROJECT CONCLUSIONS

1) The aims of this project was largely reached, however linking endocrine disruption caused by pesticides to ecological risk assessment models proved to be a bridge too far. Pesticides as a diverse group of chemicals include multiple possibilities regarding mechanism of actions related to the endocrine system and to address potential disruption of this group of locally used chemicals in a single project was a difficult task.

2) In this project research showed that pesticides, including insecticides, fungicides and herbicides, have the potential to interact with reproductive as well as thyroid endocrine systems and a single chemical may interact with more than one system at a time. Modes of action associated with insecticides and herbicides varied and individual chemicals or formulations needs comprehensive testing to predict the potential for endocrine disruption. Fungicides, on the other hand, were mostly associated with anti-androgenic activity, either by inhibiting binding of male hormone to its receptor (AR) or by inhibiting the activity of the enzyme 5α reductase. The role of fungicides as disruptors in the female reproductive system, especially, as aromatase inhibitors needs more study.

3) Laboratory studies proved valuable to perform first tier screening for potential endocrine disruption activity of individual pesticides and mixtures of pesticides. However, *in vivo* exposure experiments are needed to validate modes of action and understand real health implications.

4) Herbicides as a group of aquatic contaminants need more study. This study showed that different life stages show different sensitivities and that development could be compromised. Herbicides as thyroid system disruptors also need more study certainly may contribute as a potential contributing factor in the decline of aquatic species, including amphibians. Certain subgroups, notably, glyphosates, need more study to assess the potential interaction with the endocrine system.

5) This study confirmed the anti-androgenic nature of fungicides, with Mancozeb standing out as a potent anti-androgenic agent in *in vitro* testing. However, the *in vivo* testing did not always confirm this potent activity and more detailed research are needed to understand the potential detoxification pathways.

6) Mixture exposure experiments, on the basis of the dose addition hypothesis, may be helpful to make accurate predictions if the mode of action on the particular endocrine system is the same. But, when conducting *in vitro* and *in vivo* exposures this prediction did not always hold. Although the additive hypothesis should hold for similar mode of action pesticides, the interaction of

active ingredients and inert formulation chemicals needs more study to understand non-additivity of a response action. More attention should also be given to experimental design of setting-up mixture experiments.

7) Field collection of sentinel species proved to be valuable, but remains a complex system to understand and confirm direct links to endocrine disrupting activity being the origin of compromised physiological systems. In this regard, this study was exploratory, since we collected natural occurring amphibians and fish from impoundments and evaluated endpoints associated with the reproductive and thyroid endocrine systems for evidence that these systems may be disrupted. Clearly, in aquatic systems where EDC activity is suspected deep analyses and a focussed systems study are needed.

8) Male frogs (*Xenopus laevis*) collected from impoundments located within an intense farming area (Stellenbosch), known to use fungicides and insecticides in large quantities did not show clear disruption of the male reproductive or thyroid systems.

9) The upper Olifants catchment is known as a polluted system receiving a multitude of effluents, positive oestrogenicity was shown to occur (linked to WWTPs) and yet exposure of juvenile tilapia fish, *Oreochromis mossambicus* only showed limited evidence of potential influences linked to agricultural activity.

10) The Loskop reservoir in the Olifants River system is known for fish displaying obesity and pansteatitis. Although the thyroid systems showed signs of disruption when compared to tilapia fish collected from other systems, including Flag Boshielo reservoir (downstream), no conclusive evidence could be found that variation in thyroid histomorphology, gene expression profiles or plasma hormone profiles were the result of thyroid disruptors in the water. Rather, it seems that a nutritional link to differential thyroid activity may explain the variation. However, this was a first study of its kind in South Africa focussing on environmental factors interacting with the thyroid system and clearly more research is needed to understand the role of environmental chemicals in the modulation of the thyroid system.

11) This project confirms the functionality of quantitative PCR to study differential gene expression modulation in brief exposure set-ups using juvenile fish or tadpoles to screen for potential endocrine disruptive activity. Both model species, the African clawed frog, *Xenopus laevis* and Mozambique tilapia, *Oreochromis mossambicus*, proved to be valuable model species in laboratory testing but also for assessment of field situations (natural water sources).

