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The effects of growth stimulants used at cattle feedlots, on reproductive health and thyroid function of Sprague-Dawley rats

By

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This work is dedicated to
my daughter and late mother
for their support and encouragement,
with love and gratitude

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Summary

Reports of endocrine disrupting potential of common environmental chemicals and the effects on reproductive health are well documented in literature. It has been suggested that deteriorating male reproductive health could be due to *in utero* exposures to these chemicals. The effects mediated through endocrine disrupting chemicals (EDCs) are on the fetus and may therefore be trans-generational. Ultimately, these chemicals land up in aquatic systems and affect wildlife and humans. Humans are exposed to these chemicals through multiple routes including atmosphere, water, occupational, domestic and food consumption.

South Africa (SA) is an important livestock producer with about 13.8 million cattle within the feedlot industry contributing up to 80% of the total beef production. Veterinary growth stimulants (VGS) are used by beef producers to enhance growth in cattle. In SA, the following five VGS have been approved for use in beef products under the Register Act 36 of 1947, estradiol, progesterone and testosterone (natural), α-zearalanol and trenbolone (synthetic). These VGS and their metabolites are environmentally stable compounds. The excretions from the animals are not treated and land up in the local aquatic systems, indirectly posing a health risk. In SA no research has been done on VGS associated with feedlot activities.

The aim of this study was to investigate the effects of a mixture of VGS, as possible EDCs on the reproductive health and thyroid function in male rats *in utero*, during lactation and life-time exposure.

The (anti)estrogenic and (anti)androgenic activity in water from specific feedlots was determined by using a battery of screening bio-assays. Water samples were collected over a period of a year and assessed for EDC activity in the recombinant yeast screen (YES), the T47D-KBluc (estrogenic) and the MDA-Kb2 (androgenic) bioassays. The OECD (Organization for Economic Co-operation and Development) 415 protocol, (1983) for a one-generation reproduction toxicity study, was modified to accommodate one control and

three experimental groups. The experimental groups were orally gavaged with mixtures of: zilpaterol, diethylstilbestrol (DES) and α-zearalanol (Group 2; estogenic); with β-trenbolone and methyltestosterone (MT) (Group 3; androgenic); a combination of compounds (Group 4; estrogenic and androgenic) and the Control group received cottonseed oil only.

The bio-assay results indicated that water samples analysed from selected feedlots contained compounds with estrogenic activity.

The shorter anogenital distance (AGD) (Group 3), decreased seminal vesicle mass (Group 4), decreased prostate mass (Group 4), increased lumen diameter (Group 3 and 4), lowered sperm concentration (Group 3), and increased T_4 (Group 2 and 3) differed significantly from the control. The body weight of the males in Group 2 in the F2 generation was significantly lower than the control. The F2 females in Group 2, 3 and 4 were also significantly lower than the control.

The reduced AGD, decreased seminal vesicle and increased T_4 (thyroxine) might be the result of an estrogenic effect. The reduced sperm concentration might be the result of *in utero* and lactation exposure to these VGS.

The bio-assays confirmed estrogenic activity in the feedlot water sources. The reproductive toxicology study findings confirm the hypothesis that VGS can act as EDCs and could therefore be responsible for negative reproductive effects and thyroid function. More research is needed to investigate the effects of VGS mixtures at different concentrations on male reproductive health, thyroid function and their offspring.

Key terms: estrogen, veterinary growth stimulants, zilpaterol, diethylstilbestrol, α-zearalanol, β-trenbolone, methyltestosterone, YES, T47D-KBluc, anogenital distance, male fertility, thyroid function.

Opsomming

Goed gedokumenteerde literatuur dui aan dat chemikalieë wat algemeen in die omgewing gevind word, die potensiaal het om die manlike voortplantingstelsel aan te tas. Dit word gespekuleer dat *in utero* blootstelling verantwoordelik kan wees vir hierdie agteruitgang. Die fetus en daaropvolgende geslagte se gesondheid kan ook beÏnvloed word deur chemikalieë. Hierdie chemikalieë het die potensiaal om die watersisteme te bereik en gevolglik dier en menslike gesondheid te beÏnvloed. Blootstelling kan plaasvind deur verskeie roetes wat die atmosfeer, water, werksomstandighede, huishoudelike produkte en gekontamineerde voedsel insluit.

Suid-Afrika (SA) is ’n belangrike produsent van vleisprodukte met omtrent 13.8 miljoen beeste wat bydra tot 80% van die vleisproduksie. Veterinêre-groei-stimulante (VGS) word gebruik om die vleisproduksie te verbeter. Vyf groei stimulante naamlik estradiol, progesteroon, testosteroon (natuurlike), α-zearalanol en trenboloon (sinteties) is goedgekeur onder die Wet 36 van 1947, vir groei produksie van beeste. Hierdie VGS en hul metaboliete is stabiel in die natuur. Die fekale en urinere uitskeidingsprodukte van die diere word nie behandel nie en eindig op in ons waterstelsels. Geen navorsing is nog in SA gedoen om die potensiële bydraes wat voerkrale tot die besoedeling van water lewer, te bestudeer nie.

Die doel van die studie was om die gesamentlike effekte van mengsels VGS as moontlike endokrien-ontwrigtende chemicalieë (EOC) op die manlike voortplantingstelsel en tiroïdhormone van mannetjiesrotte na *in utero*-, gedurende laktasie- en na ’n leeftyd-blootstelling te bepaal.

Die (anti)estrogeniese en (anti)androgeniese aktiwiteit in water vanaf spesifieke voerkrale is met behulp van ’n reeks biologiese seltoetse bepaal. Watermonsters is geanaliseer met die gisseltoets (YES)(estrogenies), die T47D-KBluc (estrogenies) en die MDA-Kb2 (androgenies). Die OECD 415 protokol (1983) vir een generasie reproduktiewe toksologie toets was

aangepas om een kontrole en drie eksperimentele groepe te huisves. Die eksperimentele groepe rotte is oraal gedoseer met ’n mengsel van zilpaterol, dietielstilbestrol (DES) en α-zearalanol (Groep 2); β-trenboloon en metieltestosteroon (Groep 3); ’n kombinasie van al bogenoemde (Groep 4); en ’n kontrole groep wat katoensaad olie VGS ontvang het nie.

Estrogeniese aktiwiteit en sitotoksisiteit was teenwoordig in die water vanaf die voerkrale. Die verkorte anogenitale afstand (AGD) (Groep 3), kleiner seminale vesikel (SV) massa (Groep 4), kleiner prostaat massa (Groep 4), groter lumen deursneë (Groep 3 en 4), laer spermtelling (Groep 3), verhoogde T_4 (Groep 2 en 3), het almal statisties-betekenisvol van die kontrole groep verskil. In die F2 generasie het die liggaamsmassas van die mannetjies in Groep 2 en liggaamsmassas van die wyfies in Groepe 2, 3 , 4, almal statisties-betekenisvol laer as die kontrole Groep.

Die verkorte AGD, kleiner SV en verhoogde T_4 kan moonlik wees as gevolg van ’n estrogeniese effek en die verlaagde sperm konsentrasie weens ‘n *in utero* en laktasie blootstelling.

Die biologiese seltoetse het die teenwoordigheid van estrogeniese aktiwiteit in voerkrale se water bevestig. Die gevolge van die blootstelling van EOC mengsels op voortplantings-parameters bevestig die moontlikheid van EOC effek geassosieer met VGS. Verdere navorsing is nodig om die dosisresponsverhoudings van verskillende VGS te ondersoek.

Sleutelwoorde: estrogeen, veternêre-groei-stimulant, zilpaterol, β-trenbolone diethylstilbestrol, α-zearalanol, methyltestosterone, YES, T47D-KBluc, anogenitale afstand, manlike voortplantingstelsel, tiroïdhormoon.

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Abbreviations

A_{al}	$A_{aligned}$
ACTH	Adrenocorticotropin
AFP	Alpha-fetoprotein
AGD	Anogenital distance
A_{iso}	$A_{isolated}$
ANOVA	Analysis of Variance
A_{pr}	A_{paired}
ARs	Androgen receptors
β_2-AR	β_2-adrenergic receptor
BPA	Bisphenol A
CBG	Corticosteroid binding globulin
CNS	Central nervous system
CPRG	Chlorophenol red-β-D-galactopyranoside
CRH	Corticotropin-releasing hormone
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
Di	Diplotene cells
DNA	Deoxyribonucleic acid
DSD	Disorders of sex differentiation
E_1	Estrone
E_2	17-β estradiol
E_3	Estriol
EC	European Community
EDCs	Endocrine disrupting chemicals
EEq	Estradiol equivalents
EPA	Environmental Protection Agency
ERE	Estrogen response element
Ers	Estrogen receptors
ERα	Estrogen receptor-α
ERβ	Estrogen receptor-β
EU	European Union

F1	First litter
F2	Second litter
FDA	Food and Drug Administration
FDA	Food and Drug Assurance Laboratories
Fisher's LSD	Least significant difference method
FLCs	Fetal Leydig cells
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
Her	Human estrogen receptor
HPA	Hypothalamic-pituitary-adrenal axis
HPG	Hypothalamic-pituitary-gonadal axis
HPT	Hypothalamic-pituitary-thyroid axis
L	Leptone cells
LH	Luteinizing hormone
MIS	Müllerian inhibiting substance
LPS	Lipopolysaccharide
MMTV	Mouse mammary tumour virus
MT	Methyltestosterone
MTT	Methythioazol Tetrazolium
OECD	Organization for Economic Co-operation and Development
P1	Parental generation
PAH	Polycyclic aromatic hydrocarbons
PAS	Periodic Acid-Schiff's reaction
PBBs	Polybrominated biphenyls
PBS	Phosphate buffered saline
PCBs	Polychlorinated biphenols
PI	Preleptotene
PRs	Progesterone receptors
SA	South Africa
SHBG	Sex hormone binding globulin
SPE	Solid phase extraction
SRY	Sex region Y

StAR	Steroidogenic acute regulatory
T_3	Triiodothyronine
T_4	Thyroxine
TBG	Thyroxine-binding globulin
TBT	Tributyltin
TDS	Testicular dysgenesis syndrome
TNF-α	Tumor necrosis factor alpha
TRH	Thyrotropin-releasing hormone
TRs	Thyroid receptors
TSH	Thyroid stimulating hormone
UPBRC	University of Pretoria Biomedical Research Centre
USA	United States of America
VGS	Veterinary growth stimulants
VTG	Vitellogenin
WHO	World Health Organization
YES	Recombinant yeast screen
Z	Zygotene cells
ZH	Zilpaterol hydrochloride

CHAPTER 1

1 Introduction

1.1 Background

Over the last few decades the environment has been exposed to excessively large numbers of natural and synthetic compounds (Colborn *et al*., 1993; Safe, 2005). These compounds have the ability to interfere with the endocrine system and produce adverse effects in humans, wildlife, fish and birds (Colborn *et al*., 1993; NIEHS, 2006; Diamanti-Kandarakis *et al*., 2009). Because these compounds have the ability to alter the endocrine system, they are termed endocrine disrupting chemicals (EDCs) (Colborn 1993; NIEHS, 2006; Phillips *et al*., 2008; Diamanti-Kandarakis *et al*., 2009).

The first definition of what constitutes an endocrine disrupter was defined by Kavlock *et al*. (1996) as: “an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes” (Kavlock *et al*., 1996; Evans et *al.*, 2011).

