

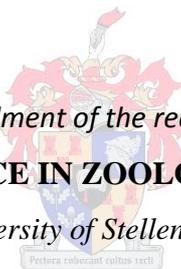
**ANDROGEN CONTROLLED SECONDARY SEXUAL  
CHARACTERS IN THE MALE AFRICAN CLAWED FROG,  
*XENOPUS LAEVIS*, AS POTENTIAL BIOMARKERS FOR  
ENDOCRINE DISRUPTOR CONTAMINANTS (WITH  
SPECIAL REFERENCE TO FUNGICIDES) IN AQUATIC  
SYSTEMS**

by  
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## ABSTRACT

Endocrine disrupting contaminants (EDCs) coming from households, industrial parks, wastewater (sewage) treatment and agricultural areas have been shown to pollute our freshwater systems. These contaminants may disrupt early development and reproductive systems in freshwater organisms (fish, frogs and crocodile species) as well as humans. Agricultural pesticides are shown as a large contributor to endocrine disruption activity in water catchment areas through spray drift, runoff, and/or groundwater leeching. Although South Africa is recognized as the largest consumer of agricultural pesticides in Africa, few studies have been undertaken to assess the prevalence and impact of endocrine disorders activities of pesticides in local freshwater systems. Recent studies have suggested that various agricultural pesticides, especially fungicides, might have adverse effects on the male endocrine system. There is therefore a need to test for a wider range of endocrine disrupting activities (mechanisms) in environmental waters other than conventional estrogenic (feminising) activities. Furthermore, there is a need to establish biomarkers in endemic species (bio-indicators) to show endocrine disruption in vertebrates (therefore also apply to humans). The specific objectives of the study were to: (1) describe and confirm the use of androgen-controlled breeding glands in male African clawed frogs (*Xenopus laevis*) as a biomarker for (anti)androgenic endocrine disruption activity (Chapter 2), (2) to investigate the premature development of breeding glands in *X. laevis* tadpoles (pre-metamorphic) and young froglets (post-metamorphic) (Chapter 2), (3) to investigate the disruption of male reproductive traits in adult *X. laevis* frogs by exposure to substances disrupting two different anti-androgenic endocrine disruption pathways (Chapter 3), (4) screen for (anti)androgenic activity of individual and binary mixtures of pesticides, which are regularly used in agricultural areas in the Western Cape Province of South Africa (Chapter 4), and (5) to test for (anti)androgenic and estrogenic endocrine disrupting activities by making use of *in vitro* assays as well as adult male *X. laevis* frogs collected from selected ponds surrounded by vineyards and fruit orchards in the Stellenbosch Winelands. The present study confirmed that male breeding glands can serve as biomarkers for (anti)androgenic endocrine disruption and that male reproductive and secondary sexual characteristics can be disrupted through two different biochemical control pathways. The study also confirmed that the expression of androgen-regulated breeding glands can be stimulated in pre-metamorphic tadpoles and immature, post-metamorphic frogs, and can thus be used for (anti)androgenic testing. The

rapid testing and predictive value of an *in vitro* recombinant yeast screen for androgen receptor binding inhibition of selected individual or binary mixtures of pesticides was also confirmed. However, the current study showed that the predicted *in vitro* (anti)androgenic activity did not always correspond with *in vivo* (anti)androgenic biomarker outcomes. This It also confirmed that single-cell *in vitro* assays can be used as a first-level prediction for (anti)androgenic activities of individual or mixtures of agricultural pesticides. This study provides a better understanding for potential mixture interactions of commonly used agricultural pesticides, the hormonal control of secondary sexual characteristics in male frogs and the use of reproduction biomarkers to study long-term effects of endocrine disruptors in local water supplies.

## UITTREKSEL

Endokriene versteurings-kontaminante (EVKe) wat vanaf huishoudings, industriële parke, afvalwater(riool)-behandeling en landbougebiede kom, besoedel ons varswaterstelsels. Hierdie kontaminante mag versteuring van vroeë ontwikkeling- en voorplantingstelsels in varswater-organismes (vis-, padda- en krokodil-spesies) sowel as die mens inhou. Landbou-plaagdoders word uitgesonder as 'n bydraer van endokriene versteuring-aktiwiteit in wateropvangs-gebiede deur spuitnewel, afloop-water en/of grondwater-deurvloei. Hoewel Suid-Afrika erken word as die grootste verbruiker van landbou-plaagdoders in Afrika, word min studies onderneem om die voorkoms en impak van endokriene versteurings-aktiwiteit van plaagdoders in plaaslike varswaterstelsels te ondersoek. Onlangse studies het voorgestel dat verskeie landbou-plaagdoders, veral swamdoders, nadelige uitwerkings kan hê op die manlike endokriene stelsel. Daar bestaan dus 'n behoefte om te toets vir 'n wyer verskeidenheid van endokriene versteurings-aktiwiteit (meganismes) in omgewingswater anders as konvensionele estrogeniese (vervroulikings) aktiwiteit. Verder bestaan daar 'n behoefte om biomerkers in endemiese spesies te gebruik as bio-indikatore van endokriene versteuring in werweldiere (daarom ook van toepassing op die mens). Die spesifieke doelwitte van die studie het ingesluit om: (1) die gebruik van androgeen-beheerde parings-velkliere ("breeding glands") in manlike platannas (*Xenopus laevis*) as 'n biomerker vir (anti)androgeniese endokriene versteuring-aktiwiteit te beskryf en bevestig (Hoofstuk 2); (2) ondersoek in te stel na die voortydige ontwikkeling van parings-kliere in *X. laevis* paddavisse (pre-metamorfose) asook jong paddas (post-metamorfose) as biomerkers van androgeniese (vermanlikheids) aktiwiteit (Hoofstuk 2); (3) ondersoek in te stel na die versteuring van manlike geslags-eienskappe in volwasse *X. laevis* paddas deur middel van blootstelling aan stowwe wat twee verskillende androgeniese endokrien reaksie-weë verteenwoordig (Hoofstuk 3); (4) toets vir (anti)androgeniese aktiwiteit van individuele en binêre mengsels van landbou-plaagdoders wat gereeld in die Westelike Provinsie van Suid Afrika gebruik word (Hoofstuk 4) en (5) te toets vir (anti)androgeniese en estrogeniese endokriene versteurings aktiwiteit deur gebruik te maak van *in vitro* toetse asook volwasse manlike *X. laevis* paddas wat uit geselekteerde damme (omring deur wingerde en vrugte boorde in die Stellenbosch wynland distrik) versamel was. Die huidige studie het bevestig dat die manlike parings-velkliere as biomerkers vir (anti)androgeniese versteuring kan dien en dat manlike voortplanting en sekondêre geslagskenmerke deur twee verskillende biochemiese beheer-weë

ontwrig kan word. Die studie het verder bevestig dat die uitdrukking van androgeen-gereguleerde parings-velkliere voortydig gestimuleer kan word in pre-metamorfose paddavissies asook onvolwasse, post-metamorfose paddas. Die vinnige toetsing en voorspellingswaarde van 'n rekombinante *in vitro* gis toets om binding-inhibisie van die androgeen reseptor deur geselekteerde individuele of binêre mengsels van plaagdoders aan te toon is ook bevestig. Alhoewel, die huidige studie het getoon dat die voorspelde *in vitro* (anti)androgeniese aktiwiteit nie altyd ooreenstem met *in vivo* (anti)androgeniese biomerker uitkomstes nie. Hierdie studie bevestig dat enkel-sel *in vitro* toetse aangewend kan word as eerste vlak- en voorspelling-toetse vir (anti)androgeniese aktiwiteite van enkel of mengsels van landbou-plaagdoders. Sodoende is 'n beter begrip verkry vir potensiële mengsel-interaksies van algemeen-gebruikte landbou plaagdoders, die hormonale beheer van sekondêre geslagskenmerke in manlike paddas asook die aanwending van voortplantingsbiomerkers om langtermyn effekte van endokriene versteurders in plaaslike waterbronne te ondersoek.

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**LIST OF ABBREVIATIONS**

11-KT	11-ketotestosterone
17 $\alpha$ -MT	17 $\alpha$ -methyltestosterone
5 $\alpha$ R	5-alpha reductase
ANOVA	One-way analysis of variance
anti-YAS	Yeast Anti-androgen Screen
AR	Androgen Receptor
ARE	Androgen Response Element
AVMA	American Veterinary and Medical Association
B-gal	Beta-galactosidase
BG	Breeding gland
BPH	Benign Prostatic Hyperplasia
BSA	Bovine Serum Albumin
CA	Concentration Addition
CAF	Central Analytical Facility
CDI	Condition Index
CHO	Chinese Hamster Ovary
CPRG	Chlorophenol Red Galactopyranoside
CYP19	Human Aromatase P450 gene
D	Duct
DBD	DNA-Binding Domain
DES	Di-ethyl-stilbestrol
DHT	Dihydrotestosterone
DHT-EQ	Dihydrotestosterone Equivalent
DDE	Dichloro-diphenyl-dichloro-ethylene
DDT	Dichloro-diphenyl-trichloroethane
DMSO	Dimethyl Sulphoxide
DNA	Dioxyribonucleic Acid
DWAF	Department of Water Affairs and Forestry
E <sub>2</sub>	Estradiol
E <sub>2</sub> -EQ	Estradiol Equivalent

EE <sub>2</sub>	Ethinyl-estradiol
EC <sub>50</sub>	Concentration Giving 50% Effect
EDC	Endocrine Disrupting Contaminant
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EDTA	Endocrine Disrupters Testing and Assessment
EIA	Environmental Impact Assessment
ELISA	Enzyme-Linked Immunosorbent Assay
EMU	Electron Microscopy Unit
EP	Epidermis
ER	Estrogen Receptor
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
GSI	Gonadosomatic Index
GWRC	Global Water Research Coalition
H295R	Female adrenocortical carcinoma cell line
hCG	Human Chorionic Gonadotropin
HPA	Hypothalamic Pituitary Adrenal
HPG	Hypothalamic Pituitary Gonadal
HPT	Hypothalamic Pituitary Thyroid
HRT	Hormone Replacement Therapy
HSD	Honest Significant Difference
HIS	Hepatosomatic Index
KH	Keratinized Hooks
IA	Independent Action
IC <sub>50</sub>	Concentration Giving 50% Inhibition
IR	Intermediate Region
LBD	Ligand Binding Domain
LH	Luteinizing Hormone
Lu	Lumen
MG	Mucous Gland
Mi	Mitochondria
MOA	Mode of Action

mRNA	Messenger Ribonucleic Acid
NF	Nieuwkoop and Faber Stage
NOEL	No Observed Effect Level
NP	Nuptial Pad
NTD	Nuclear-Terminal Domain
NTMP	National Toxicity Monitoring Program
OECD	Organisation for Economic Co-operation and Development
OMG	Ordinary Mucous Gland
PAS	Periodic Acid Schiff
PBS	Phosphate Buffered Saline
RER	Rough Endoplasmatic Reticulum
POP	Persistent Organic Pollutant
RO	Reverse Osmosis
SAWIS	South African Wine Information System
SC	Secretary Epithelial Cells
SDSG	Sexually Dimorphic Skin Glands
SMG	Specialised Mucous Gland
SPC	Spermatocyte
SPG	Spermatogonia
SPT	Spermatid
SPZ	Spermatozoa
Srd5a1	Type 1 5 $\alpha$ -reductase isomer
Srd5a2	Type 2 5 $\alpha$ -reductase isomer
Srd5a3	Type 3 5 $\alpha$ -reductase isomer
SSC	Secondary Sexual Characteristic
STW	Sewage Treatment Works
T	Testosterone
TDS	Testicular Dysgenesis Syndrome
UNEP	United Nations Environment Programme
USEPA	United States Environmental Protection Agency
VIN	Vinclozolin
VIN-EQ	Vinclozolin Equivalent

VTG	Vitellogenin
WHO	World Health Organisation
WRC	Water Research Commission
WWTW	Wastewater Treatment Works
XEMA	Xenopus Metamorphosis Assay
YAS	Yeast Androgen Screen
YES	Yeast Estrogen Screen

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**Figure 3.5:** 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 single asterisk (\*) indicates a significant difference of T concentrations of treatment groups compared to control frogs (ANOVA:  $F_{3,16} = 100.7$ ; Tukey's HSD *post hoc* test:  $p < 0.05$ ). The double asterisk (\*\*) indicates a significant difference of DHT concentrations of treatment groups compared to control frogs (ANOVA,  $F_{3,16} = 8.19$ ; 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.

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**Figure 5.2:** Sigmoidal standard curves of dihydrotestosterone (DHT), estradiol ( $E_2$ ) and vinclozolin in the yeast androgen (YAS), estrogen (YES) and anti-androgen screen (anti-YAS) respectively. Values (means  $\pm$  SE) are expressed relative to untreated (blank) cells in the assays. The series dilution of vinclozolin was spiked with 6.25 nM DHT ( $EC_{50}$ ) in each well to obtain a dose-response relationship of the competitive AR binding between DHT and vinclozolin in the assay.

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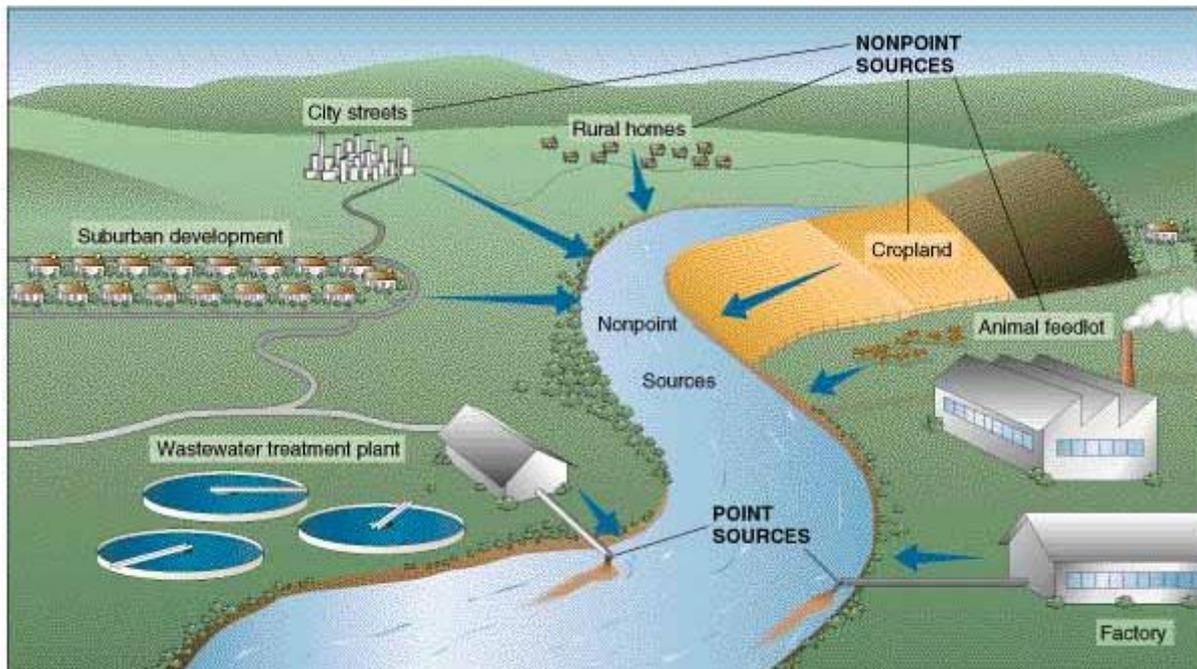
## CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

### *Endocrine disrupting chemicals (EDCs): Sources and effects on humans and wildlife*

Human populations are predicted to grow exponentially on a global scale. Along with the rise in population dynamics comes a need for increased crop production (increased food demand), sanitation, industrial development and housing. These activities have been shown to put severe pressure upon freshwater supply and quality by means of point and non-point source pollution (Falconer *et al.*, 2006; Bollmohr *et al.*, 2008; Bollmohr & Schulz, 2009; Pal *et al.*, 2010; Ansara-Ross *et al.*, 2012; Fig. 1.1). Reports on human health disorders such as hormone-related cancers, altered thyroid function, neuro-developmental disorders in children, adrenal disorders, metabolic disorders, infertility and immune disorders have also been associated with environmental pollutants which contaminate water resources (Dalvie *et al.*, 2003; Ansara-Ross *et al.*, 2012; WHO, 2012). In light of the various health effects of freshwater pollutants on non-target species, several contaminants have been shown to interact with endocrine systems of vertebrates, which are collectively referred to as endocrine disrupting chemicals (EDCs). Disruption of the endocrine system, which is especially associated with the reproductive system, has been the focus of many studies around the world since the publication of “Silent Spring” (Carson, 1962) and “Our Stolen Future” (Colborn *et al.*, 2000). Initial reports linked individual and population level effects associated with the reproductive endocrine system with contaminants entering water systems. For example, in male and female alligators of Lake Apopka in the USA, disrupted development and reproductive endocrinology was found, which was associated with extensive agricultural activities using the insecticide DDT and other persistent organic pollutants (POP) (Guillette *et al.*, 1994; 1995). Since then, several man-made chemicals has been reported to act as EDCs by potentially modulating wildlife species and human endocrine system pathways.

An EDC is described by the United States Environmental Protection Agency (USEPA) as “an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance of homeostasis, reproduction, development and or behaviour” (EDSTAC, 1998). The World Health Organisation (WHO) and United Nations Environment Programme (UNEP) also define EDCs as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny,

or (sub) populations” (WHO, 2012). To date, research has shown that the sources of EDCs span the full range of pollution sources, including industrial effluents and byproducts (Parks *et al.*, 2001; Waring & Harris, 2005; Kortenkamp & Faust, 2010), pesticides and fertilizers in agriculture (Colborn *et al.*, 1993; McKinlay *et al.*, 2008), personal care products (Witorsch & Thomas, 2010) and contaminants from sewage and medical facilities (Jobling, 1998; Bögi *et al.*, 2003; Jobling *et al.*, 2009; Sowers *et al.*, 2009; Ole Kusk *et al.*, 2011).



**Figure 1.1:** Diagramme showing point- and non-point sources of water pollution. Contaminants entering water systems via non-point sources can come from spray drift, rainwater, run-off and groundwater leaching. One major pathway of non-point source pollution is from agricultural practices such as pesticides and fertilisers ending up in mixtures in the environment. Source: <http://apesnature.homestead.com/chapter17.html>

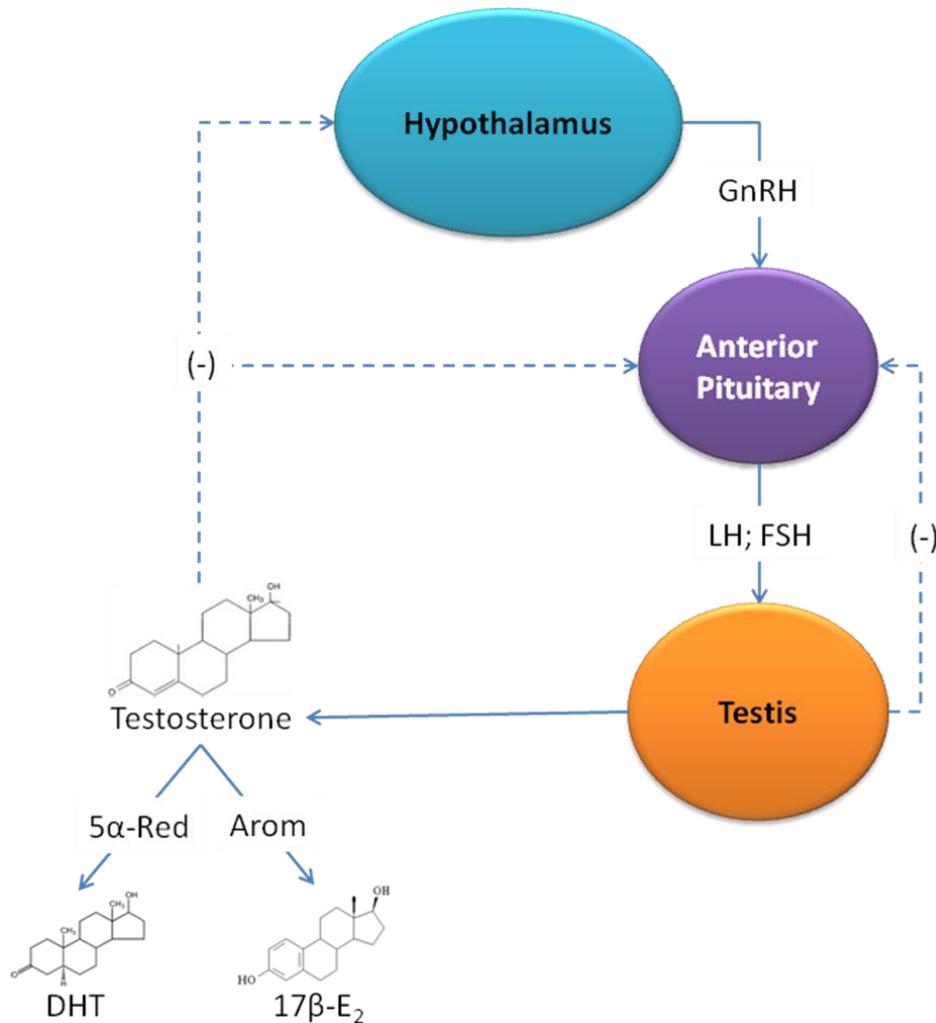
The main endocrine pathway that has received the most attention in EDC studies is the hypothalamic-pituitary gonadal (HPG) endocrine axis (Hayes, 2011). Contaminants that disrupt or facilitate modulation of systems associated with this axis can cause altered reproductive functioning as well as altered development in several vertebrate species (Kelce *et al.*, 1998; Urbatzka *et al.* 2007a; Kloas *et al.*, 2009; Langlois *et al.*, 2010b). Examples of disrupted gonadal endocrine systems (such as altered reproductive functioning as well as altered development) associated with contaminated water sources have been shown in several wildlife species, including fish (Jobling, 1998; Jobling *et al.*, 2009; Knacker *et al.*, 2010;

Morthorst *et al.*, 2010; Skolness *et al.*, 2011), amphibians (Carey & Bryant, 1995; Van Wyk *et al.*, 2003; Hecker *et al.*, 2005; Boone, 2008), rodents (Kelce *et al.*, 1998; De Angelis *et al.*, 2009; Rider *et al.*, 2009) and primates (Prahlada *et al.*, 1997).

#### *The HPG endocrine axis and HPG endocrine disruption*

The gonadal endocrine system has been shown to be moderately conserved between vertebrate species (McCoy *et al.*, 2008; Kloas *et al.*, 2009) and of particular importance for reproductive success (Behrends *et al.*, 2010; Urbatzka *et al.*, 2010). Production of the luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the pituitary gland (under control of GnRH) leads to the synthesis of androgens and estrogens in the gonads (Fig. 1.2; Kloas *et al.*, 2009; Urbatzka *et al.*, 2010). In higher vertebrates, including amphibians, steroid hormones (androgens and estrogens) such as testosterone (T), dihydrotestosterone (DHT) and estradiol (E<sub>2</sub>) are synthesised and play key roles in sexual development, differentiation, spermatogenesis, induction and maintenance of secondary sexual characters during development and normal hormonal maintenance (Kortenkamp & Faust, 2010).

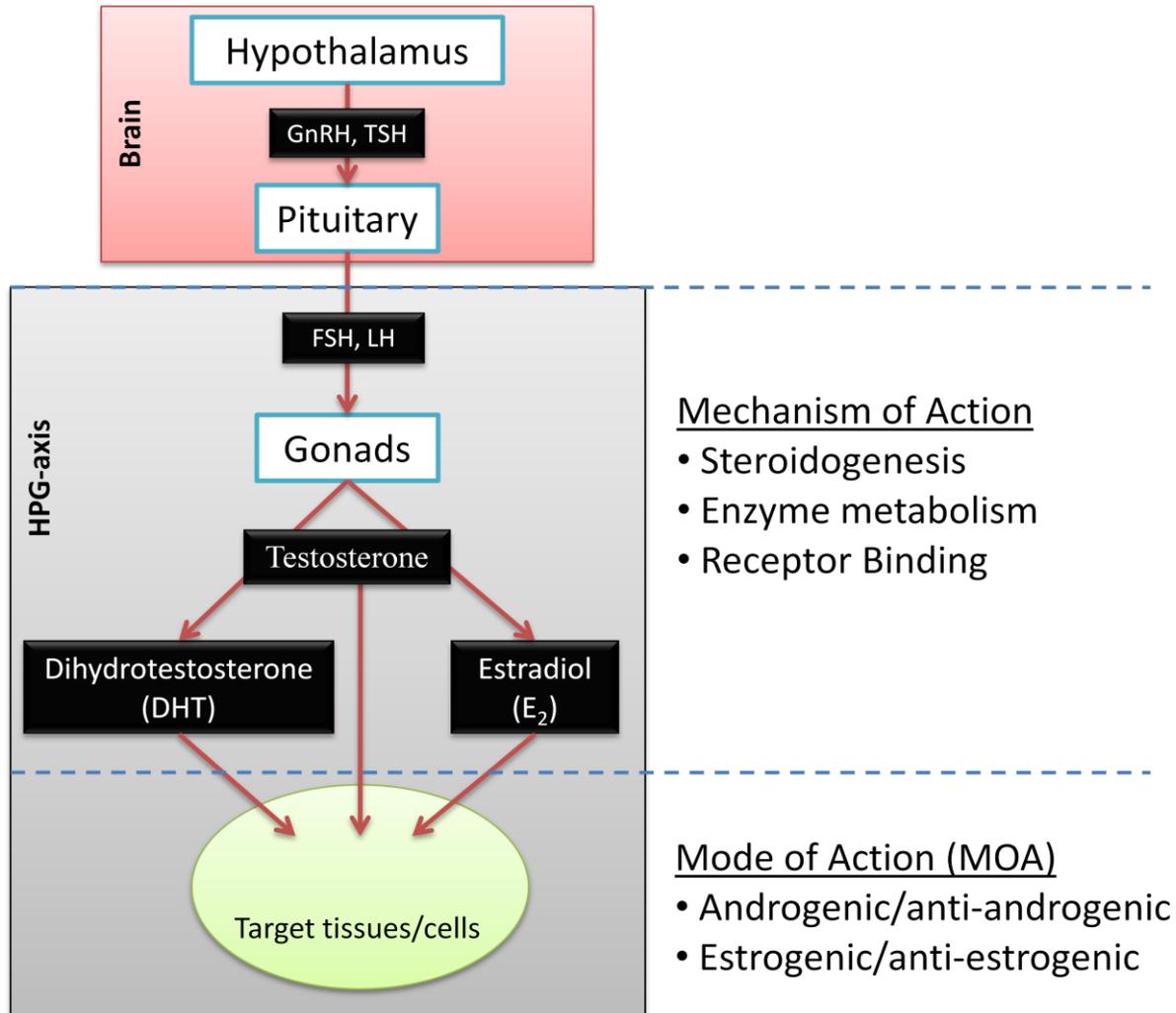
In all vertebrate species as well as humans, androgens are not only responsible for primary reproductive functions, but also responsible for regulating male secondary sexual characteristic (SSC) which are associated with courtship behaviour and sexual dimorphism (Thomas *et al.*, 1993; Hoffman & Kloas, 2010). Modulation of gonadal endocrine systems by exposure to EDCs will largely affect early life development of the gonads and urogenital tracts of vertebrates, which are hormonally regulated (Gray *et al.*, 1999; Wolf *et al.*, 1999; Falconer *et al.*, 2006). Disruption of reproductive systems can therefore alter reproductive success and eventually fitness, general health and survival of exposed individuals.



**Figure 1.2:** Hormonal pathways along the male hypothalamic-pituitary-gonadal (HPG) axis. GnRH, Gonadotropin-releasing hormone; LH, Luteinizing hormone; FSH, Follicle stimulating hormone; 5α-Red, 5α-Reductase; Arom, Aromatase; DHT, Dihydrotestosterone; 17β-E<sub>2</sub>, 17β-Estradiol. Solid lines represent stimulation and dashed lines represent inhibition (negative feedback).

### *Gonadal endocrine disruption (Androgens & Anti-androgens)*

Gonadal endocrine modulation has initially been studied at the mode of action (MOA) level, which are broadly characterized as either being estrogenic, anti-estrogenic, androgenic or anti-androgenic (Fig. 1.3; Behrends *et al.*, 2010; Hoffmann & Kloas, 2010). Within these specific MOAs, contaminants may affect endocrine systems by means of different mechanisms of actions. These include mechanisms such as either modulating the production, synthesis or metabolism of hormones, by modulating hormone transport to target organelles, or by modulating hormone action at target organelles by affecting ligand-steroid receptor binding (Fig. 1.3; Kelce *et al.*, 1998; Waring & Harris, 2005).



**Figure 1.3:** Pathways of endocrine disrupting contaminants in vertebrate systems through the hypothalamic-pituitary-gonadal (HPG) endocrine axis. Synthesis of LH and FSH in the brain acts on the gonads to synthesise the primary androgen, testosterone. Metabolising enzymes such as aromatase and 5 $\alpha$ -reductase (5 $\alpha$ R) leads to the metabolism of testosterone to the estrogen estradiol (E<sub>2</sub>) and to the more reactive androgen dihydrotestosterone (DHT). These steroid hormones can then be transported through the blood to target tissues or cells to exert their physiological function. Contaminants which modulate the HPG-axis can have a broad mode of endocrine disrupting action (MOA) which can be observed in endpoints from target tissues or cells. However, the broad MOA can be caused by modulating different mechanisms in the HPG-axis.

The majority of EDC studies conducted to date focused on EDCs which modulate the estrogenic response system in one way or the other (Sohoni & Sumpter, 1998; Urbatzka *et al.*, 2007b; Behrends *et al.*, 2010). The synthetic estrogens, ethynyl-estradiol (EE<sub>2</sub>) and diethylstilbestrol (DES), either historically or presently being used as contraception and/or

hormone replacement therapy (HRT) are among the best studied environmental pharmaceuticals showing endocrine disruption in humans as well as aquatic organisms (Colborn *et al.*, 1993; Behrends *et al.*, 2010). Furthermore, natural chemicals (phytoestrogens) and anthropogenic compounds with estrogenic modulating activities (such as agricultural pesticides, pharmaceuticals, personal care products, plastic byproducts, etc.) have now been identified in several freshwater pollution sources (Koponen *et al.*, 2004; Swart & Pool, 2007; Aneck-Hahn *et al.*, 2009; Bornman *et al.*, 2009; Witorsch & Thomas, 2010; Orton *et al.*, 2011; Swart *et al.*, 2011; Ansara-Ross *et al.*, 2012; WHO, 2012).

In contrast to the intensive research focus on man-made chemicals that potentially may disrupt normal estrogenic function (or related systems), relatively few studies addressed the interaction and potential modulation caused by chemicals having androgenic or anti-androgenic endocrine disrupting activities (Urbatzka *et al.*, 2007a; 2007b; Hoffmann & Kloas, 2010). Research suggests that exposure to environmental chemicals (EDCs) during early life stages may be causative factors to some male human disorders such as testicular dysgenesis syndrome (TDS), cryptorchidism, hypospadias, decreased penile length, reduced sperm quality and testicular cancer. These disorders have been linked to EDCs coming from sources associated with chlordecone-producing companies (Waring & Harris, 2005), PCBs (Aneck-Hahn *et al.*, 2007) and insecticides such as p,p'-DDE and endosulfan (Kelce *et al.*, 1998; Dalvie *et al.*, 2003; Waring & Harris, 2005; Aneck-Hahn *et al.*, 2007). 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; Waring & Harris, 2005; Kortenkamp & 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, 1997; 1998). Apart from anti-androgenic properties of some contaminants, certain compounds might also act as androgenic endocrine disruptors. Although limited information is present for androgenic EDCs in the environment, the growth promoter trenbolone, used in livestock feedlots, has been shown to contaminate water systems and cause reproductive disorders in wildlife species (Ankley *et al.*, 2003; Sone *et al.*, 2005; Cripe *et al.*, 2010; Olmstead *et al.*, 2012). Furthermore, androgenic or anti-androgenic contaminants might also accumulate at wastewater treatment works (WWTWs) in the same manner as found for estrogenic pollutants (Santos *et al.*, 2008; Sebire *et al.*, 2011; Metcalfe *et al.*, 2013). However

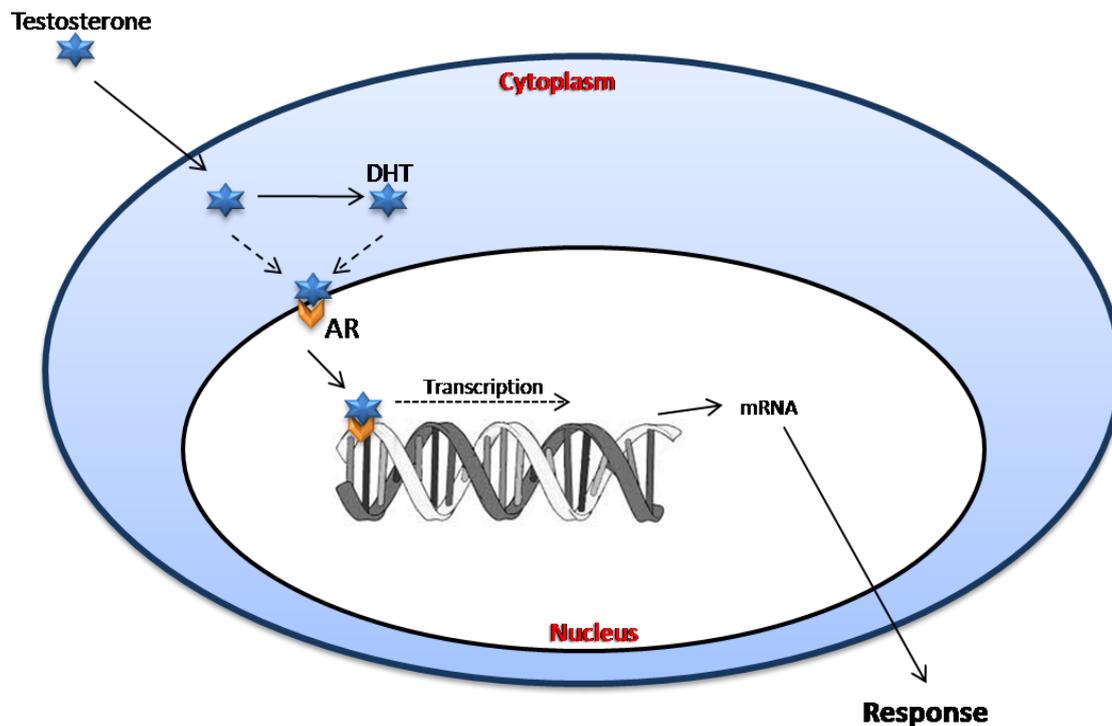
contaminants which modulate androgen endocrine pathways are less screened compared to the overwhelming information available of estrogenic pollutants in the environment. It is therefore evident that several types of contaminants from various sources can modulate even more types of reproductive endocrine systems of wildlife and humans than previously thought.

EDCs can inhibit the normal function of the HPG axis by interfering with signaling pathways such as the activation or blocking of androgen or estrogen hormone receptors (Kelce *et al.*, 1998; Urbatzka *et al.*, 2007b; Chen *et al.*, 2009; Orton *et al.*, 2011) or by the interference with enzymes (such as CYP19 and/or 5 $\alpha$ -reductase) necessary for required steroid biosynthesis and metabolism (Langlois *et al.*, 2010a; Hecker *et al.*, 2011; Ankley *et al.*, 2012; Hass *et al.*, 2012). Androgenic EDCs are contaminants that mimic the action of natural androgens, while an anti-androgen is a substance that inhibit or prevent the action of a natural androgen (Chen *et al.*, 2009). Apart from androgenic EDCs, it has been estimated that 8% of all anthropogenic chemicals may have anti-androgenic properties such as phthalates (Christiansen *et al.*, 2009; Kortenkamp & Faust, 2010), plasticizers (Kortenkamp & Faust, 2010), pesticides and heavy metals (McKinlay *et al.*, 2008), and pharmaceuticals (Christiansen *et al.*, 2009). Furthermore, research has revealed that several compounds that have been regarded as estrogenic EDCs are also anti-androgenic EDCs (Sohoni & Sumpter, 1998) and that anti-androgenic EDC contamination in freshwater systems may be more abundant than estrogenic EDC contamination (Jobling *et al.*, 2009). Regardless of the broad MOA of certain contaminants (estrogenic, anti-estrogenic, androgenic, anti-androgenic), it is important to have knowledge on the specific mechanism of disruption. For example, some anti-androgenic compounds will activate or block androgen receptor binding or related pathways (Kelce *et al.*, 1998; Urbatzka *et al.*, 2007a; Chen *et al.*, 2009). Alternatively, other anti-androgenic compounds will have direct effects on the production of steroid hormones as well as altering enzyme activation for steroid metabolism (Urbatzka *et al.*, 2009).

### *The androgen receptor (AR)*

The role of the androgen receptor is to bind circulating androgens in a specific tissue and activate the transcription of target genes to perform or express a certain androgenic trait (Thornton & Kelley, 1998). The AR consists of an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region and a ligand binding domain (LBD) (Thornton &

Kelley, 1998; Chen *et al.*, 2009). Binding of an androgen to the receptor will initiate nuclear translocation, DNA binding and co-activator recruitment, which will activate the receptor (Figs. 1.4 & 1.5c; Chen *et al.*, 2009). This ligand-receptor interaction at target organelles has key functions in male vertebrate development, reproduction and mating behaviour (Thornton & Kelley, 1998).

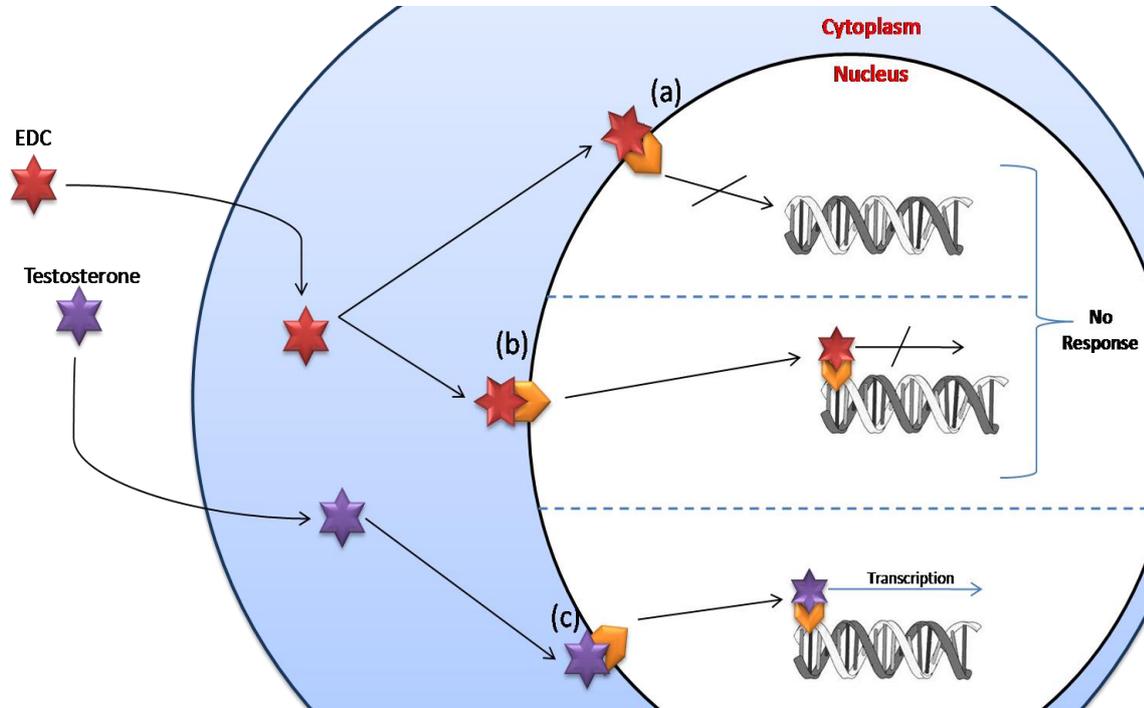


**Figure 1.4:** Diagramme showing the function of normal androgen receptor (AR) binding by natural androgens. An androgen (such as testosterone) is transported through the blood and enters the cytoplasm of the target cell, where it can be metabolised to dihydrotestosterone (DHT) by the enzyme 5α-reductase (5αR). The androgens bind to androgen receptors (AR) and initiate transcription of target genes in the nucleus. Messenger RNA (mRNA) is transcribed and initiates the coding for the desired androgenic response.

#### *AR antagonism*

Pharmaceuticals targeting AR binding have been developed to specifically combat diseases linked with an over-expression of androgen-related functions. Binding of an anti-androgenic EDC to the AR can have two mechanisms by which it exerts its antagonistic effect. An AR antagonist may either bind competitively along with an androgenic compound, initiating nuclear translocation and DNA binding, but does not recruit co-activators for transcription of

target genes (Fig. 1.5; Chen *et al.*, 2009). Alternatively, a chemical may bind to the AR and prevent stabilization of the N-terminal domain and completely seize nuclear translocation and DNA binding, also leading to inhibited co-activator recruitment for transcription of target genes (Fig. 1.5; Chen *et al.*, 2009).



**Figure 1.5:** Diagram to show normal androgen receptor (AR) binding by a natural androgen testosterone (c) and binding/interaction of an AR antagonistic EDC with the AR in a target organelle (a & b). An AR antagonistic EDC may have the same binding affinity as a natural androgen and bind to the AR competitively, but will change the conformation of the AR so that nuclear translocation may not continue (a). Alternatively, an AR antagonistic EDC may bind competitively to the AR and initiate nuclear translocation and DNA binding, but do not continue with transcription (b). Both these AR antagonistic mechanisms of action will indicate an AR antagonistic response and evidently an anti-androgenic response in a vertebrate.

Flutamide is the best known pharmaceutical AR antagonist used in treatment of prostate cancer patients. It prevents androgens from binding to the AR in target cells, initiating nuclear translocation and DNA binding without continuation of transcription of target genes (Imperato-McGinley *et al.*, 1992; Chen *et al.*, 2009; Hoffmann & Kloas, 2010). In vertebrate species, flutamide has been shown to disrupt the development of several secondary sexual characteristics (SSCs). In adult male rats, flutamide exposure resulted in a decline of seminal vesicles, prostate weight as well as a reduction in AR numbers expressed in epididymal

nuclei compared to unexposed males (Imperato-McGinley *et al.*, 1992; Kelce *et al.*, 1997). In juvenile Asian catfish, flutamide exposure led to a decrease in expression of testis-related transcription factors and steroidogenic enzymes (Rajakumar *et al.*, 2012). In anurans, calling activity and breeding gland development was inhibited by exposing adult male frogs to flutamide (Van Wyk *et al.*, 2003; Behrends *et al.*, 2010). The usage of flutamide as a model AR antagonistic EDC has led to the investigation of environmental pollutants which might mimic the mechanism of endocrine disrupting effect of flutamide and therefore pose environmental health effects of individuals contaminated to these pollutants.