5. RECOMMENDATIONS

- More South African used pesticides should be studied for endocrine disrupting activity to understand the dose-response relationships, before hoping to embark on predicting health or environmental risk.
- Clearly, pesticides as potential endocrine disruptors needs more research specifically focussed on understanding the details of interaction with the diversity of facets presented by the endocrine system. Although herbicides as a subgrouping stand out as being understudied, both fungicides and insecticides need more attention in South Africa.
- Biomarkers representing a larger part of the endocrine response system should be studied and validated. In particular, molecular (gene expression) biomarkers should be used more widely since this sensitive response system could be used following brief exposure experiments.
- Modes of action associated with insecticides and herbicides varied and individual chemicals or formulations needs comprehensive testing to predict the mechanism of action. Fungicides, on the other hand, were mostly associated with anti-androgenic activity, either by inhibiting binding of male hormone to its receptor (AR) or by inhibiting the activity of the enzyme 5α-reductase. The role of fungicides as disruptors in the female reproductive system, especially, as aromatase enzyme inhibitors needs more study.
- More research is needed regarding the behaviour of pesticides in mixture, to understand the interaction of these chemicals when attempting to predict endocrine disruption when dealing with mixtures.
- Pesticide-linked epidemiology should be investigated by including or linking to endocrine disruption research.
- The capacity to determine environmental concentrations of pesticides is urgently needed in South Africa. Dedicated, but affordable analytical facilitates are needed to validate working concentrations as well as environmental concentrations of pesticides.
- Research regarding potentially affected wildlife populations needs more studies. Although population effects needs more attention, understanding the complex interactions when exposed to complex mixtures in the field at individual levels must continue.
- Pesticides as developmental and reproductive modulators must not be ignored when considering the development of ecological or health risk models to manage the use of these chemicals in South Africa.

- The use of differential relevant gene expression in conjunction (part of a battery of tests) should be encouraged in future EDC related studies in South Africa.
- Linkage and cross-cutting research projects including pesticides should be facilitated on an ongoing basis.

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APPENDIX A

CAPACITY BUILDING AND RESEARCH OUTPUTS

Student dissertations

Archer, E. 2014. Androgen-controlled secondary sexual characters in the male African clawed frog, *Xenopus laevis*, as potential biomarkers for endocrine disruptor contaminants (with special reference to fungicides) in aquatic systems. MSc thesis, Stellenbosch University, Stellenbosch.

Babalola, O.O. 2014. Ecotoxicological and potential endocrine effects of selected aquatic herbicides on life stages of the African clawed frog, *Xenopus laevis*. PhD Dissertation, Stellenbosch University, Stellenbosch.

Dabrowski, J. 2014. Thyroid and nutritional status of *Oreochromis mossambicus* from two sub-tropical African reservoirs. Chapter in PhD Dissertation, University of Pretoria, Pretoria.

Jansen van Rensburg, E. 2014. Endocrine disruption effects of agrichemical mixtures in the aquatic environment: Bio-responses and linkage to population-level impacts. Chapter in PhD Dissertation, Stellenbosch University, Stellenbosch University.

Truter, J.C. 2014. Testing for endocrine disruptors in South African waters: A comparative study to evaluate *in vitro* and *in vivo* testing approaches in laboratory and field situations. Chapter in PhD Dissertation, Stellenbosch University, Stellenbosch University.

Manuscripts in preparation, submitted or published

Archer, E, Van Wyk, J.H. 2014. *The potential anti-androgenic effect of agricultural pesticides used in the Western Cape: In vitro investigation for mixture effects*. Accepted by the journal “Water SA”.

Archer, E., Van Wyk, J.H. 2014. *Effects of the androgen receptor-binding inhibitor, Flutamide, and dual 5α-reductase inhibitor, Dutasteride, on epidermal breeding glands and reproductive endpoints in male Xenopus laevis frogs.* In preparation.

Archer, E., Van Wyk, J.H. 2014. *Histological and immunological comparison of androgen stimulated and inhibited breeding glands in the African clawed frog, Xenopus laevis: A biomarker for screening endocrine disrupting compounds.* In preparation.

Archer, E., Van Wyk, J.H. 2014. *Premature development of androgen-dependent breeding glands in the African clawed frog, Xenopus laevis.* In preparation.

Archer, E., Van Wyk, J.H. 2014**.** *Exposure of two regularly-used fungicides in Western Cape agriculture to the African clawed frog, Xenopus laevis: In vivo biomarker investigation of individual and mixture effects*. In preparation.

Archer, E, Van Wyk, J.H. 2014. *Environmental screening for gonadal endocrine disrupting contaminants (EDCs) of selected water impoundments among vineyards of the Western Cape: Using in vitro and in vivo biomarkers (African clawed frog, Xenopus laevis)*. In preparation.