In 2002 the World Health Organization (WHO) defined EDCs as follows: “An endocrine disrupter is 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.” (Damstra *et al*., 2002; Evans *et al.*, 2011).

The mechanisms through which EDCs can act, were investigated in various animal studies. It was therefore concluded that EDCs can mimic or partly mimic naturally occurring hormones in the body like estrogens, androgens and thyroid hormones, potentially producing over-stimulation (NIEHS, 2006). EDCs can bind to a receptor within a cell and block the endogenous hormone from binding (NIEHS, 2006) and interfere or block the way natural hormones or their receptors are made or controlled (NIEHS, 2006; Evans *et al.*, 2011).

EDCs can be present in a variety of forms and synthetic compounds. Identified EDCs include industrial solvents and their by-products [polychlorinated biphenols (PCB), polybrominated biphenyls (PBBs), and dioxins], plastics [bisphenol A (BPA)], plasticizers (phthalates), pesticides [methoxychor, DDT (dichlorodiphenyltrichloroethane)], fungicides (vinclozolin) and pharmaceutical agents. Natural chemicals such as phytoestrogens, also have the ability to act as EDCs (Colborn *et al*., 1993; Gray *et al*., 2006; NIEHS, 2006; Hotchkiss *et al*., 2008; Woodruff *et al*., 2008; Diamanti-Kandarakis *et al*., 2009; Burkhardt-Holm, 2010; Woodruff *et al*., 2010).

Evidence exists that a variety of compounds, some of which can disrupt endocrine development in wildlife and laboratory animals is found in the aquatic environment including oceans, freshwater, marine and terrestrial food products (Colborn *et al*., 1993; Sumpter, 2005). The aquatic environment is particular vulnerable as EDCs enter rivers, lakes, and sea through the release of effluent from sewage treatment works and some industries. Water sources also receive a lot of accidental releases of chemicals particularly through spills, run-off and atmospheric deposition. The pressure on our water sources has increased due to a growing population and a greater need for water usage (Sumpter, 2005). Therefore, humans may be exposed to mixtures of environmental contaminants on a daily basis through multiple routes including the atmosphere, water, occupational, domestic and food consumption (Woodruff *et al*., 2008, 2010). These effects are evident in various wildlife studies done over the years (Damstra *et al*, 2002; Woodruff *et al*., 2008, 2010).

For over a century, wildlife and laboratory animals have been used to predict the human health effects of various environmental compounds (Woodruff *et al.*, 2008). Although each species has its unique attributes, a growing literature indicates that substantial conservation exists in the underlying molecular, cellular, and physiological systems associated with vertebrate reproduction (Guillette and Edwards, 2008; Woodruff *et al.*, 2008). Estrogen, androgen, and thyroid signalling are essential for normal embryonic development and reproductive activity in all vertebrates (Woodruff *et al*.,

2008). Wildlife studies demonstrated the effects of levels and mixtures of exposures on our environment in genetically diverse populations (Guillette and Edwards, 2008; Woodruff *et al.*, 2008). Therefore wildlife observations are directly relevant to assessing potential influences on human reproduction (Woodruff *et al.*, 2008).

Studies in the early 1990's and onwards began to associate environmental contamination with altered reproductive performance in wild populations of fish, amphibians, reptiles, birds and mammals (Colborn *et al*., 1993; Woodruff *et al.*, 2008, 2010). Studies in fish demonstrated increased rates of feminized males phenotype and reduced fertility from exposure to estrogenic effluents (Colborn *et al*., 1993; Sumpter, 2005; Woodruff *et al.*, 2008, 2010). Alligators inhabiting pesticide-contaminated lakes showed reduced fertility and increased occurrence of multioocyte follicles (Guillette *et al.*, 1994; Woodruff *et al.*, 2008). Studies done on mammals indicate that the decline in Adélie penguins could be due to DDT exposure (Woodruff *et al*., 2010). Various animal studies done over time confirm the adverse reproductive health effects found in wildlife (Woodruff *et al*., 2010).

EDCs can cause a broad spectrum of effects, which depend not only on the route of exposure and dose, but on the susceptibility of the individual to the compound (Woodruff *et al.*, 2008). Age, gender, and genotype can influence susceptibility to disorders, anatomic abnormalities, and diseases from exposures (Damstra *et al.*, 2002; Woodruff *et al.*, 2008). It has been suggested that sperm counts in human males may have declined by nearly 50% during the past 50-60 year (Carlsen *et al*., 1992; Dalgaard 2001; Damstra *et al.*, 2002; Woodruff *et al*., 2010; Evans *et al.*, 2011). Sharp and Skakkebaek, (1993) proposed a hypothesis that agents that interfere with normal development of the reproductive system via an endocrine mechanism could plausibly be related to increases noted in human male reproductive disorders over a number of years (Damstra *et al.*, 2002). There is a relatively high incidence of male reproductive disorders that manifest at birth (cryptorchidism, hypospadia) or in young adulthood (testicular germ cell cancer and infertility) (Woodruff *et al.*, 2008; Sharpe, 2009). These four

disorders are increasing and are risk factors for each other. Skakkebaek *et al*. (2001) hypothesize that testicular germ cell cancer; cryptorchidism, hypospadia, and low sperm count may all be symptoms of testicular dysgenesis syndrome (TDS) with a common origin in fetal life (Skakkebaek *et al.*, 2001; Woodruff *et al.*, 2008; Jorgensen *et al.*, 2010). The hypothesis proposes that "abnormal testis development which could have numerous primary causes, leads secondarily to hormonal or other malfunctions of the Leydig and Sertoli cells during male sexual differentiation, leading to increased risk of reproductive disorders of the testicular system" (Skakkebaek *et al.*, 2001; Sharpe and Skakkebaek, 2003, 2008; Woodruff *et al.*, 2008).

Other than cancers, reproductive problems in men are generally not life threatening. But, in the last five years there has been growing recognition that male reproductive function and risk of cardio-metabolic disorders, including abnormal obesity, type II diabetes and hypertension are interlinked. A late-onset of hypogonadism (low/subnormal testosterone levels) in men is an important determinant and/or consequence of these disorders (Kupelian *et al.*, 2008; Laughlin *et al.*, 2008; Makarow and Højgaard, 2010).

Female reproductive health is also affected by EDC exposure. From 1938 to 1971 DES, a potent synthetic estrogen, was given to pregnant women to prevent miscarriages in the United States States (USA) (Damstra *et al.*, 2002; Hotchkiss *et al.*, 2008; Woodruff *et al.*, 2008, 2010). It was later discovered that the daughters of women exposed to DES developed clear cell adenocarcinoma of the vagina and gross structural abnormalities of the cervix, uterus and fallopian tube (Damstra *et al.*, 2002; Hotchkiss *et al*., 2008; Woodruff *et al*., 2008, 2010).

Research in the past has mainly focused on various other EDCs that contaminated the environment, but in America the Environmental Protection Agency (EPA) has established that estrogens from dairy and livestock farms are of major environmental concern (Knowlton *et al*., 2010). Steroid hormones, including estrogens are synthesized by the endocrine system and excreted in urine and faeces (Khan *et al*., 2008; Knowlton *et al*., 2010). If

these hormones enter surface water, they may disrupt the endocrine system of the organisms exposed to them (Knowlton *et al*., 2010). It is estimated that over 330 metric tons per year of natural hormones for example estrogens, androgens and progestagens are excreted by farm animals in the US (Khan *et al*., 2008; Zhao *et al*., 2010). VGS residues can persist for weeks to months in manure and in feedlot runoff (Knowlton *et al*., 2010). These hormones may be retained in soil or transported to ground and surface water (CECBP, 2008).

Large amounts of natural hormones from livestock farms are released into the environment and detectable concentrations have been reported in USA rivers (Soto *et al*., 2004; Khan *et al*., 2008; Knowlton *et al*., 2010) as well as in countries like the United Kingdom (Johnson *et al*., 2006; Matthiessen *et al*., 2006). In addition to this natural hormone burden are synthetic hormones used as VGS to enhance growth production of meat. The European Union (EU) has banned the use of growth stimulants since 1988, however they are still widely in use by large cattle producing countries world wide (CECBP, 2008; Starmer and Mald, 2009). These countries include the USA, Australia, Argentina and Canada (CECBP, 2008; Knowlton *et al*., 2010). VGS are usually given in the form of implants (Soto *et al*., 2004). Three natural steroid hormones, estradiol, testosterone and progesterone and three synthetic hormones α-zearalanol, melengestrol acetate and trenbolone are used as VGS either alone or in combination (Swan *et al*., 2007) in the USA and Canada (Soto *et al*., 2004; Swan *et al*., 2007; Starmer and Mald, 2009).

Humans are ultimately exposed to synthetic hormones/VGS by consumption of meat products and by environmental exposure related to animal waste (CECBP, 2008). A study done by Soto *et al.* (2004) on water bodies receiving cattle feedlot effluent, in Eastern Nebrasaka, USA confirmed that feedlot operations released significant estrogenic and androgenic activity into the water. The presence of these hormones is evident in animal studies done by Orlando *et al*. (2004) where reproductive defects were found in fish at various sites in the Elkhorn River, Nebraska, USA. There is further concern that exposure to VGS can cause cancer, reproductive effects or other endocrine disrupting outcomes (CECBP, 2008), because VGS like trenbolone acetate

and α-zearalanol can cross the placenta (Lange *et al*., 2002a; CECBP, 2008). In the fetus, postmenopausal women and pubertal children, the natural feedback systems may be less effective, leaving them more vulnerable to the effects of exogenous hormone exposure (CECBP, 2008). A study done by Swan *et al*. (2007) on maternal beef consumption concluded that consumption during pregnancy and possibly the xenobiotics in beef may alter testicular development *in utero* and adversely affect the male reproductive capacity.

In SA scientific studies have indicated that EDCs are present in our aquatic environment/water sources and its health effects have been confirmed in various wildlife studies. (Aneck-Hahn 2003; Aneck-Hahn *et al*., 2008, 2009; Bornman *et al*., 2010; Pieterse *et al*., 2010, Botha *et al*., 2011). The possible endocrine disrupting effects of VGS on the male reproductive health and thyroid functions must be investigated.

In SA, VGS are also used by livestock producers to increase lean meat production (SAFA, 2006; Jonker, 2008). Only five hormones have been approved for use in beef products in SA under the Register Act 36 of 1947 (SAFA, 2006; Jonker, 2008). The three natural hormones are estradiol, progresterone and testosterone and the two synthetic hormones are α-zearalanol and trenbolone acetate (SAFA, 2006; Jonker, 2008). The feedlot industry contributed up to 80% of the total beef production in SA. SA is also an important livestock producer with about 13.8 million cattle (SAFA, 2006; Jonker, 2008). In SA feedlots are fairly small areas, containing large number of animals and the feedlots are situated on a slope to manage cattle waste (Jonker, 2008). Thus, due to the large number of cattle that can be confined to a small area the possibility for environmental contaminations of VGS are increased.

1.2 Problem statement

Various scientific studies were done in the USA and Europe to confirm the presence of estrogenic and androgenic active compound in water sources. These compounds have their origin from livestock/cattle feedlots. In SA, to our knowledge there is no research previously done to determine the effects of VGS on the reproductive endpoints in males.