A study by Guillette *et al.* (1994) has shown demasculinizing effects in male alligators from Lake Apopka, which were linked to contamination of the lake with the organochloride insecticide DDT. These authors suggested that pesticides, such as DDT, caused reduced plasma testosterone (T) levels in hatchling males and elevated plasma estradiol (E<sub>2</sub>) levels and ovarian synthesis in hatchling females (Guillette *et al.*, 1994; 1995). In sham-operated rats, the metabolite of DDT (p,p'-DDE) has also been shown to reduce seminal vesicle and prostate weight in sham-operated rats in the same manner as flutamide, suggesting an anti-androgenic mechanism of action by modulating AR binding. These initial studies investigating the link between pesticide pollution and endocrine disruption in wildlife have set the benchmark for other studies investigating environmentally relevant pesticide pollutants, which might cause the same demasculinizing effects as flutamide and the insecticide DDT and its metabolites.

The DDT metabolite, p,p'-DDE, and the dicarboximide fungicide, vinclozolin, are two compounds that received much attention in terms of their anti-androgenic activity (Kelce *et al.*, 1994; 1997; 1998; Van Wyk *et al.*, 2003; Aneck-Hahn *et al.*, 2007; Urbatzka *et al.*, 2007a; McKinlay *et al.*, 2008; Hofmann & Kloas, 2010; Bouwman *et al.*, 2012). Vinclozolin has been shown to block AR binding in rat epididymal cultures as well as to reduce seminal vesicle and prostate weight in sham-operated rats in the same manner as flutamide (Kelce *et al.*, 1994; 1997). In male *X. laevis* frogs, vinclozolin exposure has been shown to suppress male-specific SSC development such as breeding gland development (Van Wyk *et al.*, 2003) and vocalization (Hoffmann & Kloas, 2010) in the same manner as with flutamide exposure. Due to the illustriousness of vinclozolin as an AR antagonistic anti-androgen, this chemical is regularly used in experimental studies to serve as an environmental control substance to

illustrate AR antagonism (Hass *et al.*, 2007; Kollé *et al.*, 2011). Apart from the fungicide vinclozolin, several other fungicides including fenarimol, prochloraz, procymidone and other azole fungicides have been suggested to be potent AR antagonists as well (Kojima *et al.*, 2004; Kjørstad *et al.*, 2010; Orton *et al.*, 2011). Other pesticide types, such as the herbicides atrazine, diuron, linuron and pyridate as well as the insecticides aldrin, chlordane, chlorpyrifos, dieldrin, endosulfan, endrin, fenitrothion and methoxychlor also act as an AR antagonist (*see* McKinlay *et al.*, 2008). However, from all the pesticides tested for anti-androgenic endocrine disruption, fungicides stand out as a group of pesticides which has the highest potential to alter AR binding.

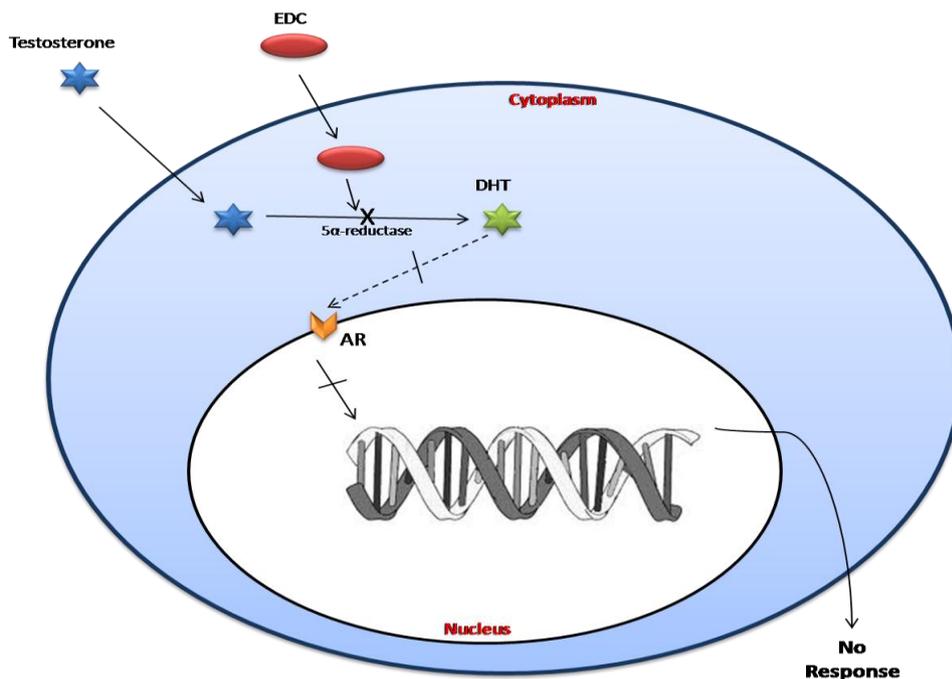
#### *5 $\alpha$ -reductase (steroidogenesis)*

As mentioned previously, the steroid hormone testosterone (T) is metabolized in target tissues to a more active androgen, 5 $\alpha$ -dihydrotestosterone (5 $\alpha$ -DHT) by the enzyme 5 $\alpha$ -reductase (5 $\alpha$ R) (Prahalada *et al.*, 1997; McConnel & Stoner, 2001; Chen *et al.*, 2009; Wu *et al.*, 2013). This conversion of T to DHT may be a crucial 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 (Prahalada *et al.*, 1997; McConnel & Stoner, 2001; Urbatzka *et al.*, 2009). Apart from the function of 5 $\alpha$ R to convert T to DHT, it is also known to metabolise progesterone and corticosterone to 5 $\alpha$ -dihydrocorticosterone (5 $\alpha$ -DHC; Tesone *et al.*, 2012), which has been shown to be essential in mating behaviour (Lephart, 1993). The gonads and the prostate of mammals have been shown to be the primary source of 5 $\alpha$ -reductase enzymes (Lephart, 1993). Two isoforms of the enzyme 5 $\alpha$ R exist, namely type 1 (srd5a1) and type 2 (srd5a2) and are differentially expressed in different target tissues (Xu *et al.*, 2006; Chen *et al.*, 2009). In humans, srd5a1 is mostly expressed in the liver and sebaceous skin glands whereas srd5a2 is mostly found in the prostate, liver and the root sheaths of scalp hair follicles (McConnel & Stoner, 2001). The importance of steroid metabolism by the enzyme 5 $\alpha$ R is therefore crucial to maintain certain physiological functions.

#### *5 $\alpha$ -reductase (5 $\alpha$ R) modulation*

Apart from the necessity of steroid hormone metabolism, an overproduction of certain steroid hormones can also lead to human health problems and modulation of developmental traits such as prostate cancer, benign prostatic hyperplasia and hair loss in men (Prahalada *et al.*,

1997; Amory *et al.*, 2008; Chen *et al.*, 2009). The established function of the 5 $\alpha$ R enzymes and differential isoform expression in different tissues has initiated the development of synthetic chemicals to inhibit the activity of the enzyme, and in so-doing, reduce male DHT-dependent reproductive disorders (Fig. 1.6). For example, finasteride is a pharmaceutical inhibitor of the *srd5a2a* isoform and has been established to be used for treatment for patients with benign prostate hyperplasia (Chen *et al.*, 2009) or patients suffering from hair loss (Prahalada *et al.*, 1997). Dutasteride, an inhibitor of both 5 $\alpha$ R isoforms, *srd5a1* and *srd5a2*, is also used for prostate-related and skin disorders and is therefore referred to as a dual 5 $\alpha$ R inhibitor (Gisleskog *et al.*, 1999; Xu *et al.*, 2006). A pharmacodynamic study in men revealed that treatment with dutasteride lowered DHT concentrations significantly after four weeks of exposure (Gisleskog *et al.*, 1999). Dutasteride inhibit tumor growth in a rat prostatic adenocarcinoma model and mouse large-T antigen prostate tumor model (Chen *et al.*, 2009). Furthermore, serum T levels increase when T-DHT conversion is inhibited, therefore reducing health risks such as acne, male pattern hair loss and benign prostate hyperplasia, which is mostly associated with DHT regulation (Amory *et al.*, 2008).



**Figure 1.6:** Diagram illustrating the function of an EDC affecting the enzyme 5 $\alpha$ -reductase (5 $\alpha$ R), which converts testosterone to the more biologically active 5 $\alpha$ -DHT. If an EDC mimics a pharmaceutical 5 $\alpha$ R inhibitor such as finasteride or dutasteride, hormone metabolism is affected and an androgenic trait (the response) dependent on DHT-AR binding cannot be expressed.

Beside the beneficial properties of these pharmaceuticals to treat for androgen-related disorders, these chemicals may also end up in the environment and cause harmful effects on non-target organisms, including humans. Although environmental pollution data is limited on contamination of water sources by pharmaceuticals such as finasteride, studies have shown that pharmaceuticals may contaminate water sources via point-source pollution from domestic sewage, livestock agriculture or hospital effluents (Gracia *et al.*, 2007; Maletz *et al.*, 2013). Laboratory studies using finasteride as an anti-androgenic treatment have shown that high oral doses (2 mg/kg/day) exposed *in utero* to pregnant female Rhesus monkeys, resulted in external genital abnormalities in male fetuses (Pralhada *et al.*, 1997). High dosages of finasteride in young adult rats resulted in inhibited hypothalamic and pituitary 5 $\alpha$ R activity (Lephart, 1993). Finasteride has also been shown to have antagonistic effects upon AR binding in the Chinese hamster ovary (CHO) cells (Kjærstad *et al.*, 2010). Finasteride exposure in male *X. laevis* tadpoles reduced spermatogenic germ cell development and increased Cyp-P450 and srd5a2 mRNA expression in the testis, while causing increased LH mRNA expression and decreased FSH mRNA expression in the brain (Urbatzka *et al.*, 2009).

Apart from the clinical effects recorded in wildlife species, the anti-androgenic mechanism of 5 $\alpha$ R inhibitors has also led to initial studies developing a male hormone contraceptive (Amory *et al.*, 2008). Potentially, the development of a male contraceptive and hormonal replacement treatment for male reproductive disorders may lead to the accumulation of androgenic and anti-androgenic pharmaceuticals in water systems via sewage sources in the same manner as reported for estrogenic pharmaceuticals (female contraceptives and hormonal replacement therapy drugs) present in water sources. Apart from these 5 $\alpha$ R inhibitors entering water systems, several other compounds (pharmaceuticals, pesticides, industrial waste products) may potentially have a similar mechanism of action. Since secondary sexual traits in vertebrate species are known to be regulated by 5 $\alpha$ R as well as the health effects recorded in non-target organisms, compounds modulating the androgen system should also be screened for their potential to mimic pharmaceuticals such as finasteride and dutasteride in the same manner as shown for EDCs modulating AR binding.

#### *Agricultural pesticides as androgen-modulating EDCs*

From all the sources of EDC accumulation in water systems, pesticide-related exposure data showed that contaminants from agricultural sources may contribute greatly towards EDC

accumulation in water systems and gonadal endocrine modulation in vertebrate organisms (Jobling *et al.*, 2009, Urbatzka *et al.*, 2007a; Grover *et al.*, 2011). Pesticides may end up in water systems by non-point source exposure, spray drift or runoff (Fig. 1.1; London *et al.*, 2000; Ansara-Ross *et al.*, 2012; Dabrowski *et al.*, 2014) where they can have varying persistence depending on several environmental and chemical factors. Agricultural pesticides possessing ED properties are especially of concern due to the high exposure potential of these chemicals to aquatic wildlife and farm workers living near agricultural fields (Guillette *et al.*, 1994; Dalvie *et al.*, 2003; 2006; WHO, 2012; Dabrowski *et al.*, 2014).

#### *EDCs in South African water systems*

South Africa is the highest produce-producing country on the African continent (Ansara-Ross *et al.*, 2012; Dabrowski *et al.*, 2014). With the high demand in food productivity due to increased population and high food quality demand, South Africa forms 60% of the pesticide market in Africa (Dabrowski *et al.*, 2011). Due to the high demand in better food quality, South African water systems face an increase in contamination due to the over-use or incorrect usage of pesticides (Dabrowski *et al.*, 2011; 2014; Ansara-Ross *et al.*, 2012). As with several developing countries in the world, there is limited information about specific EDC levels or evidence of endocrine disruption activity in South African water systems (Aneck-Hahn, 2005; Dabrowski *et al.*, 2011). However, research funded by the South African Water Research Commission (SA-WRC) has begun addressing these problems by screening for potential EDCs in various South African water systems. High levels of pesticides were detected in various river systems within South Africa, such as in the Crocodile River (Heath & Claassen, 1999), Olifants River (Heath & Claassen, 1999), Berg River (Heath & Claassen, 1999), Lourens River (Dabrowski *et al.*, 2011) and Hex River (London *et al.*, 2000). Several vertebrate species in these river systems have also been found to accumulate the pesticides found in the water and therefore could potentially show endocrine disrupting effects on population level (Heath & Claassen, 1999; Van Wyk *et al.*, 2005; Bornman *et al.*, 2009). 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 (DWAFF, 1996; DAFF, 2013) does not consider the environmental consequences or pollution of water from agricultural pesticides (London *et al.*, 2000; Mensah *et al.*, 2013).

Although, chemical determination of pesticides in water sources has been reported and pesticide use and contamination potential through run-off and spray drift have been modelled (Schulz, 2001; Dabrowski *et al.*, 2002; Thiere & Schulz, 2004), limited research still exist that report on endocrine disruption in wildlife and humans in South Africa. From a pesticide use perspective, the total usage volume may be lower than in developed countries but the range/diversity of pesticides in use may be very much similar.

#### *The mixture hypothesis*

It is increasingly 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 complex environmental mixtures as often found in rivers and manmade impoundments (Blake *et al.* 2010). For example, pulp and paper mill discharges containing androgenic and estrogenic compounds may combine with other pollutants from sources such as spray drift or leeching of agricultural pesticides and 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 & Schulz, 2009; Dabrowski *et al.*, 2011; 2014), potentially containing chemicals having similar modes of endocrine disrupting effects (therefore additive) or dissimilar modes of action (therefore independent or antagonistic).

In the Lourens River region of the Western Cape (South Africa) for example, different pesticides are applied to combat pests on apple and pear orchards as well as on vineyards (Bollmohr & Schulz, 2009; Dabrowski *et al.*, 2011). The different chemicals which accumulate in water systems at environmentally relevant aquatic mixtures may not have strict similar acting responses upon vertebrate endocrine systems (Backhaus & Faust, 2012) and may therefore interact differently than expected laboratory EDC mixture interactions. However, *in vitro* investigation of a directed endocrine disrupting mechanism of action (such as AR antagonism and steroidogenesis inhibition) will help understanding the interaction of contaminants, which might occur 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 a mixture. It has been stated in the literature that when investigating the combined effect of anti-androgens, having the same MOA, it is mostly assumed that they have a dose addition response on one another, also known as the additivity null hypothesis (Boone, 2008; Christiansen *et al.*, 2009; Ermler *et al.*, 2011). 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). Silva *et al.* (2011) also showed that estrogenic pesticide mixtures revealed to have weaker estrogenic mixture responses than the expected additive response. Furthermore, it was reported that a mixture of compounds at low concentrations can be as potent as an individual compound at a high concentration (Crago & Klaper, 2012). Such a dose-dependent mixture interaction at different concentration ranges has been found in red-eared slider turtles exposed to a mixture of chlordane and p,p'-DDE, where low doses of the mixtures had greater (synergistic) than the expected additive mixture response (Willingham, 2004).

Another toxicological mixture interaction which is overlooked in EDC mixture investigations is the occurrence of potentiation. Some antimicrobials, such as triclocarban, which is often found in the environment, may not have an endocrine disrupting action on their 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 modeling programs.

#### *EDC investigation and regulation*

Several global regulatory bodies such as the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) of the United States Environmental Protection Agency (USEPA) and a task force on Endocrine Disruptors Testing and Assessment (EDTA) by the Organisation for Economic Co-operation and Development (OECD) has mandated the

development and validation of testing (screening) methods for standardized assessments. The tiered approach suggested by US-EPA was accepted globally.

The tiered approach includes a battery of assays to screen or test for endocrine interactions and to identify and evaluate the potential of contaminants serving as EDCs (EDSTAC, 1998). First-tier assays were chosen to act as high-throughput screens identifying and prioritizing contaminants for second-tier testing, to understand the specific MOA (for example anti-androgenicity) and then to investigate the specific mechanism of endocrine disruption, for example modulation on receptor-binding level (EDSTAC, 1998). Second-tier screens aim to evaluate the results obtained from the first-tier screens in multi-generational or long-term *in vivo* studies to gain further support for a compound or mixture of contaminants to serve as environmental EDCs (EDSTAC, 1998). The desired outcomes from second-tier screening can therefore provide biological relevant information, which can be used in supporting of environmental monitoring programmes and environmental impact assessments (EIAs).

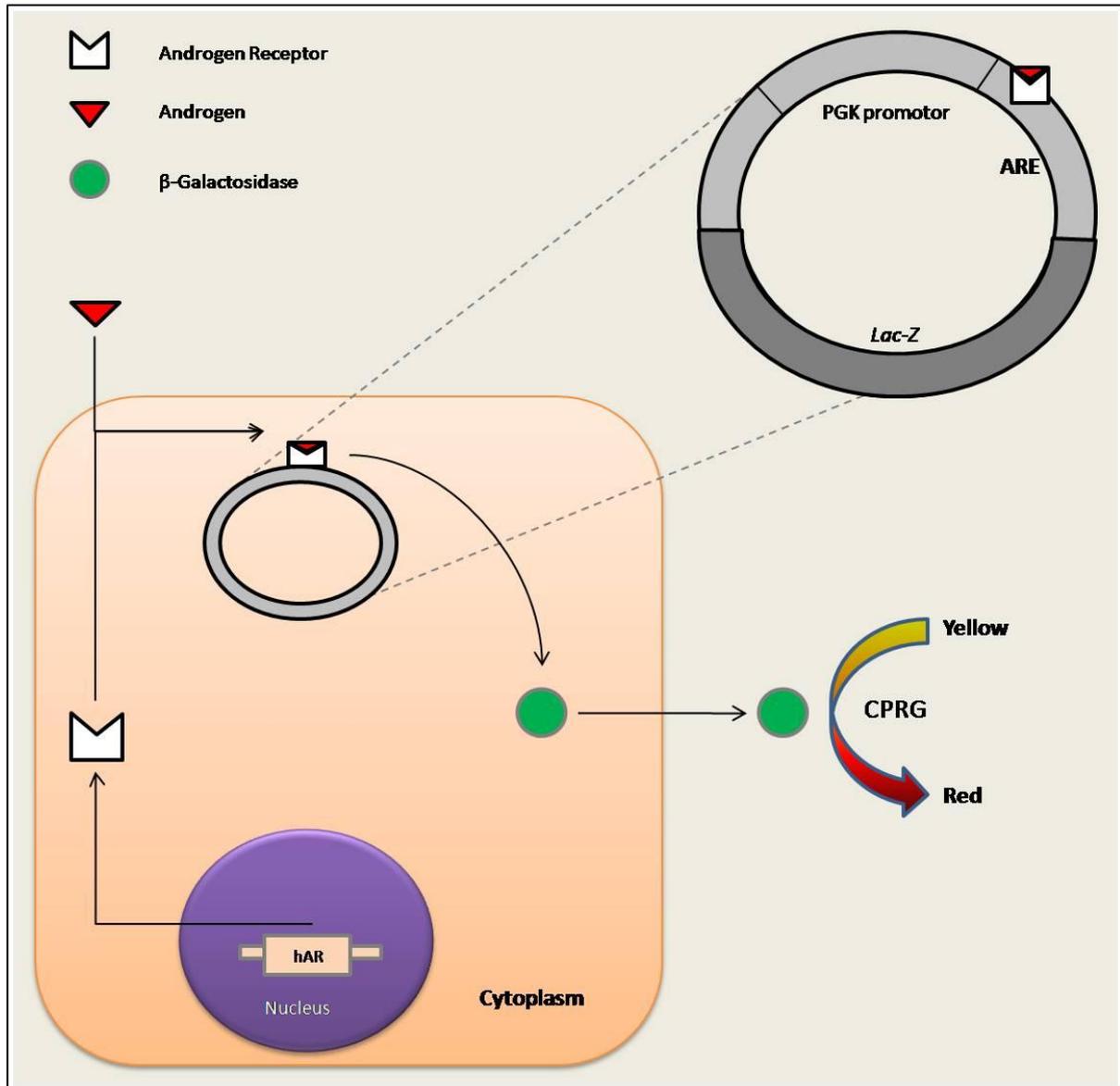
#### *In vitro screening of androgen-modulating EDCs*

Several *in vitro* assays have been described in the literature to measure and investigate EDC activity of selected pollutants or in environmental samples (EDSTAC, 1998; GWRC, 2003). It was shown that animal cell-based assays are more consistent and sensitive in the detection of endocrine disrupting endpoints compared to non-mammalian cell-based assays (GWRC, 2003; Leusch *et al.*, 2010). One of the reasons for the lower consistency of endocrine disruption detection in non-mammalian bioassays is ascribed to cell walls which may impede the transport of chemicals to the intercellular space, therefore causing false negatives (Leusch *et al.*, 2010). On the other hand, mammalian cell-based assays include metabolizing enzymes which might alter steroid hormone bioactivity (Coldham *et al.*, 1997; Akram *et al.*, 2011). However, due to the costliness and time dependency of these mammalian cell-based assays, a minced testis assay has also been shown to investigate steroidogenesis modulation (Ebrahim & Pool, 2010). Furthermore, a recombinant yeast androgen screen (YAS) allowed for a cost-effective and high throughput AR binding screen also to be used for investigating endocrine modulation upon AR binding (Sohoni & Sumpter, 1998; Kolle *et al.*, 2011). Although these assays have received some critique regarding their consistency relevant to other animal cell-based assays (EDSTAC, 1998; GWRC, 2003), these assays still function as valuable tools of detecting and investigating EDCs and their mechanism of endocrine disruption action.

*Assays investigating androgen receptor (AR) modulation**Recombinant yeast androgen/anti-androgen screens (YAS/ anti-YAS)*

Recombinant yeast screens have been evaluated and compared to mammalian cell-based assays (Coldham *et al.*, 1997; Leusch *et al.*, 2010; Akram *et al.*, 2011; Grover *et al.*, 2011; Orton *et al.*, 2011). However, recombinant yeast screens were not included in the first-tier battery of test by EDSTAC, due to the lack of standardization and the fact that yeast cells are not animal-based cells (EDSTAC, 1998). As previously mentioned, the cell wall of yeast cells may affect the uptake of chemicals and lead to false negatives being reported (Leusch *et al.*, 2010). Regardless of these constraints, many researchers use the YAS to screen chemicals or environmental water samples for (anti)androgenic activity (Sohoni & Sumpter, 1998; Urbatzka *et al.*, 2007; Grover *et al.*, 2011; Kolle *et al.*, 2011, 2012; Li *et al.* 2010; Orton *et al.*, 2011) because of its ease of use and relative short exposure time to screen a larger sample set over a wide dilution range.

The yeast androgen /anti-androgen screen (YAS/anti-YAS) protocol follows the principle of a yeast strain, *Saccharomyces cerevisiae*, which were transfected with the human androgen receptor (hAR) gene and a plasmid containing an androgen response element (ARE)-linked *lac-Z* gene (Fig. 1.7). Successful binding of ligands (steroids and test contaminants) to the human androgen receptor (hAR) in the transfected yeast cells will initiate expression of the *lac-Z* reporter gene that encode for the enzyme  $\beta$ -galactosidase (Fig. 1.7). The  $\beta$ -galactosidase metabolises chlorophenol red galactopyranoside (CPRG), which is included in the assay, resulting in a colour change in the assay medium (Fig. 1.7). The colour change, which is measured as absorbance, indicates a dose-dependent activity of the ligands to bind to the androgen receptor. The absorbance is measured at 570 nm for reactivity to the hAR and at 620 nm to measure turbidity change of the yeast cells.



**Figure 1.7:** Principle of the androgen-inducible expression system in the yeast androgen screen (YAS). Re-drawn from Routledge & Sumpter (1996).

Apart from the binding potential of ligands to the AR, contaminants may also be tested for their ability to modulate the binding of an androgen to the hAR. Ligands may bind competitively or affect AR expression in the yeast cells and therefore cause an AR antagonistic response in the assay (an anti-androgenic MOA). This is observed by exposing the yeast cells to an androgen such as T or DHT in mixture with the test compound. The expected binding to hAR by the androgen is used as the control/baseline. A decrease in binding to hAR therefore will indicate anti-androgenic activity (anti-YAS). In theory,  $\beta$ -galactosidase encoding will be suppressed when a ligand suppresses the binding of an

androgen to the AR and will therefore cause a reversed colour change as opposed to reactivity of an androgenic EDC in the YAS. Anti-androgenic activity can be evaluated between chemicals by using a known anti-androgen like the fungicide, vinclozolin or the pharmaceutical flutamide (Urbatzka *et al.*, 2007b; Li *et al.*, 2010).

#### *Reporter gene assay (MDA-kb2 cell line)*

The MDA-kb2 cell-line is derived from a breast cancer cell line, MDA-MB-453, which is stably transformed with a mammary tumor virus (MMTV) luciferase.neo reporter gene construct (Wilson *et al.*, 2002). Both androgen receptors (ARs) and glucocorticoid receptors (GRs) are present in this cell-line (Wilson *et al.*, 2002), which give this assay the advantage to investigate cytotoxicity, androgen modulation, as well as glucocorticoid mediated activities (Ait-Aïsa *et al.*, 2010). Chemicals which might modulate AR and/or GR binding in these cells may either activate or de-activate the expression of the luciferase reporter plasmid, which is driven by the MMTV promoter (Wilson *et al.*, 2002).

For androgen modulating screening, as is the case with yeast screen, both AR antagonism as well as agonism can be screened for by either exposing the cells to the test contaminants in the presence or absence of an androgen (such as DHT). Studies have shown that several pesticides (Wilson *et al.*, 2002; Ait-Aïsa *et al.*, 2010; Ermler *et al.*, 2011; Orton *et al.*, 2011) and other pollutants such as the plastic byproduct, bisphenol A, and the livestock growth promoter, trenbolone (Blake *et al.*, 2010; Ermler *et al.*, 2011) show AR agonistic or antagonistic properties. Orton *et al.* (2011) screened 134 pesticides for their ability to antagonise or induce AR binding. From these pesticides, 23 were shown to have AR antagonistic properties and only seven showed AR agonistic properties. However, it is clear that more pesticides need to be tested and that alternative interaction, to AR binding, need to be explored in the endocrine pathways

#### *Steroidogenesis*

##### *Minced testis assay*

The minced testis assay provides a system, in which androgenicity and anti-androgenicity can be detected by means of modulation of testosterone production in rat Leydig cells. The assay is conducted by using three-month old male Balb/C rat testes, minced and placed in an appropriate growth medium. Test contaminants may then be added to the testes cells in the

presence or absence of an androgen or androgen-stimulating hormone (such as luteinising hormone, LH). Steroid biosynthesis is measured by a testosterone enzyme-linked immunoabsorbent assay (ELISA). The rat minced testis assay has been shown to provide useful information to detect compounds modulating steroid biosynthesis pathways (Ebrahim & Pool, 2010). This assay is beneficial in the fact that modulation of steroid biosynthesis is detected using a biological system consisting of various cellular mechanisms (*ex vivo*) rather than conventional single-cell *in vitro* bioassays.

#### *H295R cell culture*

The female adrenocortical carcinoma cell line (H295R) has been used to study effects of contaminants on non-receptor binding effects in the steroidogenic pathways (Hecker *et al.*, 2011). These cells have the ability to synthesize steroid hormones associated with adrenal cortex. As with the minced testis assay, steroid hormone concentrations can be measured in the medium by using ELISAs for specific hormones. Furthermore, mRNA gene expression and specific enzyme concentrations can also be measured (Hecker *et al.*, 2011). Several azole fungicides have been shown to cause aromatase inhibition which is necessary to convert T to E<sub>2</sub> and therefore serve as an anti-estrogenic or a masculinizing EDC (Johansson *et al.*, 2002; Kjærstad *et al.*, 2009). In addition, antibiotic pharmaceuticals can modulate gene expression and hormone production by upregulating CYP11 and CYP19 genes and therefore causing increased E<sub>2</sub> and progesterone production (Gracia *et al.*, 2007). This assay is therefore a valuable screening tool to detect steroidogenic alterations on various levels, such as transduction of the steroidogenic signal, cholesterol transport and regulation of key enzymatic activities for steroid biosynthesis.

#### *Androgenic and anti-androgenic endocrine disruption in aquatic vertebrates*

Organisms living in or near water systems are of great risk at being exposed to EDCs. Aquatic vertebrates are in constant or direct contact with polluted water systems where different stages or their entire life cycles are within water systems. Given the fact that contaminant levels may fluctuate throughout the year, these organisms may therefore be exposed to varying and relatively low environmental contaminant concentrations. Aquatic vertebrates are therefore valuable by serving as bio-indicator species showing possible EDCs within the water system over an extended time period. Furthermore, fish and amphibians share some physiological similarities with higher vertebrates in how they regulate the HPG

axis and can therefore serve as sentinels to show gonadal endocrine disruption pollution in an aquatic system (Kloas *et al.*, 2009).

It is clear from the literature that fish species are more used as sentinel or model species in *in vivo* studies than other aquatic vertebrates (such as frogs) to test for endocrine disruption activity (Kloas & Lutz, 2006; McCoy *et al.*, 2008; Kloas *et al.*, 2009). However, with the recent global decline in amphibian populations, it is postulated that EDCs may be a contributing factor to this trend (Carey & Bryant, 1995; Kloas, 2002; Sparling & Fellers, 2007; Hayes *et al.*, 2010). Furthermore, by identifying and validating biomarkers using local species will have the added advantage that endemic aquatic systems can be assessed by studying the same biomarkers in the same endemic species (Van Wyk *et al.*, 2005).

#### *Biomarkers in frogs*

The suggested global decline in amphibian populations initiated awareness of xenobiotic contamination in the aquatic environment and the potential of these pollutants contributing to these declines. A specific link has been drawn to agrichemicals entering water systems through point and non-point pollution sources, possibly affecting health disorders in non-target vertebrates (McCoy *et al.*, 2008). Although the link between the use of pesticides and amphibian population decline has not yet been firmly established, several pesticides present in the aquatic environment have been shown to disrupt hormonal-dependent processes associated with reproduction and development (McKinlay *et al.*, 2008; Hofmann & Kloas, 2010). Amphibians in adult and pre-metamorphic tadpole stages are particularly vulnerable to aquatic contaminants since they breed and develop in ponds and rivers at times (spring and summer) when large quantities of agrichemicals may be applied. As opposed to fish models, steroid hormones and endocrine systems in frogs resemble a more close relationship to higher vertebrates (Kloas & Lutz, 2006; Kloas *et al.*, 2009). With the fairly similar conformation in the gonadal endocrine system, it seems more relevant to conduct amphibian-based endocrine disruption research. However, fewer studies have used amphibians as a bio-indicator species, with most studies involving the fully aquatic African clawed frog (*X. laevis*) as model species (Kloas & Lutz, 2006; McCoy *et al.*, 2008; Kloas *et al.*, 2009).

Declining amphibian populations may be explained by means of altered reproductive fitness and behaviour, which may be due to compounds affecting steroid hormone pathways. One

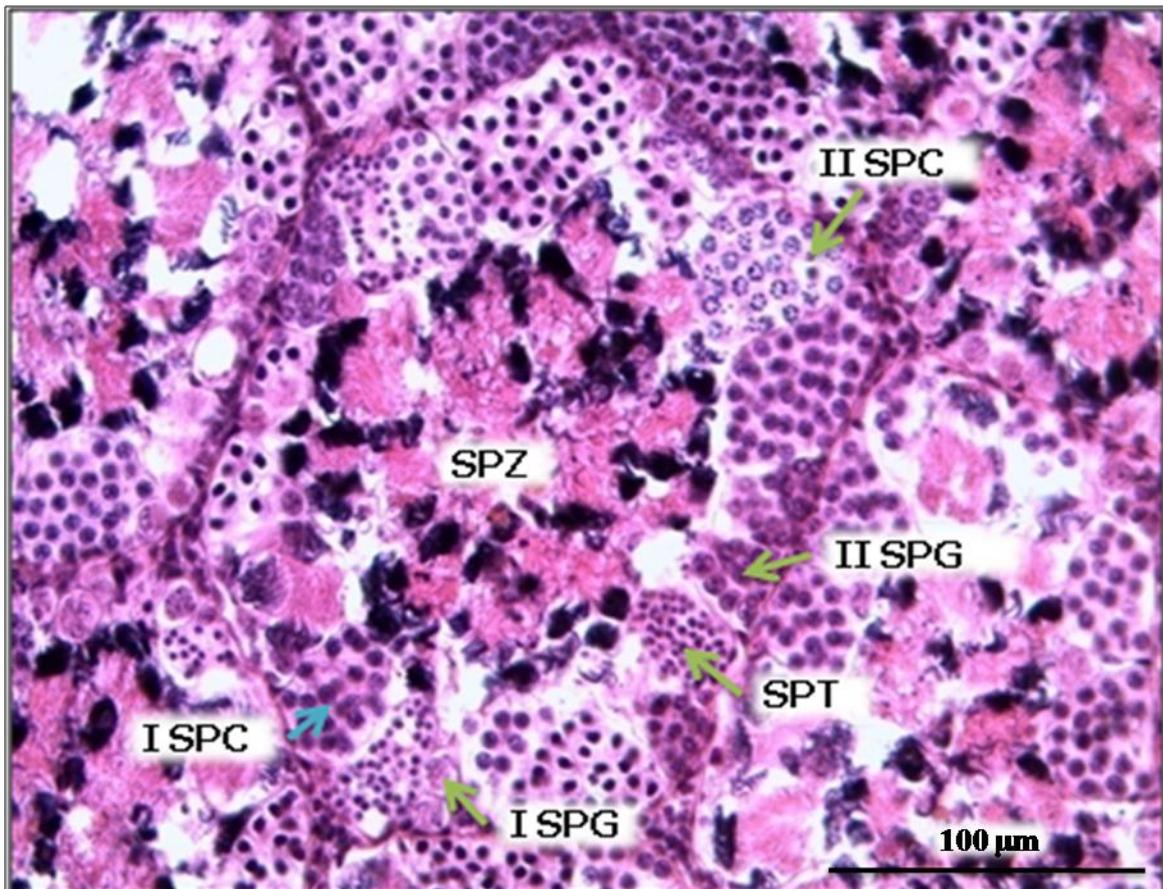
pathway in particular which has recently received attention is anti-androgenic contaminants used in agricultural practices (McCoy *et al.*, 2008). Several pesticides, especially fungicides, have been screened for their anti-androgenic endocrine disrupting properties *in vitro*, but few studies report on the endocrine disrupting effect of these contaminants upon amphibian reproductive physiology. It has been stated that the main mechanism of action for EDCs interfering with the gonadal endocrine system is direct steroid hormone receptor modulation as well as the bioavailability of sex steroids (Kloas *et al.*, 2009). This means that EDCs will most likely affect androgen and estrogen receptor binding as well as steroidogenesis in an organism.

Although the research on anti-androgenic endocrine disruption in amphibian species is limited, there have been several studies using biomarkers in amphibian species to investigate gonadal endocrine disruption (Van Wyk *et al.*, 2003; Hayes *et al.*, 2010; Hoffmann & Kloas, 2010). As with fish species, gross morphological abnormalities, steroid hormone and metabolism levels in frogs can aid in investigations of gonadal endocrine disruption caused by EDCs. For example, exposures to the organophosphate insecticide, chlorpyrifos, revealed to modulate testicular morphology in *Rana dalmatina* frogs (Bernabò *et al.*, 2011). Exposure to the fungicide prochloraz in the common frog, *Rana temporaria*, caused hermaphroditism, male-biased sex ratio, but lower T levels (Brande-Lavridsen *et al.*, 2008).

As with the biomarkers used in fish species, frog species can also be used for the induction of vitellogenin (VTG) in male- and female frogs (Van Wyk *et al.*, 2005). The hepatic-induced yolk precursor, VTG is produced in the liver of female frogs under the control of estrogenic hormones (Van Wyk *et al.*, 2005). Although the production of VTG is known to be female-specific, male frogs are also shown to be capable of producing VTG in their liver when exposed to estrogenic compounds. In male *X. laevis* and *R. temporaria* frogs, VTG levels are increased when exposed to 17 $\beta$ -estradiol (E<sub>2</sub>) and the synthetic estrogen ethinylestradiol (EE<sub>2</sub>; Van Wyk *et al.*, 2003; Brande-Lavridsen *et al.*, 2008). Elevated levels of VTG-mRNA have been shown in *X. laevis* and *R. temporaria* frogs exposed to sewage effluents, indicating estrogenicity in the effluent (Bögi *et al.*, 2003). The synthesis or upregulation of VTG can therefore be used to detect estrogenicity in environmental waters using *in vivo* biomarker systems. Furthermore, as with anti-androgenic investigation in fish using spiggin levels in females, it might be possible to identify anti-estrogenic MOAs of EDCs using VTG levels in

frogs by exposing males or females to an estrogen such as E<sub>2</sub> or EE<sub>2</sub> as well as to a test compound possibly mimicking the action of natural estrogenic hormones. The estrogen will induce VTG production and if the test compound has an anti-estrogenic MOA, it will suppress the induction of VTG and can then be compared to a control specimen which was induced with an estrogen alone.

Another organ which can be used to indicate gonadal endocrine disruption is gonad development, spermatogenesis and spermiogenesis in male anurans. The testis is necessary for the production of steroid hormones associated with reproduction and male-associated traits (Wada & Gorbman, 1977; Kelce *et al.*, 1998). Anurans have a cystic-type of testis (Fig. 10, Pierantoni *et al.*, 2002; Kaptan & Murathanoğlu, 2008). The cysts are randomly organized in the seminiferous tubules of the testis and each cyst is composed of Sertoli cells and germ cells of the same developmental stage, either being spermatogonia (SPG), spermatocysts (SPC) or spermatids (SPT) (Fig. 1.8; Pierantoni *et al.*, 2002).



**Figure 1.8:** Seminiferous tubule bearing cysts with different stages of spermatogenic development in an adult male *X. laevis* frog taken during peak spermiogenesis periods in the summer season. I SPG, primary spermatogonia; II SPG, secondary spermatogonia; I SPC, primary spermatocyte; II SPC, secondary spermatocyte; SPT, spermatid; SPZ, spermatozoa (sperm cells). Magnification: 100  $\mu\text{m}$ .

Male *X. laevis* spermatogenesis is continuous throughout the year (Kalt, 1976). Although producing spermatozoa on a continuous basis, some seasonality in spermatogenic activity of *X. laevis* has been reported (Kalt, 1976; Van Wyk *et al.*, 2003). Studies on *Ranid* frog species, also having a continuous spermatogenic cycle, have shown that pituitary gonadotropins initiate and maintain spermatogenesis (Rastogi *et al.*, 1986; Pierantoni *et al.*, 2002) and that both androgen and estrogen receptors are found in urodele testis (Pierantoni *et al.*, 2002). This then gives an indication that both androgens and estrogens are needed to maintain an active spermatogenic cycle. Testosterone is responsible for the transformation of one spermatogenic stage to a more advanced stage (Rastogi *et al.*, 1986; Pierantoni *et al.*, 2002). Follicle stimulating hormones (FSH) stimulates the proliferation of spermatogonia and promotes spermatid formation (Pierantoni *et al.*, 2002) while estrogens have a stimulatory role in mitotic/meiotic cycles of spermatogenesis (Pierantoni *et al.*, 2002). It is therefore evident that alterations that act on androgen or estrogen levels and metabolism (modulation of aromatase and 5 $\alpha$ -reductase) by EDCs will result in an altered spermatogenic cycle.

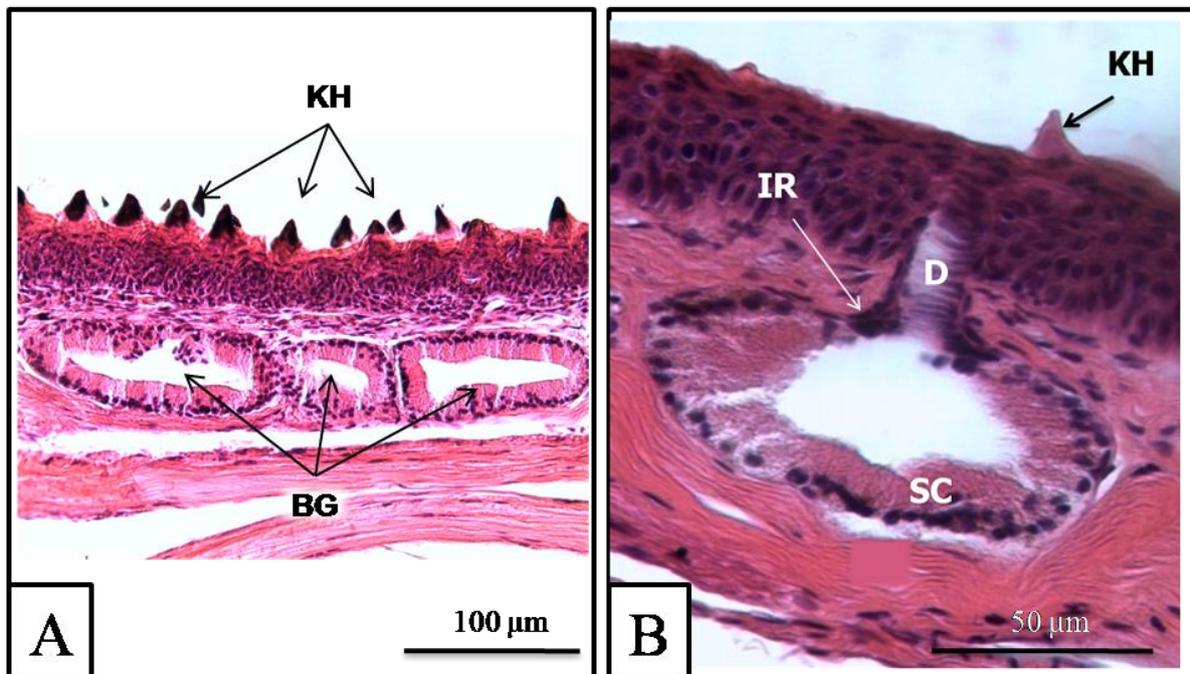
Apart from morphological and steroid hormone modulation of EDCs, it has been shown that the development of secondary sexual characteristics (SSCs) necessary for mating behaviour in anurans can also be used as biomarkers of reproductive endocrine disruption. Various biomarkers have been established to show the harmful effects that EDCs can pose to amphibians (Emerson *et al.*, 1999; Van Wyk *et al.*, 2003; McCoy *et al.*, 2008; Behrends *et al.*, 2010; Hoffman & Kloas, 2010). Disruption in SSC development of amphibians such as spermatogenesis (Rastogi *et al.*, 1986), vocalization (Kelley *et al.* 1989; Emerson *et al.*, 1999; Hoffmann & Kloas, 2010) and sexually dimorphic skin gland (SDSG) development (Emerson *et al.*, 1999; Van Wyk *et al.*, 2003; McCoy *et al.*, 2008; Hayes *et al.*, 2010) have been documented and may serve as biomarkers to give an early indication of the disruption of testicular function and hormone pathways (McCoy *et al.*, 2008).