Babalola, O.O, Van Wyk, J.H. 2014. *Comparative Toxicity and Identification of Most Sensitivity Developmental Stage of Xenopus laevis Daudin, 1802 (Anura; Pipidae) to various herbicide formulations.* In preparation.

Babalola, O.O., Van Wyk, J.H. 2014. *Lethality, Teratogenicity and Growth Inhibition of three Glyphosate-based herbicide formulations using the Frog Embryo Teratogenesis Assay- Xenopus (FETAX)*. In preparation.

Babalola, O.O., Van Wyk, J.H. 2014. *Lethal and Teratogenic Impacts of Imazapyr, Diquat dibromide and Glufosinate ammonium herbicide formulations using the Frog Embryo Teratogenesis Assay- Xenopus (FETAX)*. In preparation.

Babalola, O.O., Van Wyk, J.H. 2014. *Thyroid Perturbation and Impact of Imazapyr, Glufosinate ammonium and Diquat dibromide herbicides Formulation on Amphibian Metamorphosis using Xenopus laevis*. In preparation.

Babalola, O.O., Van Wyk, J.H. 2014. *Thyroid Perturbation and Impact of Glyphosate Formulations on Metamorphosis using the Xenopus Metamorphosis Assay (XEMA).* In preparation.

Babalola, O.O., Van Wyk, J.H. 2014. *Environmental Relevant Concentrations of Diquat, Imazapyr and Glufosinate formulations induced reproductive abnormalities and altered Sex Ratios in Xenopus laevis*. In preparation.

Babalola, O.O., Van Wyk, J.H. 2014. *Glyphosate formulations induce Reproductive Malformations and Altered Sex Ratio in Xenopus laevis*. In preparation

Babalola, O.O., Van Wyk, J.H. 2014. *Exposure Impacts of Diquat dibromide and Imazapyr formulations on Amphibian Using Xenopus laevis male as sentinel*. In preparation.

Dabrowski, J., Oberholster, P.J. and Steyl, Osthoff, J.G., Hugo, A. 2014. *The relationship between thyroid function and nutritional status of Oreochromis mossambicus from two sub-tropical African reservoirs*. In preparation for publication in “Journal of Fish Biology”.

Truter, J. C., Van Wyk, J. H., Oberholster, P. J., and Botha, A-M. *Toxicogenomic biomarkers for endocrine disruption: Land-cover dependent signatures in a major South African river*. In preparation for publication in "Water, Air and Soil Pollution".

Truter, J.C., Van Wyk, J.H., Botha, A-M and Oberholster, P.J. *The expression of selected genes linked with metabolic homeostasis in obese pansteatitis-suffering Mozambique tilapia Oreochromis mossambicus.* In preparation.

Conference contributions

Jansen van Rensburg, E. *Interaction of xenobiotic chemicals in the rivers of the Western Cape.* ***16th Biennial International Symposium for Toxicity Assessment Cape Town*** *(21-26 February 2013).*

Archer, E. *The anti-androgenic effect of commonly used agricultural pesticides in South Africa: using an in vitro screen to test for mixture effects.* ***16th Biennial International Symposium for Toxicity Assessment Cape Town*** *(21-26 February 2013).*

Truter, C. *Aspects of endocrine disruption in the Upper Olifants River and Lake Loskop, Mpumalanga Province, South Africa).* ***16th Biennial International Symposium for Toxicity Assessment Cape Town*** *(21-26 February 2013).*

Van Wyk, J.H. *Interaction of xenobiotic chemicals in the rivers of the Western Cape*. *Keynote at the* ***16th Biennial International Symposium for Toxicity Assessment Cape Town*** *(21-26 February 2013).*

Archer, E. *Androgen receptor antagonistic effects of fungicides regularly used in Western Cape agriculture: testing mixture response.* **Poster presentation:** ***3rd Regional Conference of the Southern African Young Water Professionals: SA YWP, Stellenbosch*** *(16-18 July 2013).*

Truter, J.C., Van Wyk, J.H., Oberholster, P.J., Botha, A-M. *The application of toxicogenomic biomarkers in juvenile tilapia to investigate potential endocrine disruption in a major South African river.* ***Society of Environmental Toxicology and Chemistry North America 34th annual meeting, 17-21 November 2013, Nashville, Tennessee.***

Archer, E. Using male-specific breeding glands in the African clawed frog (Xenopus laevis) as biomarkers of anti-androgenic endocrine disruption studies.**42nd Annual conference of the Anatomical Society of Southern Africa. Cell and Developmental Biology Symposium**, 13 April 2014, University of Stellenbosch, South Africa.