1.3 Aim

To determine the effects of VGS used in cattle feedlots on the reproductive health and thyroid function of male Sprague-Dawley rats.

1.4 Objectives

To determine:

1. The endocrine disrupting (anti-/estrogenic and anti-/androgenic) activity in water from specific feedlots by using a battery of screening bio-assays.
2. The endocrine disrupting effects of a combination of zilpaterol, DES, α-zearalanol, β-trenbolone and MT on the reproductive [AGD, histology (stages), semen analysis] and thyroid parameters in male rats after maternal, *in utero*, during lactation and life time exposure.

1.5 Hypothesis

The exposure to a mixture of VGS adversely affects the reproductive health and thyroid function of male rats.

Chapter 2

2. Literature Review

2.1 A brief overview of the endocrine system

The endocrine system plays an important and essential role in the body which involves the short and long term regulation of metabolic processes (Damstra *et al*., 2002). This system is composed of diverse glands that secrete hormones into the blood stream (Damstra *et al*., 2002; Evans *et al*., 2011*).* The endocrine system coordinates and programs related bodily functions and which are primarily regulated through negative feedback control (Damstra *et al*., 2002; Evans *et al.,* 2011*).*

2.2 Hormones

Hormones can be defined as chemical messengers secreted into the blood by endocrine cells or by specialized neurons (Silverthorn *et al*., 1998; Tarrant *et al*., 2005) that are responsible for long term functions. All hormones bind to target cell receptors and initiate biochemical responses which are known as the cellular mechanism of action of hormones. Furthermore, hormones can act on multiple tissues that may be far from the original gland that secreted them. Processes that fall under hormonal control include growth and development, metabolism, regulation of the internal environment (temperature, water balance, ions), and reproduction. Hormones have one of three basic ways in which they act in their target cells; they control the rates of enzymatic reactions, control transport of molecules across cell membranes or control gene expression and the synthesis of proteins (Silverthorn *et al*., 1998).

2.2.1 Function of hormones

The function of hormones includes four broad domains, reproduction, growth and development, the maintenance of the internal environment and lastly the production, utilization and storage of energy (Silverthorn *et al*., 1998; Wuttke *et al*., 2010).

2.2.2 Regulation of hormones

The hypothalamus, located at the base of the brain, releases hormones that both stimulate and inhibit the release of hormones from the pituitary gland. These hypothalamic hormones all have a polypeptide structure and act on the anterior lobe of the pituitary. The anterior pituitary produces seven specific hormones that stimulate (or inhibit) the various "target" glands or tissues associated with the endocrine system, including: adrenocorticotropin hormone (ACTH) that acts on the adrenal cortex; follicle stimulating hormone (FSH) that acts on the ovary and testes; luteinizing hormone (LH) acting on the ovary and testes and thyrotropin [thyroid stimulating hormone (TSH)] acting on the thyroid. The hypothalamus can both stimulate and inhibit the release of pituitary hormones through the action of hypothalamic "releasing factors" or "inhibiting factors", respectively. The pituitary gland then stimulates other endocrine glands and tissues by release of its hormones (Silverthorn *et al*., 1998; Ganong, 1997).

2.2.3 Classification of hormones

Hormones are divided into three types namely; peptide, steroid and amine hormones. The peptide hormones are composed of three or more amino acids where the steroid hormones are cholesterol derived. The amine hormones are derivatives of single amino acids (Silverthorn *et al*., 1998; Wuttke *et al*., 2010).

2.3 Endocrine glands

Endocrine glands are ductless and vascular, and include the pituitary, thyroid and adrenal glands, and parts of the kidney, liver, heart and gonads (Figure 2.1). Endocrine glands may signal to each other in series, and thereby form an endocrine axis. The three important endocrine axes are the hypothalamic-pituitary-gonadal (HPG) axis; the hypothalamic-pituitary-adrenal (HPA) axis and the hypothalamic-pituitary-thyroid (HPT) axis (Damstra *et al*., 2002; Tarrant *et al.,* 2005).

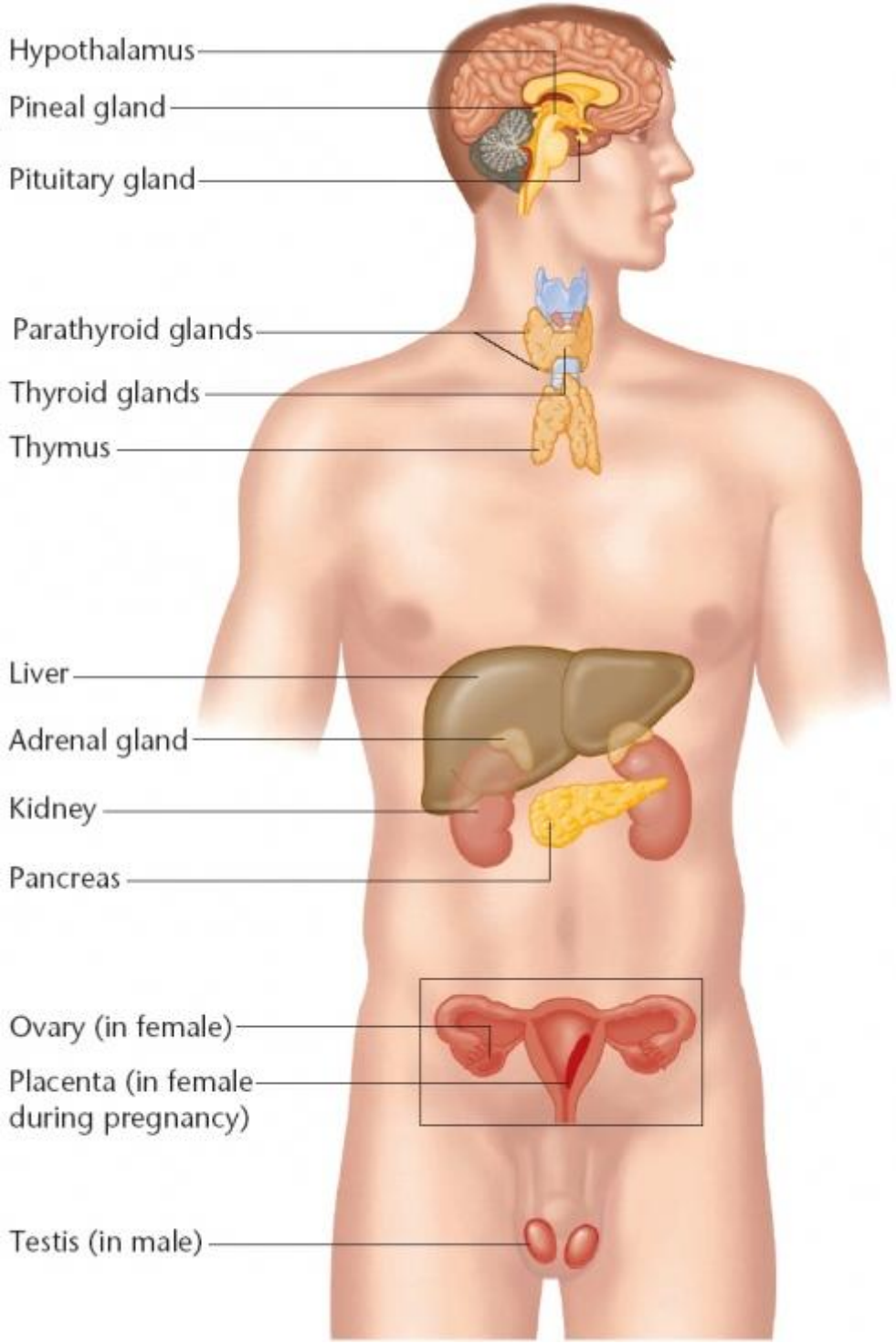

Figure 2.1: The endocrine system
(http://biomachines.files.wordpress.com/2011/04/endocrinesystem.jpg)

2.3.1 The Hypothalmic-Pituitary-Gonadal Axis (HPG)

The HPG axis involves three component parts. The first component is the gonadotropin-releasing hormone (GnRH) neurons projecting from the hypothalamus of the brain. The second component is the gonadotropes in the anterior pituitary gland (adenohypophysis), which secretes the gonadotropins LH and FSH. The final component is the somatic cells of the gonads (theca and granulosa cells in the ovary; Leydig and Sertoli cells in the testes). GnRH is secreted in pulses (Kimura and Funabashi, 1998; Terasawa, 1998; Tarrant *et al.,* 2005) from the terminals of GnRH neurons and acts on the gonadotropes to induce secretion of both LH and FSH, which then act on their respective target cells in the gonads (LH on theca/Leydig cells; FSH on granulosa/Sertoli cells). In females, LH stimulates ovulation and the conversion of the ovulated ovarian follicle into an endocrine structure called a corpus luteum. In males, LH stimulates the secretion of male sex hormones (mainly testosterone) from the interstitial cells of Leydig in the testes. FSH stimulates the growth of ovarian follicles in females and the production of sperm in the testes of males. Secretion of GnRH is modified by other neurons, and the action of GnRH on gonadotropin release may be modified by other hypothalamic or pituitary peptides (Evans, 1999; Tarrant *et al.,* 2005). As a consequence, gonadal sex steroids produced in response to FSH and LH are released into the bloodstream and these feedback to the hypothalamus and pituitary gonadotropes to reduce the secretion of GnRH, LH and FSH, with inhibin (a protein hormone produced by the testes) selectively inhibiting FSH and the sex steroids inhibiting LH secretion (Crowley *et al.*, 1991; Damstra *et al*., 2002; Tarrant *et al.,* 2005).

The role of the sex steroid binding proteins and their transport around the body via the bloodstream is of prime importance. These include albumin, alpha-fetoprotein (AFP) in the fetus/neonate and, most importantly in humans, sex hormone binding globulin (SHBG). Approximately 97-98% of the testosterone and estradiol that circulates in human blood is bound to SHBG and only 2-3% is free and biologically active (Moore and Bulbrook, 1988; Rosner, 1990; Tarrant *et al.,* 2005). This arrangement has two important consequences firstly the half-life of the sex steroid is considerably prolonged.

Secondly a new indirect pathway for regulating sex steroid action becomes evident; i.e. modulation of SHBG secretion (by the liver) can potentially alter levels of bioactive sex steroid without affecting any of the major component parts of the HPG axis (Rosner, 1990; Tarrant *et al.,* 2005).

The level of pituitary hormone production and secretion is, in general, regulated by negative feedback mechanisms. Hormone production and release by target endocrine glands such as the thyroid and adrenal cortex are stimulated directly by the pituitary (and indirectly by the hypothalamus by the release of TSH and ACTH, respectively). When the resultant hormones released by these glands (i.e. thyroxine and cortisol), reach a high blood concentration, stimulation of the target endocrine glands by both the hypothalamus and pituitary is decreased. The “long loop” feedback mechanism is where the blood concentration of hormones feed EDCs back to the hypothalamus or pituitary; the “short loop” is where the trophic hormones of the pituitary act on the hypothalamus in a feedback mechanism (Damstra *et al*., 2002; Tarrant *et al.,* 2005*)*.