It was shown that there is a direct dependence of SSC development on steroid hormones (Sassoon & Kelley, 1986; Thomas *et al.*, 1993) and high levels of especially androgen receptors are located in tissues associated with these characteristics (Kelley *et al.*, 1989; Emerson *et al.*, 1999; Kang *et al.*, 2004b). In *X. laevis* frogs, vocalization for reproductive behaviour is regulated by muscle contractions in the larynx (Kelley *et al.*, 1989). In male frogs, androgens induce the development of cartilage and muscle fibers in the larynx, and therefore, masculinise the vocal cords. Environmental compounds affecting the development of the larynx will eventually alter vocalization, and therefore, reproductive success. It has been shown that the anti-androgenic pharmaceutical flutamide suppressed calling behaviour in adult male *X. laevis* frogs and that exposure to the fungicide vinclozolin also suppressed calling behaviour in a similar manner (Behrends *et al.*, 2010; Hoffmann & Kloas, 2010). It is therefore assumed that other EDCs having the same MOA as vinclozolin and flutamide will modulate vocal behaviour. Furthermore, it has been suggested that the enzyme 5 $\alpha$ R might play an important role in vocal behaviour. A study by Hoffmann & Kloas (2012) has shown that non-aromatizable steroids such as DHT play an important role and possibly may be the primary hormone necessary for vocal behaviour. This suggests that the metabolism of testosterone to DHT is necessary for vocalization and any compound affecting the enzyme 5 $\alpha$ R will therefore also suppress vocalization in the same manner as a compound modulating AR binding. However, this assumption still needs further investigation and be tested in other SSCs such as in SDSGs.

The SDSGs are situated on the front extremities of most male anuran species, also known as the nuptial pad (NP) region (Thomas *et al.*, 1993; Epstein & Blackburn, 1997; Brizzi *et al.*, 2002; Kaptan & Murathanoğlu, 2008). The NP is characterised by the presence (association) of keratinised hooks (KH) on the surface of the epidermis and the SDSGs situated in the underlying epidermis (Fig. 1.9). These SDSGs are also referred to as specialized mucous glands (SMG), breeding glands (BG) or nuptial pad glands (NP glands) in various literature sources (Thomas *et al.*, 1993; Epstein & Blackburn, 1997; Emerson *et al.*, 1999; Brizzi *et al.*, 2002; Van Wyk *et al.*, 2003; Kaptan & Murathanoğlu, 2008). For the remainder of the thesis, I shall refer to the SDSGs as breeding glands (BGs).

Much speculation exists around the function of BGs in anuran males, and several authors suggested that these glands play an important role, together with the KHs during clasping and

amplexus behaviour, due to the development of KHS and BGs during times of breeding behaviour (Brizzi *et al.*, 2002; Emerson *et al.*, 1999). However, the function and role of BGs during mating still needs to be established (Van Wyk *et al.*, 2003; Kaptan & Murathanoğlu, 2008). It is hypothesised that the function of BGs can be a combination of secreting an adhesive mucous for clasping (Brizzi *et al.*, 2002; Van Wyk *et al.*, 2003) and/or secreting chemosignals to receptive females (Pearl *et al.*, 2000; Kaptan & Murathanoğlu, 2008). This lack of understanding the role of BGs is partly due to the lack of detailed information about the ultrastructure and chemical composition of the glands. In male *X. laevis* frogs, the BGs are shown as a specialized form of mucous glands, located in the NP region on the ventral side of the forearm and fingers (Fig. 1.9; Fujikura *et al.*, 1988; Thomas *et al.*, 1993; Van Wyk *et al.*, 2003).



**Figure 1.9:** Photomicrograph of the breeding gland (BG) in male *X. laevis* skin situated on the dorsal side of the front extremities of the frog. These glands are situated underneath the epidermis, which are lined with multiple keratinised hooks (KHS) on the surface (A). The KH present a darkened area on the front extremities of the frog. The BGs are connected to the outer surface by a duct (D) and layered by secretory epithelial cells (SC) surrounding the lumen of the gland (B). A different kind of epithelial cell is situated at the intermediate region (IR) close to the duct (B). Magnification: A, 100 µm; B, 50 µm.

Castrated male *X. laevis* frogs lost breeding glands, while androgen supplement re-initiated the development of breeding NP glands in the castrated frogs, therefore showing direct androgen dependency for their development and maintenance (Kelley & Pfaff, 1976). This androgen dependence, possible functions and structure of SDSGs in *X. laevis* has been confirmed in several other anuran species (Kurabuchi, 1993; Thomas *et al.*, 1993; Byrne & Keogh, 2007; Kaptan & Murathanoğlu, 2008; Brunetti *et al.*, 2012), implicating SDSGs in anurans have the potential to be used as a biomarker of gonadal endocrine disruption. However, validation studies using control substances to characterize the disrupted phenotype following exposure to either androgenic or anti-androgenic EDCs are still lacking. Van Wyk *et al.* (2003) presented preliminary data to suggest that exposure to anti-androgenic EDCs, such as the fungicide vinclozolin, the insecticide metabolite of DDT (p, p' DDE) and the pharmaceutical agent flutamide did indeed inhibit the functionality of the breeding glands in male *X. laevis* frogs. Breeding glands are speculated to play a key role in the mating behaviour of male frogs (Thomas *et al.*, 1993; Emerson *et al.*, 1999). These sexually dimorphic skin glands are therefore proposed to serve as a good androgen target biomarkers (Van Wyk *et al.*, 2003). In spite of initial studies showing androgen dependency of breeding glands in *Xenopus* (Fujikura *et al.*, 1988; Emerson *et al.*, 1999; Van Wyk *et al.*, 2003) and SDSGs in other anuran species (Saidapur & Nadkarni, 1975; Thomas *et al.*, 1993; Brizzi *et al.*, 2002; Kaptan & Murathanoğlu, 2008), the functionality of breeding glands is still unknown. Understanding the morphology and hormonal control of the male breeding glands will represent the first step towards using these glands as biomarkers in EDC studies. Finally, the morphology and hormonal control of breeding glands can furthermore contribute towards a better understanding of an important communication system (such as possible chemical signalling) in amphibians although its role in amphibian reproductive behaviour still needs to be assessed.

## AIMS AND OBJECTIVES OF THE PRESENT STUDY

Overwhelming published evidence suggests that chemicals in the aquatic environment may modulate the vertebrate endocrine system with potential adverse health effects. However, two clear gaps emerge. Firstly, most endocrine disruption research has been concerned with estrogenicity testing and more studies are needed to investigate the potential of (anti)androgenic activity of chemicals and environmental matrices such as surface water. Secondly, sentinel species associated with endocrine disruption in freshwater systems mostly include fish species, despite the evidence that amphibians are globally declining, which may be related to manmade EDCs contaminating freshwater systems. It is evident that there is a need for additional investigation of the androgenic and anti-androgenic potencies of pesticides which are regularly used in agriculture, and to develop and validate more biomarkers for establishing androgenic and anti-androgenic endocrine disruption in freshwater systems using sentinel species.

The aim of this thesis was to focus on these constraints by *in vitro* investigation of pesticides, especially fungicides, and mixtures which may serve as possible androgen receptor modulators as well as to validate *in vivo* biomarkers of androgen modulation in *X. laevis* frogs. Furthermore, the last chapter of my thesis includes field sampling of *X. laevis* frogs to investigate possible reproductive endocrine disruption using *in vivo* biomarkers as well as *in vitro* investigation of estrogenic, androgenic and anti-androgenic ligands present in water catchments close to agricultural practices.

Specific objectives of this study were:

- To validate male breeding gland activity in *X. laevis* adult males as an androgen-sensitive biomarker (Chapter 2).
- To investigate the use of premature development of breeding glands in pre-metamorphic *X. laevis* tadpoles and juveniles as a biomarker for exogenous (xenobiotic) androgen stimulation (Chapter 2).

- To compare histological and physiological endpoints in gonadal development and breeding glands of adult *X. laevis* males following the exposure to an AR antagonist and a 5 $\alpha$ Ar inhibitor (Chapter 3).
- To investigate AR antagonistic effects of commonly used pesticides in the Western Cape, in single or in binary using an *in vitro* yeast androgen/anti-androgen screen (Chapter 4).
- To screen and detect estrogenic and (anti)androgenic activity in water impoundments of intense farmed (vineyards, and orchards) agricultural areas around Stellenbosch in the Western Cape during different periods of agricultural activity (summer, winter and spring) using an *in vitro* yeast screen and the minced testis steroidogenesis assay (Chapter 5).
- To investigate (anti)androgenic effects in adult male *X. laevis* frogs collected from selected impoundments of intense farmed agricultural areas (vineyards and orchards), around Stellenbosch in the Western Cape, using a suite of *in vitro* and *in vivo* biomarkers (Chapter 5).

## CHAPTER 2: DEVELOPMENT OF BREEDING GLANDS IN THE AFRICAN CLAWED FROG, *XENOPUS LAEVIS*, DURING DEVELOPMENTAL STAGES: A BIOMARKER FOR SCREENING ENDOCRINE DISRUPTING COMPOUNDS<sup>1,2</sup>

### ABSTRACT

The integument of anuran skin consists of several epidermal glands which provide specific functions. One of these glandular types are specialized mucous glands (or breeding glands), which aid in mating behaviour. It has been shown that the development of these glands can be stimulated and inhibited by treatment with androgenic and anti-androgenic compounds respectively, including environmental endocrine disrupting contaminants (EDCs). However, the ultrastructure and comparison between a developed and inhibited gland has not yet been investigated for its use as an *in vivo* biomarker of endocrine disruption. Furthermore, the ontogeny of breeding gland development in premature frogs is also unknown and may provide insights into the possible use of these glands as a biomarker for androgen-linked endocrine pathway modulation. The present study investigated the ultrastructural modifications and androgen receptor (AR) immunohistochemistry of stimulated and inhibited breeding glands in adult *X. laevis* frogs. Adult male frogs treated with either the anti-androgenic compound flutamide or dithiocarbamate fungicide mancozeb showed lower incidence of AR immunolabeled nuclei in the nuptial pad (NP) integument and altered ultrastructure of breeding gland secretory cells. Tadpoles (NF stage 56-59) and juvenile frogs (5d post-metamorphic) exposed to an exogenous androgen, 17 $\alpha$ -methyltestosterone (17 $\alpha$ -MT), resulted in a significantly increased occurrence of epidermal keratinised hooks (KHs) and breeding gland development from a mucous gland precursor. The present study suggests that the development of epidermal KHs and underlying breeding glands can be used in future endocrine studies as *in vivo* biomarkers of androgen endocrine pathway modulation.

**KEYWORDS:** Breeding gland, anti-androgen, endocrine disruption

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<sup>1</sup> **Article 1:** *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* (To be submitted to African Zoology)

<sup>2</sup> **Article 2:** *Premature development of androgen-dependent breeding glands in the African clawed frog, Xenopus laevis* (To be submitted to African Journal of Aquatic Sciences)

## INTRODUCTION

Anurans possess several types of epidermal exocrine glands which aid several physiological functions such as predator defence, communication, homeostasis and reproduction (Brunetti *et al.*, 2012). Depending on the ultrastructure and innervations of the secretory cells as well as the chemical composition of the secretory product, these glands can be grouped into four types, namely mucous, serous, mixed (serous-mucous) or lipid (Brizzi *et al.*, 2002). Mucous glands serve a great role in anuran skin by keeping the skin moist, giving protection from environmental factors and reducing friction within the water (Brizzi *et al.*, 2002). Apart from the functions of ordinary mucous glands (OMG), it is found that specialized forms of mucous glands (termed specialized mucous glands, SMGs) are found to be gender-specific and localized at certain regions of anuran skin (Thomas *et al.*, 1993; Epstein & Blackburn, 1997; Brizzi *et al.*, 2002; Kaptan & Murathanoğlu, 2008; Siegel *et al.*, 2008; Brunetti *et al.*, 2012; Luna *et al.*, 2012).

Specialized mucous glands have been characterized to play some role in chemical communication and/or reproduction in several anuran species and are therefore better characterised as breeding glands (BGs) (Fujikura *et al.*, 1988; Thomas *et al.*, 1993; Emerson *et al.*, 1999; Brizzi *et al.*, 2002; Van Wyk *et al.*, 2003; Siegel *et al.*, 2008; Brunetti *et al.*, 2012; Luna *et al.*, 2012). These glands are mainly localized at specialized patches of skin on the pectoral and/or ventral region of the front extremities of the NP skin area (Van Wyk *et al.*, 2003; Siegel *et al.*, 2008; Brunetti *et al.*, 2012; Luna *et al.*, 2012). The NP can be recognized by the darkened appearance of the ventral skin area which is due to the presence of dark epidermal keratinized hooks (KH) on the skin surface (Fujikura *et al.*, 1988; Luna *et al.*, 2012). However, the underlying BGs also develop in conjunction with the epidermal KHs and are also included as a characteristic of the NP integument (Fujikura *et al.*, 1988; Van Wyk *et al.*, 2003; Brunetti *et al.*, 2012).

Both the KHs and BGs are shown to proliferate during times when blood androgen levels are high (such as in anuran breeding seasons) or when adult males are induced with androgens (Parakkal & Ellis, 1963; Rastogi *et al.*, 1986; Fujikura *et al.*, 1988; Kurabuchi, 1993; Thomas *et al.*, 1993; Epstein & Blackburn, 1997; Van Wyk *et al.*, 2003). From an endocrinological control perspective, both the KHs and the associated breeding glands have been shown to exhibit high androgen receptor (AR) expression and dependence on androgens as well as

seasonal activity for their development (Emerson *et al.*, 1999). It seems that both these structures are necessary for key reproductive behaviour in male frogs.

The NP in general is proposed to be used for clasping and amplexus behaviour in anuran males, mostly due to the locality and structure of the KHs and the dependency on androgens to develop (Thomas *et al.*, 1993; Brizzi *et al.*, 2002; Emerson *et al.*, 1999). The underlying BGs are also a key characteristic of the NP skin area (Thomas *et al.*, 1993; Brizzi *et al.*, 2002; Emerson *et al.*, 1999). In adult male African clawed frogs (*Xenopus laevis*), BGs are shown to be localized on the ventral side of the forearm and fingers where the NP is situated and develop in conjunction with the epidermal KHs (Fujikura *et al.*, 1988; Thomas *et al.*, 1993). Although these glands occur at a specialized region for reproductive behaviour, the function and role of BGs is still largely speculated (Van Wyk *et al.*, 2003; Kaptan & Murathanoğlu, 2008). It is hypothesised that the function of BGs can be a combination of secreting an adhesive mucous for clasping (Brizzi *et al.*, 2002; Van Wyk *et al.*, 2003; Luna *et al.*, 2012) and/or secreting chemical signals to receptive females (Thomas *et al.*, 1993; Pearl *et al.* 2000; Kaptan & Murathanoğlu, 2008). Regardless of their specific function, histochemical and ultrastructural findings of BGs have shown that these glands share similarities in their secretory product and structure with OMGs (Thomas *et al.*, 1993; Brizzi *et al.*, 2002; Brunetti *et al.*, 2012). However, the ultrastructural modification of BGs and the possible reduction in ARs at the NP integument in frogs exposed to anti-androgenic EDCs has not yet been reported on.

Furthermore, although the general BG structure and androgen dependency have been reported on for various anuran species (Saidapur & Nadkarni, 1975; Fujikura *et al.*, 1988; Thomas *et al.*, 1993; Emerson *et al.*, 1999; Brizzi *et al.*, 2002; Van Wyk *et al.*, 2003; Kaptan & Murathanoğlu, 2008; Siegel *et al.*, 2008; Brunetti *et al.*, 2012; Luna *et al.*, 2012), it is still unknown how and at which developmental life stage BGs develop (Brunetti *et al.*, 2012). A recent study has suggested that there are two possibilities for the development of BGs: Firstly; that BGs originate from OMGs during ontogeny when androgen levels are starting to differentiate between sexes; secondly, that BGs originate at the start of ontogeny independent of a mucous gland-type precursor (Brunetti *et al.*, 2012). Considering these suggestions, comprehensive ontogenic studies to investigate these hypotheses have not been undertaken. Olmstead *et al.* (2012) has reported on the premature development of NPs in Tropical clawed

frogs (*Silurana tropicalis*) exposed to the cattle growth promoter 17 $\beta$ -trenbolone, which is known to be an androgen agonistic compound. However, this study has only reported on the development of KHS and resultant darkening of the forelimbs of the male frogs but did not specifically report on the development of the associated BGs. Furthermore, although the androgen dependency of BGs in *Xenopus* frogs have been well-documented (Thomas *et al.*, 1993), not many studies have considered BGs as a biomarker of environmental pollutants modulating androgen endocrine pathways (Van Wyk *et al.*, 2003; Hayes *et al.*, 2010).

Limited information is available on the sensitivity and morphological response of undifferentiated BGs to anti-androgenic endocrine disrupting contaminants (EDCs). Although, BGs has been shown to develop to a functional level only after testicular maturity (Saidapur & Nadkarnai, 1975; Kurabuchi, 1993; Lynch & Blackburn, 1995; Epstein & Blackburn, 1997), it may be possible to use breeding gland development as a biomarker in adult or premature *X. laevis* frogs to indicate androgenic and/or anti-androgenic endocrine disruption. The present study investigated the ultrastructural modifications and AR immunoreception of stimulated and inhibited BGs in adult *X. laevis* frogs as well as the sensitivity of the NP area to develop along with an exogenous androgen treatment in tadpole and juvenile *X. laevis* frogs.

## **MATERIALS AND METHODS**

### *Exposure chemicals*

The chemicals used for the exposure studies included human chorionic gonadotropin (hCG; Ovidrel, 250  $\mu$ g, Serono, Darmstadt, Germany), the synthetic androgen 17 $\alpha$ -methyltestosterone (17 $\alpha$ -MT;  $\geq$  97.0%, 69240-5G, Sigma), the pharmaceutical anti-androgen flutamide (F9397-1G, Sigma) and the dithiocarbamate fungicide mancozeb (CAS No. 8018-01-7, Sigma). Dimethyl sulphoxide (DMSO, Sigma) was used as vehicle in the adult and tadpole exposure experiments. Absolute ethanol (64-17-5, Sigma) was used to dissolve the 17 $\alpha$ -MT in the juvenile exposure experiment.

*Adult male husbandry and exposure*

Adult *X. laevis* frogs were sexed by using sexual dimorphic external morphological features in the cloacal region (Brown, 1970). Female *X. laevis* frogs display cloacal labia while the absence of these labia characterizes males (Brown, 1970). If present, dark regions on the forearms and inside surfaces of the hands of male frogs, which represent the NP area, were also used to recognize males. All glass containers used in the exposure experiments and housing were pre-cleaned with Extran (MA 01 alkaline, Merck), methanol and distilled water. Water was replaced once a week. Husbandry was followed according to literature (Brown, 1970; Opitz *et al.*, 2005) and guidelines from the American Veterinary and Medical Association (AVMA, 2008).

Ten adult male and female *X. laevis* frogs were obtained from a commercial dealer collecting frogs from two local farms (33°50.075'S, 018°52.375'E and 33°49.639'S, 018°52.739'E) around the Klapmuts area near the town of Stellenbosch in the Western Cape. Frogs were transported in a sealed, aerated container filled with water. The frogs were housed in 40 L glass tanks filled with carbon-filtered tap water and quarantined (30 days) under summer conditions (24°C, 14 h light: 10 h dark) before exposure experiments started. Water pH and temperature were measured daily using a waterproof pH meter (pHTestr 30; Eutech instruments). Two breeding pairs (two males and two females) were used for breeding of tadpoles. Additionally, six sexually stimulated (hCG injected) adult male frogs were used in total for the histological and ultrastructural examination of the BGs. Frogs were exposed for 21 days to either hCG (100 i.u.), flutamide (100 ug/g body mass) or mancozeb (50 ug/g body mass) which was injected into the dorsal lymph sac (Brown, 1970) once a week. The exposure groups consisted of two frogs in each group treated with anti-androgenic EDCs (flutamide and mancozeb) in combination with hCG and two frogs treated with only hCG. DMSO (100 µL) was used as solvent for the flutamide and mancozeb stock solutions. Two frogs were used for each exposure and kept in 10 L activated charcoal filtered tap water at a temperature of  $24 \pm 2^\circ\text{C}$  and photoperiod of 14 hours light and 10 hours dark cycle. All test chemicals were injected once a week.

*Embedding of adult male NP skin samples for histology*

Upon completion of the 21 day exposure, frogs were euthanized by decapitation and pithing (AVMA, 2008) and skin sections of the NP region at the front limbs were dissected. For basic histological and ultrastructural investigation, skin samples at the NP region of the right arm in the hCG-, flutamide- and mancozeb-exposed frogs were removed and fixed in Karnovsky's fixative (Bancroft & Stevens, 1977) for 24 hours. Half of the skin section was then rinsed in water and subsequently processed for routine paraffin histology using a Shandon tissue processor 2LE (Optolabor (PTY) LTD) and embedded in paraffin wax (Paraplast-plus, 56°C melting point) for basic histological examination. The other half of the skin sections were then placed in phosphate buffer, stored overnight at 4°C, and then postfixed in osmium tetroxide (OsO<sub>4</sub>) for transmission electron microscopy (TEM) investigation. The TEM samples were rinsed in distilled water, 2% uranylacetate, ethanol and sodiumsulphate in 100% ethanol and then embedded in Spurr's epoxy resin using a tissue processor (EM Tp, Leica). For immunohistological investigation, skin from the NP area of the left arm of frogs exposed to hCG, flutamide and mancozeb were removed and embedded in Tissue-Tek OCT (Sakura Finetek) and frozen in liquid nitrogen for immunohistological examination.

*Basic histology*

Thin sections (5 µm) were cut using a rotary microtome (Reichert-Jung D6907, Cambridge Instruments GmbH) and mounted on glass slides. Sections were de-waxed using xylene, hydrated through an alcohol series and stained differentially with Hematoxylin and Eosin (H&E; Bancroft & Stevens, 1977). Following dehydration through an alcohol series, slides were cleared in xylene and a coverslip permanently mounted with a Euparal resin compound.

*Transmission electron microscopy (TEM)*

All procedures were done at the electron microscopy unit (EMU) at the University of Cape Town (RW James Building, UCT upper campus, Cape Town, South Africa). Skin samples in the Spurr's epoxy resin capsules were taken for TEM to describe detailed ultrastructural features of cells in the BGs. The ultrastructure of the BGs of stimulated (hCG-exposed) and inhibited (flutamide-exposed) frogs (n = 2 per exposure group) were compared. Semi-ultrathin sections (200 nm) were cut using an ultramicrotome (EM UC7, Leica), placed on a glass slide and stained with toluidine blue. Ultrathin sections (100 nm) were then cut and

placed on grids for electron microscope imaging. Sections were observed using the JEM-1011 transmission electron microscope (JEOL) and imaging software (iTem, Olympus).

#### *Immunostaining of adult male breeding glands (BGs)*

The frozen skin samples embedded in Tissue-Tek were taken to the Central Analytical Facility Imaging Unit of Stellenbosch University (SU-CAF). Thin sections (8  $\mu\text{m}$ ) of the frozen Tissue-Tek skin samples of the hCG-, flutamide- and mancozeb-exposed frogs were sectioned using a cryostat microtome and placed on gelatin-coated glass slides. Immunostaining procedures of the samples followed the protocol supplied by the manufacturer of the antibodies used (Upstate Cell Signalling Solutions, Millipore). Staining was done at room temperature except when otherwise mentioned, and freshly stained sections placed in a humidified box to avoid evaporation. Slides were blocked with PBA, which consists of phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA) for one hour followed by a blocking step using 2% (v/v) normal goat serum (PAA, Cat no. B15-035) diluted in PBA for 30 minutes. Tissue samples were covered with a 1:50 dilution of the rabbit anti-androgen receptor polyclonal antibody, PG-21 (Millipore; Cat. # 06-680) using PBA and left overnight at 4°C. The PG-21 antibody is an anti-peptide antibody, which corresponds to amino-acids 1-21 of the human androgen receptor, has high specificity for only the androgen receptor and has been shown to have cross reactivity for non-mammalian vertebrates, including frog (Emerson *et al.*, 1999), newt (Matsumoto *et al.*, 1996) and lizard (Moga *et al.*, 2000). Selected skin sections on the slides that were not stained with the primary antibody served as a negative control of AR reactivity. After overnight incubation, slides were gently rinsed twice in PBS for five minutes. Sections were covered with a 1:500 dilution of the secondary antibody (Goat anti-rabbit IgG-FITC, Sigma-Aldrich, F9887) in PBA for 1 hour. Sections were rinsed twice in PBS for 5 minutes and mounted for fluorescent microscopy.

#### *Tadpole and juvenile frog housing*

The two adult *X. laevis* breeding pairs obtained from the local dealer (*see above*) were used to obtain tadpoles through an artificial breeding procedure as set out by Brown (1970). In brief, adult female and male frogs were primed with 100 i.u. hCG (Ovidrel) twelve hours prior to pairing. On the day of pairing, females received 300 i.u. hCG and males 100 i.u. hCG. The breeding pairs were transferred to a tank containing 10 L reverse osmosis (RO) water,

supplemented with 2.5 g iodized sea salt (Seepo, 0.004 g iodine/100 g NaCl). The tank contained a false bottom to allow eggs not to be harmed or consumed by the adults. The fertilized eggs were identified by visualization using a binocular dissecting microscope. Eggs, larvae and developing tadpoles were maintained at  $22 \pm 1^\circ\text{C}$ , photoperiod of 12 hours light and 12 hours dark and pH  $7.0 \pm 0.5$  (Opitz *et al.*, 2005) and kept in a aerated 40 L aquarium containing distilled water supplemented with 10 g iodized sea salt (Opitz *et al.*, 2005).

Tadpoles were fed Sera Micron (Sera, USA) twice daily (OECD, 2008). Temperature and pH levels of the water were measured on a daily basis using a pH and temperature meter (pHTestr 30, Eutech instruments). Staging of the tadpoles throughout metamorphosis was done by following the anatomical description of developing *X. laevis* tadpoles (Nieuwkoop & Faber, 1994). 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 & Faber, 1994). Remaining tadpoles from the in-house breeding program were cultured through metamorphosis until a post-metamorphic juvenile stage was achieved (Nieuwkoop & Faber, 1994), which were then used for the juvenile exposure experiments. Prior to the exposure, the juvenile frogs were selected and acclimated in 50 L tanks filled with carbon-filtered tap water. Tanks were aerated, filtered and water temperature maintained at  $21 \pm 2^\circ\text{C}$  and monitored on a daily basis. Water pH and temperature were measured daily using a Waterproof pHTestr 30 (Eutech instruments) to ensure consistent exposure conditions.

#### *Premetamorph tadpole exposure*

Forty pre-metamorphic *X. laevis* tadpoles (NF stage 56-59) with well-developed front limbs were exposed to the synthetic androgen  $17\alpha\text{-MT}$ . Maintenance of the tadpoles followed the OECD guidelines for housing *X. laevis* tadpoles (Opitz *et al.*, 2005; OECD, 2008). In brief, ten tadpoles were held in a 15 L glass tank containing 10 L of RO water buffered with 2.5 g iodized sea salt (Seepo, 0.004 g iodine/ 100 g NaCl). Water quality parameters were measured weekly and conditions were maintained at  $22^\circ\text{C}$  ( $\pm 1^\circ\text{C}$ ), pH 6.5-8.5 and oxygen levels  $>3.5$  mg/L. Exposure lasted for 21 days. Tadpoles were fed Sera Micron algae powder (Sera, USA) twice daily. Tadpoles were exposed to nominal concentrations of 0, 30, 100 and 300  $\mu\text{g/L}$   $17\alpha\text{-MT}$  with ten tadpoles in each group. The stock solutions of  $17\alpha\text{-MT}$  were dissolved in DMSO at the three varying concentrations and then added to the exposure tanks

to be dissolved in the water. The final concentration of DMSO in the exposure tanks was 0.01% of the total amount of water as described in Opitz *et al.*, (2005). Aquarium water and test chemicals were statically renewed two times a week.

#### *Juvenile exposure*

Twenty juvenile (5 days post-metamorphic) *X. laevis* frogs were used to assess the development of BGs in normal frogs and frogs exposed to 17 $\alpha$ -MT. Two exposure groups were used with ten frogs in each group either exposed to 0 or 600  $\mu$ g 17 $\alpha$ -MT per 10 g food. Briefly, the stock solution of 17 $\alpha$ -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. All frogs were kept in 20 L glass tanks containing 10 L carbon-filtered tap water and measured weekly for water conditions (22  $\pm$  1 $^{\circ}$ C temperature, pH between 6.5-8.5 and oxygen levels at >3.5 mg/L). Frogs were fed clean or spiked pellets at a rate of 10 pellets per frog every second day for 21 days.

#### *Tadpole and juvenile breeding gland histological investigation*

Upon completion of the 21 day exposure, both tadpoles and juveniles were euthanized with an overdose of Benzocaine (250 mg/L; AVMA, 2008) and preserved in 10% buffered formalin (Bancroft & Stevens, 1977). Variation in developmental stage of tadpoles in the exposure groups (Nieuwkoop & Faber, 1994) were noted and compared among the different treatments. Skin samples taken from the NP area and/or whole forearms in both tadpoles and juvenile froglets were subsequently subjected to routine resin embedment (Technovit 7100). Thin sections (5  $\mu$ m) were taken using a rotary microtome and stained differentially with Hematoxilin and Eosin (H&E) or periodic acid Schiff (PAS) (Bancroft & Stevens, 1977). The NP area was then compared between exposure groups including categorical data analysing the prevalence of epidermal KHs and BGs and measurements of the number of KHs and BGs per mm<sup>2</sup> of NP skin section area using image analysis (Leica Application Suite, Version 1.5.0, Switzerland). The general structure and glandular composition of the skin sections in the tadpoles and juvenile frogs were also qualitatively compared to the integument of the NP in sexually matured adult male *X. laevis* frogs.

### *Statistical analyses*

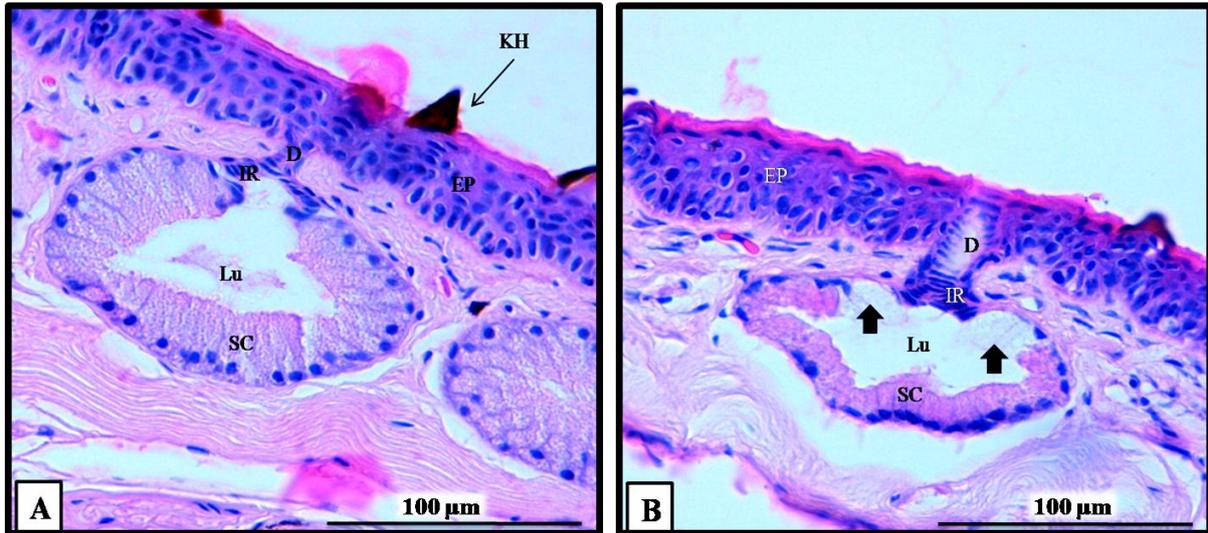
All statistical analyses were done using Statistica 10.0 (StatSoft Inc., USA). Categorical data of tadpole NP development were analysed using Fisher's exact test (a 2X2 contingency table) indicating the correlation between the prevalence (in percentage) of epidermal KHs and dermal BGs between the different testosterone treatments. Qualitative measurements of the amount of epidermal KHs and BGs per mm<sup>2</sup> NP skin surface area were compared between exposure groups using a breakdown and one-way ANOVA. Measurements were regarded significantly different from the control at a p-value < 0.05.

## **RESULTS**

### *Histology of stimulated and inhibited adult male breeding glands (BGs)*

The NP epidermis of adult male *X. laevis* frogs stimulated with hCG contained epidermal glands, which represented BGs as reported by other authors (Thomas *et al.*, 2003; Fujikura *et al.*, 1988). These glands were ovoid in shape and lined with multi-cellular secretory cell (SC) epithelium (Fig. 2.1A). These SCs were polarised (the nucleus situated at the basal portion of the cell) and columnar in shape, containing eosinophilic secretory product (Fig. 2.1A). The basal nuclei were aligned parallel to the basement membrane of the gland (Fig. 2.1A). The SCs were aligned in an orderly manner around the lumen of the gland and densely packed, as indicated by the close proximity of adjacent cell nuclei and distinctive cell membranes (Fig. 2.1A). Surface keratinized epidermal hooks (KH) were associated with breeding gland occurrence, and occur at the surface of the epidermis where these glands are situated (Fig. 2.1A).

Frogs treated with the AR antagonist (flutamide) showed a decreased SC height, which were more cuboidal in shape (Fig. 2.1B). The cytoplasm of the SCs still contained eosinophilic secretory content (Fig. 2.1B) as shown for SCs in a stimulated BG (Fig. 2.1A). Cells surrounding the intermediate region (IR) of the gland appeared to consist of a different type of single layered epithelial cells than epithelium lining the lateral and dorsal sides of the BG (Fig. 2.1B). Few or no KHs were seen at the surface of the epidermis as compared to control-treatment frogs (Fig. 2.1B).

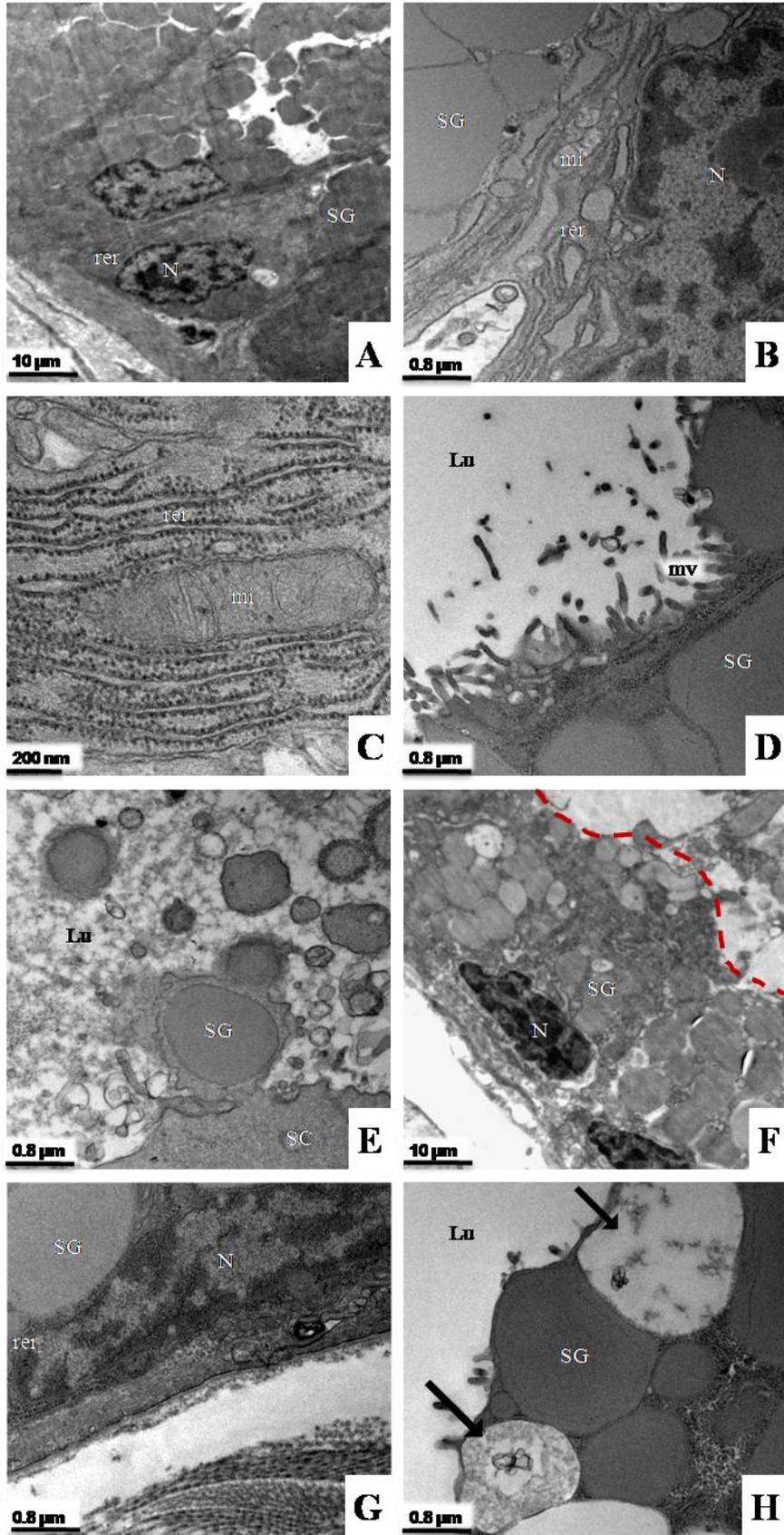


**Figure 2.1:** Photomicrographs (100x) of an induced (hCG-exposed, **A**) and inhibited (flutamide-exposed, **B**) BG in adult male *X. laevis* frogs. The hCG-exposed NP (**A**) include more keratinised hooks (KH) on the epidermis (EP) and underlying BGs present increased secretory cell epithelium (SC) development compared to flutamide-exposed frogs (**B**). A clear lumen (L) and duct (D) can be seen in both glands of the exposure groups. Regions of the SC in the flutamide-exposed frogs situated close to the IR showed a difference in epithelial cell type compared to other SCs of the BG (arrows). Scale bar: 100 µm.

#### *Transmission electron microscopy (TEM) of stimulated and inhibited adult male breeding glands (BGs)*

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. 2.2A). The nucleus located at the basal portion of BG SCs exhibited prevalent diffuse chromatin (euchromatin) content (Figs. 2.2A & B). The density of the chromatin differed between adjacent cell nuclei; indicating asynchronous cell secretion activity (Fig. 2.2A). 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. 2.2B & 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. 2.2D). 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. 2.2E).

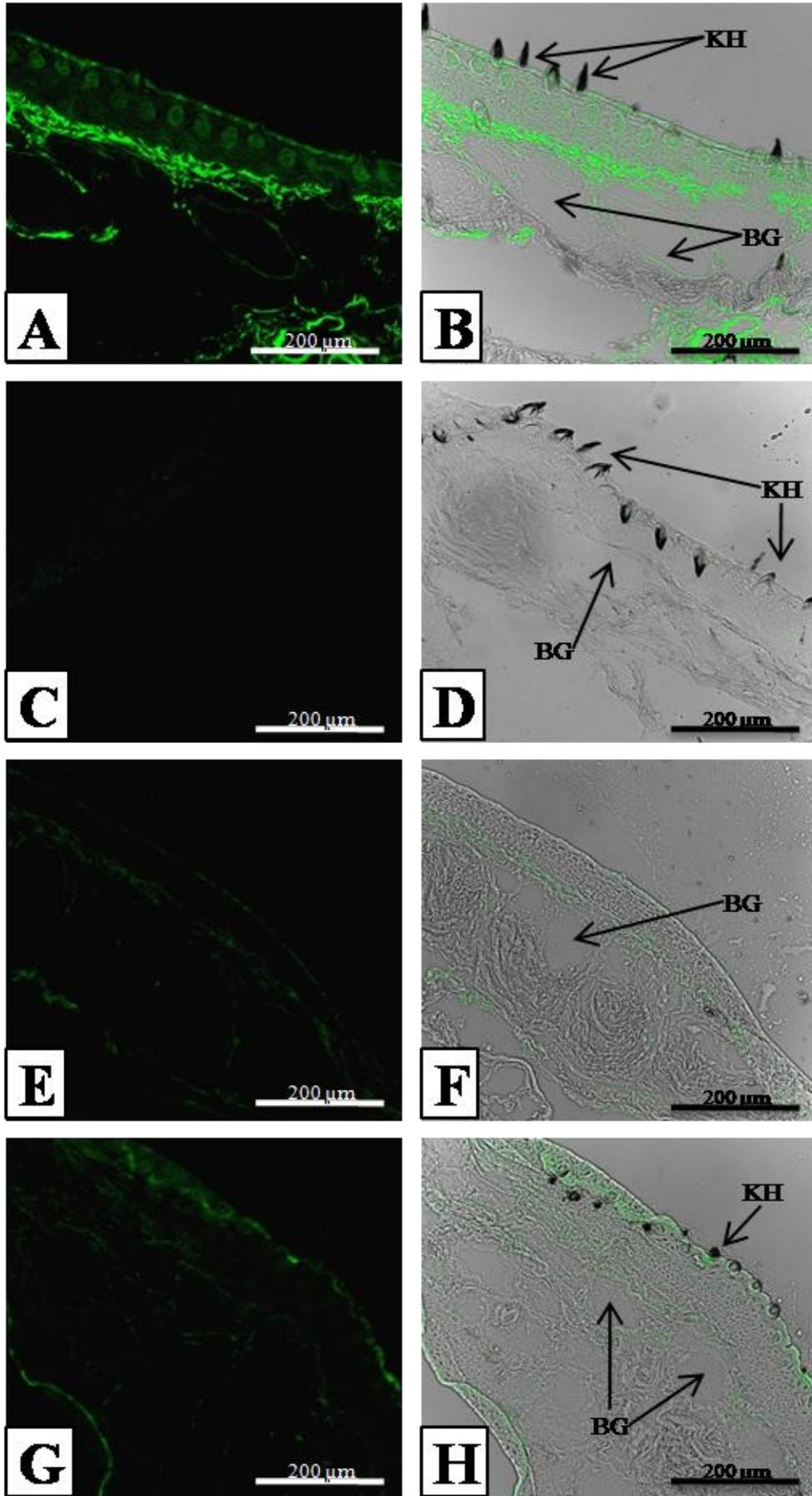
Ultrastructure of the SC epithelium in flutamide-treated frogs showed less densely packed SGs occupying the cell cytoplasm as well as a less differentiated cell membrane dividing adjacent cells (Fig. 2.2F). The basal portion of these cells showed less visible rer and mitochondria surrounding the nucleus (Fig. 2.2G). The nucleus appeared pyknotic in shape with more prevalent heterochromatin content and less visible nuclear pores (Figs. 2.2F & G). The apical region of the secretory cells showed smaller SGs as compared to a stimulated BG as well as the presence of a different type of SGs and less electron dense secretory product (Fig. 2.2H).