2.3.2 The Hypothalmic-Pituitary-Adrenal Axis (HPA)

Adrenal cortex steroids of major interest include glucocorticoids, mineralocorticoids, androgens, estrogens and progesterone. The HPA axis operates in a similar way to the HPG axis, the major difference being in the regulatory and secretory molecules involved. Corticotropin-releasing hormone (CRH) is secreted from the terminals of hypothalamic neurons and acts on corticotropes in the anterior pituitary gland to regulate the synthesis and secretion of ACTH, which is then transported via the bloodstream to the adrenal glands where it stimulates the secretion of glucocorticoid hormones (cortisol and/or corticosterone). Glucocorticoid functions include enhancement of fat lipolysis and protein catabolism while promoting the production of glucose from non-carbohydrate sources. Thus, abnormally high levels of these steroids can cause “muscle wasting” together with the redistribution of fat. The glucocorticoids exert negative feedback effects at the hypothalamic and pituitary levels to suppress CRH secretion. Similar to the sex steroid hormones, much of the glucocorticoid in circulation in blood is bound to a

binding protein in the human [corticosteroid binding globulin (CBG)], and local release of bioactive hormone from the CBG represents one mechanism of local tissue response to pro-inflammatory changes (Rosner, 1990; Tarrant *et al.,* 2005).

In the context of reproduction, the most important products of the adrenal glands are the weak androgen, dehydroepiandrosterone (DHEA), DHEA sulfate and androstenedione, the secretion of which are also stimulated by ACTH. These adrenal androgens may be converted in target tissues to more potent androgens or to estrogens and can therefore potentially affect functioning of the reproductive endocrine axis and the cell types that are responsive to androgens and estrogens (Simpson and Rebar, 1995; Damstra *et al*., 2002; Tarrant *et al.,* 2005).

2.3.3 The Hypothalmic-Pituitary-Thyroid Axis (HPT)

In the HPT axis, thyrotropin-releasing hormone (TRH) is secreted from the terminals of hypothalamic neurons and acts on thyrotropes in the anterior pituitary gland to regulate the synthesis and secretion of TSH in mammals (Reed and Pangaro, 1995). TSH is then transported via the bloodstream to the thyroid gland where it acts to stimulate the synthesis of triiodothyronine (T_3) and tetraiodothyronine (thyroxine or T_4) that are released into the bloodstream and act throughout the body to stimulate general metabolic activity. T_3 and T_4 feedback to the hypothalamus and pituitary and reduce the levels of TRH and TSH. There is 70 times more plasma T_4 circulating than T_3. T_3 is more metabolically active than T_4 and is loosely bound to a plasma carrier protein, thyroxine-binding globulin (TBG). If the level of TBG is increased (as seen in pregnancy or women taking birth control pills containing estrogen), the level of free hormone feeding back to the hypothalamus and pituitary is decreased. This event results in increased TRH and TSH, which stimulate the thyroid to increase the total hormone level to a point where the “free” hormone is again within normal limits. Other hormones such as anabolic steroids, lower the TBG levels and increase the total amount of circulating T_4, causing a reverse of the above situation. The HPT axis also plays an important role in terminal differentiation of various tissues, extending from

neurons to muscle and to Sertoli cells in the testes (Damstra *et al*., 2002; Tarrant *et al.,* 2005).

The two systems most vulnerable to endocrine disruption is the reproductive and thyroid system, because disruption can already occur *in utero*.

2.4 Human reproduction

Reproduction in humans starts with the fusion of a male gamete (spermatozoon) with a female gamete (ovum) to form a zygote. (Meij and Van Papendorp, 1997). Therefore the proper functioning of the reproductive system and its enormously complex control mechanism ensures survival not only of the individual but of the species (Thibodeau, 1987).

2.4.1 The sex chromosomes

Sex is determined genetically by two chromosomes, called the sex chromosomes to distinguish them from somatic chromosomes. In humans and many other mammals, the sex chromosomes are called the X and Y chromosomes. The Y chromosome is necessary and sufficient for the production of testes, and the testis-determining gene product is called sex region Y (SRY). SRY contains a DNA (Deoxyribonucleic acid)-binding domain and acts as a transcription factor that initiates transcription of a cascade of genes necessary for testicular differentiation. Male cells with a diploid number of chromosomes contain an X and Y, whereas female cells contain two X chromosomes. As a consequence of meiosis during gametogenesis, each normal sperm contain a single X chromosome, but half the normal sperm contain an X chromosome and half a Y chromosome. When a sperm containing a Y fertilizes an ovum, an XY pattern results, then the zygote develops into a genetic male. There are 46 chromosomes: in males 22 pairs of autosomes plus an X chromosome and a Y chromosome; in females, 22 pairs of autosomes plus two X chromosomes. The human Y chromosome is smaller than the X chromosome, and it has been hypothesized that sperm containing the Y chromosome are lighter and able to swim faster up the female genital tract (Ganong, 1997).

2.4.2 Embryology of the human reproductive system

2.4.2.1 Development of the gonads

On each side of the embryo, a primitive gonad arises from the genital ridge, a condensation of tissue near the adrenal gland. The gonad develops a cortex and a medulla. Until six weeks of development, these structures are identical in both sexes. In the males, the medulla develops during the seventh and eighth weeks into a testis and the cortex regress. Leydig and Sertoli cells appear, and testosterone and Müllerian inhibiting substance (MIS) are secreted. In females, the cortex develops into an ovary and the medulla regresses. The embryonic ovary does not secrete hormones (Ganong, 1997).

2.4.2.2 Embryology of the genitalia

In the seventh week of gestation, the embryo has both male and female primordial genital ducts. In a normal female fetus, the Müllerian duct system then develops into uterine tubes (oviducts) and uterus. In the normal male fetus, the Wolffian duct system on each side develops into the epididymis and vas deferens. The external genitalia are similarly bipotential until the eighth week. There after, the urogenital slit disappears and male genitalia form, or, alternatively, it remains open and female genitalia form. But, when there are functional testes in the embryo, male internal and external genitalia develop. The Leydig cells of the fetal testis secrete testosterone and the Sertoli cells secrete MIS (Ganong, 1997).

The gonads have a dual function in both sexes, the production of germ cells (gametogenesis) and the secretion of sex hormones. The testes secrete large amounts of testosterone that is necessary for masculinisation. The secretory and gametogenic functions are both dependant on FSH and LH secretion (anterior pituitary gonadotropin) (Ganong, 1997). FSH, LH, and TSH which are glycoprotein hormones are secreted by the anterior pituitary and are made up of two subunits. The subunits are α and β and they must be combined for maximal physiologic activity (Ganong, 1997).

2.5 The male reproductive system

2.5.1 Structure

The male reproductive system includes (Ganong, 1997; Kilian, 2004; Figure 2.2).

- the testes where the gametes (spermatozoa) and male sex hormones are formed.
- the duct system, which conveys the spermatozoa to the exterior.
- accessory glands (prostate gland, seminal vesicle and bulbourethal glands) which supply spermatozoa with their fluid medium and contribute to their maturation.
- the penis.

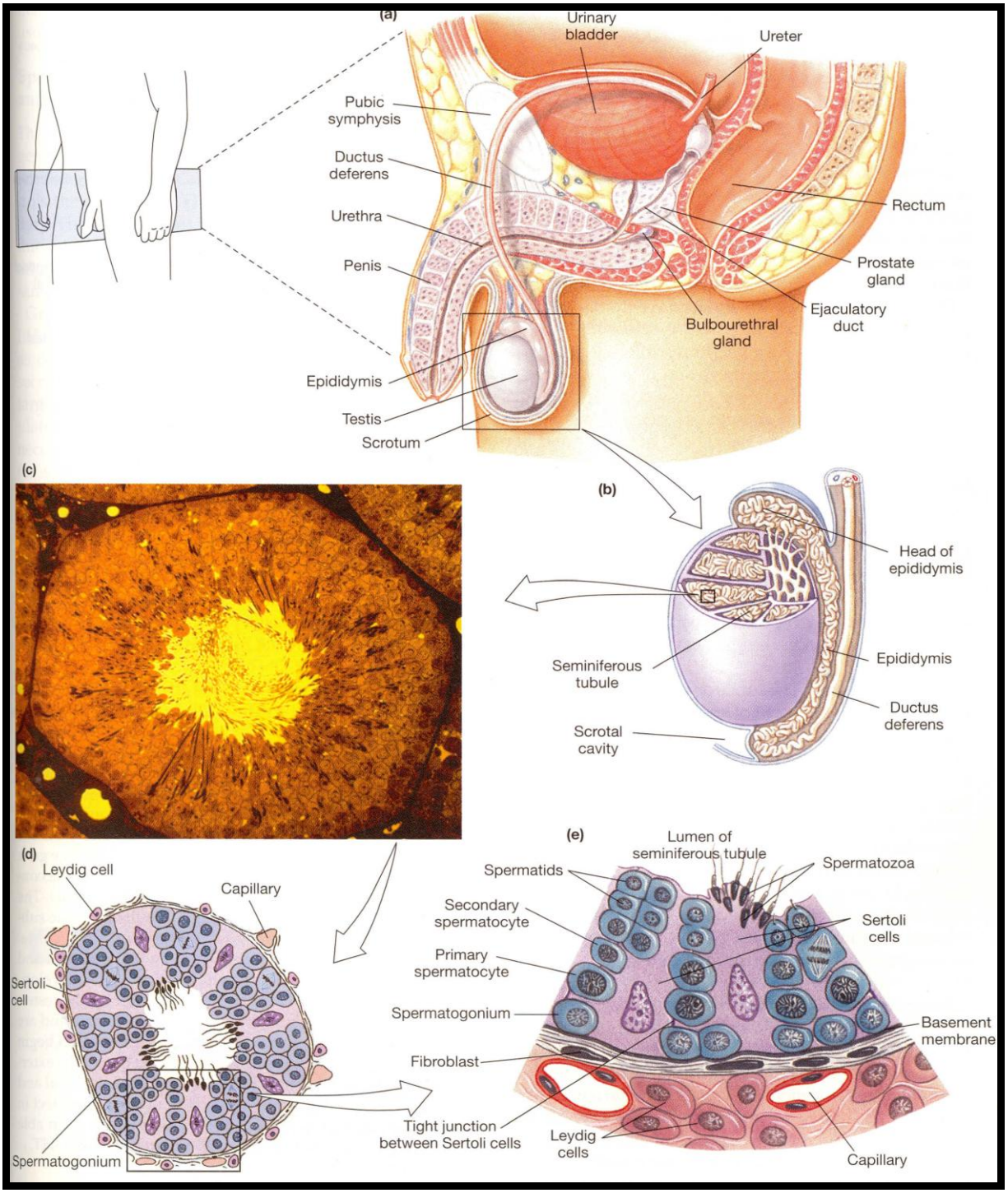

Figure 2.2: Male reproductive system (Silverthorn *et al*., 1998)

2.5.1.1 Testis

The primary sex structure in the male is the testes. The testes have a dual function which includes the production of reproductive cells and sex hormones (Meij and Van Papendorp, 1997). The testes are small ovoid glands that are somewhat flattened from side to side, measure about 4 or 5 cm in length, and weight 10 to 15g each. The left testis is generally located about 1cm lower in the scrotal sac than the right testis. The two testes are located in the sac-like scrotum in the scrotal cavity outside the abdomen. Each testis is surrounded by a fibrous membrane, the tunica albuginea and is suspended in the scrotum by a spermatic cord, which contains the vas deferens, blood vessels, nerves and a bundle of smooth muscle fibres (Meij and Van Papendorp, 1997).