**Figure 2.2:** 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.

### *Immunohistochemistry*

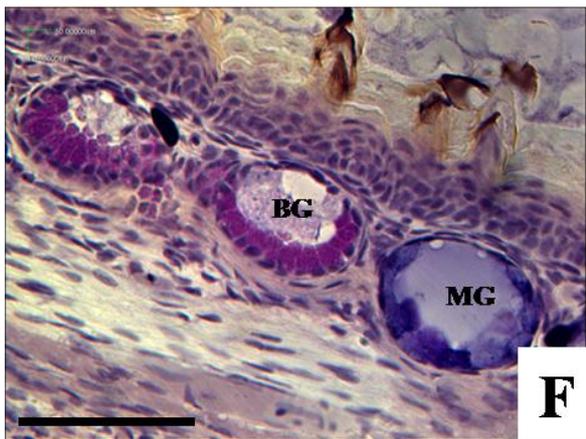
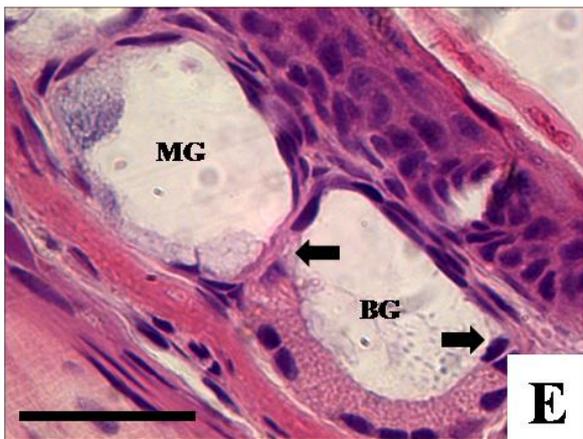
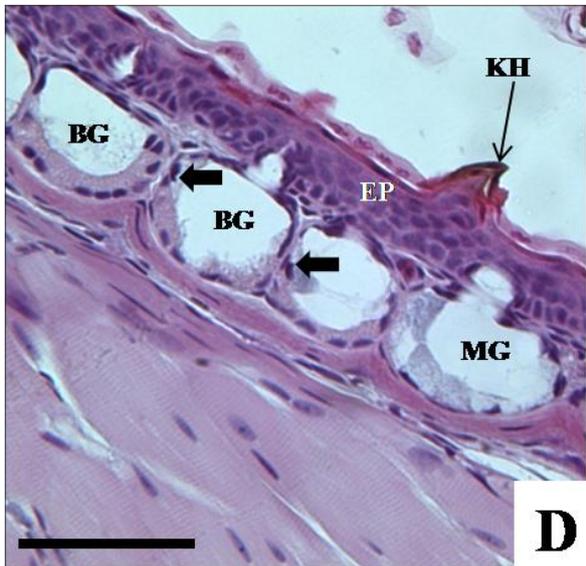
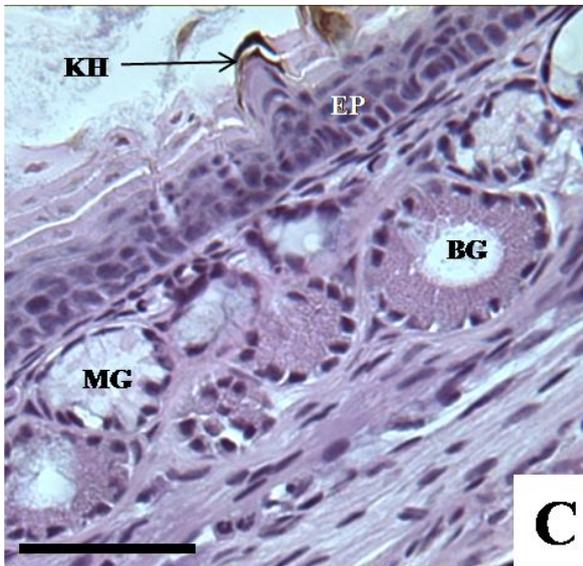
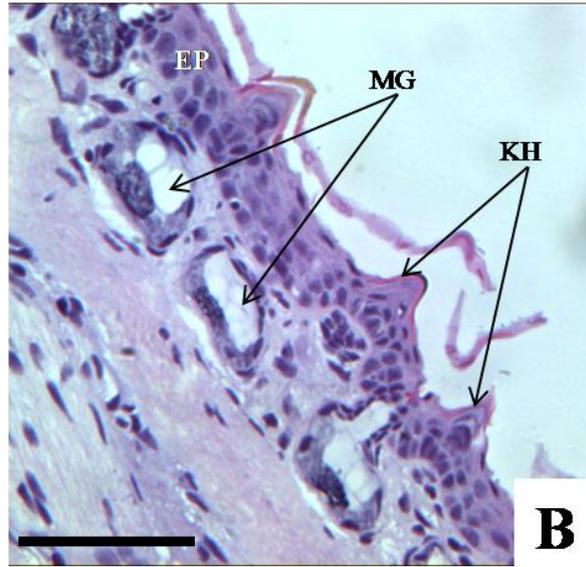
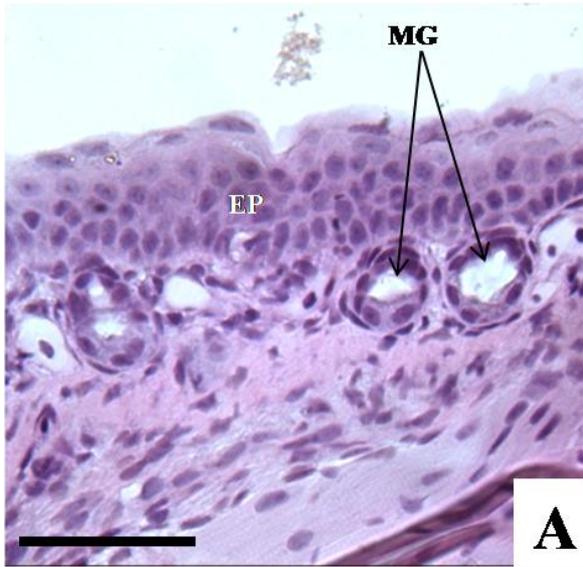
Adult male frogs exposed to hCG showed high levels of AR immunopositive nuclei in the epidermis, keratin hooks KHs and breeding gland epithelium (Figs. 2.3A & B). Skin sections which were treated with PBA lacking the primary PG-21 antibody showed to generate little or no AR-positive immunostaining (Figs. 2.3C & D). Skin sections of the NP epidermis exposed to either the AR antagonist flutamide (Figs. 2.3E & F) or the dithiocarbamate fungicide mancozeb (Figs. 2.3G & H) showed low expression of AR-immunolocalization at the epidermis, KHs and breeding gland epithelium compared to the hCG-treatment (Figs. 2.3A & B).



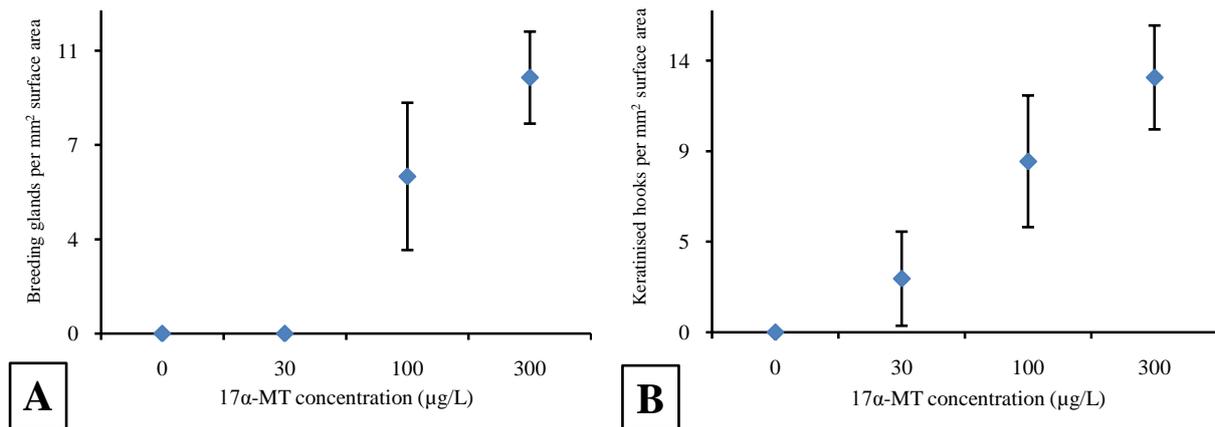
**Figure 2.3:** Immunohistochemical visualization of the distribution of AR in the NP area of adult male *X. laevis* frogs exposed to an hCG control (A & B), negative stained control (C & D), flutamide pharmaceutical (E & F) and mancozeb fungicide (G & H). The negative stained control (C & D) represents 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  $\mu\text{m}$ .

#### *Pre-metamorph tadpole breeding gland (BG) development response*

The NP region in control pre-metamorphic tadpoles (NF 59-62) showed the exclusive presence of dermal mucous glands (Fig. 2.4A). 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. 2.4A). Skin sections of the NP integument surface of tadpoles treated with 30  $\mu\text{g/L}$  17 $\alpha$ -MT reveal the prevalence of KHs with underlying glands resembling a mucous gland-type (Fig. 2.4B). Tadpoles exposed to 100 and 300  $\mu\text{g/L}$  17 $\alpha$ -MT exhibited a higher frequency of KHs on the skin surface as well as the presence of both mucous and breeding gland types (Figs. 2.4C & D). Some of the sub-epidermal glands in tadpoles from the 300  $\mu\text{g/L}$  17 $\alpha$ -MT treatment also showed a mixed-type of gland, which included secretory cell epithelium resembling both mucous and breeding gland-types (Figs. 2.4D & E, arrows). The presence of BGs in the 300  $\mu\text{g/L}$  17 $\alpha$ -MT treatment was also confirmed by showing breeding gland secretory cell epithelium stained highly positive for PAS (Fig. 2.4F). Categorical data indicated a significant correlation between the prevalence of KHs and BGs with increasing testosterone treatment (Fisher's exact test,  $p < 0.05$ ) with the highest density of BGs and KHs per  $\text{mm}^2$  NP skin surface area observed in the 300  $\mu\text{g/L}$  treatment group (Figs. 2.5A & B).



**Figure 2.4:** Development of nuptial BGs and epidermal keratin hooks in pre-metamorphic *X. laevis* tadpoles (NF 59-62) given either a DMSO supplement (**A**) or a  $17\alpha$ -MT supplement of 30  $\mu\text{g/L}$  (**B**), 100  $\mu\text{g/L}$  (**C**) or 300  $\mu\text{g/L}$  (**D, E & F**). Some of the sub-epidermal glands in tadpoles from the 300  $\mu\text{g/L}$   $17\alpha$ -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  $\mu\text{g/L}$  treatment (**F**). Labels: MG, mucous gland; BG, breeding gland; KH, keratin hooks; EP, epidermis. Scale bar: **A, B, C, D, F** (100  $\mu\text{m}$ ); **E** (40  $\mu\text{m}$ ).

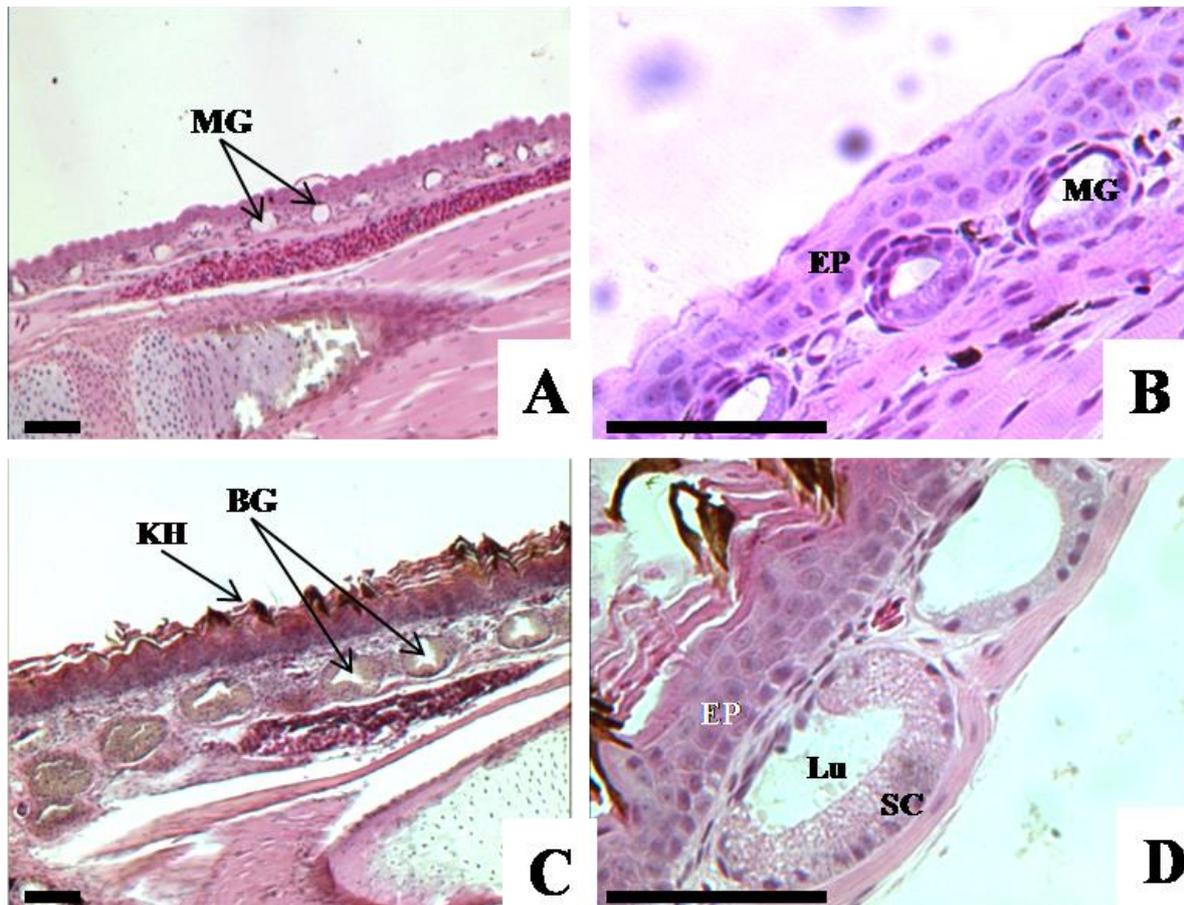


**Figure 2.5:** Occurrence of the breeding gland density per mm NP surface area (A) and epidermal KH density per mm NP surface area in pre-metamorphic tadpoles (NF stage 56-59; n = 10 per treatment group) exposed to different concentration ranges of  $17\alpha$ -MT. Values are expressed as mean ( $\pm$  SD).

#### *Juvenile metamorph (froglet) 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. 2.6A & B). Serous and/or other specialized gland types were absent in the skin sections of the control froglets (Fig. 2.6A). 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. 2.6B). Juveniles subjected to  $17\alpha$ -MT treatment developed epidermal KHS in the NP skin surface (Fig. 2.6C). Underlying glands only resembled a breeding gland type, along with the absence of mucous and serous gland types (Fig. 2.6D). 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. 2.6D). These

glandular characteristics resemble BGs such as found in the NP area of sexually mature adult male *X. laevis* frogs.



**Figure 2.6:** 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  $\mu$ m.

## DISCUSSION

### *Adult male NP development*

The description and localization of BGs in *X. laevis* observed during the present study correspond to other breeding gland studies in other frog species (Fujikura *et al.*, 1988; Thomas *et al.*, 1993; Brizzi *et al.*, 2002; Van Wyk *et al.*, 2003; Kaptan & Murathanoğlu, 2008). The influence of steroid hormones upon breeding gland development has been investigated previously (Rastogi & Chieffi, 1975; Thomas *et al.*, 1993; Lynch & Blackburn,

1995; Epstein & Blackburn, 1997; Emerson *et al.*, 1999). Androgen treatment can increase AR mRNA expression in BGs (Varriale & Serino, 1994) and also cause increased gland epithelium and epidermal thickness of the NP integument (Lynch & Blackburn, 1995). This increase in NP development caused by exogeneous androgens correlate to normal elevation of epidermal layers and breeding gland epithelium during the breeding season when androgens are naturally at higher levels (Kaptan & Murathanoğlu, 2008).

One of the aims of the present study was to validate male breeding gland activity in adult male *X. laevis* frogs as androgen-sensitive biomarkers. In the present study, flutamide exposure significantly inhibited breeding gland development. Several possible outcomes may explain the reduction of breeding gland secretory cell epithelium in flutamide-exposed frogs. Ultrastructural analyses of breeding gland secretory cells showed the presence of a larger percentage of heterochromatin in the flutamide-treated frogs, which indicates less transcriptionally active chromatin, leading to less protein synthesis in the cells. Fewer mitochondria organelles were also present at the epinuclear portion of the cell. Androgen levels have been shown to regulate mitochondria biogenesis (Traish *et al.*, 2011). Damage or loss of mitochondria can lead to membrane potential heterogeneity and oxidative stress, all of which can lead to an increase in free radicals and cell apoptosis (Traish *et al.*, 2011). Furthermore, it has been shown in AR-deficient mice that these organisms express low levels of the mitochondrial co-activator, PGC-1 $\alpha$ , which is a component necessary for mitochondrial biosynthesis (Fan *et al.*, 2005). In the current study, AR deficiency at the NP integument was also recorded in frogs exposed to flutamide and the dithiocarbamate fungicide mancozeb. If the same scenario in the present study was found as reported by Fan *et al.* (2005), the inhibited AR receptor functionality will eventually lead to less mitochondrial biosynthesis and consequently to less ATP production and oxidative stress in the cells, leading to their degradation. However, more research is needed to fully support this statement.

Histological investigation of the BGs in flutamide-exposed frogs also showed some epithelial cells and secretory granule content which more closely resemble a mucous gland-type. Mucous glands are the most abundant glandular type found in amphibian skin (Epstein & Blackburn, 1997; Brizzi *et al.*, 2002). Two different epithelial cell types have been observed in mucous glands under TEM, with the majority being the secretory cells located at the lower

region of the gland enclosed by a secretory compartment containing the secretory product of the cells. The second type of epithelial cells was located more towards the duct, and possibly plays a role in yearly cell renewal (Parakkal & Ellis, 1963). Furthermore, several studies proposed that BGs are specialised forms of mucous glands, as they share structural and histochemical similarities with BGs (Thomas *et al.*, 1993; Epstein & Blackburn, 1997; Brizzi *et al.*, 2002; Kaptan & Murathanoğlu, 2008; Siegel *et al.*, 2008; Brunetti *et al.*, 2012; Luna *et al.*, 2012). In flutamide-treated frogs of the present study, basic histology and electron microscopy indicated that some of the BGs also showed two types of epithelial cells in the same arrangement as reported for mucous glands by Parakkal & Ellis (1963). It may be possible that flutamide treatment (AR antagonism) might cause a transition of specialised mucous glands (BGs) to ordinary mucous glands or that flutamide caused premature cell renewal processes in these glands. Nevertheless, the development of BGs was successfully suppressed when frogs were treated with a model AR antagonistic EDC. Environmental pollutants which mimic the endocrine disrupting action of flutamide (AR antagonism) may therefore cause the same modulating effect upon breeding gland development.

#### *NP immunohistochemistry*

A study conducted by Emerson *et al.* (2003) indicated that high levels of AR are present in both the BGs and KHs of the NP integument in male anuran species. During the present study, it was reported that exposure to an AR antagonistic compound (flutamide) can cause a decline in nuclear AR localization in the NP area. Furthermore, the present study also showed that the dithiocarbamate fungicide, mancozeb, reduced AR immunopositive nuclei of the NP area in a similar manner than flutamide. Due to the fact that the NP as a whole is regarded as an androgen dependent characteristic, competitive binding of an AR antagonist to the AR at these regions will therefore lead to lowered transcription of androgen-related genes and therefore a reduction in NP development. Similar immunological results have been obtained in rats exposed to a xenobiotic EDC such as the carboximide fungicide, vinclozolin, which caused a similar decline in nuclear AR localization in the epididymides of rats as flutamide (Kelce *et al.*, 1997). While vinclozolin has been extensively shown as serving as an AR antagonist (both *in vitro* and *in vivo*), the anti-androgenic properties of mancozeb has been less documented. However, it was shown that mancozeb caused AR antagonism in a transfected yeast anti-androgenic screen (anti-YAS) with higher potencies than either vinclozolin or flutamide (Archer & Van Wyk, article in for review in Water SA; Chapter 4).

The present study therefore proved that the AR antagonistic response of mancozeb *in vitro* can also be shown to cause an AR antagonistic response *in vivo* within the sexually dimorphic skin of male *X. laevis* frogs.

#### *Biological function of breeding glands (BGs)*

It is suggested that due to the loss of a vocal sac in *Xenopus* species, some other specialised function for courtship is in play for these anurans (Brizzi *et al.*, 2002). Due to the androgen dependency for their development, as well as the localization of BGs at the region of clasping behaviour, it is safe to assume that these glands play some role in courtship. Breeding glands are hypothesised to be involved in either chemical signalling or reproductive behaviour (Thomas *et al.*, 1993; Brizzi *et al.*, 2002). The parallel proliferation of the secretory compartment within these glands, along with the development of the epidermal KHs suggests that both these structures are necessary for male reproductive behaviour. While several authors suggested that BGs produce an adhesive substance necessary for the clasp reflex or to produce a mucous substance to moisten the KHs of the NP (Thomas *et al.*, 1993), other authors suggested that these glands are used for social or reproductive chemical communication during reproductive periods by attracting receptive females, or to express territoriality between males (Pearl *et al.*, 2000; Brizzi *et al.*, 2002). Nonetheless, the proliferation of these glands is undoubtedly a necessary component in anuran reproduction. Modulation in the development of these glands could therefore alter courtship and eventually cause significant effects on population dynamics.

#### *Premature development of breeding glands (BGs)*

The second major aim of the present study was to investigate the premature development of BGs in pre-metamorphic *X. laevis* tadpoles and juveniles to be used as a biomarker for exogenous (xenobiotic) androgen stimulation. Qualitative and quantitative data suggested that epidermal KHs and BGs will proliferate along with increasing concentrations of  $17\alpha$ -MT. These findings are in accordance with previous studies showing the premature development of the NP area in *S. tropicalis* (Olmstead *et al.*, 2012) and *X. laevis* tadpoles exposed to exogenous androgens (Chang & Witschi, 1956). Evidence published to date therefore suggests the potential for exogenous androgens or androgen mimics to prematurely initiate the expression of the epidermal KHs and BGs.

The present study showed that secretory cells of mucous glands could be transformed to modified cells resembling breeding gland epithelium after exposure to androgens. These findings support the first hypothesis by Brunetti *et al.* (2012), which stated that mucous glands primarily occur within the NP area of immature frogs and a breeding gland-type of secretory epithelium develops afterwards during androgen stimulation, or by the normal increase of androgens in male sexual differentiation.

Although the time of development and androgen dependence of the NP is well documented in adult frogs (Saidapur & Nadkarnai, 1975; Kurabuchi, 1993; Lynch & Blackburn, 1995; Epstein & Blackburn, 1997; Van Wyk *et al.*, 2003), these studies mostly focused on the development of epidermal KHs giving the darkened appearance of the NP. The present study is the first to report on the development of underlying BGs in the NP area of sexually immature *X. laevis* frogs. Although it was noted that the epidermal KHs started to develop at lower concentrations of androgen treatment, we suggest that the underlying BGs may serve as a more definite indicator of masculinisation. Breeding glands and epidermal KHs may then be used to indicate various severities of androgen modulation and may also be used along with other SSCs as well as testicular germ cell development (spermatogenesis) and plasma hormone levels to characterise androgen modulation in *X. laevis* frogs.

Other SSCs such as the sexually dimorphic larynx has also been shown to be prematurely induced in *X. laevis* using an exogeneous androgen treatment (Sassoon & Kelley, 1986). In adult frogs, the larynx as well as the epidermal KHs and BGs (the NP) develop as circulating androgens increase in male frogs (Sassoon & Kelley, 1986). These findings are supported by the presence of high levels of androgen receptor (AR) immunopositive loci located in both the larynx and NP integument (Sassoon *et al.*, 1987; Emerson *et al.*, 1999). Androgen dependence of the NP (both breeding glands and KHs) has also been confirmed with the regression of these structures following the castration of adult males (Kelly & Pfaff, 1976; Fujikura *et al.*, 1988) and exposure to the AR antagonistic (anti-androgenic) pharmaceutical flutamide and selected environmental chemicals (Van Wyk *et al.*, 2003; Hayes *et al.*, 2010).

Based on the close relationship between the larynx and NP development during reproductive activity, it is therefore evident that reports on increased masculinisation of either the sexually dimorphic larynx or the epidermal KHs of *X. laevis* frogs will also be in conjunction with

premature development of dermal BGs in the NP region. Development of SSCs has been shown to be controlled by steroid hormones and steroid metabolising enzymes which allow their action upon target tissues (Sassoon *et al.*, 1987). Furthermore, androgen responsive tissues such as SSCs are characterised by having high levels of ARs (Sassoon & Kelley, 1986; Emerson *et al.*, 1999). Taken these factors into account, the stimulation of BGs may be explained by the presence of ARs at early tadpole stages in the NP area as well as the increasing levels of androgens which are responsible for SSC development at later-stage juveniles. An exogenous androgen treatment might then primarily trigger early reproductive development and sexual differentiation in immature frogs. Nevertheless, the development of dermal BGs associated with development during reproductive activity in adult male frogs can be prematurely induced, which holds the possibility to use this SSC as a biomarker of androgenic endocrine disruption when assessing environmental pollutants.

#### *Potential for using breeding glands (BGs) as biomarkers*

Several male characteristics in amphibians such as gonadal differentiation, secondary sexual characteristic (SSC) development and breeding behaviour have been investigated as potential biomarkers of exposure to environmental EDCs due to their role in normal reproduction (Kloas & Lutz, 2006). The modulation of steroidogenesis as the main controlling pathway during reproduction could potentially affect individual reproductive success in the short term but also population survival in the longer term (Kloas & Lutz, 2006). Aquatic animals are more likely to be exposed to EDCs due to their occurrence in polluted water systems (Carey & Bryant, 1995; Kloas *et al.*, 2009). Amphibians can serve as good sentinel species of EDC contamination in a water system due to their direct contact with contaminated water and their hormonal system being more closely related to higher vertebrates than fish. However, it is evident that the effects of anti-androgenic compounds in water systems and effects on the amphibian male reproductive system are not well understood (Behrends *et al.*, 2010; Hoffman & Kloas, 2010).

The present study provided added information to use male-specific BGs as a sufficient *in vivo* biomarker of androgenic or anti-androgenic endocrine disruption studies in both adults as well as in premature *X. laevis* frogs. Due to the regular laboratory usage of these species globally (Hecker *et al.*, 2004; Opitz *et al.*, 2005; Urbatzka *et al.*, 2007a; Kloas, 2009; Hoffmann & Kloas, 2010) as well as their endemic dispersal in their natural habitats (Van

Wyk *et al.*, 2005; Du Preez *et al.*, 2009), these frogs can serve as a suitable sentinel species showing several pathways of endocrine disrupting activities caused by environmental pollutants.

### *Conclusion*

Development of male-specific, androgen-regulated BGs in adult male *X. laevis* frogs were shown to be reduced when exposed to anti-androgenic compounds. These glands are therefore sensitive in serving as a biomarker of anti-androgenic endocrine disruption studies. Furthermore, the binding of androgens to the AR at the NP integument seem to be a key factor regulating the development of BGs. Compounds modulating breeding gland development may then be ascribed as having an AR antagonistic mechanism of endocrine disruption. A further finding was that these glands may be prematurely stimulated in tadpoles and juveniles when given an exogenous testosterone supplement, giving the possibility of using this SSC to detect androgenic or anti-androgenic endocrine disruption using various life stages of *X. laevis* frogs.

### **CHAPTER 3: EFFECTS OF THE ANDROGEN RECEPTOR-BINDING INHIBITOR, FLUTAMIDE, AND DUAL 5 $\alpha$ -REDUCTASE INHIBITOR, DUTASTERIDE, ON EPIDERMAL BREEDING GLANDS AND REPRODUCTIVE ENDPOINTS IN MALE *XENOPUS LAEVIS* FROGS.**

#### **ABSTRACT**

Several man-made compounds that may end-up in freshwater systems are known to disrupt the reproductive endocrine system of wildlife species and humans. These compounds are referred to as gonadal endocrine disrupting contaminants (EDCs). Although the disruption of the estrogenic endocrine system has been extensively studied in the past, research has shifted towards investigating compounds which might modulate androgenic endocrine pathways. Agricultural fungicides and chemicals in industrial effluents have been suggested to specifically disrupt androgenic pathways, but mostly assuming a mechanism of action related to competitive binding to the androgen receptor (AR). However, compounds with an anti-androgen action may disrupt primary and/or secondary sexual traits at levels other than binding to AR. An example is chemicals inhibiting the metabolizing enzyme 5 $\alpha$ -reductase (5 $\alpha$ R), which is known to convert testosterone (T) to the more biologically active, dihydrotestosterone (DHT). In the present study we used *in vivo* biomarkers associated with reproduction in adult male *Xenopus laevis* frogs to evaluate differential responses when exposed to an AR antagonist (flutamide) and a dual 5 $\alpha$ -reductase inhibitor (dutasteride). The biomarkers used included the development of male-specific breeding glands, spermatogenesis and plasma steroid hormone levels. Following exposure, breeding gland activity was found to be inhibited by both anti-androgenic compounds. Breeding gland size and gland epithelial height were reduced when compared to a control, suggesting that both AR binding and 5 $\alpha$ R play key roles in nuptial gland development. Flutamide treatment caused a reduction in spermatogenesis and dutasteride treatment significantly increased plasma T but led to a decrease in plasma DHT levels. These results suggest a significant blockage of androgen metabolism and potentially also an altered negative feedback effect. The present study confirms that although *X. laevis* breeding gland activity can be used as a biomarker endpoint in the screening for anti-androgenic endocrine disruption, gland histological changes may not differentiate between the specific mechanism of anti-androgenic action (AR antagonism or 5 $\alpha$ R inhibition).

**Key Words:** Anti-androgen; breeding gland; spermatogenesis; dutasteride; flutamide

## INTRODUCTION

Several natural and xenobiotic compounds found in the environment have been shown to modulate several pathways within the endocrine system in wildlife species and humans. These compounds may end up in water sources by means of spray drift, water runoff and/or direct disposal and are collectively referred to as endocrine disrupting contaminants (EDCs). Apart from the potential harm that these chemicals might pose (directly or indirectly) to human reproduction or development, aquatic animals occurring in polluted water systems are very likely to be exposed to EDCs. This prediction is supported by several studies in the laboratory and field that reported reproductive and developmental disruption in fish (Jobling, 1998; Skolness *et al.*, 2011) and amphibians (Carey & Bryant, 1995; van Wyk *et al.*, 2003; Hecker *et al.*, 2005; Hayes *et al.*, 2010). Aquatic animals can therefore serve as sentinels (early warning systems) for pollution in surface waters (Kloas *et al.*, 2009; Jobling *et al.*, 2009), contributing to the knowledge of understanding the threats of EDCs in receiving waters, sourced for wildlife and human consumption.

Pollutants which modulate the gonadal endocrine system in vertebrates have received the most attention in endocrine disruption studies. In theory, EDCs causing gonadal endocrine disruption can be classified as being either being estrogenic, anti-estrogenic, androgenic or anti-androgenic (Behrends *et al.*, 2010). Although chemicals having an estrogenic response are extensively reported on, androgen disruption-relevant data for aquatic organisms are limited (Jobling *et al.*, 2009). Research suggests that early life exposure of humans to environmental chemicals may result in endocrine disorders associated with the male reproductive system such as testicular dysgenesis syndrome (TDS), cryptorchidism, hypospadias, decreased penile length, reduced sperm quality and testicular cancer (Skakkebæk *et al.*, 2001; Orton *et al.*, 2011). These reproductive and developmental deformities can be explained by chemicals affecting normal steroid and/or thyroid hormone synthesis, transport, targeted receptor binding or metabolism.

The initial human-related reports of male gonadal endocrine disruption led to investigation into EDCs using rat models with the focus on compounds modulating the androgen receptor (AR) (Kelce *et al.*, 1994; Kelce *et al.*, 1997). The role of the AR is to bind to circulating androgens and activate the transcription of target genes, which are involved in male

development, reproduction and mating behaviour (Thornton & Kelley, 1998). Potentially, EDCs may act as AR agonists, by binding to the AR, resulting in an increased transcription of target genes (Parks *et al.*, 2001; Morthorst *et al.*, 2010; Ekman *et al.*, 2011). In contrast, EDCs may act as AR antagonists, by competing with androgens to bind to the AR, and resulting in decreased transcription of target genes. Examples of such anti-androgens include flutamide, a pharmaceutical AR antagonist used for prostate cancer treatment (Behrends *et al.*, 2010; Hoffmann & Kloas, 2010) and pesticides (specifically DDE, the DDT metabolite, and several fungicides) which have been shown to express similar anti-androgenic effects than that of flutamide (Colborn *et al.*, 1993; Kelce *et al.*, 1994; McKinlay *et al.*, 2008).

Although interactions of EDCs with the AR seem to have been the first line of most studies to date, previous studies have shown that secondary sexual characteristics (SSCs) which regulate reproductive behaviour and ensure correct gonadal endocrine functioning in males may be primarily controlled by enzymatic metabolism of steroid hormones (Chen *et al.*, 2009; Urbatzka *et al.*, 2009). The steroid hormone testosterone (T) is known to be converted to dihydrotestosterone (DHT) by the enzyme 5 $\alpha$ -reductase (5 $\alpha$ R) (Pralhada *et al.*, 1997; McConnel & Stoner, 2001; Chen *et al.*, 2009). DHT has been shown to dissociate much slower from the AR and is therefore regarded as a more potent androgen agonist than T in SSC expression and maintenance (Pralhada *et al.*, 1997; McConnel & Stoner, 2001; Urbatzka *et al.*, 2009; Wu *et al.*, 2013).

Three isoforms of 5 $\alpha$ R exists, namely type 1 (srd5a1), type 2 (srd5a2) and type 3 (srd5a3), and are shown to be present in different tissues (Xu *et al.*, 2006; Chen *et al.*, 2009; Wu *et al.*, 2013). In humans, srd5a1 is shown to be present in the liver and skin, whereas srd5a2 and srd5a3 are present in the prostate (Wu *et al.*, 2013). An over-expression of these isoforms in specific tissues has been hypothesized to lead to disorders such as acne, hair loss, benign prostatic hyperplasia (BPH) and prostate cancer (Amory *et al.*, 2008; Andriole *et al.*, 2010; Wu *et al.*, 2013). The pharmaceutical 5 $\alpha$ R inhibitor, finasteride, has affinity for only srd5a2, hence its usage for treatment of BPH. However, another 5 $\alpha$ R inhibitor, dutasteride, has been shown to inhibit both srd5a1 and srd5a2 and is therefore used for prostate cancer treatment and male-pattern hair loss (Xu *et al.*, 2006; Wu *et al.*, 2013). Dutasteride treatment in animal screening studies should therefore serve as the preferred control treatment for 5 $\alpha$ R inhibition, since it is predicted to facilitate inhibition of multiple isoforms of 5 $\alpha$ R.

With regard to environmental EDC pollution, some studies have been done investigating 5 $\alpha$ R inhibition in rats (Christiansen *et al.*, 2009) and frogs (Urbatzka *et al.*, 2009) using finasteride as the positive control. Exposing African clawed frog, *Xenopus laevis* tadpoles to finasteride resulted in the upregulation of *srd5a2* gene expression in the testis as well as altered negative feedback mechanisms on T (Urbatzka *et al.*, 2009). This opens up the possibility that some anti-androgenic environmental pollutants (EDCs) may mimic the action of 5 $\alpha$ R inhibitors and therefore modulate steroid metabolism rather than modulation of steroid binding to the AR (Christiansen *et al.*, 2009). Modulation of 5 $\alpha$ R enzymes may be another type of gonadal endocrine disruption mechanism of action, which has been largely underestimated in EDC studies concerning environmental androgen modulation. However, although some animal studies considered the 5 $\alpha$ R inhibition pathway/mechanism of action, it needs to be confirmed as biomarker for inhibitory pathway action in the male reproductive endocrine system.

Several primary male traits in amphibians, including gonadal maturation (spermatogenesis), blood-hormone levels and breeding behaviour have been investigated to serve as biomarkers for gonadal endocrine disruption (Kloas & Lutz, 2006). The modulation of these male traits could potentially affect individual reproductive success in the short term but also population structure and survival in the longer term (Kloas & Lutz, 2006). Furthermore, androgens are responsible for the development of secondary sexual characteristics (SSCs) in male frogs (Saidapur & Nadkarni, 1975; Kaptan & Murathanoğlu, 2008). These SSCs include vocal chords as well as the development of specialized patches of skin (nuptial pads; NP) on their front extremities during the breeding season (Fujikura *et al.*, 1988; Thomas *et al.*, 1993). These NP skin patches are recognized by dark keratinized hooks (KHs) on the epidermal surface and subepidermal specialized mucus glands (SMG) (Fujikura *et al.*, 1988; Thomas *et al.*, 1993; Brizzi *et al.*, 2003; Van Wyk *et al.*, 2003; Brunetti *et al.*, 2013) also referred to as breeding glands (Fujikura *et al.*, 1988). It has been shown that AR antagonists, such as finasteride and the fungicide vinclozolin, can suppress the development of breeding glands in male *X. laevis* frogs (Van Wyk *et al.*, 2003). However, although both the epidermal KHs and breeding glands have been shown to exhibit high levels of AR expression and dependence on androgens for their development and activity (Saidapur & Nadkarni, 1975; Fujikura *et al.*, 1988; Thomas *et al.*, 1993; Emerson *et al.*, 1999; Brizzi *et al.*, 2002; Van Wyk *et al.*, 2003; Kaptan & Murathanoğlu, 2008), it is still not known whether breeding gland development is

controlled by DHT rather than T. The details of the control pathway via androgen binding to ARs and/or the transformation of T to DHT facilitated by the enzyme 5 $\alpha$ R needs more research. Although the role of breeding glands in amphibian reproductive behaviour is not well-understood, knowledge about the morphology and hormonal control of breeding glands will change this situation. Understanding the control pathways of male breeding glands will also evaluate the usefulness of these male traits as biomarkers for xenobiotic anti-androgenic endocrine disruption.

The aim of this study was to expose male *X. laevis* frogs to known anti-androgens, flutamide, a pharmaceutical AR antagonist, and dutasteride, a dual isoform 5 $\alpha$ R inhibitor. Changes in plasma hormone levels along with androgenic endpoints, including spermatogenesis and breeding gland development were evaluated.

## MATERIALS AND METHODS

### *Test chemicals and animals*

The chemicals used in the study included: human chorionic gonadotropin (hCG; Ovidrel, 250  $\mu$ g, Serono), flutamide (CAS No. 13311-84-7, Sigma) and dutasteride (Avodart 0.5 mg; GalaxoSmithCline, Brentford, UK). Dimethylsulphoxide (DMSO, >99%, Sigma) was used as solvent for test chemical exposure. Thirty two adult male *X. laevis* frogs ( $\pm$  36 g) were obtained from a commercial dealer that caught the frogs from two local farms (33°50.075'S, 018°52.375'E and 33°49.639'S, 018°52.739'E) near Klapmuts in the Western Cape. The frogs were kept in an acclimatized room for four weeks under typical summer climatic conditions (20°C, 14 h light: 10 h dark cycle). Activated charcoal filtered drinking water was used as water medium and water was replaced a day after feeding them with trout pellets (NutroScience, Malmesbury, South Africa) once a week. Ethical clearance for the project was obtained from the University of Stellenbosch Research Ethics Committee for animal care and use (SU-ACUM12-00013).

### *Exposure*

Thirty-two adult male *X. laevis* frogs were injected in the dorsal lymph sac (Brown, 1970) with the anti-androgenic chemicals (flutamide and dutasteride, n = 8 per exposure group) once a week for 28 days (Fig. 3.1). In order to artificially stimulate BG development, all frogs

were treated with hCG (Ovidrel, 100 i.u. per frog/week) during the third and fourth week of exposure (Day 14 and 21, Table 3.1; Fig. 3.1). During the final two weeks of treatment, frogs (n = 8) in the control treatment (no anti-androgen exposure) were also treated with hCG (Fig. 3.1). After the two-week hCG injection, control male frogs showed a darkened area on the ventral aspect of their forearms and fingers. The darkening corresponds to the development of the breeding glands and associated epidermal KHs as reported in other literature (Fujikura *et al.*, 1988; Van Wyk *et al.* 2003). Exposure concentrations of flutamide were decided according to similar flutamide exposure of male *X. laevis* frogs in van Wyk *et al.* (2003). Due to the limited research done on dutasteride exposure in aquatic species, half the concentration than the flutamide exposure were selected for the dutasteride exposure (50 µg/g BM), as well as a 10 fold lower concentration than the first exposure concentration (50 µg/g BM).

**Table 3.1:** Chemicals and concentrations used during the 28 day exposure of adult male *X. laevis* frogs. Male frogs (n = 8 per exposure group) were exposed by injection into the dorsal lymph sac to study agonistic and antagonistic effects of two anti-androgenic mechanisms of actions on the male reproductive system as well as breeding gland development and activity. BM, body mass; MOA, mode of action.

Group	n	Trade name	MOA	Concentration (per frog)
Control	8	-	-	-
Flutamide	8	Flutamide	AR antagonist	100 µg/g BM
Dutasteride (5 µg)	8	Avodart	5αR inhibitor	5 µg/g BM
Dutasteride (50 µg)	8	Avodart	5αR inhibitor	50 µg/g BM



Stevens, 1977). Testes were removed and weighed to the nearest 0.001g to calculate a gonado-somatic index (GSI; Eq. 2) and then fixated in Bouin's fixative (Bancroft & Stevens, 1977).

$$\text{GSI (\%)} = \frac{\text{gonad mass}}{\text{body mass}} \times 100 \quad [\text{Eq. 2}]$$

### *Histology*

NP skin and testes samples were washed in water for several hours and subsequently processed for routine paraffin wax histology. Sections were taken at 8  $\mu\text{m}$  using a rotary microtome, de-waxed and stained with Hematoxylin and Eosin (H&E; Bancroft & Stevens, 1977). Digital micrographs of ten breeding glands per NP integument sample (for each individual) were captured at a magnification of 400x using a light microscope (DMD108, Leica). Image analysis (Leica Application Suite, Version 1.5.0, Switzerland) of breeding glands was used to measure gland epithelial heights (average of six measurements per gland) to the nearest micrometer ( $\mu\text{m}$ ). Glandular area was measured to the nearest square micrometer ( $\mu\text{m}^2$ ) and compared between treatments by calculating the average of two perpendicular diameters ( $R_1$  and  $R_2$ ) and using Equation 3 (Van Wyk *et al.*, 2003).

$$\text{Breeding Gland Area (A)} = \pi(R_1 \cdot R_2) \quad [\text{Eq. 3}]$$

Histology of the cystic-type testis in *X. laevis* was assessed according to Rastogi *et al.*, 1986 and Kaptan & Murathanoğlu (2008). Digital micrographs of ten seminiferous (SN) tubules per testis sample were captured at a magnification of 100x using a light microscope (DMD108, Leica) and image analysis software (Leica Application Suite, Version 1.5.0, Switzerland). Germ cell stages were classified as primary and secondary spermatogonia (1° and 2° SPG), spermatocytes (SPC) and spermatids (SPT). Cysts were classified as 1° SPG having large single cells surrounded by a membrane, whereas 2° SPG cysts were classified as having solid oval nuclei with clear nuclear envelopes and smaller in size than 1° SPG. The SPC germ cell stage was characterised as having cells with dispersed chromatin and no clear nuclear envelope. The SPT germ cell stage were identified by having condensed nuclei,

smaller in size than SPCs and darkly stained. Some SPT cell stages were also identified as having elongated cell size leading to spermatozoa (SPZ) formation which was identified as having crescent-shaped tail flagella and situated towards the lumen of the SN tubules. Germ cell stages were compared between treatment groups by calculating a ratio (in percentage) of cysts bearing 1° SPG, 2° SPG, SPC and SPT germ cell types. The average percentage of SPZ per SN tubule area was determined by calculating the area of SPZ relative to the area of the SN tubule.

#### *Plasma hormone analysis*

Plasma T and DHT levels were determined using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (96 well, DRG Instruments GmbH, Germany). For plasma T concentrations, unextracted plasma samples (in duplicate) were diluted 40 fold with PBS containing 0.1% human serum albumin (HSA). 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 has a sensitivity of 0.083 ng/ml for testosterone with low cross-reactivity for other hormones. For the DHT analysis, unextracted plasma samples (in duplicate) were diluted 200 fold with PBS containing 0.1% HSA. The DHT ELISA kit has a sensitivity of 0.019 ng/ml and also a low cross-reactivity for other hormones. Data generated for plasma T and DHT concentrations were also used to calculate the ratio of DHT relative to T (plasma DHT/T) to compare androgen turnover between the different exposure groups.

#### *Statistical Analysis*

All statistical analyses were performed using Statistica 10.0 (StatSoft Inc. USA). All data were tested for normality (Shapiro-Wilk W test) to choose parametric or non-parametric tests. Differences in the average frog BM at the onset (day 0) and completion of the test chemical exposure (day 28) for each treatment group was compared using a one-way analysis of variance (ANOVA). For breeding gland measurements and plasma hormone concentrations, a breakdown and one-way ANOVA was used to show general variance between all treatment groups. If the ANOVA indicated significant variance, a Tukey's honest significant difference (HSD) *post hoc* test was conducted to indicate specific variance between selected treatment groups. For spermatogenesis measurements, germ cell-type percentages in each SN tubule

were transformed using arcsine before analyzing the dataset using one-way ANOVA and Tukey's HSD *post hoc* test. A p-value < 0.05 was taken as significantly different.

## RESULTS

### *Morphometric measurements and organosomatic indexes*

Treatment groups did not vary in initial CDI (day 1, ANOVA,  $p > 0.05$ , Table 3.2) or snout-to-vent length (ANOVA,  $p > 0.05$ , Table 3.2) at the onset of the exposure experiment. The treatment for 28 days also had no effect on CDI among treatment groups (ANOVA,  $p > 0.05$ , Table 2) nor between body condition on day 1 and day 28 or GSI values between treatment groups (ANOVA,  $p > 0.05$ , Table 3.2).

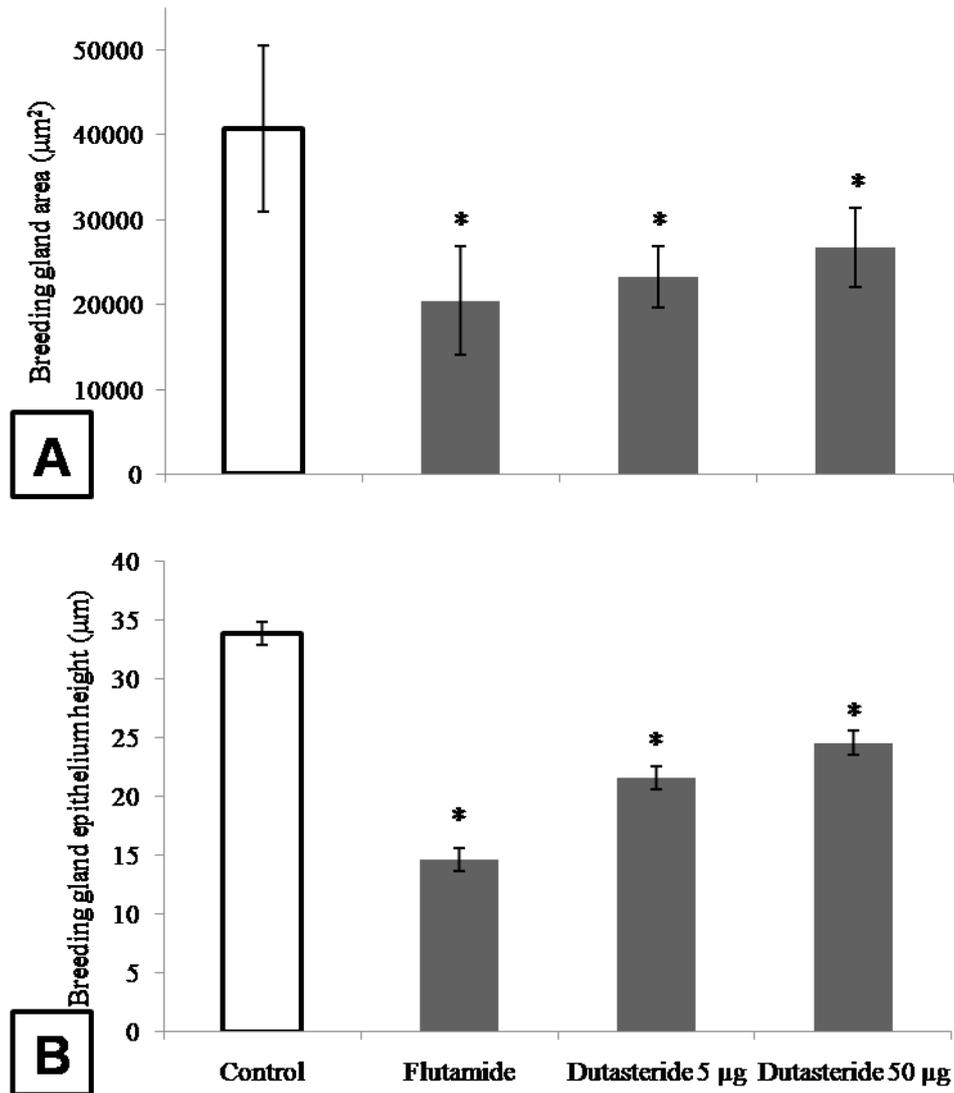
**Table 3.2:** Summary of the morphometric measurement data of adult male *X. laevis* frogs exposed to hCG (control), flutamide and two concentrations of dutasteride (average  $\pm$  SD). Test chemicals were administered by injection on a weekly basis into the dorsal lymph sac of the frog. Exposure lasted for 28 days. Labels: SVL = Snout-to-vent length; BM = Body Mass; GSIndex = Gonadosomatic index; CDIndex = Condition index. Treatment groups: Control = 100 i.u. hCG; Flutamide = 100  $\mu$ g/g body mass + 100 i.u. hCG; Dutasteride 5  $\mu$ g = 5  $\mu$ g /g body mass + 100 i.u. hCG; Dutasteride 50  $\mu$ g = 50  $\mu$ g/g body mass + 100 i.u. hCG.

Test Chemical	n	SVL	Day 0		Day-28		
			BM	CDI	BM	CDI	GSI (%)
Control	8	7.00 $\pm$ 0.38	36.72 $\pm$ 1.61	0.16 $\pm$ 0.02	34.08 $\pm$ 5.49	0.15 $\pm$ 0.05	0.27 $\pm$ 0.06
Flutamide	8	7.08 $\pm$ 0.58	39.65 $\pm$ 6.78	0.17 $\pm$ 0.01	38.05 $\pm$ 6.59	0.16 $\pm$ 0.06	0.27 $\pm$ 0.11
Dutasteride (5 ug)	8	7.31 $\pm$ 0.37	37.89 $\pm$ 7.76	0.16 $\pm$ 0.02	36.79 $\pm$ 5.83	0.15 $\pm$ 0.03	0.21 $\pm$ 0.05
Dutasteride (50 ug)	8	7.13 $\pm$ 0.52	38.70 $\pm$ 7.20	0.16 $\pm$ 0.03	37.81 $\pm$ 6.85	0.16 $\pm$ 0.05	0.29 $\pm$ 0.04
ANOVA		$F_{3,26} = 13.24$ $p > 0.05$	$F_{3,26} = 8.37$ $p > 0.05$	$F_{3,26} = 2.33$ $p > 0.05$	$F_{3,26} = 5.57$ $p > 0.05$	$F_{3,26} = 0.43$ $p > 0.05$	$F_{3,26} = 23.5$ $p > 0.05$

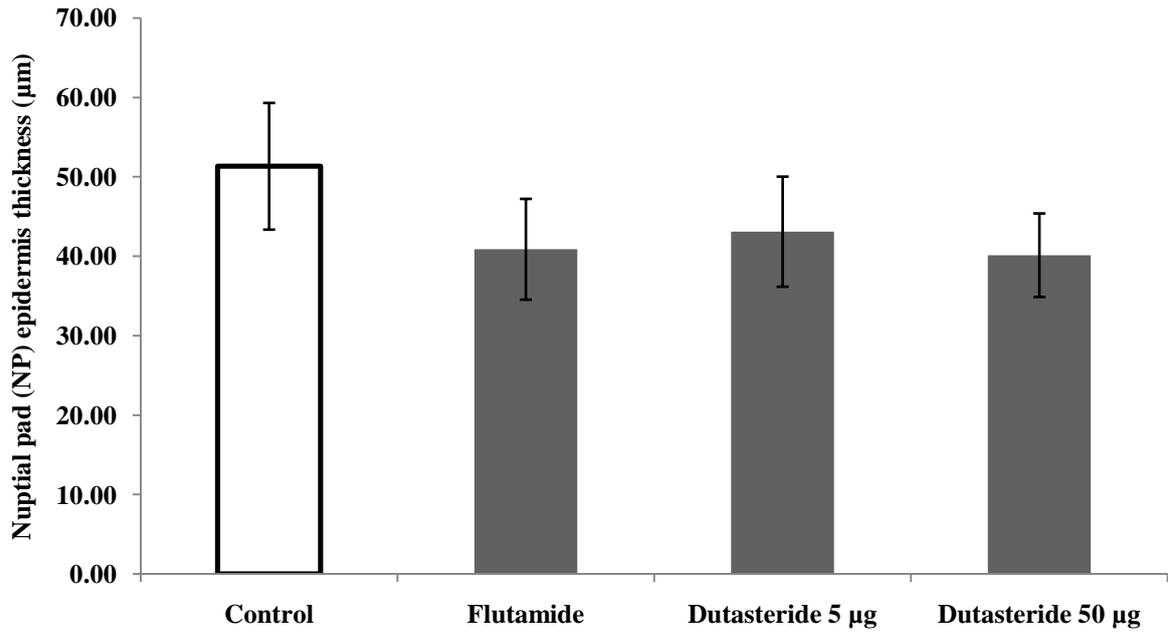
### *Breeding gland morphology*

All frogs showed significant variation in breeding gland morphology. 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. 3.2). Flutamide and dutasteride treatment significantly reduced breeding gland cross-sectional areas (Figs. 3.2A & 3.4; ANOVA,  $F_{3,26} = 7.54$ ; Tukey's HSD,  $p < 0.05$ )

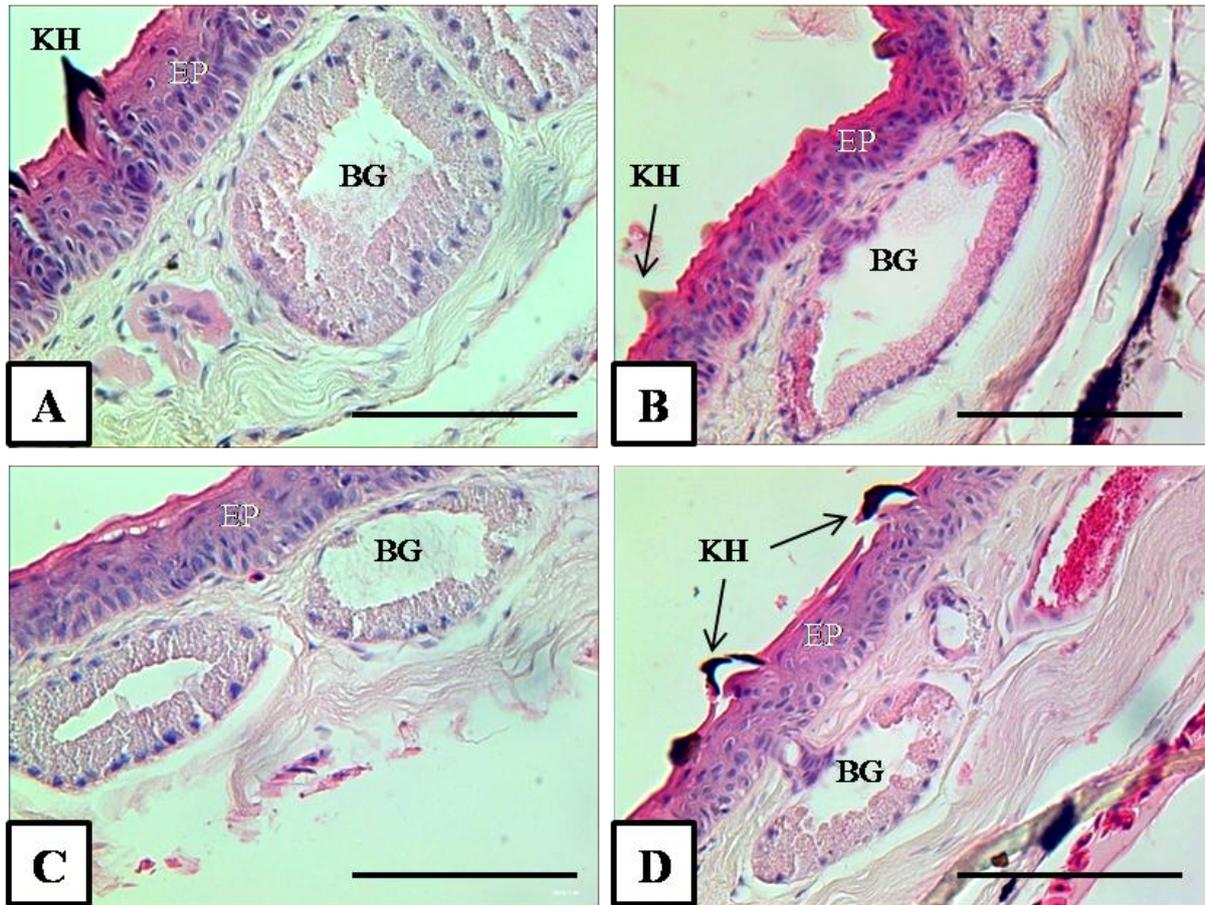
as well as gland secretory cell epithelium height (Figs. 3.2B & 3.4; ANOVA,  $F_{3,26} = 20.45$ ; Tukey's HSD,  $p < 0.05$ ) compared to frogs injected with only hCG (control treatment). No significant differences were measured in either cross-sectional breeding gland areas or secretory cell epithelium 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 (Figs. 3.3 & 3.4; ANOVA,  $F_{3,26} = 5.47$ ,  $p > 0.05$ ). 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. 3.4A). The stimulated state of the breeding glands in these frogs was also accompanied by the development of well-defined keratinized epidermal hooks (Fig. 3.4A). 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. 3.4).



**Figure 3.2:** Variation in the overall size (area) of BGs (A;  $\mu\text{m}^2 \pm \text{SD}$ ) and BG epithelium height (B;  $\mu\text{m}, \pm\text{SD}$ ) among male *X. laevis* frogs exposed to hCG (control), flutamide (AR antagonist) and two concentrations of dutasteride (5 $\alpha$ R antagonist). Six BGs and the length of ten epithelial cells within each gland were measured per frog ( $n = 6$  per exposure group). The asterisk indicates a significant difference of treatment groups compared to control frogs (ANOVA; Tukey's HSD *post hoc* test:  $p < 0.05$ ). Bars indicate average BG area (A;  $\pm \text{SD}$ ) and average epithelium height (B;  $\pm \text{SD}$ ) per treatment group. Treatment groups: Control = 100 i.u. hCG; flutamide = 100  $\mu\text{g/g}$  body mass + 100 i.u. hCG; dutasteride 5  $\mu\text{g}$  = 5  $\mu\text{g/g}$  body mass + 100 i.u. hCG; dutasteride 50  $\mu\text{g}$  = 50  $\mu\text{g/g}$  body mass + 100 i.u. hCG.



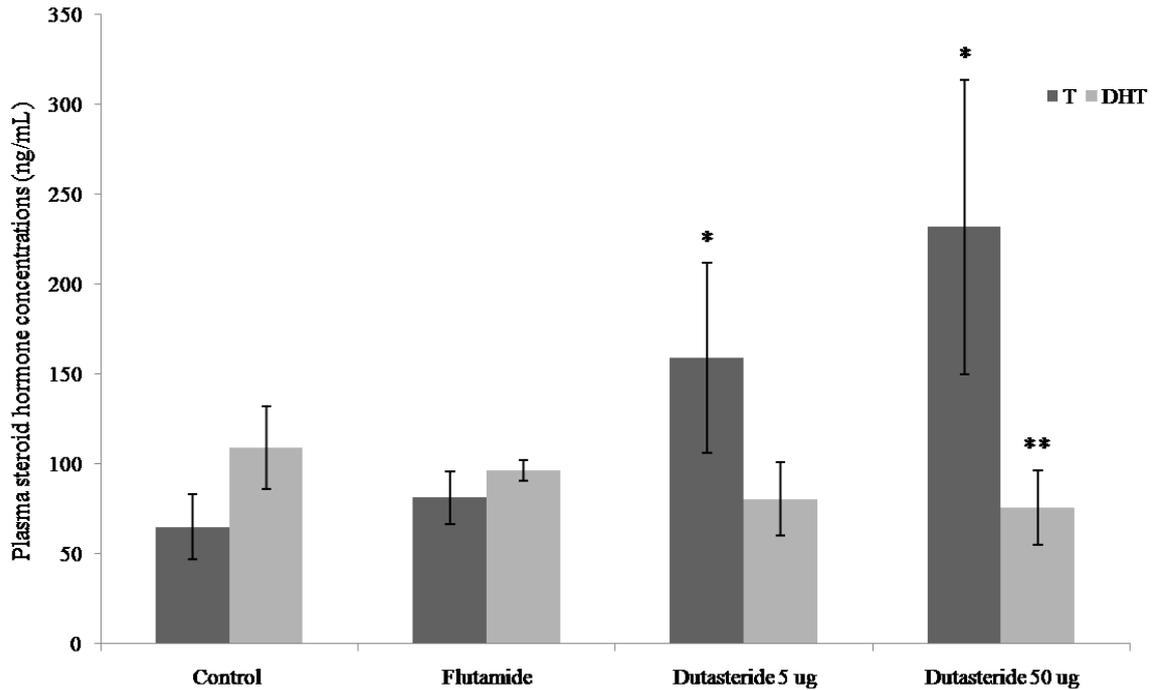
**Figure 3.3:** Variation in the average thickness of the epidermis in the NP region ( $\mu\text{m}$ ,  $\pm\text{SD}$ ) among male *X. laevis* frogs exposed to hCG (control), flutamide and two concentrations of dutasteride. Six measurements were taken per cross-sectional skin area of each frog ( $n = 6$ ) in the different treatment groups. Treatment groups: Control = 100 i.u. hCG; flutamide = 100  $\mu\text{g/g}$  body mass + 100 i.u. hCG; dutasteride 5  $\mu\text{g}$  = 5  $\mu\text{g/g}$  body mass + 100 i.u. hCG; dutasteride 50  $\mu\text{g}$  = 50  $\mu\text{g/g}$  body mass + 100 i.u. hCG.



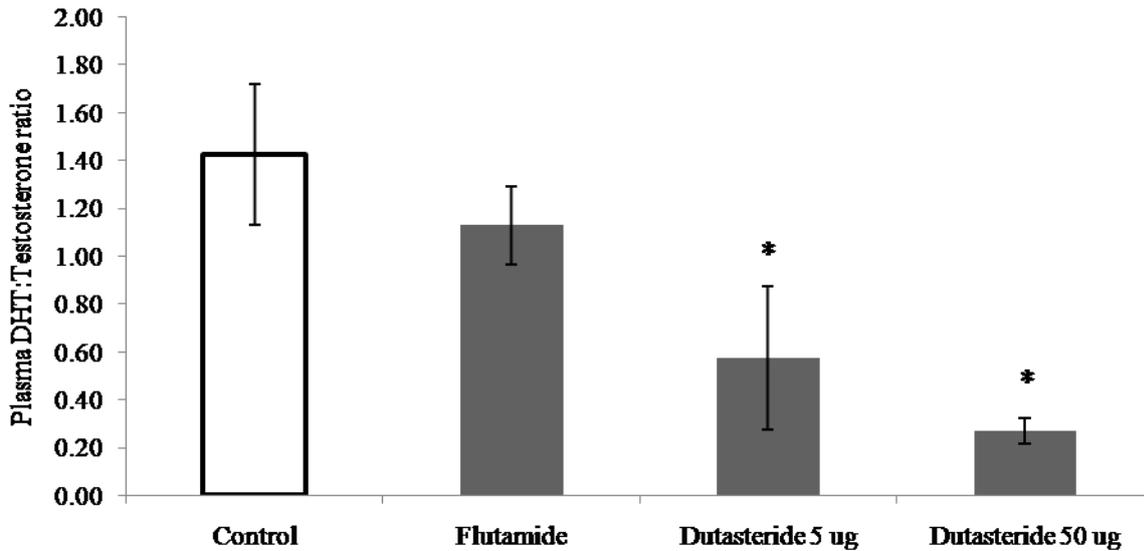
**Figure 3.4:** Cross sections (400x) of the NP area on the ventral forearm of male *X. laevis* frogs containing breeding glands. Treatment groups: (A) 100 i.u. hCG (control); (B) 100 ug/g flutamide + 100 i.u. hCG, (C) 5 µg/g dutasteride + 100 i.u. hCG and (D) 50 µg/g dutasteride + 100 i.u. hCG. Labels: KH, keratinized hooks; EP, epidermis; BG, breeding gland. Scale bar: 100 µm.

#### *Steroid hormone analysis*

Flutamide treatment did not cause significant modulation of plasma T or DHT concentrations relative to the control (Fig. 3.5; ANOVA,  $F_{3,16} = 8.187$ ;  $p > 0.05$ ). In contrast, plasma T levels were significantly elevated in the dutasteride treatments (Fig. 3.5; 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. 3.5; 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. 3.6; ANOVA,  $F_{3,16} = 48.34$ ; Tukey's HSD,  $p < 0.05$ ).



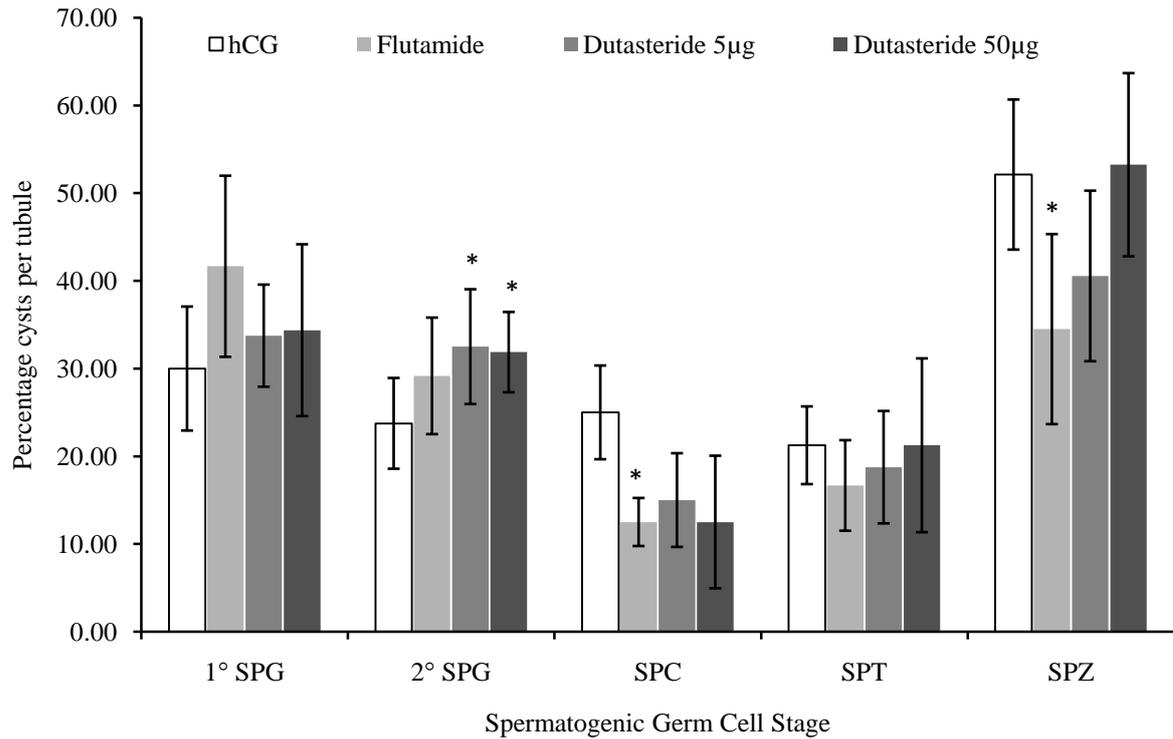
**Figure 3.5:** Variation in plasma testosterone (T) and dihydrotestosterone (DHT) concentrations (ng/ml  $\pm$  SD) among male *X. laevis* frogs (n= 6) exposed to hCG (control), flutamide and two concentrations of dutasteride. The single asterisk (\*) indicates a significant difference of T concentrations of treatment groups compared to control frogs (ANOVA:  $F_{3,16} = 100.7$ ; Tukey's HSD *post hoc* test:  $p < 0.05$ ). The double asterisk (\*\*) indicates a significant difference of DHT concentrations of treatment groups compared to control frogs (ANOVA,  $F_{3,16} = 8.19$ ; Tukey's HSD *post hoc* test,  $p < 0.05$ ) Treatment groups: Control = 100 i.u. hCG; flutamide = 100  $\mu$ g/g body mass + 100 i.u. hCG; dutasteride 5  $\mu$ g = 5  $\mu$ g /g body mass + 100 i.u. hCG; dutasteride 50  $\mu$ g = 50  $\mu$ g/g body mass + 100 i.u. hCG.



**Figure 3.6:** 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  $\mu\text{g/g}$  body mass + 100 i.u. hCG; dutasteride 5  $\mu\text{g}$  = 5  $\mu\text{g/g}$  body mass + 100 i.u. hCG; dutasteride 50  $\mu\text{g}$  = 50  $\mu\text{g/g}$  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. 3.7). 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. 3.7; 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. 3.7; 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. 3.7; ANOVA,  $F_{3,26} = 5.95$ ; Tukey's HSD,  $p < 0.05$ ).



**Figure 3.7:** Variation in the average percentage (%  $\pm$ 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 5 $\alpha$ R inhibitor dutasteride. The asterisk indicates a significant difference from the hCG treated control (ANOVA,  $p < 0.05$ ; Tukey's HSD *post hoc* test,  $p < 0.05$ ).

## DISCUSSION

The present study provides evidence that xenobiotics could inhibit androgen-dependent breeding glands (a SSC in anuran males) through different pathways, either by competitively occupying the AR (flutamide treatment) or by inhibiting 5 $\alpha$ R activity (dutasteride treatment) necessary for the transformation of T to DHT. Furthermore, the present study showed differential disruption of the primary gonadal system, with flutamide exposure (AR antagonism) affecting spermatogenic germ cell development and dutasteride treatment (5 $\alpha$ R inhibition) mostly affecting blood plasma androgen hormone concentrations and turnover.

### *Anti-androgenic effects on male breeding gland development*

Flutamide has been extensively used as a model AR antagonist in several mammalian and aquatic vertebrate exposure studies to investigate gonadal endocrine disruption (Kinnberg &

Toft, 2003; León *et al.*, 2007; Maschio *et al.*, 2010; Sen *et al.*, 2011; Baudiffier *et al.*, 2013). In the present study, this compound decreased breeding gland development in male *X. laevis* frogs in the same manner as reported in a study by Van Wyk *et al.* (2003). High levels of AR immunopositive nuclei have been shown to be localized at male-specific breeding glands (Emersson *et al.*, 1999) and are therefore dependent on androgens for their increased activity (Thomas *et al.*, 1993; Lynch & Blackburn, 1995; Epstein & Blackburn, 1997; Van Wyk *et al.*, 2003). Breeding glands have also been shown to be reduced in male *X. laevis* frogs when treated with environmental anti-androgens, such as the carboximide fungicide vinclozolin and the triazine herbicide atrazine in the same manner as an exposure to the AR antagonist flutamide (Van Wyk *et al.*, 2003; Hayes *et al.*, 2010). It is therefore concluded that anthropogenic pollutants inhibiting normal AR binding by an anti-androgenic MOA may result in decreased breeding gland activity. Although the specific biological function of the breeding gland is not yet well known (either for clasping behaviour and chemical communication), this androgen-dependent SSC is recognized as playing an important role in reproductive behaviour (Thomas *et al.*, 1993; Emerson *et al.*, 1999). Modulation of breeding glands by environmental AR antagonistic EDCs may therefore potentially lead to a reduced activity with consequences of altered reproductive behaviour in male anurans.

Similar to the treatment with an AR antagonist (flutamide), exposure in the present study to the 5 $\alpha$ R inhibitor, dutasteride, also decreased breeding gland development in male *X. laevis* frogs. This result shows that 5 $\alpha$  reduction of T to DHT plays an active role in controlling breeding gland development and function. The clinical use of dutasteride is to treat for benign prostatic hyperplasia (BPH; enlarged prostatic nodule formation in aging men) and androgenic alopecia (hair loss), which are influenced by DHT concentrations regulating epithelial cell function in the prostate and decreasing hair growth in the scalp (McConnel & Stoner, 2001). Due to the presence of the *srd5a2* isoform of 5 $\alpha$ R in the skin (McConnel & Stoner, 2001), it is expected that modulation of breeding gland epithelium cell proliferation by dutasteride in the present study may be due to the presence of the *srd5a2* isoform of 5 $\alpha$ R localized in the NP integument of male anurans. In several anuran species, breeding gland development have been shown to correlate with increased plasma androgen levels (Thomas *et al.*, 1993, Kaptan & Murathanoğlu, 2008). Dutasteride treatment is therefore expected to inhibit the transformation of T to DHT and cause decreased secretory cell epithelium in breeding glands. However, although dutasteride has been shown to primarily target the

metabolism of T to DHT, this compound may also secondarily modulate other androgen pathways. For example, it was shown in the mammalian prostate (which is dependent on DHT for development) that dutasteride treatment targeted multiple pathways such as metabolism of T to DHT by 5 $\alpha$ R, the uptake and retention of androgens in target cells or by directly affecting AR binding of androgens, regardless of steroid metabolism (Wu *et al.*, 2013). The possibility therefore arises that dutasteride may also modulate AR binding of either T or DHT in breeding glands, hence resulting in a similarly regressed appearance of the breeding gland epithelium and glandular area compared to flutamide treatment.

#### *Anti-androgenic effects on plasma steroid hormone levels*

Plasma hormone levels in flutamide-treated male *X. laevis* frogs in the current study showed no modulating effects upon steroid hormone biosynthesis. These results are supported by other studies, which stated that flutamide does not alter steroid biosynthesis in the testis (Van Wyk *et al.*, 2003), neither does it modulate the expression of LH and FSH in the pituitary of male *X. laevis* frogs (Behrends *et al.*, 2010). In contrast, both dutasteride treatments significantly increased plasma T concentrations, and decreased plasma DHT in the highest treatment group. Dutasteride exposure at both exposure concentrations significantly altered the ratio of plasma DHT/T compared to the levels in frogs from the hCG-induced control group. Although dutasteride exposure resulted in increased plasma T levels in male frogs, the resulting elevated T levels did not necessarily cause increased development of SSC development such as breeding gland size and gland epithelium height, which suggested that development of BGs is stimulated by DHT rather than its precursor (T). The metabolism of T to DHT is therefore vital for normal SSC development in frogs and must therefore receive the same attention in endocrine disruption studies as contaminants modulating AR binding pathways.

#### *Anti-androgenic effects on spermatogenesis*

Differentiation of germ cell-types (spermatogenesis) in flutamide-exposed frogs was significantly reduced compared with control frogs in the present study. In normal sperm production, the developmental stages of spermatogenesis consist of a mitotic phase of the spermatogonia, meiotic phase of the spermatocytes and spermiogenesis of the spermatids creating spermatozoa (Anahara *et al.*, 2008). It has been shown that an increase in primary spermatogonia can be related to molecular changes of Sertoli cells, which are rich in AR

mRNA and respond to steroid hormones such as 11-ketotestosterone (11-KT) in fish and T in higher vertebrates (Baudiffier *et al.*, 2013). Although there was no modulation of steroid hormone levels in the present study of flutamide-exposed frogs, a slight increase in primary spermatogonia germ cell stages and a significant reduction in spermatocyte cysts and spermatozoa (post-meiotic germ cell phases) could imply that spermatogenesis as well as spermiogenesis may be delayed by means of AR antagonism at the Sertoli cells. This was also found in other aquatic species such as in male medaka (*Oryzias latipes*) and guppies (*Poecilia reticulata*) exposed to flutamide which resulted in delayed spermatogonial differentiation, germ cell necrosis (inflammation at the spermatid stage) and absent/deformed spermatozoa (Kinnberg & Toft, 2003; León *et al.*, 2007). In higher vertebrates, flutamide exposure has been shown to induce spermatogonial differentiation and cause degeneration of primary spermatocytes and spermatids in male pubertal guinea pigs (Maschio *et al.*, 2010). Flutamide exposure in rats has revealed abnormalities in spermatids such as irregular shaped acrosomes and deformed spermatozoa (Anahara *et al.*, 2008) while a decreased percentage of seminiferous tubules containing spermatozoa was observed in mice exposed to flutamide (Vojtísková *et al.*, 1978). In the present study, it was evident that flutamide already altered male fertility at testicular level by disrupting germ cell production which can be attributed to interference in an AR antagonistic mechanism (competitive binding with steroid hormones to the AR).

Furthermore, it was also shown in other studies that flutamide increased the volume of Leydig cells in adult guinea pig testes (Maschio *et al.*, 2010). The increase in Leydig cell number could be attributed to hyperactivity of Leydig cell development in response to T, which is not able to bind to the AR due to the competitive binding of flutamide to the AR (Maschio *et al.*, 2010). The blockage of T to the AR can also affect premature germ cell development of spermatocytes and spermatids due to occlusion junctions in the Sertoli cells, which are affected by flutamide (Maschio *et al.*, 2010). This means that flutamide exposure might also have an indirect affect on the T to DHT turnover ( $5\alpha$ -reduction) due to the fact that flutamide alters the function of Sertoli cell function that is responsible for steroid hormone metabolism (Maschio *et al.*, 2010). In summary, although flutamide exposure in *X. laevis* did not cause a significant modulation of steroid hormone synthesis, affects on spermatogenesis level were observed. Along with the decrease in SSC development such as BG development in these frogs, it is hypothesized that modulation of reproductive

characteristics by AR antagonistic mechanisms may lead to reduced fertility as well as reproductive behaviour in male frogs.

In the present study, dutasteride treatment did not significantly alter later-stage spermiogenesis (SPC & SPT), although more cysts bearing the secondary spermatocyte germ cell type were observed in these frogs relative to hCG-induced frogs. This finding corroborates the results of a study using male Wistar rats, showing no clear effects on spermatogenesis when treated with finasteride, a  $5\alpha$ R inhibitor (Rhoden *et al.*, 2002). In the male gonadal endocrine system, it is mostly assumed that T is the primary hormone controlling spermatogenesis whereas DHT rather controls the development of secondary sexual traits (Overstreet *et al.*, 1999; McConnel & Stoner, 2001; Amory *et al.*, 2008). Due to the primary role of T for germ cell development (spermatogenesis) and sperm formation (spermiogenesis),  $5\alpha$ R inhibitors may not alter the pituitary controlling pathway and testicular responsiveness to gonadotropin releasing hormones (GnRH), consequently having little effect on the development of spermatogenic germ cells (Overstreet *et al.*, 1999). Furthermore, exposure to  $5\alpha$ R inhibitors in rats, dogs and rabbits have also indicated no significant modulation of testicular morphology or spermatogenesis during adulthood (Overstreet *et al.*, 1999; Rhoden *et al.*, 2002). However, Urbatzka *et al.*, (2009) reported that finasteride treatment increased LH mRNA expression in the pituitary of *X. laevis* tadpoles, which resulted in lowered occurrences of spermatid germ cells and developing spermatozoa in the testis of male frogs. However, this has not been done in the present study and needs further investigation.

#### *Effect of hCG upon biomarker outcomes*

Considering the outcomes of the present study, administration of anti-androgens along with hCG indicated that hCG treatment may not act independent from testicular secretions, hormones or enzymes, as also shown by Pozzi & Ceballos (2000). These authors used testicular fragments of the frog *Bufo arenarum* and suggested that increased spermiogenesis caused by hCG administration is not necessarily mediated by steroid biosynthesis mechanisms. In the current study, administration of the anti-androgenic contaminants in combination with hCG reduced the development of breeding glands, modulated plasma steroid hormone levels and partially delayed spermiation in male *X. laevis* frogs. If hCG acted independently from steroid biosynthesis, development of breeding glands would not have

been reduced in these treatment groups due to hCG which would have targeted the development of breeding glands (an androgen-mediated characteristic) regardless of anti-androgens causing effects on the proliferation of breeding gland secretory epithelium cells. Both anti-androgens did affect some androgen pathways such as plasma steroid hormone levels and a delay in spermiogenesis, which suggests that the administration of hCG in these groups still could not counter the effects of the anti-androgenic compounds. These results obtained from the present study indicated that hCG is safe to use as an androgen-stimulant for anti-androgen exposure studies in that this compound would not directly affect androgen outcomes caused by anti-androgen administration. Exposure to hCG can therefore be used to obtain a uniform breeding stage in experimental organisms, such as to maintain regular spermatogenic processes and to investigate androgen-dependent biomarkers such as male BGs.

### *Conclusion*

During the present study, androgen-dependent breeding glands in male *X. laevis* frogs were inhibited in a similar manner when exposed to two EDCs having different mechanisms of anti-androgenic endocrine activity. Circulating plasma steroid hormones (T and DHT) were differentially affected and limited inhibitory effects were present in spermatogenesis and spermiogenesis. Although similar results were shown in breeding gland development between the anti-androgenic treatment groups, investigation of spermatogenesis and plasma hormone levels revealed marked differences between frogs exposed to the different anti-androgenic MOAs. Furthermore, the current study showed that hCG may not induce androgen-mediated processes independent from steroid biosynthesis, as proposed by Pozzi & Ceballos (2000). The possibility of dutasteride (a 5 $\alpha$ R inhibitor) to modulate AR binding directly or by affecting androgen uptake in the blood (as mentioned by Wu *et al.*, 2013) can also not be ruled out for the results obtained in the current study and needs more detailed investigation. Future studies should therefore include AR-specific biomarkers to establish whether a 5 $\alpha$ R inhibitor such as dutasteride will directly affect AR binding rather than primarily modulating steroid hormone metabolism. The outcomes from the current study can provide useful tools for detecting gonadal endocrine disrupting activity in the environment.

## CHAPTER 4: THE POTENTIAL ANTI-ANDROGENIC EFFECT OF AGRICULTURAL PESTICIDES USED IN THE WESTERN CAPE: *IN VITRO* INVESTIGATION OF MIXTURE EFFECTS<sup>3</sup>

### ABSTRACT

Several xenobiotic pollutants from anthropogenic sources have been shown to end up in water systems and disrupt vertebrate endocrine pathways. Although it is known that environmental chemicals can also affect the androgenic system, far less attention has been paid to chemicals interacting with the androgen receptor (AR). Pesticides, particularly fungicides, have been shown to competitively bind or affect expression of the AR in an inhibiting manner. In spite of an extensive agricultural industry in South Africa, few studies addressed anti-androgenic effects of locally used agrochemicals. The aim of this study was to characterize pesticides; mainly fungicides used in the Western Cape region, and investigate their AR antagonistic potential, using a recombinant yeast androgen screen (YAS). Secondly, we investigated the potential of binary pesticide mixtures to generate a predicted additive mixture response. The fungicides vinclozolin, folpet, procymidone, dimethomorph, fenarimol, mancozeb and the insecticide chlorpyrifos independently all antagonized the binding of the androgen dihydrotestosterone (DHT) to the AR in a dose-dependent manner. The AR antagonistic potency of the test chemicals ranged from 143% to 64%. The fungicide mancozeb was found to be the most potent anti-androgen in the assay. Binary, equimolar mixtures of the pesticides also antagonized the binding of DHT to the AR at IC<sub>50</sub> concentration potencies relative to vinclozolin ranging from 138% to 85%. The mixtures of the majority selected test pesticides did not conform to an additive mixture interaction. However, a mixture between dimethomorph and mancozeb did show an additive mixture response at IC<sub>50</sub> concentrations, and therefore, showed a more severe AR antagonistic effect compared to their individual counterparts. In conclusion, this study confirmed that pesticides regularly used in agriculture inhibit the binding of androgens to the AR, but when in mixture do not always conform to the predictive addition mixture response model.