The testes are made up of seminiferous tubules where spermatozoa are formed from germ cells (spermatogenesis). Both ends of the seminiferous tubules drain into a network of ducts in the head of the epididymis. From the epididymis the spermatozoa pass through the epididymis tail into the vas deferens (Meij and Van Papendorp, 1997; Ganong, 1997). Spermatozoa enter through the ejaculatory ducts into the urethra in the body of the prostate at the time of ejaculation. The seminal vesicle, a saccular gland opens into each ejacular duct and the upper part of the urethra is surrounded by the prostate. The prostate secretion is conveyed to the urethra by means of a number of ducts during ejaculation (Meij and Van Papendorp, 1997; Ganong, 1997).

Between the tubules in the testes are nests of cells containing lipid granules namely the interstitial cells of Leydig, which secrete testosterone into the bloodstream. The principle hormone of the testes is testosterone. Testosterone is synthesized from cholesterol in the Leydig cells and is also formed from andostenedione secreted by the adrenal cortex. Testosterone secretion is also under the control of LH (Ganong, 1997).

The testis is both an endocrine gland and a reproductive organ. It is responsible for the production of hormones and male gametes and may therefore be an important target for endocrine disruption. The testis consists of two types of tissue: seminiferous tubules, supported by Sertoli cells, and

the interstitial compartment, comprised of Leydig cells (Fisher, 2004; Akingbemi 2005; Phillips and Tanphaichitr, 2008). Testicular functions are regulated by the hypothalamic-pituitary-testicular axis, which involves the pituitary gonadotropins LH and FSH (Amory *et al*., 2001, Phillips and Tanphaichitr, 2008).

2.5.1.2 Seminiferous tubules

The tough outer fibrous capsule of the testis encloses masses of coiled seminiferous tubules clustered into 250-300 compartments. Between the tubules is interstitial tissue consisting primarily of blood vessels and Leydig cells. The seminiferous tubules constitute nearly 80% of the testicular mass in adults. Each individual tubule is 0.3-1m long. The seminiferous tubules leave the testis and join the epidydimis. The seminiferous tubule is composed of two cells: spermatocytes in various stages of becoming sperm and the Sertoli cells (Silverthorn *et al*., 1998).

2.5.1.3 Sertoli cell

The Sertoli cell is situated along the basal lamina of the seminiferous tubules and is the first somatic element to differentiate in the testis in fetal life (Auharek *et al*., 2010). The Sertoli cell also mediates the actions of FSH and LH-stimulated testosterone production in the testis in a stage-dependent manner (Hess and de Franca, 2008). The basic function of the Sertoli cell is to create an appropriate microenvironment for germ cell development (Li and Heindel, 1998; Dalgaard, 2001; Walker and Cheng, 2005). The Sertoli cell is responsible for orchestrating germ cells through sequential phases of mitosis, meiosis and differentiation. Clones of germ cells pass through several stage specific cell associations until the highly differentiated elongated spermatids are released into the lumen of the seminiferous tubules, a process called spermiation. The Sertoli cell is a fundamental component of the architectural structure of the seminiferous epithelium (Walker and Cheng, 2005; Woodruff *et al*., 2010). It provides physical support to the germ cells which are enveloped in the Sertoli cell cytoplasm. The tight junctions between the Sertoli cells outline the blood-testis-barrier and form a basal compartment containing spermatogonia and early spermatocytes and an adluminal compartment

isolating haploid spermatocytes and the spermatids from the surrounding tissue and the blood stream. The Sertoli cell is also responsible for the synchronised lifting of early meiotic spermatocytes through the tight junctions from the basal compartment to the adluminal compartment (Sharpe *et al*., 2003; Woodruff *et al*., 2010).

Apart from these physical properties, the Sertoli cell is also responsible for the nutrition of germ cells by transporting nourishment such as sugars, amino acids, lipids and metallic elements. Also of importance to spermatogenesis, the Seroli cell secretes paracrine factors such as inhibin, androgen binding protein, sulphated glucoprotein 1 and 2, and metal carrier protein (Clermont, 1993; Dalgaard, 2001). The Sertoli cell has phagocytotic properties and eliminates dead germ cells as well as residual bodies, the remains of the sparse cytoplasm of the released spermatid (Russell and Shina Hikim, 1995; Dalgaard, 2001). A well functioning Sertoli cell population is crucial for the development and maintenance of the spermatogenetic process at a normal-physiological level (Sharpe *et al*., 2003; Woodruff *et al*., 2010). Each Sertoli cell can only support a fixed number of germ cells during the development into spermatids (Hess and de France, 2008; Woodruff *et al*., 2010). The number of Sertoli cells in adult testis determines both testis size and daily sperm production (Sharpe *et al*., 2003; Hess and de Franca, 2008). Using rats as an example that have 14 stages, a toxic insult to a rat Sertoli cell may affect at least 10 elongated spermatids nursed by this specific Sertoli cell (Russell and Peterson 1984; Dalgaard, 2001). A compound affecting Sertoli cell population may result in a massive germ cell dysfunction and eventually lead to the death of these cells (Dalgaard, 2001; Woodruff *et al*., 2010).

2.4.1.4 Leydig cells

Between the tubules in the testes are nests of cells containing lipid granules, namely the interstitial cells of Leydig. They secrete testosterone into the bloodstream (Silverthorn *et al*., 1998). Two distinct generations of Leydig cells have been identified: fetal Leydig cells and adult Leydig cells. Fetal and adult Leydig cells share the same principal function to produce androgens, despite

their differences in morphological and biochemical properties (Svechnikov *et al.*, 2010).

2.4.1.4.1 Fetal Leydig cells

The primary source of androgens during the prenatal period is the fetal Leydig cells (FLCs). These cells secrete testosterone which is required for masculinisation and proper development of male reproductive organs. Experimental studies using animals indicated that disruption of FLC development resulted in feminization of external genitalia due to the lack of androgens necessary for their masculinization (Svechnikov *et al*., 2010).

2.4.1.4.2 Adult Leydig cells

Adult Leydig cells originate postnatally and produce testosterone required for pubertal development of external genitalia and onset of spermatogenesis (Svechnikov *et al*., 2010). Adult Leydig cells are not derived from pre-existing FLCs, but rather from undifferentiated peritubular-like stem cells that express both stem and peritubular cell markers (Ge *et al.*, 2006; Svechnikov *et al*., 2010). Adult Leydig cells have low numbers of lipid drops, high number of LH receptors and testosterone is the major androgen produced (Svechnikov *et al*., 2010).

2.5.2 Spermatogenesis

Spermatogenesis is the transformation of spermatogonial cells into spermatozoa over an extended period of time within the seminiferous tubules boundries of the testis (Hess and de Franca, 2008). The process of spermatogenesis is only initiated by puberty and then maintained throughout adult life (Woodruff *et al*., 2010).

The seminiferous tubules are lined with sperm-producing cells and are called spermatogonia. Spermatogonia are found during fetal life, but are basically dormant till puberty. From puberty they undergo mitotic division continuously. It is only during this period that the spermatogenic process is directly vulnerable to adverse effects. However the foundation for spermatogenesis is laid during fetal development (Sharpe, 2010).

Spermatogenesis comprises a precisely timed and synchronized development of several generations of germ cells. Throughout this complex development the germ cells are embedded within the cytoplasmic processes of the Sertoli cell, which structurally supports and moves germ cells from the base of the lumen of the tubule. Therefore, spermatogenesis relies on the coordinated support and interactions of germ cells, Sertoli cells, Leydig cells, peritubular cells, interstitial macrophages and the blood vasculature (Creasy, 2001; Hess and de Franca, 2008; Woodruff *et al*., 2010).

Spermatogenesis may be divided into three phases based upon functional considerations (Figure 2.3):

1) the proliferative phase (spermatogonia), in which cells undergo rapid successive divisions,
2) the meiotic phase (spermatocytes) in which genetic material is recombined and segregated,
3) the differentiation or spermiogenic phase, in which spermatids transform into cells structurally equipped to reach the ovum (Russell *et al*., 1990; Hess and de Franca, 2008; Phillips and Tanphaichitr , 2008; Singh *et al*., 2011).

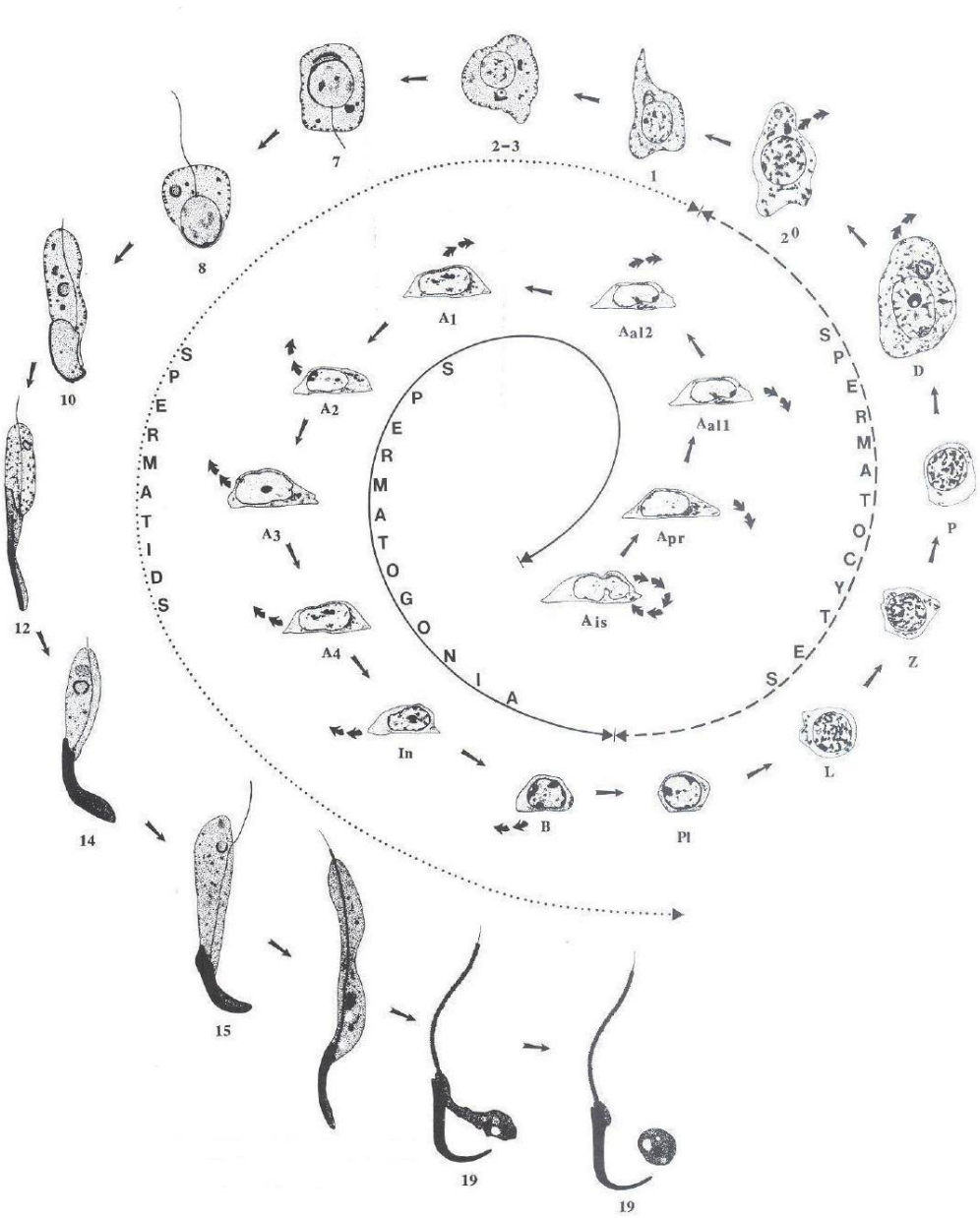

Figure 2.3: Diagrammatic representation of rat spermatogenesis showing the three major developmental phases (Russell *et al.*, 1990).