**Keywords:** Androgen Receptor; Anti-androgen; Fungicides; Mixtures

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<sup>3</sup> Submitted to the journal Water SA for review

## INTRODUCTION

Several natural and man-made compounds such as agricultural pesticides, industrial waste products, pharmaceuticals and household chemicals have been shown to modulate endocrine activity which might affect physiological homeostasis in vertebrates (Heath & Claassen, 1999; Urbatzka *et al.*, 2007a; Blake *et al.*, 2010). Compounds acting in this way are collectively referred to as endocrine disrupting contaminants (EDCs). Although the endocrine modulation may be subtle in cases, several of these EDCs have been shown to adversely disrupt the gonadal endocrine system of non-target organisms, potentially resulting in altered reproductive fitness and eventually may show effects on a population level (Blake *et al.*, 2010). Chemicals having endocrine disrupting (ED) effects on the reproductive system can in general be categorized in one of four mode-of-action (MOA) groups (responses), namely estrogenic, anti-estrogenic, androgenic or anti-androgenic (Behrends *et al.*, 2010; Hoffmann & Kloas, 2010). The majority of EDC studies conducted to date focused on EDCs modulating the estrogenic response system in one way or the other (Sohoni & Sumpter, 1998; Urbatzka *et al.*, 2007a, 2007b; Behrends *et al.*, 2010). In contrast to intensive research focus on the effect of man-made chemicals that potentially may disrupt normal estrogenic function in individuals, relatively few studies have addressed interaction with the androgenic endocrine systems, either from an agonistic or antagonistic perspective (Urbatzka *et al.*, 2007a).

Contaminants having endocrine disrupting activity by an anti-androgenic MOA have been shown to disrupt mammalian foetal androgen synthesis, leading to incomplete masculinisation and reproductive organ development (Cristiansen *et al.*, 2009). Chemicals modulating the androgen receptor (AR), resulting in an anti-androgenic response, have been shown to adversely affect male reproductive health, such as causing testicular dysgenesis syndrome, cryptorchidism, testicular cancer and altered semen quality (Kortenkamp & Faust, 2010). Although modulation of the androgenic system may not be limited to interaction with the AR, competitive binding at the AR level seems to have been the focus of most previous studies (Kelce *et al.*, 1998; Ermler *et al.*, 2010, 2011; Orton *et al.*, 2011). Certain EDCs may serve as AR agonists, therefore binding to the AR in an additive way in the presence of normal androgens, resulting in an increased transcription of target genes (Morthorst *et al.*, 2010; Ekman *et al.*, 2011) while others may act as AR antagonists, competing with androgens to bind to the AR, resulting in decreased transcription of target genes (Birkøj *et al.*, 2004; Ermler *et al.*, 2010, 2011). An example of a direct AR antagonist is flutamide, a clinical drug

used to treat prostate cancer (Behrends *et al.*, 2010; Hoffmann & Kloas, 2010). From an environmental chemical perspective, several pesticides, including the DDT metabolite, DDE (Kelce *et al.*, 1997), and several fungicides such as vinclozolin (Kelce *et al.*, 1997; Orton *et al.*, 2011) have been shown to antagonize an androgenic response similar to flutamide (Kelce *et al.*, 1994).

Of all pesticides to date screened for anti-androgenic activity, fungicides stand out as the best studied pollutant group (Christiansen *et al.*, 2008, 2009). In agricultural practice, however, pesticides are seldom applied individually and/or farmers rarely keep to one specific compound (in fear of acquired resistance) (Boone, 2008). In South Africa alone, more than 300 registered fungicide trade products are available and are either made up of a single active ingredient or more than one active ingredient (DAFF, 2013). Furthermore, as in most agriculture countries in the world, water pollution (through runoff, spray drift and/or leeching) may be regarded as one of the major exposure threats to aquatic wildlife, domesticated stock and human health (Heath & Claassen, 1999; Dabrowski *et al.*, 2011). It is therefore inevitable that water sources close to agricultural practices will be contaminated with a mixture of chemicals (Feron & Groten, 2002; Boone, 2008; Blake *et al.*, 2010; Kortenkamp & Faust, 2010; Hass *et al.*, 2012).

Two of the major challenges in the study of environmental compounds interacting with the endocrine system include that these chemicals mostly act at low concentrations, far below their reported no-observed-effect-level (NOEL) and may well persist in complex mixtures for long periods of time in aquatic systems. Apart from the establishing endocrine mode (or mechanism) of action of an individual chemical, it is also important to understand the interaction of chemicals when in mixture. There are two dimensions to consider in mixture studies, firstly characterizing the broad MOA of individual chemicals (androgenic, anti-androgenic, estrogenic and anti-estrogenic) but secondly, to establish their specific mechanism of actions (e.g. receptor binding or steroidogenesis modulation) (Woodruff *et al.*, 2008). Previous studies have stated that EDCs having similar mechanisms of action (e.g. AR antagonism) will be expected to have an additive mixture response (e.g. elevated anti-androgenic activity) while EDCs having dissimilar mechanisms of action will be expected to have independent mixture responses (no increased or reduced endocrine activity) (Birkhøj *et al.*, 2004; Blake *et al.*, 2010; Ermler *et al.*, 2011). However, it is clear from the literature that

few *in vitro* studies report on anti-androgenic EDC mixtures of commonly used pesticides in South African agriculture. Disregarding the combined effects of EDCs may lead to an underestimation of the potential environmental and health risk that pollutants in our water regimes may pose (Kortenkamp & Faust, 2010).

Several *in vitro* assays have been proposed for inclusion in an endocrine disruptor screening and testing program (EDSTAC, 1998) by the United States Environmental Protection Agency (US-EPA). These assays serve as first tier screens to identify the potential EDC activity and suggested MOA of man-made chemicals detected in the environment (Hecker *et al.*, 2011). Although the recombinant yeast assay (using hAR) has not been included in the battery of first tier tests suggested by the USA-EPA, the YAS is a recognized assay (Sohoni & Sumpter, 1998; Urbatzka *et al.*, 2007b; Kolle *et al.*, 2011; Orton *et al.*, 2011) with the advantage that it is a relatively inexpensive and robust screening tool with a standardized protocol (Sohoni & Sumpter, 1998; De Jager *et al.*, 2011).

The aim of this study was to screen selected pesticides, mostly fungicides, commonly used in the Western Cape agricultural areas of South Africa, for their ability to antagonize the binding of an androgen (DHT) to the human AR (hAR) and to test the additivity hypothesis when commonly used pesticides having similar MOAs are exposed in mixture.

## **MATERIALS AND METHODS**

### *Recombinant yeast and test chemicals*

The yeast strain, *Saccharomyces cerevisiae*, transfected with the human androgen receptor (hAR) gene and a plasmid containing an androgen response element (ARE)-linked lac-Z gene was obtained from the laboratory of Prof. J. Sumpter, Brunel University, UK. Test chemicals 5 $\alpha$ -androstan-17 $\beta$ -ol-3-one (DHT, CAS No. 1521-18-6), flutamide (CAS No. 13311-84-7), vinclozolin (CAS No. 50471-44-8), folpet (CAS No. 613-045-00-1), procymidone (CAS No. 32809-16-8), dimethomorph (CAS No. 613-102-00-0), flusilazole (CAS No. 85509-19-9), fenarimol (CAS No. 60168-88-9), chlorpyrifos (CAS No. 2921-88-2) and mancozeb (CAS No. 8018-01-7) were all purchased from Sigma Aldrich (Taufkirchen, Germany). All medium components for the yeast assay were prepared according to Sohoni & Sumpter (1998) and were obtained from Sigma Aldrich, except for the chlorophenolred- $\beta$ -D galactopyranoside

(CPRG) which was obtained from Roche Diagnostics (Mannheim, Germany). Absolute ethanol (99.8% HPLC grade, Sigma) was used as solvent for the chemical solutions.

#### *Recombinant Yeast Anti-Androgen Screen (anti-YAS) Procedure*

We followed the basic protocol described in Sohoni & Sumpter (1998), but with modifications for the yeast anti-androgen screen (anti-YAS) as described in later studies (Urbatzka *et al.*, 2007b; Kolle *et al.*, 2011). In brief, assay medium was prepared by adding 0.5 mL CPRG to 50 mL of freshly prepared growth medium. The medium was seeded with  $4 \times 10^7$  yeast cells from a previously incubated 24 hour culture. To generate a standard curve of a positive control, a stock solution of DHT (1  $\mu$ M) dissolved in absolute ethanol was made up and serially diluted (10  $\mu$ L/well) into a sterile 96-well flat bottom assay plate with a low evaporation lid (Costar, 3370). Blank wells containing no ligands were supplemented with only absolute ethanol (10  $\mu$ L/well). Test chemicals were evaluated in the presence of DHT at a concentration of 6.25 nM ( $EC_{50}$  from the DHT standard curve). All pesticides were tested for their ability to inhibit the agonistic action of the androgen spike (Table 4.1). 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 (Greiner bio-one, 655161). Each diluted concentration was transferred to a sterile 96-well flat bottom assay plate with a low evaporation lid (Costar, 3370) at 10  $\mu$ L/well along with the DHT ( $EC_{50}$ ) spike. Assay medium was added (200  $\mu$ L/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 using equation 1 (Sohoni & Sumpter, 1998).

$$\text{Corrected value} = \frac{\text{chemical absorbance (570 nm)} - [\text{chemical absorbance (620 nm)} - \text{blank absorbance (620 nm)}]}{\text{blank absorbance (620 nm)}} \quad [\text{Eq.1}]$$

To firstly validate the usage of the known AR antagonistic fungicide vinclozolin as a negative control in this study, the dose-response curve of vinclozolin was compared to that of the pharmaceutical flutamide, which is regarded as a model AR antagonist. The fungicide vinclozolin was then used as the negative control in each assay in which the corrected absorbencies of the test chemicals were log transformed and compared relative to the dose-response curve of vinclozolin (eq. 2).

$$\text{Log \% Max Vinclozolin} = \frac{\text{Log corrected abs.}_{\text{chemical}}}{\text{max Log corrected abs.}_{\text{Vinclozolin}}} * 100 \quad [\text{Eq. 2}]$$

The corrected absorbance of the test chemical dilutions was also used to calculate the  $\text{IC}_{50}$  (inhibition concentration at 50% relative to the control respectively) for each chemical. The relative potency (RP, eq. 3) was calculated from the  $\text{IC}_{50}$  for each pesticide (De Jager *et al.*, 2011).

$$\text{RP (\%)} = \frac{\text{IC}_{50, \text{chem}}}{\text{IC}_{50, \text{vinclozolin}}} * 100 \quad [\text{Eq. 3}]$$

RP indicates the efficiency (%) of the chemical to antagonize the AR binding of 6.25 nM DHT relative to the AR antagonistic potency of the control (vinclozolin).

**Table 4.1:** 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 <i>et al.</i> , 2011
Fenarimol*	Pyrimidinyl carbinol	AR binding antagonist	Vinggaard <i>et al.</i> , 2005
Mancozeb*	Dithiocarbamate	AR binding antagonist	Viswanath <i>et al.</i> , 2010
Folpet*	Dicarboximide	Expected AR binding antagonist (same chemical group as vinclozolin and procymidone)	n/a
Dimethomorph*	Cinnamic acid	AR binding antagonist	Orton <i>et al.</i> , 2011
Procymidone*	Dicarboximide	AR binding antagonist	Ostby <i>et al.</i> , 1999
Flusilazole*	Triazole	Decrease androgen biosynthesis	Trösken <i>et al.</i> , 2004
Chlorpyrifos	Organophosphate	Decreased testosterone biosynthesis, AR binding antagonist	Viswanath <i>et al.</i> , 2010

#### *Fungicide mixture assay calculations*

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  $\mu\text{M}$  due to its high individual potency in the assay. The concentration giving 50% inhibition ( $\text{IC}_{50}$ ) for each mixture was calculated and used to determine the isobole coefficient of the mixture as described by Birkhøj *et al.* (2004) (eq. 4).

$$\text{Isobole coefficient} = \sum \frac{p \cdot c_{i,\text{mix}}}{c_{i,\text{single}}} \quad [\text{Eq. 4}]$$

Where  $p$  is the fraction of the single chemical in the mixture and  $c_i$  is the concentration of 50% inhibition ( $\text{IC}_{50}$ ) of the mixture or the single chemical. The isobole coefficient estimates three mixture effect levels. A value equal to 1 suggests an additive response, a value above 1 suggests an antagonistic response and a value below 1 suggests a synergistic response of the mixture. Apart from measurement of the mixture interaction, we investigated the additivity null hypothesis which estimates the predicted  $\text{IC}_{50}$  concentrations of the pesticide mixtures assuming concentration addition (CA) or independent action (IA) (Ermler *et al.*, 2011). For the CA prediction, equation 5 was used:

$$\text{CA} = \sum \left( \frac{p_i}{\text{EC}_i} \right)^{-1} \quad [\text{Eq. 5}]$$

Where  $p_i$  is the fraction of the compound in the mixture and  $\text{EC}_i$  is the effect concentration of the  $i^{\text{th}}$  compound, which produces the same effect as in the mixture ( $\text{IC}_{50}$ ). For independent action, equation 6 was used for the predicted  $\text{IC}_{50}$  of the mixture interactions.

$$\text{IA} = 1 - \prod [1 - E(C_i)] \quad [\text{Eq. 6}]$$

Where  $E(C_i)$  is the effect concentration of the  $i^{\text{th}}$  compound which produces the same effect as in the mixture ( $\text{IC}_{50}$ ).

### *Statistical analyses*

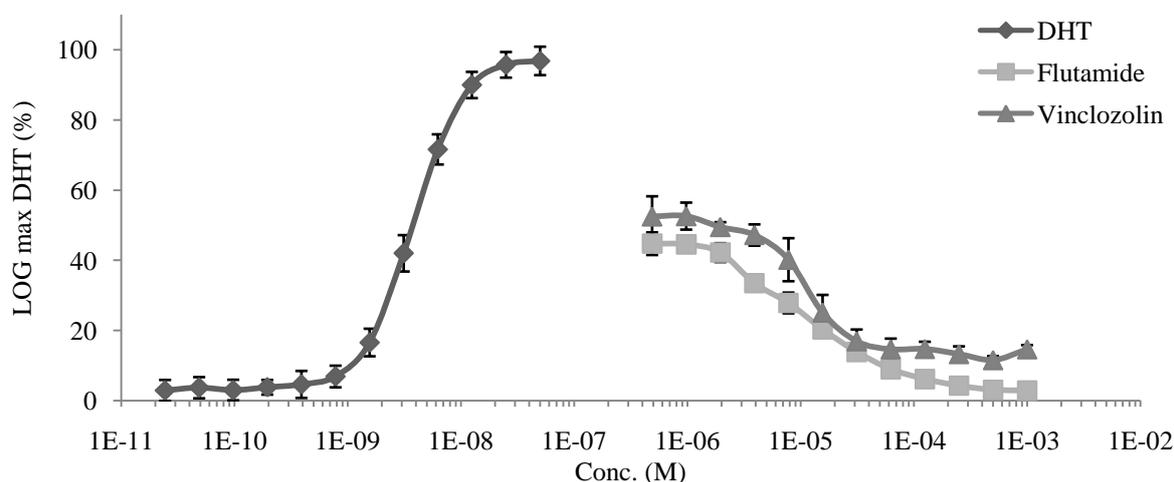
Statistical analyses were done by the statistics division at the University of Stellenbosch. The data were analyzed using Statistica (version 10.0, StatSoft) and tested for homogeneity of

variance and normal distribution. A one-way analysis of variance (ANOVA) was performed followed by pairwise comparisons between test compounds and control groups using Bonferroni's *post hoc* test. Values obtained for  $IC_{50}$  concentrations, slope and lower parameters of the test compounds and mixtures were compared using a Student's t-test with differences from the control (vinclozolin exposure) indicating significant variances at p-values smaller than 0.05.

## RESULTS

### *Specificity of the YAS and anti-YAS*

The specificity of DHT to bind to the AR in a dose-response manner was confirmed in the  $\beta$ -galactosidase response in the YAS assay (Fig. 4.1). Androgenic reactivity initiated at 1 nM and AR binding continued to increase until the saturation level was reached at about 50 nM DHT (Fig. 4.1). The AR binding reached 50% saturation ( $EC_{50}$ ) at a DHT concentration of 6.25 nM. The inhibition of the androgenic spike ( $EC_{50}$  concentration of DHT, 6.25 nM) response was confirmed through addition of flutamide (AR antagonist) (Fig. 4.1). Both vinclozolin and flutamide showed a similar AR antagonistic response and vinclozolin was subsequently used as the equivalent control for the selected test pesticides and mixtures thereof (Fig. 4.1).



**Figure 4.1:** Sigmoidal standard curve of DHT, flutamide and vinclozolin in the yeast androgen and anti-androgen screen respectively (YAS/anti-YAS). The series containing flutamide and vinclozolin were spiked with 6.25 nM DHT in each well. Values (means  $\pm$  SE) are expressed relative to the maximum log-transformed absorbance obtained for DHT.

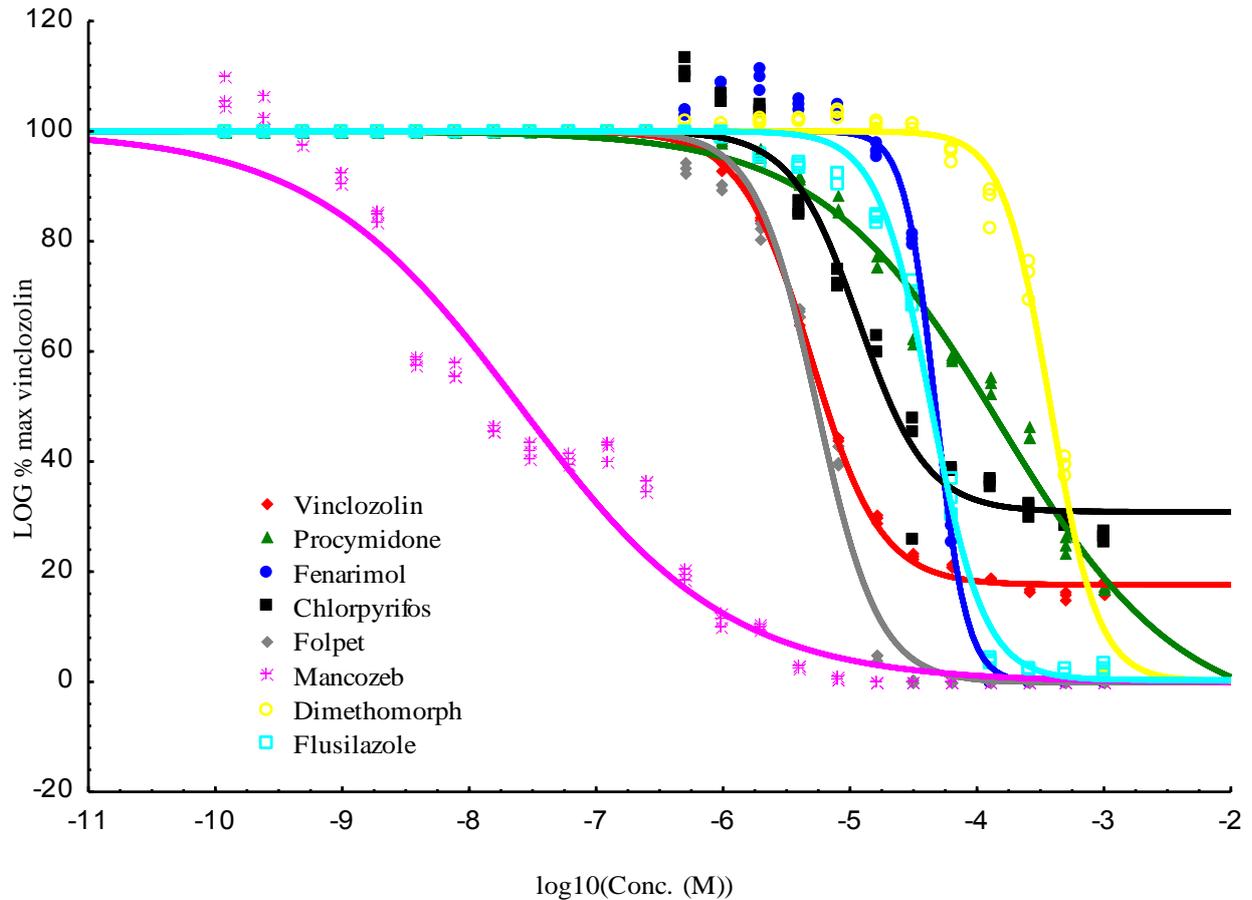
*Pesticide and mixture exposure in the anti-YAS*

A clear dose-response of all test pesticides upon inhibiting the binding of DHT to AR (AR antagonism) was observed between 0.3  $\mu\text{M}$  and 3 mM with the exception of mancozeb, which had a dose-response concentration range between 0.03 nM and 32  $\mu\text{M}$  (Fig. 4.2). The dose-response curves provided  $\text{IC}_{50}$  concentrations of vinclozolin at 0.0049 mM (1.4 mg/L, Table 4.2). The carboximide fungicide folpet generate the same AR antagonistic potency as vinclozolin (RP, 99.1%) with an  $\text{IC}_{50}$  concentration of 0.0055 mM (1.6 mg/L, Table 4.2). The  $\text{IC}_{50}$  values of the two carboximide fungicides did not vary significantly (t-test,  $p > 0.05$ ). The other caboximide fungicide procymidone had a weaker AR antagonistic potency than vinclozolin (RP, 71.9%, Table 4.2) and an AR antagonistic  $\text{IC}_{50}$  concentration of 0.15 mM (42.6 mg/L, Table 4.2). The dose-response curve of fenarimol provided an  $\text{IC}_{50}$  concentration of 0.0472 mM (156 mg/L, Table 4.2), which is higher than vinclozolin, but still generated a high AR antagonistic potency relative to vinclozolin (RP, 81.5%, Table 4.2).

Mancozeb was shown to be the most potent AR antagonist in the anti-YAS (RP, 143.2%, Table 4.2; Fig. 4.2) with the  $\text{IC}_{50}$  of mancozeb being  $2.5 \times 10^{-5}$  mM in the assay (7  $\mu\text{g/L}$ ; Table 4.2). Dimethomorph had a low AR antagonistic potency relative to vinclozolin (RP, 64.5%, Table 4) and was only anti-androgenic at higher concentrations compared to the other test compounds, with an  $\text{IC}_{50}$  concentration of 0.38 mM (147.4 mg/L, Table 4.2). Dimethomorph also generated a steep slope in the dose-dependent responses indicating a small concentration range of AR antagonistic activity (Fig. 4.2). Similar steep dose-dependent slopes for AR antagonistic activity were also recorded in exposures to the fungicides fenarimol and flusilazole (Fig. 4.2). Flusilazole showed lower AR antagonistic potency relative to vinclozolin (RP, 82.2%), with an  $\text{IC}_{50}$  concentration of 0.043 mM (13.6 mg/L, Table 4.2). Apart from the fungicides tested in the study, the insecticide chlorpyrifos had a fairly high AR antagonistic potency relative to vinclozolin (RP, 92.9%, Table 4.2) and an  $\text{IC}_{50}$  concentration of 0.0118 mM (4.1 mg/L, Table 4.2). The slope parameters of chlorpyrifos generated from the dose-response curves did not vary significantly from the model AR antagonist vinclozolin (t-test,  $p > 0.05$ ; Fig. 4.2), but the lower parameters from the dose-response curve of chlorpyrifos was high compared to other test pesticides (Fig. 4.2), indicating incomplete AR antagonism throughout the tested concentration range.

**Table 4.2:** The IC<sub>50</sub> 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<sub>50</sub> concentrations for the binary pesticide mixtures assuming concentration addition (CA) or independent action (IA) were also estimated.

Pesticides	IC <sub>50</sub> Conc. (mM)	RP			
			Isobole Coefficient (IC <sub>50</sub> )	Predicted concentration addition (CA; IC <sub>50</sub> )	Predicted independent action (IA; IC <sub>50</sub> )
Vinclozolin	4.94e <sup>-03</sup>	100.00			
Procymidone	1.53e <sup>-01</sup>	71.88			
Fenarimol	4.72E <sup>-02</sup>	81.54			
Chlorpyrifos	1.18e <sup>-02</sup>	92.90			
Folpet	5.50e <sup>-03</sup>	99.13			
Mancozeb	2.54e <sup>-05</sup>	143.16			
Dimethomorph	3.80e <sup>-01</sup>	64.47			
Flusilazole	4.34e <sup>-02</sup>	82.22			
Mancozeb + Dimethomorph	4.65e <sup>-05</sup>	138.19	0.92	5.07e <sup>-05</sup>	3.80e <sup>-01</sup>
Dimethomorph + Folpet	2.49e <sup>-02</sup>	86.78	2.29	1.08e <sup>-02</sup>	3.83e <sup>-01</sup>
Vinclozolin + Folpet	2.98e <sup>-02</sup>	85.30	5.72	5.21e <sup>-03</sup>	1.04e <sup>-02</sup>
Vinclozolin + Mancozeb	7.70e <sup>-03</sup>	96.38	152.55	5.05e <sup>-05</sup>	4.97e <sup>-03</sup>
Vinclozolin + Fenarimol	2.36e <sup>-02</sup>	87.22	2.63	8.95e <sup>-03</sup>	5.19e <sup>-02</sup>
Vinclozolin + Chlorpyrifos	1.23e <sup>-01</sup>	73.73	17.59	6.96e <sup>-03</sup>	1.67e <sup>-02</sup>

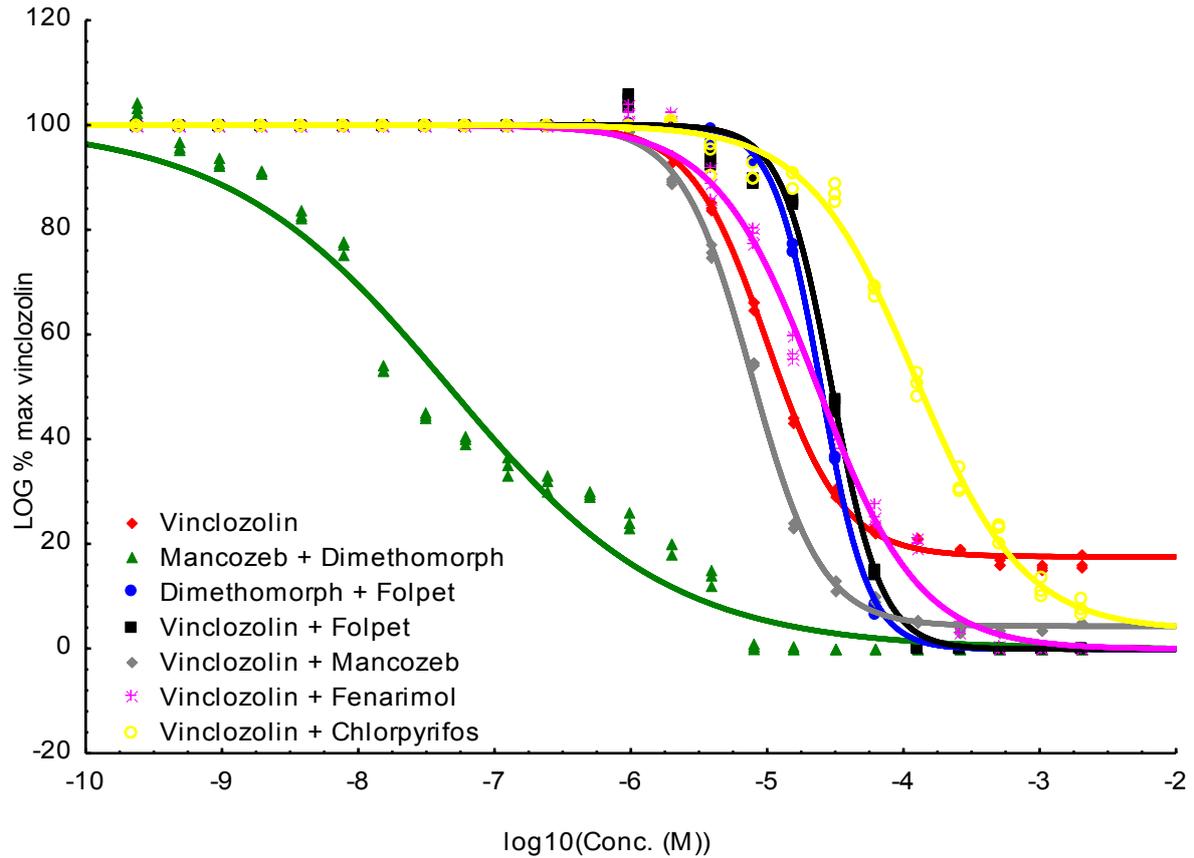


**Figure 4.2:** AR antagonistic dose-response of the test pesticides regularly used in agriculture in the Western Cape. Values are expressed as a percentage relative to the model AR antagonistic fungicide vinclozolin.

A dose-response concentration range between binary, equimolar mixtures of selected test pesticides was generated between 1  $\mu$ M and 1 mM with the exception of a binary mixture between mancozeb and dimethomorph, which ranged between 0.1 nM and 32  $\mu$ M in the assay (Fig. 4.3). The binary mixtures did not follow the expected additive mixture response at  $IC_{50}$  concentrations (isobole coefficient  $> 1$ , Table 4.2; Fig. 4.3) with the exception of a mixture between mancozeb and dimethomorph which generated an additive mixture response at  $IC_{50}$  concentrations (isobole coefficient  $\approx 1$ , Table 4.2; Fig. 4.3). This mixture also generated an observed  $IC_{50}$  concentration closer to the predicted  $IC_{50}$  concentrations assuming concentration addition (CA, Table 4.2). The mixture also had a higher AR antagonistic potency relative to vinclozolin alone (RP, 138.2%, Table 4.2). Binary mixtures of folpet and dimethomorph showed higher AR antagonistic potencies than dimethomorph

alone, but weaker than *folpet alone* (Table 4.2) and therefore generated an antagonistic mixture response at  $IC_{50}$  concentrations (isobole coefficient  $> 1$ , Table 4.2, Fig. 4.3). However, the mixture had observed  $IC_{50}$  concentrations closer to the predicted  $IC_{50}$  concentrations assuming concentration addition (CA, Table 4.2).

The observed  $IC_{50}$  concentrations of all other binary mixtures were closer to the predicted  $IC_{50}$  concentrations assuming independent action (IA, Table 4.2). A mixture between *folpet* and *vinclozolin* had an AR antagonistic potency of 85.3% which is less than the relative potency of *folpet alone* (Table 4.2). Although the fungicide *mancozeb* was a potent AR antagonist in the assay, a mixture of *vinclozolin* and *mancozeb* generated an  $IC_{50}$  concentration closer to the  $IC_{50}$  of *vinclozolin* (Table 4.2; Fig. 4.3). The mixture therefore showed a non-additive mixture response (isobole coefficient  $\gg 1$ , Table 4.2; Fig. 4.3) and was slightly less potent at antagonizing AR binding than *vinclozolin* alone (RP, 96.4%, Table 4.2). In the binary mixture of *vinclozolin* and *chlorpyrifos*, the mixture revealed a lower AR antagonistic potency relative to both *vinclozolin* and *chlorpyrifos* alone (RP, 73.7%, Table 4.2) and also did not conform to the expected additivity mixture response at  $IC_{50}$  concentrations (isobole coefficient  $> 1$ , Table 4.2; Fig. 4.3). This pesticide mixture was the least reactive in the anti-YAS.



**Figure 4.3:** 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.

## DISCUSSION

In this study, we confirm that fungicides used widely in the Western Cape agricultural region, all have the ability to antagonize AR binding in an *in vitro* yeast-based screen. These chemicals may then potentially disrupt the normal functioning of the androgenic endocrine system, which has been reported on in several *in vivo* vertebrate exposure studies (Kelce *et al.*, 1997; Gray *et al.*, 1999; Joshi *et al.*, 2005; Vinggaard *et al.*, 2005; Viswanath *et al.*, 2010). The fungicides vinclozolin, procymidone, folpet, flusilazole, dimethomorph, mancozeb, fenarimol and insecticide chlorpyrifos all exerted anti-androgenic activity by decreasing the androgen response expected from the presence of DHT, as assessed by using the anti-YAS. Since many of these fungicides may occur in mixture in water sources, we investigated the additivity null hypothesis (Christiansen *et al.*, 2008, 2009; Ermler *et al.*, 2011), and showed that this model mixture interaction may not always apply for chemical mixtures having similar MOAs.

Vinclozolin (a carboximide fungicide) has been extensively studied, and its anti-androgenic properties have been well-recorded. Therefore, vinclozolin have been included in most studies as a model AR antagonist (Gray *et al.*, 1999, Kelce *et al.*, 1997; Kojima *et al.*, 2004). The similar potency of this fungicide to flutamide has also been well-documented in both *in vitro* and *in vivo* studies (Hoffmann & Kloas, 2010; Kolle *et al.*, 2011). Perinatal exposure in rats has shown that vinclozolin applied at concentrations below the no-observed-effect-level (NOEL) did alter external genital morphology, seminal vesicle size and ventral prostate size (which are androgen-dependent traits) (Kelce *et al.*, 1997; Ostby *et al.*, 1999). In aquatic vertebrates, vinclozolin has been shown to modulate secondary sexual characteristic development and reproductive behaviour of male African clawed frogs, *Xenopus laevis* (Van Wyk *et al.*, 2003; Hoffmann & Kloas, 2010). The other carboximide fungicides screened in the present study (procymidone and folpet) also revealed AR antagonistic properties upon AR binding. The similar chemical structure of these fungicides therefore predicts a similar mode of action to vinclozolin. Procymidone, for example has been shown to cause an AR receptor-binding-dependent increase in LH and testosterone levels in rats and mice (Crisp *et al.*, 1998), and was shown to increase the incidence of hypospadias in newborn rats (Ostby *et al.*, 1999; Baskin *et al.*, 2001). However, folpet has not been studied for its potential anti-androgenic action, which has shown to be a potent AR antagonist when screened using anti-YAS. Folpet is a carboximide fungicide, like vinclozolin and promycidone, and a similar mechanism of action was predicted. However, due to the limited information on folpet as an anti-androgenic EDC, both *in vitro* and *in vivo* research is needed to confirm its MOA and specific mechanism of action.

It is evident that information regarding the specific mechanism of endocrine action is also lacking for other test chemicals such as the fungicide dimethomorph used in the present study. Not many studies have considered dimethomorph as an anti-androgenic EDC. However, Orton *et al.* (2011) reported that dimethomorph did antagonize AR binding in both a yeast-based assay as well as a mammalian cell-based assay (MDA-kb2), but that there was a large divergence between inhibition concentrations (IC) in the two assays. However, dimethomorph has a high environmentally equivalence ratio (ERR) and therefore, need to be included in future studies (Orton *et al.*, 2011). Furthermore, the dithiocarbamate fungicide mancozeb was found to be a potent AR antagonist in the present study; exceeding the AR

antagonistic potency of vinclozolin. Although, mancozeb is largely known to act as a thyroid inhibitor (Cocco, 2002; Pickford, 2010), recent research investigated its reproductive toxicity potential (Viswanath *et al.*, 2010). However, the anti-androgenic MOA of mancozeb needs more study since inconsistent results were reported, such as reports that showed mancozeb did not exhibit AR antagonistic activity in Chinese hamster ovary (CHO) cells nor did it reduce testosterone synthesis in human adenocarcinoma (H295R) cells (Hass *et al.*, 2012). However, an *in vivo* study using Wistar rats exposed to mancozeb reported lowered testis, epididymis and ventral prostate weights as well as lowered sperm counts (Joshi *et al.*, 2005). Results from the present study confirm the anti-androgenic MOA of mancozeb through a mechanism of AR antagonism. However, more comprehensive research (on both *in vitro* and *in vivo* level) is needed to verify the fungicide mancozeb as an AR antagonistic EDC. Apart from the fungicides tested in this study, the organophosphate insecticide chlorpyrifos is extensively used in agriculture and applied alongside fungicides and has been reported to show anti-androgenic activity in mammalian and amphibian species (Kang *et al.*, 2004a; Viswanath *et al.*, 2010; Bernabò *et al.*, 2011). Organophosphorus pesticides are commonly used in pest control and it is reported that several organophosphorous insecticides may have anti-androgenic and estrogenic properties (Kojima *et al.*, 2004). The present study confirms the anti-androgenic activity of chlorpyrifos with a dose-response activity similar to that of vinclozolin. Although, chlorpyrifos may act as an anti-androgen, several lines of evidence suggest that this pesticide may also disrupt the thyroid system (De Angelis *et al.*, 2009) and therefore has the potential to act as both a reproductive and thyroid endocrine disruptor which may complicate investigations of pesticide mixtures.

It is assumed that pollutants from various point sources eventually end up in complex mixtures in freshwater systems (Blake *et al.*, 2010). This has been confirmed, for example in pulp and paper mill discharges containing both androgenic and estrogenic compounds (Blake *et al.*, 2010) which combine with other pollutants from sources such as agricultural pesticides and effluents from wastewater treatment plants (WWTPs). In the agricultural sector itself, compounds from different agricultural crops located near each other and treated with different pesticide types (insecticides, nematocides, herbicides and fungicides) and will eventually reach water catchments (Bollmohr & Schulz, 2009). In complex mixtures, these cocktails of chemicals may include contaminants that may or may not have similar mechanisms of endocrine modulation (Backhaus & Faust, 2012).

For chemicals having the same mechanism of endocrine action, it is mostly assumed that a dose-additive response will be the outcome (Boone, 2008; Christiansen *et al.*, 2009; Ermler *et al.*, 2011). Since all the individual pesticides included in this study showed a dose-dependent inhibition effect of DHT binding to the AR, an additive response was the default prediction. The binary mixture of dimethomorph and mancozeb did provide an additive mixture response. This may be ascribed to dimethomorph (a less potent AR antagonist), which might increase the AR antagonistic potency of mancozeb. Interestingly, commercial agricultural fungicides are available which consist of a mixture of dimethomorph and mancozeb (for example Acrobat WG, MZ; DAFF, 2013).

In light of the findings during this study, further analyses are needed to investigate other commercial mixtures where the active ingredients may have a larger probability to accumulate in freshwater systems. However, it is evident in this study that chemical mixtures having similar MOAs will not always generate an additive mixture response. For example, the antagonistic mixture response between vinclozolin and chlorpyrifos in this study revealed that the mixture was far less potent than either one of the individual chemicals. The mixture between vinclozolin and folpet (which are both carboximide fungicides) also did not generate an additive mixture response. However, the binary mixture of vinclozolin and mancozeb raised a very interesting response, in which the inhibitory activity (capacity) of the vinclozolin/mancozeb mixture corresponded more to the vinclozolin inhibition response curve. The mixture interaction in this case therefore could be considered as being antagonistic, but the mixture was almost as potent at antagonizing the AR as vinclozolin and not the more potent mancozeb. These results suggest that the AR antagonistic potency of mancozeb was lowered when combined with vinclozolin due to vinclozolin itself, which might have a higher affinity to bind to the AR than mancozeb.

The non-additive response of the pesticide mixtures in this study is supported by other mixture studies as well. Equimolar mixtures of four parabens all revealed individual AR antagonism in CHO cells, but in mixture also had lower AR antagonistic properties than the predicted additivity model (Kjærstad *et al.*, 2010). This was also a case in another study where mixtures of 10, 11 and 16 estrogenic pesticides respectively revealed to have weaker estrogenic mixture responses than the expected additive response (Silva *et al.*, 2011). However, 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. Ermler *et al.* (2011) suggested that their approach using a balance mixture ratio design, as opposed to the equimolar mixture ratio design (Kjærstad *et al.*, 2010), accounted for the variation in AR antagonistic potency by the different parabens. Clearly, the lack of conformation of the CA model in the present study needs more research to confirm the deviation from the CA prediction as in other studies.

Clearly, the fate and presence of anti-androgenic pesticides is not well investigated in environmental samples (Urbatzka *et al.*, 2007b). Along with this conclusion, the pesticides tested for their AR antagonistic potential in this study have all been recorded to be used in South African agriculture and found in close-by river catchment systems (Dabrowski *et al.*, 2002; Dalvie *et al.*, 2003). To our knowledge the present study is the first to evaluate their AR antagonistic potential alone and in mixture. Most gonadal endocrine research studies in South Africa were concerned about estrogenic activity of environmental contaminants (Van Wyk *et al.*, 2005; Ansara-Ross *et al.*, 2012). However, the present study confirms the potential anti-androgenic activity of agricultural chemicals in local aquatic environments in the Western Cape regions of South Africa. Due to the cross-reactivity of certain compounds to several gonadal endocrine systems (e.g. targeting both the ER and AR), it has been noted that some compounds regarded as estrogenic EDCs may well be even more potent anti-androgenic EDCs (Sohoni & Sumpter, 1998). This phenomenon has been observed in an environmental screening study in the United Kingdom (Jobling *et al.*, 2009). Therefore, more research is needed to assess the situation in South African water systems receiving similar pollution-types than any developed country in the world.

It is also clear from the literature that there are limited data available on the environmental monitoring of some of the pesticides used in this present study (Jooste *et al.*, 2008; Ansara-Ross *et al.*, 2012). Several factors, including the pesticide's environmental fate, bio-availability and bioaccumulation/bio-concentration in a biotic system as well as geographical conditions and climatic periods in which the pesticide is applied might also modulate the field potency of a chemical (Dabrowski *et al.*, 2011). Therefore, we argue that knowledge

regarding temporal (seasonal) and spatial (different agricultural activities and river catchments) variation in environmental concentrations of fungicides must be considered in future studies. The present study has shown that the individual pesticides (alone or in mixture with other fungicides) can antagonize AR binding at concentrations well below the reported IC<sub>50</sub> levels and may therefore pose environmental threats well below reported NOAEL levels of some of the pesticides. In South Africa, government has initiated the national toxicity monitoring program (NTMP) which focuses on identifying chemicals of interest that causes toxic effects upon wildlife with the ultimate goal to isolate or phase-out these toxic substances found in inland water resources (Jooste *et al.*, 2008). It is however evident that this program does not propose to include EDC endpoints (Jooste *et al.*, 2008; Ansara-Ross *et al.*, 2012) even though contaminants (especially fungicides) present at low concentrations in the aquatic environment as well as in treated water may well be potent sources of anti-androgens. Although *in vitro* studies pose several advantages in establishing the mechanism of endocrine disruption of a chemical or when in mixture with other chemicals, the nature of these one-cell assays serves as valuable first-tier screens. However, the use of *in vivo* studies is still necessary to confirm the ED effects in functional biological systems. We have suggested the importance of, and deviations from the predicted additive model when exposing different concentrations of contaminants together. The finding that mixture predictions did not always apply when using equimolar mixtures needs to be further investigated, as this may complicate extrapolation of mixture results to environmental concerns. It is evident that a more comprehensive screen of regularly used pesticides on a tiered approach is needed, which includes more *in vitro* and *in vivo* mixture investigations of presently-used pesticides (Ansara-Ross *et al.*, 2012) both using equimolar and a mixed ratio design.