2.5.2.1 Proliferative phase

Spermatogonia are relatively immature cells that undergo numerous mitoses to build a large population of cells that will subsequently undergo meiosis and differentiation to form sperm. There are three types of spermatogonia: stem cell spermatogonia, proliferative spermatogonia and differentiating spermatogonia. The first two groups are also known as undifferentiated spermatogonia (Russell *et al.,* 1990; Kilian, 2004; Hess and de Franca, 2008). The proliferative and differentiating spermatogonia show a high mitotic rate and consequently, are more susceptible to agents that affect spermatogenesis (Russell *et al*., 1990). In the rat, these spermatogonia divide approximately nine times a week (Russell *et al.,* 1990; Kilian, 2004; Hess and de Franca, 2008).

The spermatogonia that are most widely thought to be stem cells are called $A_{isolated}$ (A_{iso}) spermatogonia, whereas the other Type A spermatogonia are proliferative (A_{paired}, A_{pr}, and $A_{aligned}$, A_{al}) and differentiated [A1, A_2, A_3, A_4, Intermediate (In), and Type V (B)] spermatogonia (Figure 2.2). The paired and aligned spermatogonia have been designated as such to refer to their connection to other spermatogonia of the same type by areas of open cytoplasmic continuity as intercellular bridges. Only some spermatogonial cell types can be distinguished from one another based on morphological criteria (Russell *et al.,* 1990; Kilian, 2004; Hess and de Franca, 2008).

2.5.2.2 Meiotic phase

At the end of the differentiation phase, the most mature spermatogonia divide to form the young primary spermatocytes. Specifically, Type B cells divide to form preleptotene spermatocytes (PI). The morphology of the preleptotene cells, or resting spermatocytes, is very similar to B cells except that the preleptotene cells are slightly smaller, having less chromatin situated along the nuclear envelope (Figure 2.2) (Russell *et al.,* 1990; Kilian, 2004; Hess and de Franca, 2008).

Chromosomes are recombined and genetic material is halved in each cell during the two meiotic divisions. Characteristically, a long meiotic prophase in

which recombination occurs is followed by two rapid divisions, the end result being the production of haploid spermatids. Prophase of the first meiotic division is exceptionally long (lasting about three weeks). There is a gradual morphological transition from one phase of prophase to another, rather than clearly defined stepwise changes (Russell *et al.,* 1990; Kilian, 2004; Hess and de Franca, 2008).

The presence of the leptone cells (L) signals the initiation of the meiotic prophase (Russell *et al.,* 1990). In the transition from preleptotene to leptone, nuclei gradually lose their peripheral chromatin and form fine chromatin threads that can be seen by light microscopy. In zygotene cells (Z), the homologous chromosomes have become paired. In pachytene cells (P) of the rat, the chromosomes have become fully paired and remain so for almost two weeks. Late pachytene nuclei are generally ovoid, whereas their pachytene predecessors are rounded. The diplotene phase of meiosis in the male is brief. Diplotene cells (Di) are the largest primary spermatocytes and also the largest of any of the germ cells types (Russell *et al.,* 1990; Kilian, 2004).

The metaphase, anaphase and telophase are referred to as the first meiotic division, or meiosis 1 (M-I). The cells formed are secondary spermatocytes (2°). The second meiotic division, or meiosis II (M-II), follows rapidly to produce spermatids (Russell *et al.,* 1990; Kilian, 2004; Hess and de Franca, 2008).

2.5.2.3 Spermiogenic phase

In the rat, about three weeks are required for cells to evolve from young spermatids to spermatozoa. This process occurs without cell division and is one of the most phenomenal cell transformations in the body (Russell *et al.,* 1990; Kilian, 2004).

2.5.2.3.1 Development of a flagellum

In rats, the first evidence of a flagellum is seen in the youngest spermatid. After migration of the centriole pair to the cell surface, one of the two centrioles at the surface forms an axoneme, a structure containing

microtubules that causes the spermatid plasma membrane to protrude from the cell. Accessory compounds are added to the flagellum to form its middle, principal, and end pieces. Outer dense fibres form both in the midpiece and the principal piece (Russell *et al.,* 1990; Kilian, 2004).

2.5.2.3.2 Development of the acrosome

Although the general features of acrosomal development are similar in all mammals, each species differs from others in the fine details of acrosome formation and in the final shape of the acrosome over the sperm head. Acrosomal formation is a slow but continuous process that is not complete until late spermatogenesis (Russell *et al.,* 1990; Kilian, 2004). The most immature rat spermatids contain no acrosome, but show only a perinuclear Golgi apparatus. Shortly after the spermatids are formed, the Golgi apparatus is involved in producing small condensing vacuoles or proacrosomal vesicles that contain dense material or proacrosomal granules. The shape of the spermatid head and its overlying acrosome change during the two weeks before sperm release, although the mechanism of shape change is not known (Russell *et al.,* 1990; Kilian, 2004). During spermatogenesis, part of the rat acrosome (ventral acrosome) separates from the main acrosome, a feature not seen in most species. The progression of changes in the acrosome is the primary basis for classifying rat spermiogenesis into steps and for using these steps to classify cell associations into stages (Russell *et al.,* 1990; Kilian, 2004).

2.5.2.3.3 Nuclear shaping and nuclear condensation

Up to a certain point in spermatogenesis, the nucleus of the spermatid is roughly spherical. The rat spermatid head is almost sickle-shaped or falciform. Nuclear shape changes may be, in part, due to the manchette, a cytoskeletal complex formed around the nucleus by a sleeve of microtubules. The changes seen in the shape of the spermatid hear during spermatogenesis provide a secondary means to classify spermiogenesis in to steps (Russell *et al.,* 1990; Kilian, 2004).

2.5.2.3.4 Elimination of cytoplasm

The spermatid is reduced in volume to approximately 25% of its original size before sperm release (Russell *et al.,* 1990). Since the cell is smaller and streamlined, the motility apparatus is capable of propelling it through the fluid environment. There are at least three phases in the process of making spermatids smaller. First, water may be eliminated from the nucleus and cytoplasm during the elongation of the spermatid. Second, some cytoplasm is eliminated just before sperm release by minute structures called tubulobulbar complexes. Third, the separation of a cytoplasmic package (residual body) is responsible for about one-fourth of the volume reduction (Russell *et al.,* 1990). The cytoplasmic fragments are phagocytized by the Sertoli cell and transported to the base of the tubule where they are then digested by the Sertoli cell (Russell *et al.,* 1990). After cytoplasmic elimination, a small amount of cytoplasm, the cytoplasmic droplet, remains around the neck of the spermatid (Russell *et al.,* 1990; Kilian, 2004).

2.5.2.3.5 Spermiation

Spermiation is the complex process by which sperm are disengaged from the Sertoli cell to be released into the tubular lumen. The number of stages of spermatogenesis differs depending on the species. In the rat spermatogenesis encompasses 14 stages (I-XIV), in the man it develops through only 6 stages (I-VI). The succession of stages through time is called the spermatogenic cycle. The duration of the spermatogenic cycle (16 days) is characteristic of the human species (Russell *et al.,* 1990; Kilian, 2004).

The development and differentiation of an A spermatogonium into a mature sperm requires at least four spermatogenic cycles. In the rat the overall duration of spermatogenesis is calculated as 51-53 days and in human males at least 64 days (Russell *et al.,* 1990; Kilian, 2004).

2.6 Thyroid

The thyroid is a butterfly-shaped gland that lies across the trachea at the base of the throat, just below the larynx (Ganong, 1997; Brown, 2003). It weighs between 15-20g. The thyroid gland maintains the level of metabolism in the

tissue that is optimal for their function. Thyroid hormones stimulate O_2 consumption of most of the cells in the body, help regulate lipid and carbohydrate metabolism, and are necessary for normal growth and maturation (Ganong, 1997; Silverthorn *et al*., 1997)

2.6.1 Thyroid and brain development

The thyroid affects the adult body's major systems and it is crucial for fetal development. In adults, environmental exposures may be less significant as the thyroid negative feedback system helps the adult body to restore balance. However the developing fetus depends upon maternal thyroid hormones until around the beginning of the second trimester. The thyroid is especially crucial during brain development (Jugan *et al*., 2010).

Thus, it is in fetal and childhood development that environmental factors may have the greatest impact. Within the first weeks of gestation the fetal brain is already developing. At this point the spinal cord and hindbrain components grow and the cerebral cortex structures begin to take shape about halfway through gestation. As early as the second month of gestation neural synapses begin to form, peaking in the child's first year of life. Many parts of the brain continue to develop postnatally and even into adulthood. The thyroid hormones are essential for neuron formation, synapse development, formation of myelin, and migration of neurons to their proper place in the brain. The fetal thyroid begins to grow around the end of the first trimester, but only begins to produce its own products by the second trimester. The hypothalamic-pituitary-thyroid axis has only matured by the last trimester. Thus, maternal thyroid hormones must be continuously available until birth because crucial brain development takes place before the fetus thyroid system is fully functional. Extreme maternal hypothyroidism can lead to neurological cretinism, which can induce spastic diplegia (a form of cerebral palsy), deafness, and severe mental retardation. Maternal hyperthyroidism on the other hand can result in low birth, prematurity, and in the case of maternal Graves disease, an increased incidence of congenital malformations (Brown, 2003).

2.6.2 The thyroid and male reproduction

It was always thought that the testis was essentially independent of thyroid effects, but over the past 15 years it has become clear that the thyroid has critical actions on the ovary and testis especially during development in the male (Cooke *et al*., 2004). A number of investigations have demonstrated that an altered thyroid hormone environment can produce abnormalities in the production of reproductive hormones, ejaculate volume, sperm motility, and a variety of other indices in animals and humans (Krassas *et al*., 2002; Cooke *et al*., 2004; Krassas *et al*., 2010).

2.6.2.1 Thyroid hormone and testicular development

Sertoli cell proliferation begins fetally in rodents, and is complete by postnatal day 16. FSH is a major regulator of neonatal Sertoli cell proliferation, but various studies over the past years have concluded that thyroid hormones play a crucial role in the Sertoli cell development. They may be responsible for the developmental changes that decrease and finally eliminate mitogenic responses to FSH. A study done by van Haaster *et al*. (1992, 1993) reported that hypothyroidism lengthened and hyperthyroidism shortened the period of Sertoli cell proliferation in the rat (Cooke *et al*., 2004). The effects of hypothyroidism on Sertoli cells appear to be predominantly direct (Cooke *et al*., 2004). Furthermore, it appears that T_3 normally inhibits Sertoli cell proliferation directly while stimulating differentiation (Cooke *et al*., 2004).

2.6.2.2 Thyroid hormone and Leydig cells

Leydig cells are also important for thyroid hormones during development and adulthood (Cooke *et al*., 2004). It has been suggested that one critical molecular target of thyroid hormones in the Leydig cell may be the steroidogenic acute regulatory (StAR) protein. It is responsible for cholesterol transport across the outer mitochondrial membrane in Leydig cells. A lack of thyroid hormones result in the impaired testosterone production observed in these cells (Manna *et al*., 1999, 2001a, 2001b; Cooke *et al*., 2004).

2.6.2.3 Other effects

T_4 levels must be controlled because hyperthyroidism can lead to thyrotoxicosis, which may compromise sperm motility (Krassas *et al*., 2010; Singh *et al*., 2011). Hypothyroidism has been linked to abnormalities in sperm morphology (Krassas *et al*., 2010; Singh *et al*., 2011).

2.7 Endocrine disrupting chemicals (EDCs)

2.7.1 Definition of EDCs

The USA EPA defines endocrine disrupting compounds as "an exogenous agent that interfere with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process" (Diamanti-Kandarakis *et al.,* 2009).

The WHO has also defined an endocrine disrupter as an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or (sub) population (Damstra *et al.,* 2002; Burkhardt-Holm, 2010; Evans *et al*., 2011).

2.7.2 General Mechanisms of Endocrine Disruption

To establish the modes of action of EDCs is made difficult by the fact that a range of factors (e.g. duration, level and timing of exposure; nutritional status, age and gender of individual; cell or tissue type in which EDC acts) may result in different effects being produced (Lister and Van der Kraak, 2001; Tarrant *et al.,* 2005). EDCs were originally thought to exert actions primarily through nuclear hormone receptors, including estrogen receptors (ERs), androgen receptors (ARs), progesterone receptors (PRs), thyroid receptors (TRs), and retinoid receptors, among others. Today, basic scientific research shows that the mechanisms are much broader than originally recognized (Tarrant *et al.,* 2005). The mechanisms by which endocrine disrupters act include nuclear receptors, non-nuclear steroid hormone receptors, non-steroid receptors (eg. neurotransmitter receptors such as serotonin receptor), orphan receptors (eg. aryl hydrocarbon receptor), enzymatic pathways involved in steroid

biosynthesis and/or metabolism and numerous other mechanisms that converge upon endocrine and reproductive systems (Diamanti-Kandarakis *et al.,* 2009). Therefore endocrine disrupters can mimic or partly mimic naturally occurring hormones, bind to a receptor within a cell and block the endogenous hormone from binding or interfere or block the way natural hormones or their receptors are made or controlled (NIEHS, 2006).

2.7.3 Main Classes of EDCs

Research has led to the recognition of five main classes of EDCs which include (anti)estrogens; (anti)androgens; (anti)progestins; aryl hydrocarbon receptor agonists and thyroid hormone disruptors. In general, the activity of estrogenic, androgenic and thyroid-like chemicals is usually mediated by their respective nuclear receptors. But other mechanisms of action such as interactions with binding globulins, inhibition of steroidogenic enzymes and binding to membrane receptors, or other nuclear receptors cannot be excluded (Zacharewski *et al.*, 2002; Tarrant *et al.,* 2005).

2.7.4 Compounds known to be EDCs

The group of molecules identified as endocrine disruptors is highly heterogeneous and endocrine disruptors comprise different groups (Damstra *et al.,* 2002; Markey *et al*., 2003; Byrne *et al.,* 2009; Burkhardt-Holm, 2010):

- natural hormones and metabolites [e.g., 17-β estradiol (E_2) and metabolites estriol (E_3) and estrone (E_1)];
- artificial/ synthetic hormones (e.g., DES; sex steroids in contraceptive pill);
- phyto- and mycoestrogens (e.g., isoflavones, lignans, coumestans, stilbenes; zearalenone);
- drugs with hormonal side effects (e.g., clofibrate);
- industrial and household chemicals (PCBs, flame retardants, paints, plasticizers (such as BPA, phthalates), alkylphenol ethoxylate detergents, UV screens);
- pesticides and metabolites (e.g., DDT, methoxychlor, kepone, dieldrin, lindan, endosulfan, toxaphene);

- side products of industrial and household processes (polycyclic aromatic hydrocarbons (PAH), dioxins, etc.);
- metals (e.g., cadmium, arsenic, lead and mercury).

These compounds are persistent in the environment and the effects are evident in various wildlife studies.

2.7.5 Evidence in wildlife

Mammals:

Exposure to organochlorines [PCBs, Dichlorodiphenyldichloroethylene (DDE)] has been shown to adversely impact the reproductive and immune function in Baltic seals, resulting in marked population declines. These seals exhibit a compromised endocrine system (Damstra *et al.,* 2002; Woodruff *et al*., 2008, 2010). Park *et al*. (2009) indicated that there is a decline in a number of species of insectivorous bats using sewage works as principle foraging sites.

Birds:

Eggshell thinning and altered gonadal development have been observed in birds of prey exposed to DDT. Exposure to PCB may be directly linked to a syndrome of embryonic abnormalities as it has been observed in fish-eating birds (Damstra *et al.,* 2002; Evans *et al.,* 2011).

Reptiles:

In 1980 a chemical spill contaminated Lake Apopka (Florida, US) with large quantities of dicofol and sulphuric acid. Lake Apopka is a well-publicized example of potential EDC effects on population decline in alligators. Studies on the alligators revealed altered steroidogenesis, abnormal circulating hormone levels, hepatic transformation of androgen and endocrine organ morphology in juvenile alligators living in polluted lakes (Iguchi *et al*., 2006; Woodruff *et al.,* 2010).

Amphibians:

Amphibians represent a suitable model for monitoring reproductive performance, advanced development including metamorphosis, and sexual

maturation (Kloas, 2002; Iguchi *et al*., 2006). Studies done over the recent decade indicated a population decline that may be related to pesticide applications during breeding seasons (Woodruff *et al.,* 2010; Evans *et al.,* 2011). Pesticides individually inhibited larval growth and development were pesticide mixtures induced damage to the thymus (Hayes *et al*., 2006).

Fish:

There is extensive evidence that chemical constituents present in pulp and paper mill effluents as well as sewage treatment effluents can affect reproductive endocrine function in fish, which may contribute to alteration in reproductive development (Stumpter, 2005; Orrego *et al*., 2010). Fish exposed to pulp and paper mill effluents revealed reduction in gonad size, depression of circulating sex steroids and an increase in plasma vitellogenin (VTG) (Orrego *et al*., 2010. Furthermore, fish exposed to sewage treatment effluents revealed feminaztion of male roach, altered kidney development, modulated immune function and cause genotoxic damage (Liney *et al*., 2006). However a variety of mechanisms (e.g., hormone-receptor interactions, interference with sex steroid biosynthesis, altered pituitary function) maybe involved (Damstra *et al.,* 2002; Stumpter, 2005; Orrego *et al*., 2010).

Invertebrates:

Exposure of marine gastropods to TBT (Tributyl tin - a biocide used in antifouling paints) provides the clearest example in invertebrates of an endocrine-mediated adverse effect caused by exposure to an environmental contaminant (Sumpter, 2005; Evans *et al*., 2011).This led to masculinisation of marine gastropods exposed to TBT which has resulted in a worldwide decline of gastropods. The endocrine mechanism probably involves elevated androgen levels possibly through altered aromatase activity. (Damstra *et al.,* 2002; Iguchi *et al*., 2006; Woodruff *et al*., 2008).

Although the mechanism of sex determination can vary among species, endocrine control of the testis and the role of androgens in male secondary sex development and functioning are highly conserved among vertebrates.

This is an indication that wildlife is effective and important sentinels of human public health (Milnes *et al*., 2006).

2.7.6 Human Health Effects

Endocrine disrupters are so named for their ability to modulate or dysregulate the endogenous endocrine system. In humans the following health effects have been reported: (Phillips *et al.,* 2008; Woodruff *et al.,* 2010; Evans *et al*., 2011).

- Infertility (fecundity, reduced sperm quality, endometriosis)
- Abnormal prenatal and childhood development (spontaneous abortions, male reproductive-tract abnormalities, and other birth defects, altered sex ratio, precocious puberty)
- Reproductive cancers of the prostate, breast, ovaries, endometrium, or testis
- Thyroid cancer

2.8 Possible effects on male reproductive health

Male reproductive health and exposure to EDCs can be divided into three groups of adverse health outcomes. Firstly, a decline in reproductive function manifested by reduced semen quality and compromised male fertility. Secondly, disruption of male fetal development resulting in congenital malformations of the urogenital tract for example cryptorchidism (undescended testes) and hypospadia (abnormal positioning of the opening of the urethra) and thirdly testicular germ cell tumours (Evans *et al*., 2011). These adverse health outcomes all share a common aetiological origin and constitute a syndrome termed TDS (Sharpe and Skakkebaek, 1993; Sharpe 2009; Jorgensen *et al*., 2010; Evans *et al*., 2011,). Animal experiments have shown that substances with estrogenic and antiandrogenic properties may cause hypospadias, cryptorchidism, reduction of sperm density and an increase of testicular tumours (Sharpe and Skakkebaek, 1993; Pflieger-Bruss *et al.*, 2004; Sharpe 2009).

2.8.1 Cryptorchidism

Cryptorchidism is the most common congenital malformation in male babies at birth. Normal testis descent occurs in two phases. The fetal testes migrate from their point of origin near the kidneys into the pelvis during early gestation (transabdominal phase). Towards the end of gestation, descent from the pelvis into the scrotum is accomplished (transinguinal phase). The second, transinguinal phase is androgen dependant, and disruption of this phase appears to be the most common cause of cryptorchidism at birth (Sharpe and Skakkebaek, 1993; Sharpe 2009; Jørgensen *et al*., 2010; Evans *et al*., 2011).

2.8.2 Hypospadias

Hypospadias affect around 0.2 – 4% of boys at birth. In fetal life, androgen action is crucially important to ensure proper location of the urethral opening at the tip of the glans penis. If androgen action is diminished, the urethra opens on the underside of the glans penis (mild, glandular hypospadia). In severe case, the opening is positioned on the shaft of the penis, or even near the scrotal sack (Sharpe and Skakkebaek, 1993; Sharpe 2009; Jørgensen *et al*., 2010; Evans *et al*., 2011).

2.8.3 Reduced semen quality

Semen quality, as determined by sperm counts, sperm motility, sperm concentration, ejaculation volume and other parameters, is variable. Semen quality can be affected by numerous factors, including abstinence, ethnicity, infectious disease, season, clothing and drug abuse (Sharpe and Skakkebaek, 1993; Evans *et al*., 2011).

2.8.4 Testicular germ cell tumours

Hypospadias and cryptorchidism are frequently seen in newborn patients with disorders of sex differentiation (DSD). This is associated with severe testicular dysgenesis (Kalfa *et al.*, 2009, Jorgensen *et al*., 2010). DSD patients are later in life at increased risk to develop germ cell tumours and most often have severely impaired spermatogenesis (Jorgensen *et al.,* 2010).

2.9 Veterinary growth stimulants (VGS)

In 1956 hormone implants containing estradiol benzoate/progesterone were approved by the US Food and Drug Administration (FDA) for increasing growth, feed efficiency, and carcass leanness of cattle. Later the FDA approved other implants containing testosterone, α-zearalanol, trenbolone acetate, and combinations of these hormones. Currently five hormones (progesterone, testosterone, E_2, α-zearalanol and trenbolone acetate) are approved for implants in cattle in the USA (Doyle, 2000). These implants have however been officially prohibited in Europe since 1989 (Doyle, 2000; CECBP, 2008).