In conclusion, the present study confirmed the value of a recombinant yeast bioassay to screen for the specific mechanism of anti-androgenic action in locally-used 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 also reported 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 commonly used agricultural pesticide

mixtures needs further study in order to eventually apply predictive risk assessment models to complex environmental mixtures.

**CHAPTER 5: ENVIRONMENTAL SCREENING FOR GONADAL ENDOCRINE DISRUPTING CONTAMINANTS (EDCs) OF SELECTED WATER IMPOUNDMENTS AMONG VINEYARDS OF THE WESTERN CAPE (SOUTH AFRICA): USING *IN VITRO* AND *IN VIVO* BIOMARKERS (AFRICAN CLAWED FROG, *XENOPUS LAEVIS*).**

**ABSTRACT**

The Western Cape Province of South Africa is known for its intensive agriculture practices, including vineyards, orchards and wheat farming activities. With the high productivity of agricultural products, pesticides are applied in large quantities throughout most of the year. These agricultural pesticides reach and may accumulate in surface waters through spray drift, water runoff and/or groundwater leaching. In spite of the high usage of pesticides in the Western Cape region, very little is known about the activity of hormone mimics and endocrine modulators (so-called endocrine disrupting contaminants, EDCs) in water resources. The effects of gonadal EDCs on reproductive health of vertebrates in freshwater sources in this region also received little attention to date. The study included main aims and outcomes. The first aim was to screen for gonadal endocrine disruption activity (estrogenicity, androgenicity and anti-androgenicity) in ten water impoundments located at the bottom of converging slopes planted with vineyards and orchards in the Stellenbosch agricultural area, using recombinant yeast-receptor assays (for steroid receptor binding) and a rat minced testis bioassay (for steroidogenesis). The second aim was to collect adult male *X. laevis* frogs from four selected impoundments and assess several androgen-responsive biomarkers associated with the male reproductive system. Biomarker outcomes of male *X. laevis* frogs were compared with frogs collected from an impoundment site that is relatively secluded from agricultural practices and surrounded by natural vegetation. The yeast-based receptor assays showed variability in detection of androgenicity, anti-androgenicity and estrogenicity between the selected impoundments as well as between seasonal sampling (summer and winter). The minced testis assay showed increased steroidogenic (androgenic) activity in one of the water impoundments. Histological analyses of male-specific breeding gland activity (an androgen-regulated secondary sexual trait) did not vary significantly between adult male *X. laevis* frogs collected from four selected impoundments during the summer season. However, histological investigation of the testis of the adult male *X. laevis*

frogs showed some differences in spermatogenic germ cell types as well as a reduced percentage of spermatozoa in one impoundment site. Plasma testosterone (T) and dihydrotestosterone (DHT) concentrations varied significantly in frogs collected from one of the impoundment sites compared to the reference site. Plasma estradiol (E<sub>2</sub>) concentrations were elevated in frogs collected from two impoundment sites with males also showing expression of plasma Vtg in the blood. This study linked *in vitro* screening of environmental impoundment samples to actual endocrine disruption in adult male *X. laevis* frogs collected from selected impoundments. The androgenic and anti-androgenic activity recorded at the different test impoundments did not clearly correspond to variation in plasma hormones in adult frogs nor did this study show corresponding responses in breeding glands or spermatogenesis, although the percentage spermatozoa was reduced in frogs collected from Site 3. Estrogen receptor activity corresponded to the reproductive endpoints *in vivo*. Taken together, in this study the *in vivo* assessment of adult male frogs did not reflect the receptor interaction or steroidogenesis results obtained from the *in vitro* screening. However, estrogenicity detected in the yeast-based assays did correspond to increased plasma E<sub>2</sub> and Vtg levels in male frogs. Although some variation in spermatogenesis was observed between frogs in the water catchments, proliferation of breeding glands did not seem to be affected. This study propose that reproductive endpoints such as the development of secondary sexual characteristics (SSCs) during breeding periods are less affected in spite of *in vitro* analysis which reported gonadal endocrine disruption in the impoundments.

**Key words:** Impoundments, YAS/anti-YAS/YES, *Xenopus laevis*, breeding glands

## INTRODUCTION

It has been reported on a global scale that several widely used agricultural pesticides may modulate endocrine system pathways in vertebrate and invertebrate species (WHO, 2012). Such contaminants are collectively referred to as endocrine disrupting contaminants (EDCs). An EDC is defined as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or sub-populations” (WHO, 2012). One group of EDCs in particular that has received the most attention are contaminants that modulate reproductive queues and gonadal development of wildlife species as well as humans. Although it has been stated that EDCs modulating the gonadal endocrine system can come from various point and non-point pollution sources (WHO, 2012), agricultural activity is proposed to be a major contributor to environmental EDC contamination of water resources. Several agricultural pesticides have been linked to reproductive health effects in wildlife species (McKinlay *et al.*, 2008; Orton *et al.*, 2011; Hass *et al.*, 2012). However, the majority of research regarding reproductive endocrine disruption has been focused on contaminants affecting estrogenic endocrine pathways, due to the ubiquity of synthetic estrogens (female contraceptives) and insecticides such as DDT (Colborn *et al.*, 1993; Guillette *et al.*, 1994). However, several cases of male-specific gonadal disorders such as testicular dysgenesis syndrome (TDS), cryptorchidism, hypospadias, decreased penile length, reduced sperm quality and testicular cancer have been postulated to be linked to *in utero* exposure to EDCs other than conventional estrogenic pollutants (Skakkebaek *et al.*, 2001; Orton *et al.*, 2011). Therefore, in light of broadening the knowledge of gonadal EDCs in the environment as well as identifying the outcomes of these pollutants on human health and wildlife species, contaminants having androgenic and anti-androgenic endocrine disrupting properties have come into focus as well (Urbatzka *et al.*, 2007a).

Most EDC studies and monitoring programs (EDSTAC, 1998; McKinlay *et al.*, 2008) focused on screening single man-made chemicals for their effects on reproductive endocrine system pathways, with few mixture or environmental studies having been conducted to date. In spite of laboratory studies showing disruption of normal endocrine system functioning when exposed to pesticides, understanding the effects of complex mixtures in field situations need more attention. Single pollutants are seldomly found in the environment and typically

end up in complex mixtures together with several pollutants coming from diverse sources (Sowers *et al.*, 2009; Orton *et al.*, 2013; Pottinger *et al.*, 2013). Pollutants in mixtures are shown to cause combination effects with each other by either increasing or reducing the magnitude of an endocrine disruption outcome (Christiansen *et al.*, 2009; Hass *et al.*, 2007; Rider *et al.*, 2009). It has been hypothesized that chemicals having similar modes of endocrine disrupting actions (MOAs) may interact additively (an increased endocrine disrupting activity compared to their individual counterparts) when combined in mixtures or when these pollutants accumulate in water systems (Ermler *et al.*, 2011; Orton *et al.*, 2013). Alternatively, chemicals having dissimilar MOAs might interact independently from one another, therefore reducing or having no elevated mixture effect of an endocrine disrupting outcome when the individual chemicals are combined (Ermler *et al.*, 2011; Orton *et al.*, 2013). Due to the multiple mechanisms by which environmental pollutants exert their endocrine disrupting effect as well as the different mixture interaction hypotheses, the presence, activity and fate of EDCs ending up in complex mixtures in the environment are not well understood (Feron & Groten, 2002; Urbatzka *et al.*, 2007b). Although the majority of studies support the concept of concentration addition and independent action, depending on the mechanism of an individual chemical's endocrine disrupting action, (Birkøj *et al.*, 2004; Ermler *et al.*, 2011; Hass *et al.*, 2007; Orton *et al.*, 2012), some pesticides mixtures were found to deviate from these proposed mixture interaction models (Christiansen *et al.*, 2009; Kjærstad *et al.*, 2010). These results clearly support the assumption that mixture interaction investigations in the environment may be more complex than previously thought (Feron & Groten, 2002; Orton *et al.*, 2013).

Several *in vitro* assays have been described in the literature to measure and investigate gonadal endocrine disruption of selected pollutants or environmental water samples (EDSTAC, 1998; GWRC, 2003). Agricultural pesticides, in particular, are largely screened for their mechanism of modulating androgen endocrine pathways (Birkøj *et al.*, 2004; Kojima *et al.*, 2004; McKinlay *et al.*, 2008; Ermler *et al.*, 2011; Orton *et al.*, 2011; 2012; 2013). Furthermore, these assays are also used to screen environmental water samples for gonadal endocrine disrupting activity (Urbatzka *et al.*, 2007b; Aneck-Hahn *et al.*, 2009; Grover *et al.*, 2011; Jarosova *et al.*, 2012; Jállová *et al.*, 2013) and can provide valuable information on the occurrence of gonadal EDCs in sediment and water systems.

Although *in vitro* analyses gives a good idea of the presence of pollutants affecting certain endocrine pathway mechanisms, it is necessary to incorporate these results with *in vivo* endpoints, which report on the effect of the pollutants on a biological level. In light of gaining knowledge regarding the occurrence of gonadal EDCs in surface waters, as well as the effects of these pollutants in wildlife species and humans, several aquatic species have been used as bioindicators or sentinel species to detect endocrine disruption in water systems. Anurans are regularly used for endocrine disruption studies due to their direct contact to pollutants entering water systems (Kloas *et al.*, 2009). The African clawed frog, *X. laevis*, has been extensively studied in the laboratory and field to serve as a bioindicator species of endocrine disruption (Hecker *et al.*, 2004; Urbatzka *et al.*, 2007a; Kloas *et al.*, 2009; Behrends *et al.*, 2010; Hoffmann & Kloas, 2010). In its natural habitat, this species has also been shown to serve as a sentinel species in polluted areas to detect endocrine disrupting activity (Hecker *et al.*, 2004; Van Wyk *et al.*, 2005; Du Preez *et al.*, 2009). Furthermore, due to the reported decline of amphibian populations on a global scale (Carey & Bryant, 1995), EDCs may serve as one of the factors leading to these declines. It has been reported, for example, that a US wastewater effluents can modulate the growth and sexual development of the northern leopard frog, *Rana pipiens* (Sowers *et al.*, 2009). Another study reported on gonadal disorders such as ovarian dysgenesis, testicular oocytes and female-biased sex ratios, which were found in Plain leopard froglets (*Lithobates blairi*) living in a US wetland close to agricultural areas (Papoulias *et al.*, 2013). Collection of endemic anuran species therefore gives a good indication on the effects of EDCs within a biological system in the environment, such as water catchments and man-made impoundments exposed to endocrine disrupting pollutants.

South Africa is reported to be the largest produce-producing country in sub-Saharan Africa (Ansara-Ross *et al.*, 2012; Dabrowski *et al.*, 2014). With the high productivity of agricultural crops and demand for quality yield, South Africa also has the highest usage of pesticide active ingredients in southern Africa (Dabrowski *et al.*, 2014). Along with the high pesticide use, agricultural practice has been shown to be a major contributor to surface water pollution (Heath & Claassen 1999; Ansara-Ross *et al.*, 2012). Surface waters such as rivers and man-made impoundments are regularly situated in close proximity or at the bottom of converging slopes of sprayed areas (e.g. vineyards and orchards), leading to these water sources systems being polluted by agricultural pesticides through spray drift, leaching and groundwater

runoff, (Dabrowski *et al.*, 2002, 2011, 2014; Ansara-Ross *et al.*, 2012). Although the harmful effects of pesticide pollution on wildlife species are well known (Heath & Classen, 1999; Barnhoorn *et al.*, 2004; Bornman *et al.*, 2009; Swart *et al.*, 2011; Wagenaar *et al.*, 2012; Kruger *et al.*, 2013), the occurrence and outcomes of pollutants affecting endocrine pathways of non-target organisms are not monitored under the South African water quality guidelines (DWAF, 1996; Jooste, 2008). The clinical implications of EDC contamination in surface waters therefore received little attention to date (Van Wyk *et al.*, 2005; Bollmohr *et al.*, 2008; Olujimi *et al.*, 2010; Bornman & Bouwman, 2012).

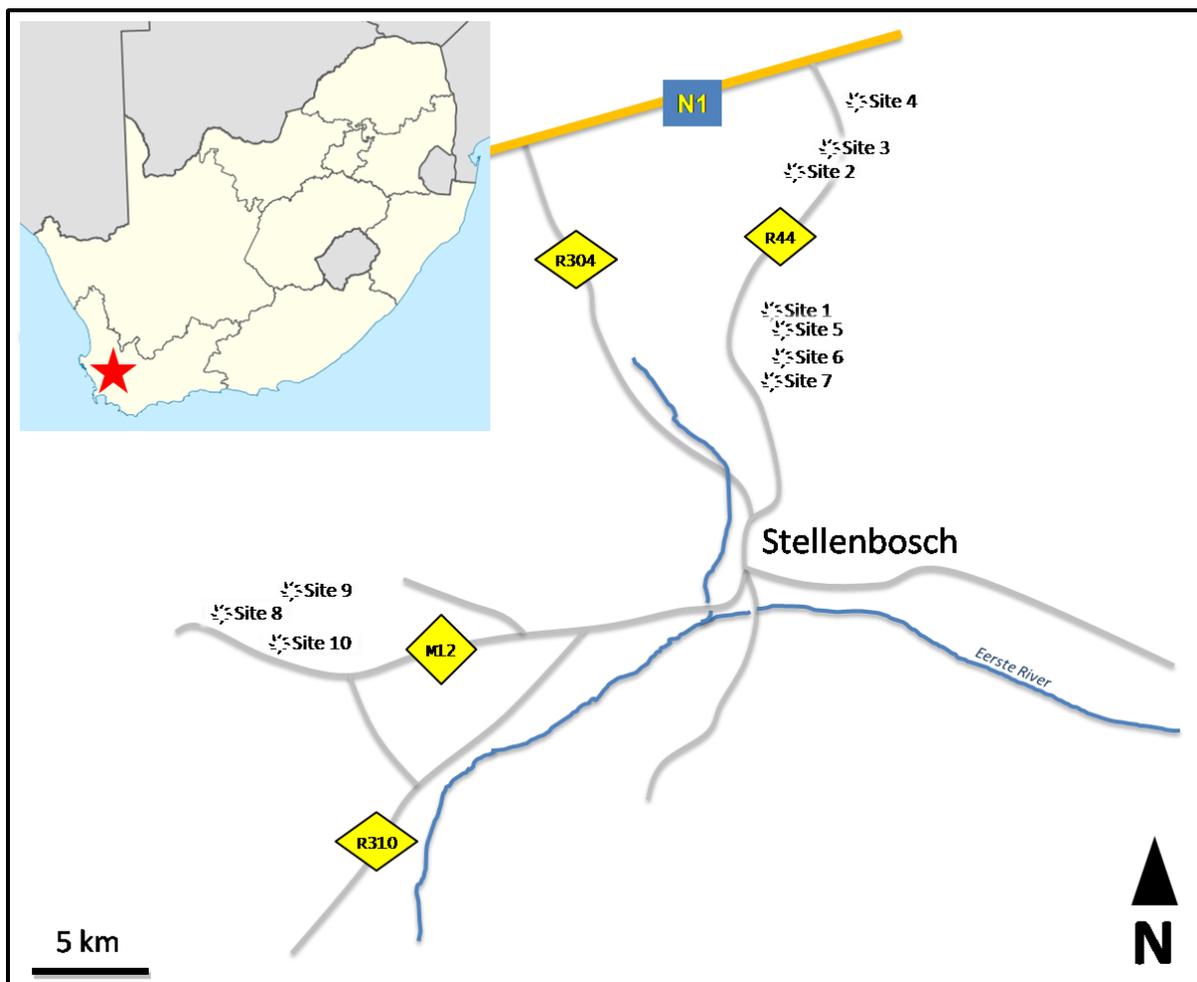
The aim of the current study was therefore to screen for estrogenic, androgenic or anti-androgenic endocrine disrupting activity in impoundments located within intensive farmed areas, primarily those surrounded by vineyards and orchards. In light of the relative high use of pesticides, especially fungicides, associated with the local farming practices (Dabrowski *et al.*, 2014.) and the anti-androgenic activity of several of these regularly used pesticides (results obtained from chapter 4 in the present thesis), water samples were collected and screened for their endocrine disruption activity. Secondly, adult male *X. laevis* frogs were collected from a subset of sites to investigate whether male reproduction is compromised, by using primary and secondary sexual endpoints, indicating the reproductive state of the organisms. The *in vitro* results were then correlated with the *in vivo* results to see whether the *in vitro* analyses can serve as a predictor of the observed *in vivo* endocrine disrupting effects in the frogs.

## **MATERIALS AND METHODS**

### *Areas of study and extraction of water samples*

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. 5.1). Nine impoundments were selected to be situated close to agricultural practices and receiving water runoff from converging slopes (Sites 2-10; Fig. 5.1) 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. 5.1). Sampling was done twice during the year of 2013 in two different seasons; during the pre-harvest period (summer, late February) and early winter (end of May). Water samples (500 mL) were taken at two different source

points per locality and combined to obtain a final 500 mL sample for each impoundment. Water was extracted in the laboratory using a standard solid phase extraction (SPE) protocol (Swart & Pool, 2007). Briefly, water samples were filtered (Whatmann No. 1) to remove large particles and extracted using Supelclean™ ENVI 18-SPE cartridges (Supelco, Sigma-Aldrich). The columns were left to dry overnight, eluted using a phase B solvent (40% hexane, 45% methanol and 15% 2-propanol) and suspended in 0.5 mL 100% absolute methanol, resulting in a 1:1000 dilution of the water samples.

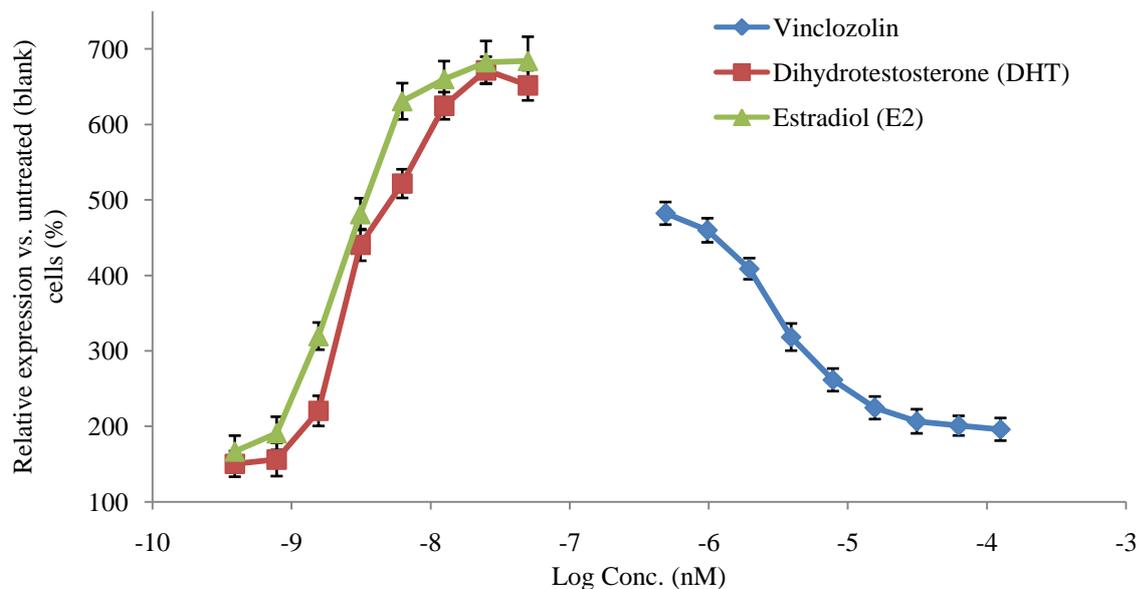


**Figure 5.1:** Localities of water samples taken from water impoundments located close to agricultural practices. Site 1 was selected as relatively secluded from agricultural practices, surrounded by natural vegetation and not receiving runoff from converging slopes whereas the other sites (Site 1-3 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.

*Recombinant yeast androgen/anti-androgen (YAS/anti-YAS) and estrogen screen (YES)*

To assess the presence of pollutants modulating androgen and estrogen receptor binding, a recombinant yeast estrogen- (YES), androgen- (YAS) and anti-androgen screen (anti-YAS) were used. These assays detect ligands in the water samples to: 1) agonistically bind to the human androgen receptor (hAR) indicating androgenicity; 2) antagonistically bind to the hAR showing anti-androgenic activity, and 3) agonistically bind to the human estrogen receptor (hER) indicating estrogenicity. The protocol followed was according to guidelines as set out by Sohoni & Sumpter (1998) and De Jager *et al.* (2011) with some slight modifications (Urbatzka *et al.*, 2007b; Kolle *et al.*, 2011). In brief, growth medium supplemented with the chromogenic substrate chlorophenol red- $\beta$ -D-galactopyranoside (CPRG) was added to *Sacharromyces cerevisiae* yeast transfected with either hAR or hER. Ligands may then bind to the hAR or hER in the yeast, which binds to the androgen response element (ARE) or estrogen response element (ERE) respectively, which then encode for the enzyme  $\beta$ -galactosidase and metabolize CPRG, indicating a dose-dependent activity of the ligands upon receptor binding. Standard curves of a model AR agonist (dihydrotestosterone; DHT), AR antagonist (vinclozolin; VIN) and ER agonist (estradiol; E<sub>2</sub>) were used as positive controls in the YAS, anti-YAS and YES respectively (Fig. 5.2).

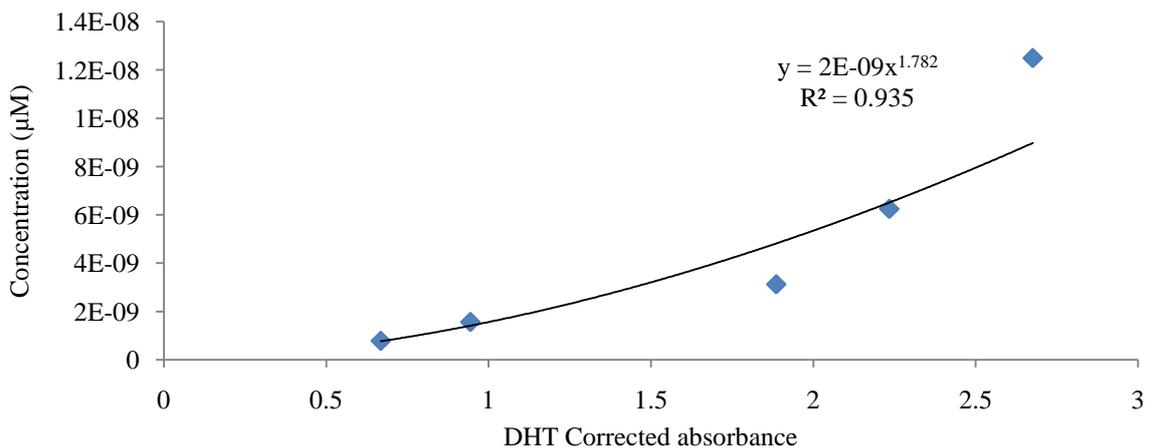
A stock solution of 1  $\mu$ M DHT was dissolved in absolute ethanol and serially diluted to generate a standard curve in the YAS. A stock solution of 0.2 mM VIN was serially diluted in absolute ethanol along with a DHT concentration (spike) of 6.25 nM (EC<sub>50</sub> from the DHT standard curve in the YAS) to generate a standard curve in the anti-YAS. For the YES, a stock solution of 0.2  $\mu$ M E<sub>2</sub> was dissolved in absolute ethanol and serially diluted to generate a standard curve in the YES. Validation of standard curves generated for the YAS, anti-YAS and YES showed suitability of the assays to detect ligands causing androgenicity, anti-androgenicity and estrogenicity respectively through steroid receptor binding (Fig. 5.2).



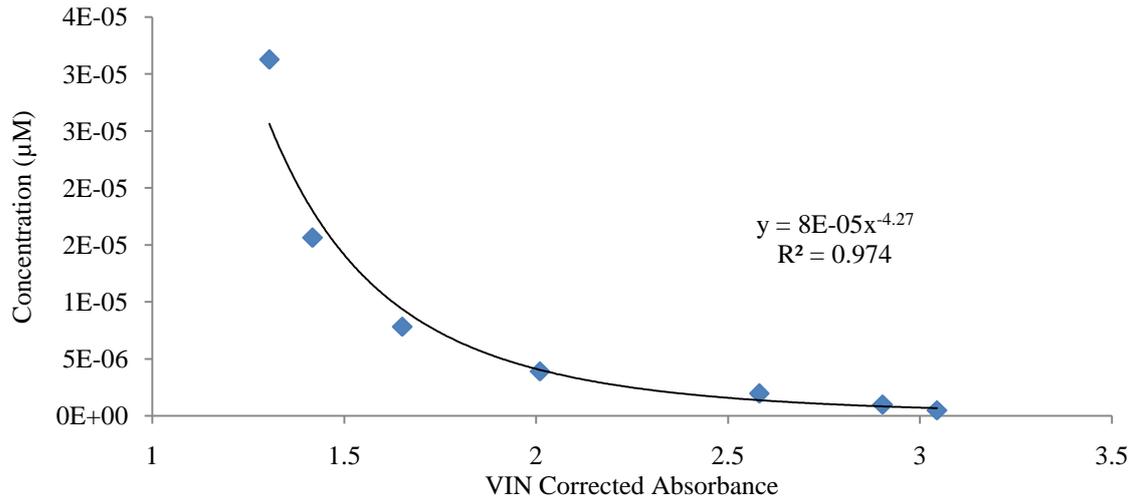
**Figure 5.2:** Sigmoidal standard curves of dihydrotestosterone (DHT), estradiol (E<sub>2</sub>) and vinclozolin in the yeast androgen (YAS), estrogen (YES) and anti-androgen screen (anti-YAS) respectively. Values (means ± SE) are expressed relative to untreated (blank) cells in the assays. The series dilution of vinclozolin was spiked with 6.25 nM DHT (EC<sub>50</sub>) in each well to obtain a dose-response relationship of the competitive AR binding between DHT and vinclozolin in the assay.

Water samples were tested in the presence or absence of 6.25 nM DHT (EC<sub>50</sub>) in the anti-YAS and YAS respectively to identify ligands in the water which might competitively bind or interfere with hAR binding. Samples were also exposed in the YES to identify ligands in the water which might agonistically bind with the hER in the assay. Water samples were transferred to a sterile 96-well flat bottom assay plate with a low evaporation lid (Costar, 3370). 10 µL of the sample were added per well and allowed to evaporate until dryness. Blank untreated cells were supplemented with absolute ethanol only (10 µL per well). Assay medium (containing CPRG) was added (200 µL/well) to give a final 1/20 dilution of the test chemicals in the assay. 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 Lab systems). These measurements were used to calculate the final absorbance (corrected value) of each water samples using the equation: Corrected value = sample absorbance (570 nm) – [sample absorbance (620 nm) – blank absorbance (620 nm)]. Corrected absorbances of water samples were log-transformed and compared relative to the log-transformed absorbance of the blank (untreated) cells.

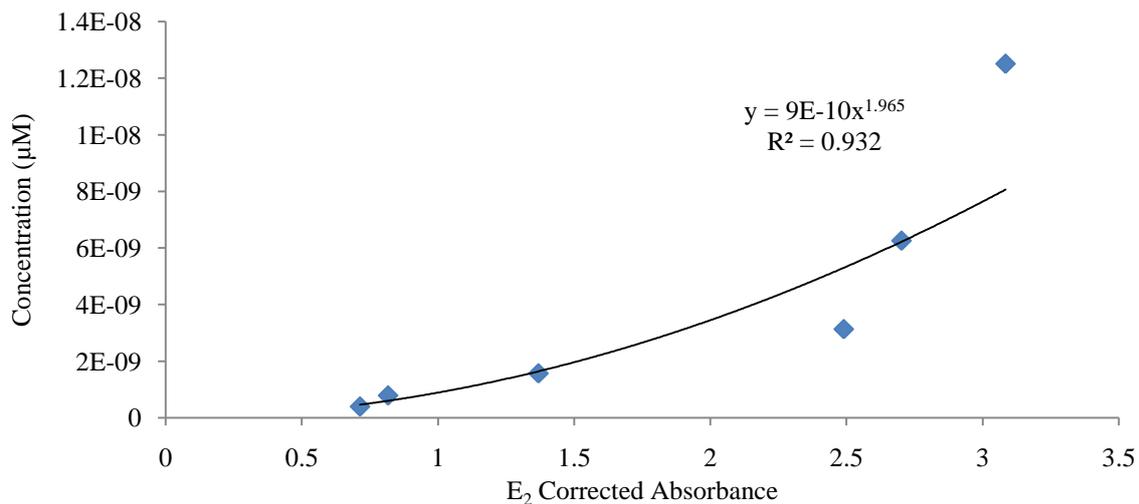
After absorbance was calculated, the water samples were measured for their equivalent concentrations relative to the standard curves generated for each assay, namely; a DHT-equivalent concentration (DHT-EQ, generated from the YAS), a vinclozolin-equivalent concentration (VIN-EQ, generated from the anti-YAS), and an estradiol-equivalent concentration (E<sub>2</sub>-EQ, generated from the YES). Each water sample absorbance was then incorporated to each exponential trendline function of each generated standard curve (Figs. 5.3; 5.4; 5.5). For DHT-EQ concentrations, absorbancies of the water samples were incorporated into the function,  $y = 2E-09x^{1.782}$  (Fig. 5.3). For VIN-EQ concentrations, absorbancies of the water samples were incorporated into the function,  $y = 8E-05x^{-4.27}$  (Fig. 5.4). For E<sub>2</sub>-EQ concentrations, absorbancies of the water samples were incorporated into the function  $y = 9E-10x^{1.965}$  (Fig. 5.5).



**Figure 5.3:** Standard curve generated for dihydrotestosterone (DHT) in the yeast androgen screen (YAS). The y-function was generated from a power trendline, which was used to measure DHT-equivalent concentrations (DHT-EQ) of water samples used in the assay.



**Figure 5.4:** Standard curve generated for vinclozolin (VIN) in the yeast anti-androgen screen (anti-YAS). The y-function was generated from a power trendline, which was used to measure VIN-equivalent concentrations (VIN-EQ) of water samples used in the assay.



**Figure 5.5 :** Standard curve generated for vinclozolin (E<sub>2</sub>) in the yeast estrogen screen (YES). The y-function was generated from a power trendline, which was used to measure E<sub>2</sub>-equivalent concentrations (E<sub>2</sub>-EQ) of water samples used in the assay.

### *Steroidogenesis assay*

Steroid biosynthesis induced or inhibited by the environmental samples was measured by conducting a minced testis bioassay as described by Ebrahim & Pool (2010). Three-month old male Balb/C mice were obtained from the University of Cape Town Animal Unit (Cape Town, South Africa). The mice were housed in a well ventilated animal house at the University of Western Cape Physiological Department (UWC, Belville, South Africa) under

a light-dark cycle of 12h:12h and fed standard mouse feed (Medical Research Council, Cape Town, South Africa) and normal drinking water. The mice were sacrificed by cervical dislocation and the testes removed aseptically. Prior to the removal of the testes, a serum-free medium was prepared using RPMI-1640 medium (Sigma-Aldrich) with the addition of 0.2% bovine serum albumin (BSA), 1% glutamax (Invitrogen) and a 1% Penicillin/Streptomycin/Fungizone mixture (Sigma-Aldrich). The testes were minced in 10 mL of the serum-free medium and testis debris was allowed to settle. The supernatant containing cells were transferred to a 50 mL test tube containing additional serum-free medium. The cells were incubated for four hours (37°C with 5% CO<sub>2</sub>) and centrifuged at 8000 rpm for 10 minutes. The supernatants were discarded and cells re-suspended in 10 mL serum-free medium and then incubated at 37°C with 5% CO<sub>2</sub> for another 30 minutes. Cells were centrifuged once more and supernatant discarded and the cell pellet re-suspended in 10 mL serum-free medium to a cell concentration of 3.4x10<sup>6</sup> cells/ml. Water samples were transferred to a sterile 96-well plate at a 10x concentrated dilution range. Changes in plasma testosterone synthesis by cells after exposure to the different water samples were subsequently determined using a testosterone ELISA kit (DRG Instruments, GmbH, Germany).

#### *Cell viability assay*

The effects of the water sample extracts on the viability of the minced testis cells were also determined according to Ebrahim & Pool (2010). Briefly, cells exposed to the water samples, as well as untreated cells, were analyzed for their ability to reduce yellow tetrazolium salt (XTT) to an orange formazan product. The cell viability assay was done under the same conditions as done during the same time period as the minced testis assay. Cells were seeded into a sterile 96-well plate and incubated for 1 hour. After the incubation period, an XTT reagent mix (Roche Diagnostics GmbH, Germany) was added to the cells at 50 µL per well and incubated for 4 hours at 37°C and 5% CO<sub>2</sub>. The formation of formazan was then measured using a microtitre plate reader (Thermo multiscan, type 355, Ascent, version 2.6, Thermo Lab systems) and quantified at a 450 nm absorbance wavelength.

#### *Field sampling of male frogs*

The necessary ethical clearance (SU-ACUM13-00038) for using *X. laevis* frogs in experimental procedures was obtained prior to the sampling of the frogs. 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 (Fig. 5.1). Upon arrival at the laboratory, body mass (BM) was measured to the nearest 0.01 g and snout-to-vent length (SVL) to the nearest 0.01 cm to calculate a condition index (CDIndex; Janssen *et al.*, 1995) for each frog using equation 1. 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 heparinised syringe and needle. Blood was centrifuged at 8,000 rpm for 2 min, plasma extracted and stored at -80°C for plasma hormone (testosterone, T; dihydrotestosterone, DHT and estradiol, E<sub>2</sub>) and Vtg (vitellogenin) 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 using equation 2 and 3, respectively. Gonads from six frogs per impoundment were then placed in Bouin's fixative (Bancroft & Stevens, 1977) for histological analyses.

$$\text{CDI} = \frac{\text{Body mass (BM)}}{[\text{Snout-to-vent (SVL)}]^3} \times 100 \quad [\text{Eq. 1}]$$

$$\text{HSI (\%)} = \frac{\text{Liver mass}}{\text{Body mass}} \times 100 \quad [\text{Eq. 2}]$$

$$\text{GSI (\%)} = \frac{\text{Gonad mass}}{\text{Body mass}} \times 100 \quad [\text{Eq. 3}]$$

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

### *Histology*

The NP skin and gonads were washed in water for several hours and subsequently processed for routine paraffin wax histology. Embedded samples were sectioned at 8 - 10 µm using a rotary microtome, de-waxed and stained with hematoxylin and eosin (H&E; Bancroft & Stevens, 1977). Digital micrographs of ten breeding glands per NP integument sample (for

each individual) were recorded at a magnification of 400x using a light microscope (DMD108, Leica). Image analysis (Leica Application Suite, Version 1.5.0, Switzerland) of breeding glands were used to measure gland epithelial heights (average of six measurements per gland) to the nearest micrometer ( $\mu\text{m}$ ) and glandular area to the nearest square micrometer ( $\mu\text{m}^2$ ).

Histology of the cystic-type testis in *X. laevis* was assessed according to literature (Rastogi *et al.*, 1986; Hu *et al.*, 2008; Kaptan & Murathanoğlu, 2008). Digital micrographs of ten seminiferous (SN) tubules per testis sample were captured at a magnification of 100x using a light microscope (DMD108, Leica) and image analysis software (Leica Application Suite). Spermatogenic germ cell stages were assessed according to Hu *et al.* (2008). Germ cell stages were classified as primary and secondary spermatogonia (1° and 2° SPG), spermatocytes (SPC) and spermatids (SPT). Cysts representing 1° SPG were recognized as large single cells surrounded by a membrane, whereas 2° SPG were identified as having solid oval nuclei with clear nuclear envelopes and smaller in size than 1° SPG. The SPC germ cell stage was classified as having cells with dispersed chromatin and no clear nuclear envelope and SPT germ cell stages were identified by having condensed nuclei, darkly stained with hematoxylin and smaller in size than SPCs. Some SPT cell stages were also identified as having an elongated cell size leading to spermatozoa (SPZ) formation, which was identified as having crescent-shaped tail flagella and being more situated towards the lumen of the SN tubules. Germ cell stages were compared between treatment groups by calculating a ratio (in percentage) of cysts bearing 1° SPG, 2° SPG, SPC and SPT germ cell types. The average percentage of SPZ per SN tubule area was determined by calculating the area of the SN tubule containing SPZ relative to the total area of the SN tubule.

#### *Circulating hormone and vitellogenin (VTG) determinations*

Plasma testosterone (T), dihydrotestosterone (DHT) and estradiol ( $\text{E}_2$ ) 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> concentrations were also used to calculate the ratio of DHT relative to T (plasma DHT/T ratio) and E<sub>2</sub> relative to T (plasma E<sub>2</sub>/T ratio) of each frog in the different water catchments to compare androgen turnover between the different impoundment sites.

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 exposure to estrogens 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 sampling sites (see Fig. 5.1) and expressed as relative fold change from the levels obtained from the control site (Site 1, Fig. 5.1).

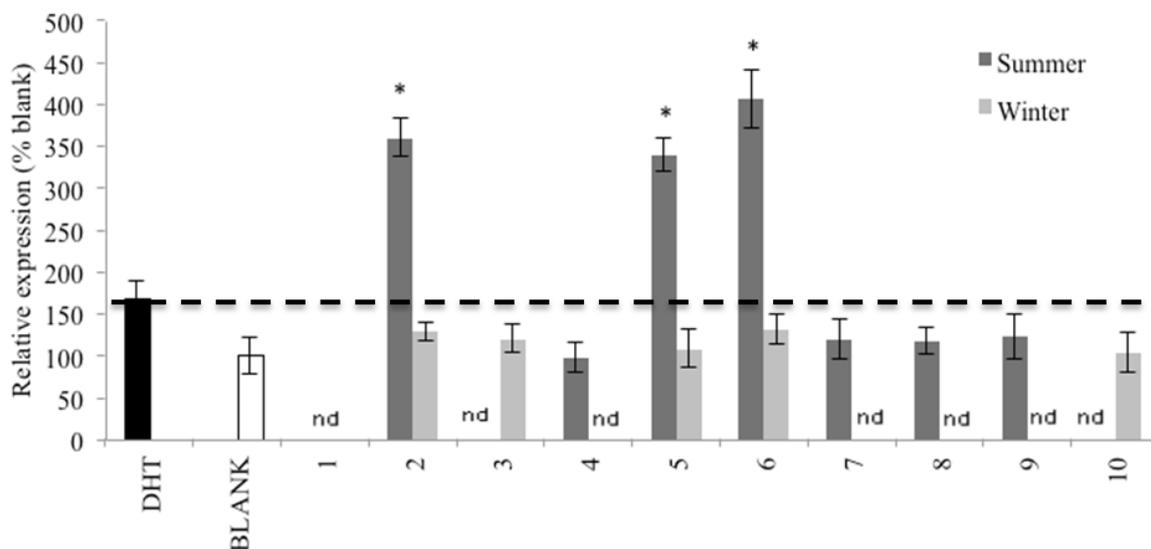
#### *Statistical analysis*

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.

## RESULTS

### *Recombinant yeast screens*

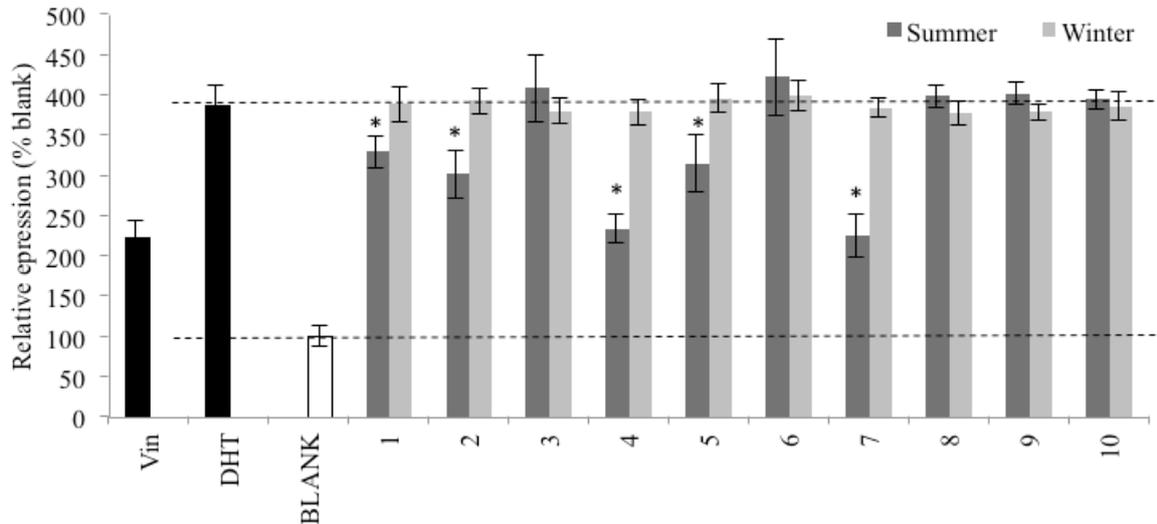
Most impoundments showed low inducibility or no detection (nd) of agonistic AR binding between impoundment sites during the two seasonal sampling periods (Fig. 5.6). However, in the summer period, three locations (Sites 2, 5 and 6; Fig. 5.1) showed elevated levels of ligand-induced AR binding compared to blank (untreated) yeast cells (ANOVA,  $F_{9,39} = 3.48$ ; Tukey,  $p < 0.05$ , Fig. 5.6). Androgen concentrations in the three water impoundments ranged between 0.22 and 0.14  $\mu\text{g/L}$  (Site 6 > Site 2 > Site 5) relative to a serial dilution range of DHT in the assay (Fig. 5.6).



**Figure 5.6:** 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.

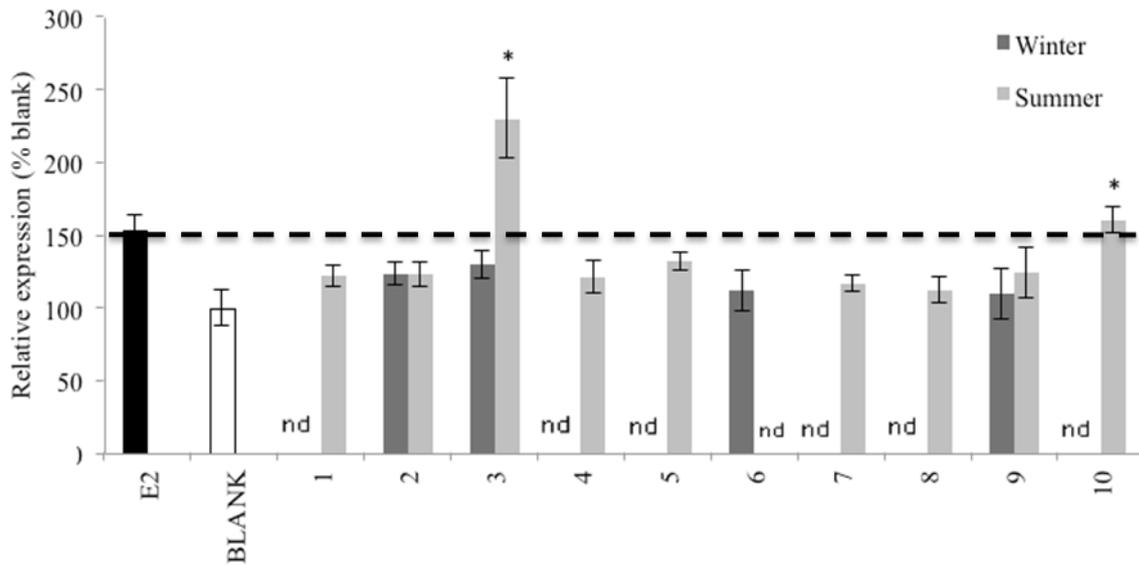
Water collected from five impoundments (Sites 1, 2, 4, 5 and 7; Fig. 5.1) significantly inhibited AR-mediated  $\beta$ -galactosidase activity (anti-androgenic) effects in the anti-YAS (ANOVA,  $F_{9,39} = 0.44$ ; Tukey,  $p < 0.05$ , Fig. 5.7). Vinclozolin-equivalent concentrations of the water samples were calculated to vary between 2.57 and 0.092 mg/L (Site 7 > Site 4 >

Site 2 > Site 5 > Site 1) relative to the standard curve generated for vinclozolin (an AR antagonistic fungicide) in the assay (Fig. 5.7). Most AR antagonistic activities 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. 5.7).



**Figure 5.7:** 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  $IC_{50}$  of DHT-induced antagonizing activity 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).

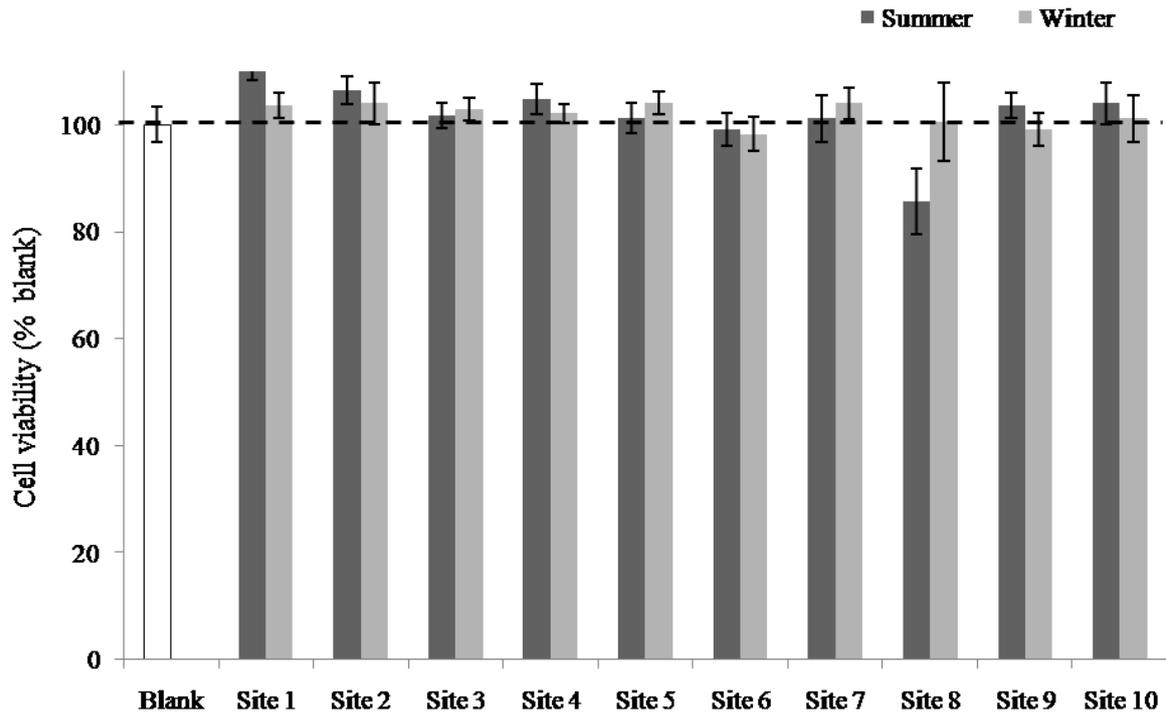
The *in vitro* YES assay showed estrogenic activity in water samples collected from two sampling locations compared in summer (ANOVA,  $F_{9,39} = 2.29$ ; Tukey,  $p < 0.05$ ; Site 3 > Site 10; Fig. 5.8), which corresponded to  $E_2$  equivalent concentrations ranging between 0.082 and 0.029  $\mu\text{g/L}$ .



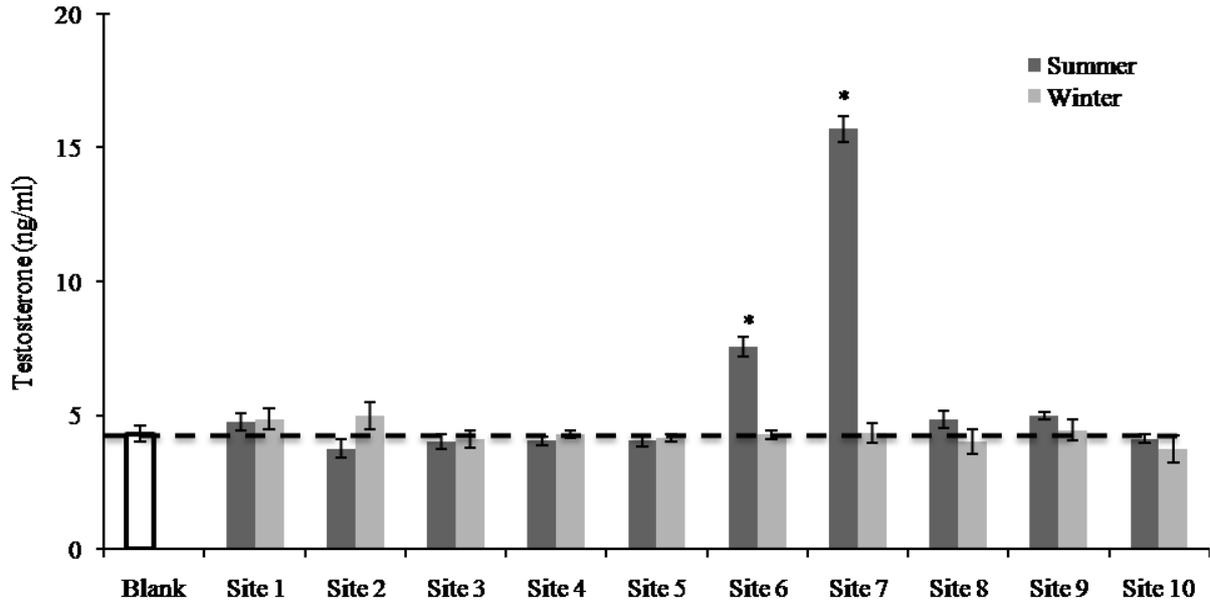
**Figure 5.8:** Estrogenic activity in water samples collected from impoundments situated close to agricultural practices (Sites 2-10) and an impoundment isolated from agricultural practices (Site 1) in the YES. Samples were taken during summer and winter seasons and expressed relative to untreated (blank) cells in the assay. The concentration shown for E<sub>2</sub> represents the EC<sub>50</sub> concentration of E<sub>2</sub> 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. 5.9). Water samples collected from two impoundments (Sites 6 & 7; Fig. 5.1) stimulated testosterone production significantly in the *in vitro* rat minced testes culture ranging from 7.58 to 15.72 ng/mL as compared to untreated (blank) cells (Fig. 5.10; ANOVA,  $F_{9,39} = 5.59$ ; Tukey,  $p < 0.05$ ). Exposure of the two water impoundment samples (Sites 6 & 7) in the culture resulted in a 76-265% increase of testosterone production compared to untreated (blank) cells (Fig. 5.10).



**Figure 5.9:** 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 ( $\% \pm \text{SD}$ ) relative to control treated (blank) cells. The asterisk indicates significant difference from the blank cells (ANOVA,  $p < 0.05$ ).



**Figure 5.10:** Effect of water samples at the catchment sites during summer and winter sampling on testosterone secretion (ng/ml  $\pm$  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,  $p < 0.05$ ).

**Table 5.1:** 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	hAR (androgenicity)		Anti-hAR (anti-androgenicity)		hER (estrogenicity)		Mince testis (androgenicity)	
	S	W	S	W	S	W	S	W
1	-	-	+	-	-	-	-	-
2	+	-	+	-	-	-	-	-
3	-	-	-	-	+	-	-	-
4	-	-	+	-	-	-	-	-
5	+	-	+	-	-	-	-	-
6	+	-	-	-	-	-	-	-
7	-	-	+	-	-	-	+	-
8	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-
10	-	-	-	-	+	-	-	-

*Field-collected male Xenopus laevis frogs*

*Body Condition, GSI and HSI comparison*

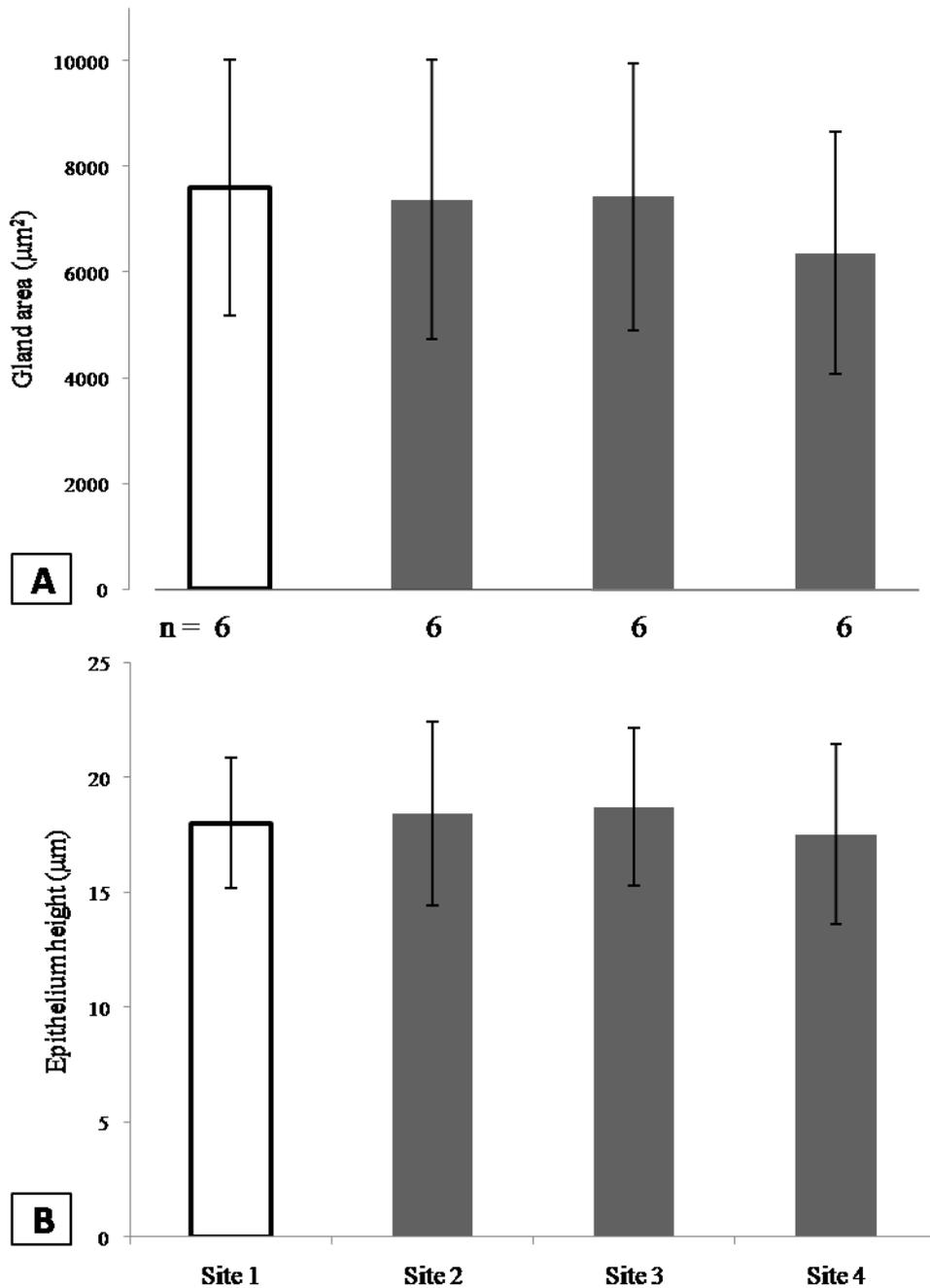
Frogs collected from the water impoundments did not differ statistically in body mass (BM), snout-to-vent length (SVL), body condition (CDI), mean GSI, or mean HSI among catchment sites (ANOVA,  $p > 0.05$ ; Table 5.2).

**Table 5.2:** Morphometric measurements of male *X. laevis* frogs collected from four selected impoundments (n = 10). The bottom row of the table indicates the results from the breakdown and one-way analysis of variance (ANOVA) investigating variance of the measurements between frogs in the water impoundments.

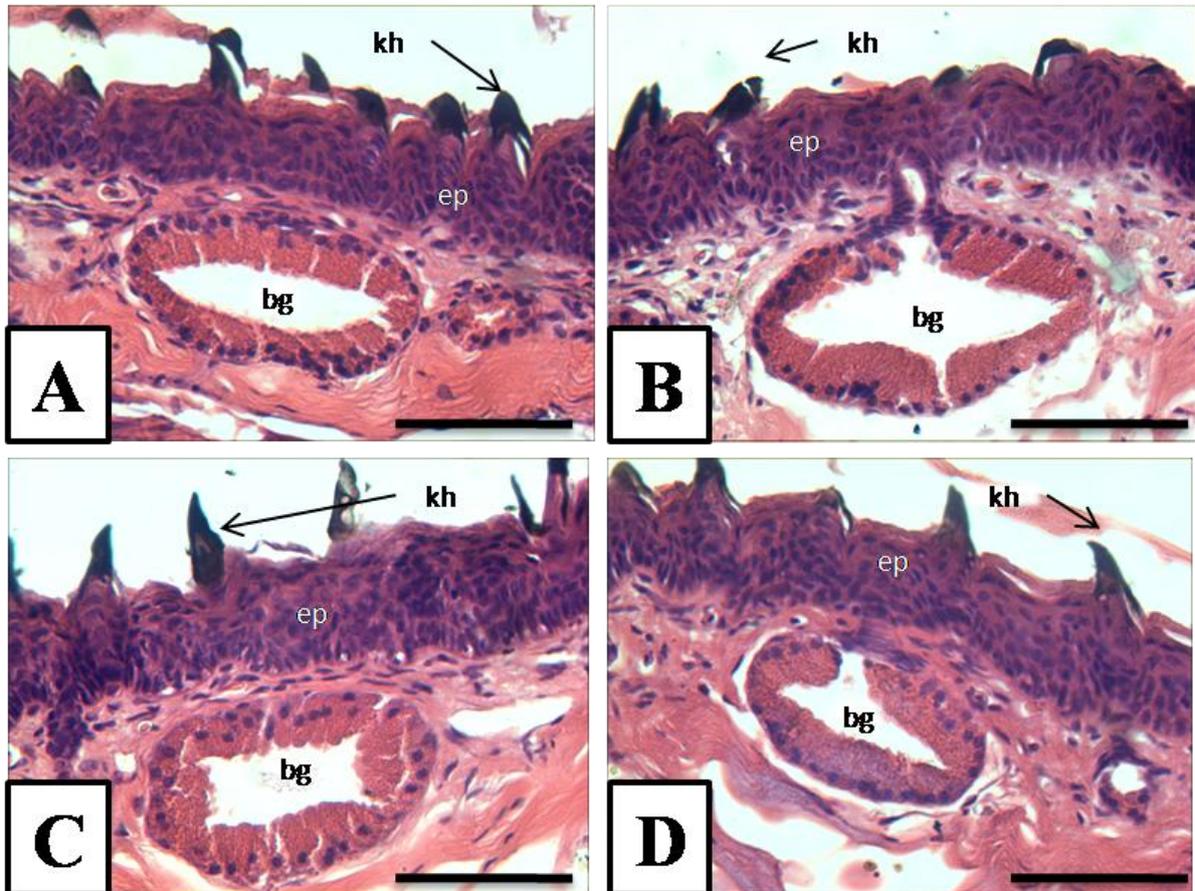
Sampling site	n	Body Mass (g)	SVL (cm)	CDI	GSI (%)	HSI (%)
1	10	46.74 ± 5.26	7.47 ± 0.52	187.53 ± 12.40	0.27 ± 0.05	2.73 ± 1.02
2	10	33.48 ± 4.90	7.26 ± 0.29	151.09 ± 18.06	0.25 ± 0.08	2.30 ± 0.30
3	10	41.54 ± 3.36	7.22 ± 0.23	172.44 ± 9.90	0.28 ± 0.10	2.00 ± 0.41
4	10	37.00 ± 3.66	6.63 ± 0.49	159.23 ± 11.93	0.26 ± 0.07	2.53 ± 0.98
ANOVA		$F_{3,39} = 0.63$ p > 0.05	$F_{3,39} = 3.06$ p > 0.05	$F_{3,39} = 1.19$ p > 0.05	$F_{3,39} = 5.34$ p > 0.05	$F_{3,39} = 1.43$ p > 0.05

#### *Breeding gland histology*

The NP skin sections of male frogs (n = 6) sampled from the different water impoundments (Sites 1, 2, 3 & 4; Fig. 5.1) did not show significant variation in glandular area (Fig. 5.11A; ANOVA,  $F_{3,23} = 12.34$ , p > 0.05) or breeding gland secretory cell epithelium height (Fig. 5.11B; ANOVA,  $F_{3,23} = 15.72$ , p > 0.05) among sites. Qualitative data of the cellular integrity of the breeding gland in frogs from the different water impoundments did not show variation in the structure of breeding gland secretory cell epithelium, where all breeding gland epithelial cells of all frogs from the different impoundments were columnar in shape with nuclei situated at the basal region of the cell and the cytoplasm stained dark eosinophilic (Fig. 5.12). No other glandular types, such as mucous or serous glands were observed in the nuptial area normally only containing breeding glands (Fig. 5.12). All nuptial area skin samples from frogs in the various impoundments exhibited keratinized epidermal hooks (KHs) on the surface of the epidermis, which is characteristically an indication of normal NP integument development in the male frogs (Fig. 5.12). The NP skin samples in male frogs from the various impoundments also did not show variation in epidermal thickness of the integument (Fig. 5.12).



**Figure 5.11:** Morphological variation in breeding gland area (A; average  $\pm$  SD) and breeding gland secretory cell epithelium height (B; average  $\pm$  SD) in adult male *X. laevis* frogs from the different water impoundments. Male frogs (n = 6 per sampling locality) 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).

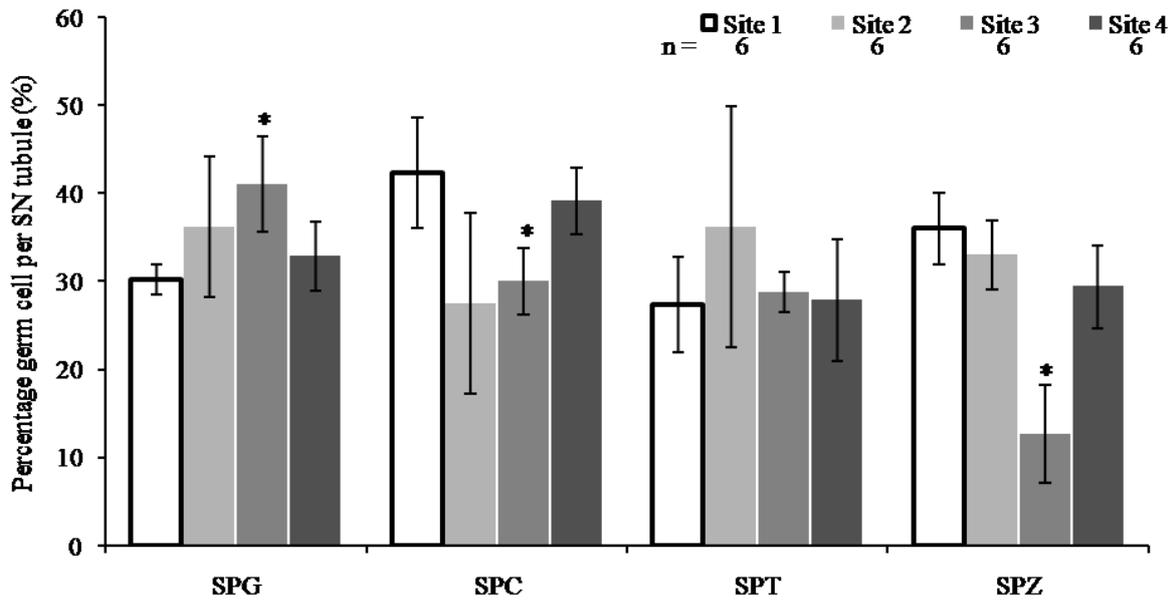


**Figure 5.12:** Breeding gland morphology (400x magnification) of adult male *X. laevis* frogs (n = 6) captured from a water impoundment surrounded by natural vegetation and not receiving runoff from converging slopes with the impoundment isolated from agricultural practice (A; Site 1, control) and 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  $\mu$ m.

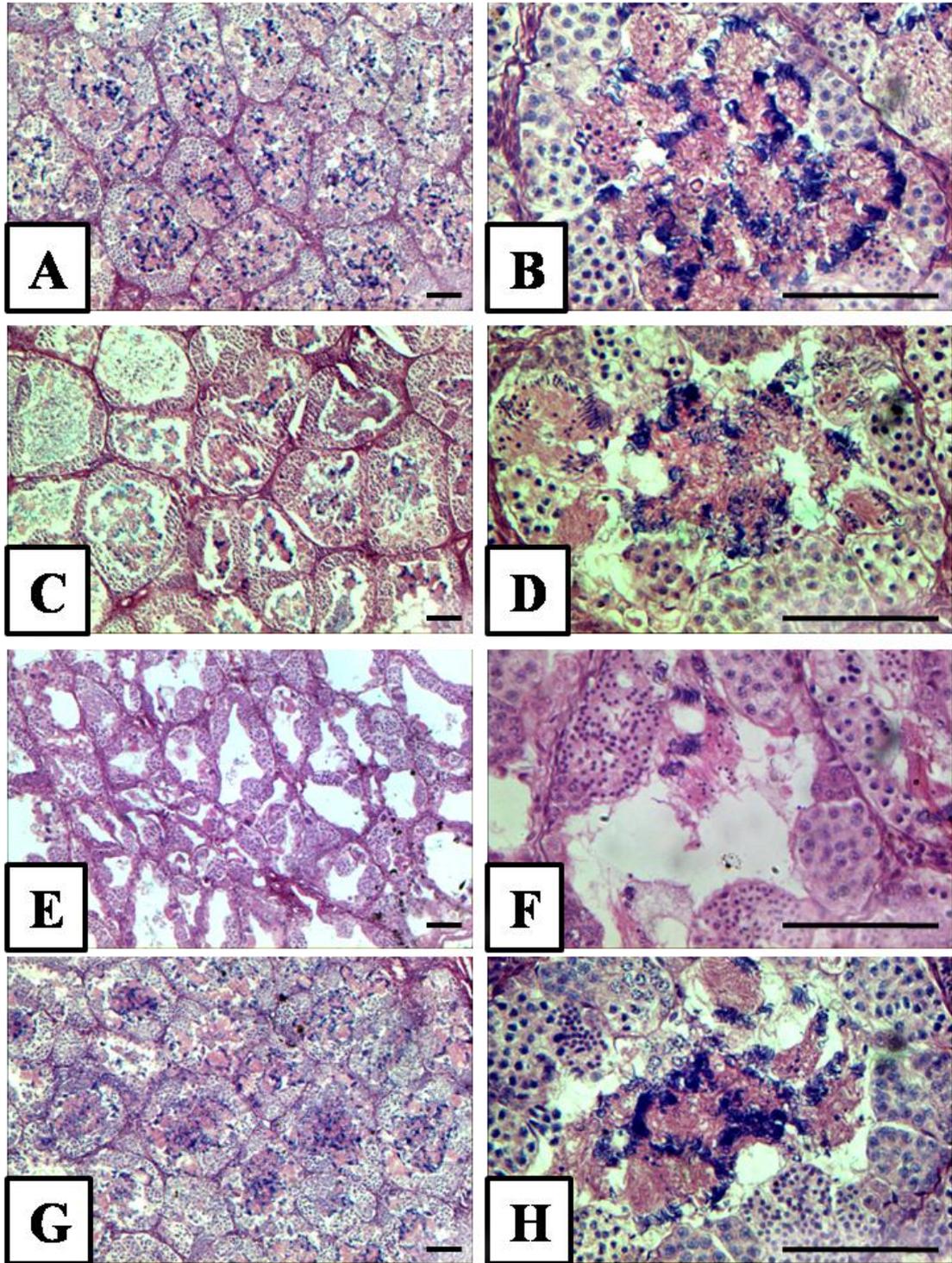
### *Spermatogenesis*

Histological investigation of the testes taken from male frogs (n = 6) from the four water impoundments showed active spermatogenesis by means of the presence of all cystic germ cell stages in frogs from all sampling localities (Figs. 5.13 & 5.14). Frogs collected from impoundment Site 3 showed an increase in the percentage of SPG germ cell stages (Fig. 5.13; ANOVA,  $F_{3,23} = 0.46$ ; Tukey's HSD,  $p < 0.05$ ) and a significant reduction in SPC germ cell stages (Fig. 5.13; 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. 5.13 & 5.14E; ANOVA,  $F_{3,23} = 2.55$ ; Tukey's HSD,  $p < 0.05$ ).



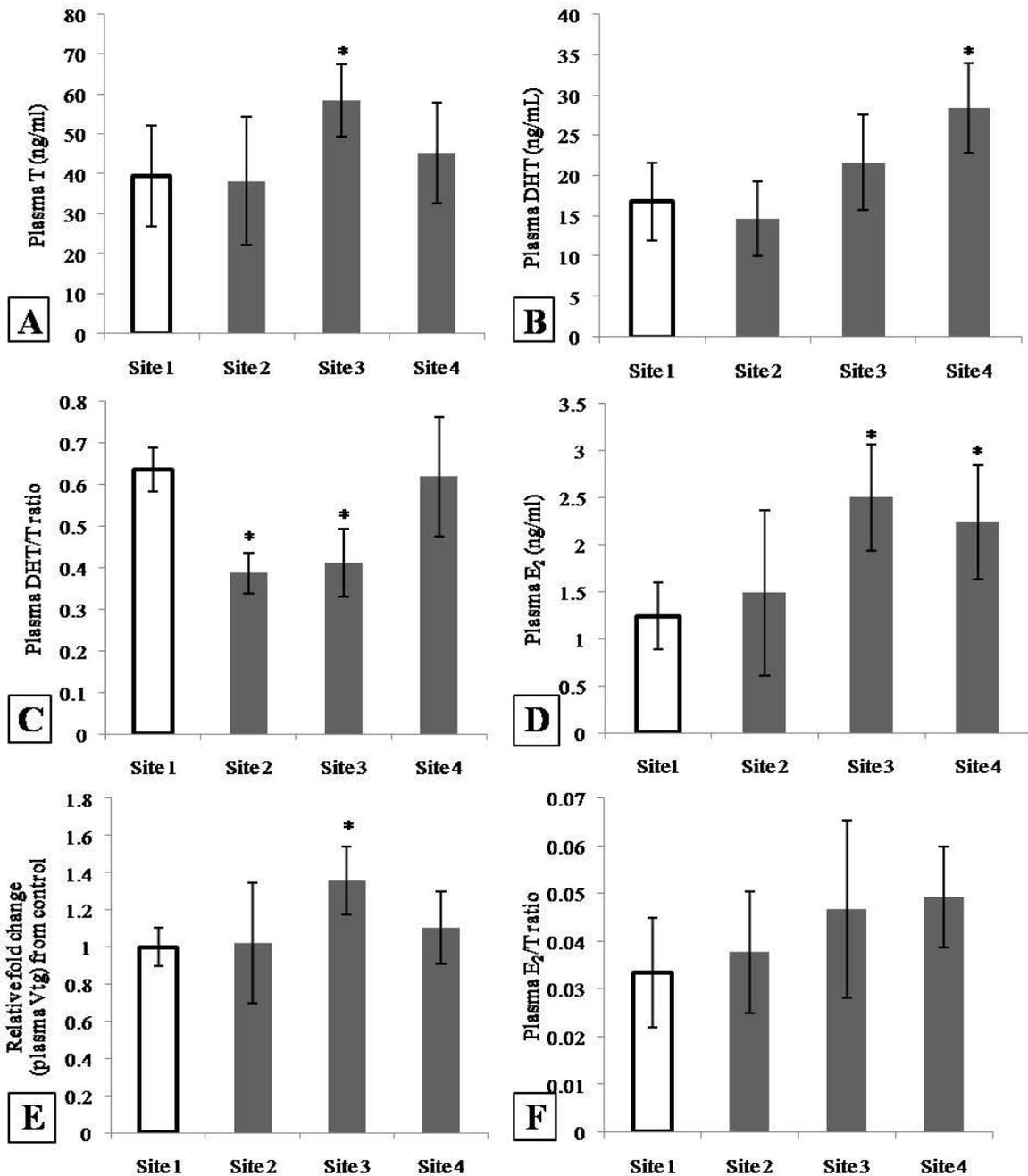
**Figure 5.13:** Variation in the average percentage ( $\% \pm SD$ ) of germ cell cysts and spermatozoa per seminiferous tubules in male *X. laevis* testes ( $n = 6$ ) obtained from water impoundments isolated from agricultural practice (Site 1, Fig. 5.1) and close to agricultural practices (Sites 2, 3 & 4, Fig. 5.1). 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 5.14:** 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  $\mu$ m

*Plasma steroid hormone levels and plasma VTG expression*

A significant difference in plasma T concentrations was found 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. 5.15A). Significant differences were also shown for 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. 5.15B). Frogs from both impoundment Sites 2 and 3 ( $n = 6$  per site) showed a significant difference in the 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. 5.15C). Frogs collected from impoundment Sites 3 & 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. 5.15D). 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. 5.15E). 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 among sites (ANOVA,  $F_{3,23} = 0.94$ ,  $p > 2.48$ ; Fig. 5.15C).



**Figure 5.15:** Concentrations (average  $\pm$  SD) of plasma T (A), DHT (B), DHT:T ratio (C), E<sub>2</sub> (D), fold change in plasma VTG expression (E) and E<sub>2</sub>: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, p < 0.05) from frogs in the control water impoundment (Site 1).

## DISCUSSION

Water impoundments are surrounded with agricultural practices, and several aquatic species inhabit these water resources. Moreover, consumption of untreated water and domestic use from natural sources in rural farms occur regularly and human exposure to harmful pollutants may therefore be a concern (Dalvie *et al.*, 2003; 2011; Mandiki *et al.*, 2014). In contaminated water systems, such as impoundments, aquatic species are exposed during parts or throughout their whole life cycle to contaminants, and should therefore represent good sentinels to study the health implications of acute and chronic exposure to these chemicals. During spray periods of agricultural fields, pesticides have been reported to end up in surface waters via spray drift, groundwater leeching or disposal of unwanted pesticides (Dabrowski *et al.*, 2002; 2011; Thiere & Schulz, 2004; Dalvie *et al.*, 2006). Regardless of the information available on the ecotoxicological effects of certain man-made compounds to vertebrate species, the link between pesticide application intensity, accumulation of pollutants and effects on inhabiting biota is poorly documented. In the current study, several water impoundments surrounded by vineyards and orchards were screened for the presence of androgenic, anti-androgenic and estrogenic potentials. Endocrine disrupting activity was detected by using a combination of *in vitro* bioassays and *in vivo* biomarkers sourced from a sentinel amphibian species (*X. laevis*) occurring naturally in these water bodies.

The *in vitro* analyses in the present study showed variable levels of androgenic, anti-androgenic and estrogenic endocrine disruption activity between impoundment sites. Furthermore, gonadal endocrine disrupting activities as determined by the *in vitro* screens only occurred during the summer period of sampling. Although it may be possible that EDCs will accumulate in water impoundments to a greater extent after rainfall periods (due to groundwater leeching and runoff), the activity of pesticide application is lower during the winter periods in the Western Cape, which is during the cold and rainy season. In the present study, it seems therefore that a dilution-effect occurred at the water impoundments during the winter season in that pollutants are present at lower concentrations in the winter months, possibly due to a higher inflow of freshwater into the impoundments during rainfall periods. The observed fluctuations of EDCs between seasons in the water impoundments suggested that there might not be a build-up of EDCs in surface waters over extended time periods.

The reason for the fluctuations in gonadal endocrine disrupting activity in *X. laevis* collected from the various impoundments may partly be explained by several environmental factors, such as topography, sources of water inflow into the impoundment, geological composition of the soil as well as the angles of surrounding slopes. Furthermore, the type of pesticides and spray programs utilized by the farmers for agricultural fields close to the impoundments might also influence the fluctuations in endocrine disruption activity observed in the different sampling localities. A diverse range of pesticides (including insecticides, fungicides and herbicides) are used in spray programs implemented during various agricultural practices, which is mostly applied during early spring and summer seasons. A study by Dabrowski *et al.* (2014) recommended that commonly used pesticides should be more thoroughly monitored for their toxicological effects in South African agricultural practices. In the study by Dabrowski *et al.* (2014), several of the listed pesticides were shown to be used regularly on agricultural crops cultivated in the study area, including the fungicide mancozeb and insecticides chlorpyrifos, carbaryl, simazine and iprodione. These pesticides have already been shown to cause gonadal endocrine disrupting properties to non-target organisms (Hanioka *et al.*, 1998; Burger, 2005; Viswanath *et al.*, 2010; Hass *et al.*, 2012; Sugeng *et al.*, 2013). The frequent detection of gonadal endocrine disrupting pollutants from the various impoundments in the current study area may be associated with pesticide application events, resulting in agricultural pollutants entering the water system via non-point sources (Schulz, 2001; Dabrowski & Schulz, 2003; Thiere & Schulz, 2004). Such non-point source pollution events might affect aquatic organisms during critical time periods of their reproductive cycle (such as breeding stages involving clasping behaviour or sperm development), leading to lower reproductive fitness as well as reproductive development and survival of offspring

During the period of frog collection in the current study, agricultural fields close to the impoundments were sprayed with pesticides following the budding and development of crops, especially vineyards, during spring and summer seasons. Male *X. laevis* frogs captured in the selected impoundments during the beginning of the warm season (summer) showed a darkened appearance on the ventral forearm, which resembled NP proliferation and therefore indicated reproductive maturity. It is mentioned that development of SSCs in vertebrates are regulated by the androgen DHT rather than T (Meachem *et al.*, 2007). Furthermore, BG development in male *X. laevis* frogs has been shown to be suppressed by estrogenic EDCs

(Van Wyk *et al.*, 2003). Although frogs captured from impoundment Site 3 showed elevated levels of plasma E<sub>2</sub> and DHT, no modulation of BG development was observed in the frogs from any of the impoundment localities. Furthermore, although androgenic, anti-androgenic as well as estrogenic endocrine disruption activities were detected in the *in vitro* screens (especially at impoundment site 3), occurrences of these EDCs did not seem to affect the development of BGs or epidermal KHs in any of the male frogs. These results show that although previous studies showed that BG development is sensitive to changes in steroid hormone profiles (Thomas *et al.*, 1993; Epstein & Blackburn, 1997; Emerson *et al.* 1999; Van Wyk *et al.*, 2003), other biological mechanisms in the organism might be in place to break down exogenous pollutants, compensate for changes in hormone levels and maintain SSC development, such as breeding glands, during reproductive activity.

Analysis of testicular germ cell development and spermiogenesis of male *X. laevis* frogs collected during the study also showed the presence of all germ cell stages in all frogs, which indicated that all frogs underwent active spermatogenesis. However, some variation in the number of spermatogenic germ cell stages as well as a reduction of sperm cell in frogs from the impoundment Site 3 were detected compared to frogs from a relatively unpolluted impoundment (Site 1). One possibility for these modulating effects on testis functionality may be attributed to the detected estrogenic and anti-androgenic activity found in the *in vitro* analyses for impoundment Site 3, which might have led to altered mitotic and meiotic division of germ cells and therefore cause less sperm cell development. Increased plasma E<sub>2</sub> and VTG concentrations further support estrogenic endocrine disruption in frogs captured from impoundment Site 3. These results correlated with findings that have shown increased occurrences of dilated testis tubules, dividing testicular gonocytes, inhibition of meiotic germ cell divisions and a lesser development of sperm cells in E<sub>2</sub>-exposed *X. laevis* frogs (Hecker *et al.*, 2005; Oka *et al.*, 2006; Wolf *et al.*, 2010). The lowered ratios of plasma DHT:T in frogs from impoundment Sites 2 and 3 also suggested anti-androgenic endocrine disrupting activity in male frogs captured from these impoundments. For frogs at impoundment Site 3, the lowered DHT:T ratio could be attributed to the significant increased plasma T concentrations in these frogs. However, neither anti-androgenic nor androgenic endocrine disruption activity was shown for impoundment Site 3 in the *in vitro* analyses, leading to the conclusion that reduced testicular development in frogs from this impoundment is likely due to the elevated observed estrogenic activity.

In conclusion, *in vitro* screens in the present study showed variation in the presence of androgenic, anti-androgenic and estrogenic EDCs among the several impoundments screened during the present study. Seasonal variation in endocrine disrupting activity was also shown in the *in vitro* analyses among impoundments, which compromises the overall image of endocrine disrupting activity in these impoundments during seasonal changes throughout the year. Although frogs were collected from two of the impoundments showing anti-androgenic endocrine activity in the anti-YAS, only a slight decrease in testicular function of frogs from one impoundment site was shown. No other biomarker endpoints used in the wild-caught male frogs suggested anti-androgenic effects. By combining the data from the *in vitro* screening and field-caught animal data, the study showed that *in vitro* analysis data may not always give a true reflection of the endocrine impacts on aquatic organisms living within these water systems. The results generated from the present study show the importance to include animal-based (*in vivo*) endpoints in EDC investigations along with multiple *in vitro* screens to obtain a broad objective of the state and presence of EDCs in the environment.

## CHAPTER 6: GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

During the various experimental procedures of this thesis, it was shown that:

- 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 with different mechanisms of anti-androgenic action, either by AR antagonism (flutamide and mancozeb) or by 5 $\alpha$ R inhibition (dutasteride). This suggests that the development 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. These findings 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.
- *In vitro* screening of anti-androgenic activity of commonly-used agricultural pesticides, especially fungicides, in the Western Cape Province of South Africa was confirmed and raised the importance of screening environmental water sources for anti-androgenic endocrine disruption activity to the same extend as estrogenic endocrine disruption activity. Furthermore, screening of mixture interactions between commonly-used agricultural pesticides has shown to deviate from the suggested additive mixture hypothesis for EDCs having similar mechanisms of endocrine disruption action. These results confirmed the value of using a recombinant *in vitro* yeast assay to predict (anti)androgen activity of compounds (single or in mixture) as well as to detect gonadal endocrine disruption from impoundments among vineyard and orchard plantations. Clearly more research are needed to understand the complex interactions in pesticide mixtures entering natural environments.
- 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 endocrine disruption activity predicted by *in vitro* assays, especially (anti)androgenic activity, did not always correlate with *in vivo* biomarkers in wild caught male frogs. These findings confirm the need for investigating both *in vitro* as well as *in vivo* endpoints when assessing environmental endocrine disruption. Only using *in vitro* endpoints may give a false reflection of endocrine disruption activity in the environment.

Information generated from this study contributed to a better understanding of the potential gonadal endocrine disruption activity in local water systems in the Western Cape region of South Africa, hormonal control of male anuran secondary sexual characteristics (SSCs) and mixture interactions of commonly-used agricultural pesticides. Since *X. laevis* is recognized as an international model species for testing endocrine disruption activity, the current study extended its use for screening of (anti)androgenic activity and also allowed for more extensive health evaluation of wild caught frogs.

Future studies should consider including a battery of tests (or tiered approach as set out by the USEPA and OECD), using both *in vitro* and *in vivo* analyses such as those done in the present study when investigating endocrine disruption activity in the environment. Such endpoints can contribute to local environmental impact assessments (EIAs) and monitoring programmes, such as the South African National Toxicity Monitoring Programme (SANTMP), to include more comprehensive monitoring of toxicity in surface waters and effects of environmental EDCs on indigenous wildlife species and humans. Furthermore, extended screening of long-term effects of EDC pollutants in environmental surface waters should be done to include investigations of multi-generational (second-tier screening) effects of man-made pollutants on aquatic organisms. Also, the effectiveness of freshwater treatment facilities, such as sewage treatment plants (STPs) and wastewater treatment works (WWTWs) to effectively remove EDCs from surface waters must be done using both *in vitro* and *in vivo* analyses, investigating multiple pathways of endocrine disruption activities.

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## APPENDIX – RESEARCH OUTPUTS

- Archer E., Van Wyk J. H. The potential anti-androgenic effect of agricultural pesticides used in the Western Cape: *in vitro* investigation for mixture effects (Scientific paper submitted for review to Water SA).
- Archer E., Van Wyk J. H. 2013. The potential anti androgenic effect of agricultural pesticides: Using a recombinant yeast screen (YAS) for mixture investigations. South African Young Water Professionals conference (SAYWP 2013), Stellenbosch, South Africa (poster presentation).
- Archer E. 2013. The anti-androgenic potential of agricultural pesticides in South Africa: using an anti-androgen yeast screen (YAS) to test for mixture effects. 16<sup>th</sup> International Symposium for Toxicity Assessment (ISTA16), Cape Town, South Africa (Oral presentation).
- Archer E., Van Wyk J. H. 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 (Scientific paper prepared to be submitted to African Zoology).
- Archer E., Van Wyk J. H. Premature development of androgen-dependent breeding glands in the African clawed frog, *Xenopus laevis* (Scientific paper prepared to be submitted to African Journal of Aquatic Sciences).