In SA, five hormones have been approved for use in beef production by the Registrar under Act 36 of 1947. These hormones include E_2, progesterone and testosterone which are natural hormones and two synthetic hormones, α-zearalanol and trenbolone acetate (Jonker, 2008).

High density cattle feeding operations have a demand for high volumes of water and that implies huge amounts of organic waste production (Jonker, 2008). In order to manage cattle waste products feedlots are ussaully situated on a slight slope. (Jonker, 2008). VGS and their metabolites have the potential to find their way into our water resources and affect wildlife/animal and human life (CECBP, 2008; Kolok and Sellin, 2008).

2.9.1 Health risks to humans

Safety of hormonal implants has been questioned on the basis that residues from these implants in beef may significantly increase exposure to humans, particularly children, which may adversely affect health. Children and the fetus *in utero* have been considered at greater risk from exposure to hormones because their normal physiological hormone levels are low compared to adults (Doyle, 2000). Humans are potentially exposed to GS by consumption of commercial meat products and from environmental exposure related to animals waste and use of contaminated water sources. Significant amounts of synthetic and natural hormones and their metabolites are excreted in animal waste (CECBP, 2008; Kolok and Selin, 2008). VGS excreted by animals are

present in manure applied as fertilizer and in feedlot retention ponds, and subsequently may be retained in soil or transported to ground and surface water (Soto *et al*., 2004; CECBP, 2008; khan *et al*., 2008, 2008b).

Although hormones are essential for various physiological processes in the body, excessive amounts may have adverse effects. For example E_2 stimulates cell division in hormonally sensitive tissue thereby increasing the possibility for accumulation of random errors during DNA duplication (Doyle, 2000). This increased cell proliferation also has the effect of stimulating growth of mutant cells (Doyle, 2000; Henderson and Feigelson, 2000).

2.9.2 Examples of veterinary growth stimulants

2.9.2.1 Zilpaterol

In recent years β-agonists have been used in cattle to increase feeding efficiency, increase carcass leanness and promote animal growth (Ricks *et al*., 1984; Birkelo, 2003; Verhoeckx *et al*., 2005). They enhance growth efficiency by stimulating the β-adrenergic receptors on cell surfaces (Courtheyn *et al*., 2002; Macias-Cruz *et al*., 2010). In the muscle tissue, β-agonists promote protein synthesis and cell hypertrophy by inhibition of proteolysis, whereas in adipose tissue they promote lipolysis. Residues of β-agonists with high oral bioavailability, long plasma half-life and slow rates of elimination, represent a potential health risk to consumers. Cases of severe food poisoning resulting from the consumption of contaminated meat products have been documented (Ricks *et al*., 1984; Birkelo, 2003; Verhoeckx *et al*., 2005).

Zilpaterol hydrochloride (ZH) is licensed as Zilmax™ and approved as a feed additive for cattle in Mexico, South Africa and the USA (Macias-Cruz *et al*., 2010), but is completely banned in the European Community (EC). Studies by Stachel *et al*. (2003) found high concentrations of zilpaterol in urine of pigs and heifers right through the treatment peroid. Four days after withdrawal levels as low as 10µg/kg was still detected and 0.4 µg/kg after 10 days.

In a study done by Verhoeckx *et al.* (2005) they indicated that zilpaterol inhibits tumor necrosis factor alpha (TNF-α) release from U937 macrophages exposed to lipopolysaccharide (LPS) in a concentration-dependant manner and this inhibitory effect is mainly mediated by the β_2-adrenergic receptor (β_2-AR). Zilpaterol is a β_2-AR agonist and inhibitor of THF-α release, both *in vitro* and *in vivo*.

2.9.2.2 α-Zearalanol

α-Zearalanol is a non-steroidal synthetic anabolic VGS. It has a potent estrogenic activity and is widely used as a VGS in the beef industry (Zhong *et al*., 2011), in various countries but is banned in all member states of the EU (Leffers H, 2001). Zeranol (α-zearalanol) is also an estrogenic derivative produced naturally from the mycoestrogen zearalenone (European Commission, 1999. Leffers *et al*., 2001). Furthermore, α-zearalanol binds to the estrogen receptor in swine, rats and chickens with a binding affinity similar to that of DES, which is much greater than that of E_2 (Fitzpatrick *et al*., 1989). In rat liver, α-zearalanol was shown to bind to the estrogen receptor and to DNA in a manner similar to that of E_2 (Mastri *et al*., 1985, European Commission, 1999).

Most exogenous hormone-like chemicals including α-zearalanol and other synthetic growth stimulating hormones exhibit limited or no binding to carrier proteins (Mastri *et al*., 1985; Shrimanker *et al*., 1985; Nagel *et al*., 1998; Leffers *et al*., 2001). Their potential potency may be much larger than their actual concentrations suggest, and all may be available to the cells. Analyses of American beef products have shown that α-zearalanol and some of its metabolites can be detected in the finished consumer product (Stephany and Andre, 1999; Leffers *et al*., 2001). α-Zearalanol has a significantly higher estrogenic potency than other potential endocrine disruptors, and its presence in meat may be considered a major source of exposure to exogenous estrogens (Aksglaede *et al*., 2006).

α-Zearalanol is administered to beef heifers by subcutaneous implantation in the ear of six pellets containing a total of 72mg α-zearalanol. Consumption of

beef containing residues of α-zearalanol and its metabolites may pose a breast cancer risk because studies have shown that α-zearalanol is able to bind to the active site of human ERα and ERβ in a similar manner to E_2 (Takemura *et al*., 2007; Zhong *et al*., 2011).

Effects of α-zearalanol exposure were evident in workers working in a pelleting plant that showed symptoms of breast irritation, including sharp pain, tingling, burning, aching and irritation (Aw *et al*., 1989; Doyle, 2000). Two outbreaks, one of breast enlargement in young children in Italy and another of precocious sexual development in Puerto Rico (Saenz de Rodriguez *et al*., 1985; Doyle, 2000), were reported. The symptoms disappeared entirely after 8 months in the Italian children and researchers suspected that a consignment of meat or poultry might have contained high levels of some estrogenic compound, possibly α-zearalanol. In the case of the Puerto Rican children it was suspected that local children contained high levels of estrogenic compounds and symptoms gradually disappeared after children stopped consuming local chicken, beef and milk (Doyle, 2000).

Deleterious effects of α-zearalanol exposure have been reported in reproduction of both male and female mammals of various species (European Commission, 1999). In the adult male, a sustained increase in the plasma level of α-zearalanol administered by ingestion, injections or implants induces a decrease in testis, seminal vesicle and prostate weights and alterations or arrest of spermatogenesis. In the adult female, such treatments induce alteration or suppression of ovarian cycles and endometrial hyperplasia. In rodents, a decrease in ovulation rate and litter size has been reported (European Commission, 1999).

In a three generation study of rats receiving α-zearalanol at levels up to 0.20 ppm throughout gestation, it has been concluded that fertility of the offspring was not affected (European Commission, 1999).). However, male mice exposed *in utero* to α-zearalanol (150 µg/kg of body weight injected on days 9 and 10 of gestation) showed testicular abnormalities (regressive changes in the germinal epithelium and Sertoli cells, and immature morphology of Leydig

cells) when testes are examined at 45 days of postnatal life (Perez-Martinez *et al*., 1997). Leffers *et al*. (2001) established that α-zearalanol had potency similar to that of E_2 and DES. This includes stimulation of proliferation of breast tissue (Sheffield and Welsh, 1985; Leffers *et al*., 2001), induction of lesions in the testes (Perez-Martinez *et al*., 1996) and induction of hepatic neoplasia (Leffers *et al*., 2001). Silcox *et al*. (1986) found that α-zearalanol suppressed spermatogenesis and estosterone production in young bulls if the implant was inserted before 200 days of age (Silcox *et al*., 1986). Exposure of pregnant rats to 4mg α-zearalanol /kg body weight depressed maternal weight gain, prolonged gestation period and decreased number of live births in some animals (Rands *et al*., I & II, 1982). α-zearalanol administrated to mice during pregnancy also decreased fetal size and caused some abnormalities in testicular development (Pylkkanen *et al*., 1991; Perez-Martinez *et al*., 1997; Doyle, 2000). α-Zearalanol could pose a risk to humans and especially to pre-pubertal children where the endogenous E_2 concentrations are low (Leffers *et al*., 2001).

2.9.2.3 Diethylstilbestrol (DES)

DES exposure is considered an important model of endocrine disruption and provides proof-of-principle for exogenous estrogenic agents as disruptors of multiple end-organs (Pfaffl *et al*., 2002; Woodruff *et al*., 2010). DES is a synthesized stilbene and was first produced in London by Dodds and Associated in 1938 (Veurink *et al*., 2005; Henley and Korach, 2010). Its biological properties are similar to those of naturally occurring estrogens. In humans DES was used to treat symptoms associated with menopause, menstrual disorders, postpartum breast engorgement, primary ovarian failure and chemotherapy of advanced breast and advanced prostate cancer (Metzler, 1981; Marselos and Tomatis, 1992, 1993; Guisti *et al*., 1995; Pfaffl *et al*., 2002). DES was prescribed to as many as 2 million Americans during pregnancy, from the time period of 1947-1971, when the first adverse effects become evidant (Titus-Ernstoff *et al.,* 2008; Woodruff *et al*., 2010). Physicians continued to prescribe DES until 1971 in the US and until 1978 in Europe (Berendes, 1993; Guisti *et al*., 1995). No adverse effects were suspected until 1970 when vaginal clear-cell adenocarcinoma was reported in six young

women aged 14-21 years (Herbst and Scully, 1970; Guisti *et al*., 1995). In human males, DES is rapidly absorbed from the proximal gastrointestinal tract, and peak plasma concentrations are reached 20-40 min after an oral dose. DES is lipid-soluble and is readily distributed throughout the body compartments. It is primarily excreted in the urine and readily crosses the placenta and is conjugated and oxidatively metabolized in the fetus (Metzler, 1981; Guisti *et al*., 1995).

In the rat, days 17-19 of pregnancy are a critical period for estrogen exposure as it is critical for the proliferation of both Leydig cell precursor cells and Sertoli cells (Huhtaniemi and Pelliniemi, 1992; Pelliniemi *et al.,* 1993; Behrens *et al.,* 2000). Estrogen may influence the development of the male reproductive tract by altering the hormonal control of the fetus testis (Sharpe and Skakkebaek 1993). The Sertoli cells may play a central role in this distribution because estrogens are produced by Sertoli cells during their proliferation and the number of Sertoli cells in adult life is of importance for the number of germ cells (Behrens *et al.,* 2000).

DES was extensively utilized as VGS for cattle and sheep and as treatment for estrogen-deficiency disorders in veterinary medicine. It was licensed in the USA until 1979, when it was banned for use as a VGS. It has been replaced by E_2 and α-zearalanol implants which are registered in the USA and many other countries, including SA. The mode of action of DES seems quite similar to E_2, but there are several fundamental differences in estrogen receptor binding, oral bioactivity and elimination DES is slightly transformed in the organism and is highly active, but the elimination is slower than for E_2. It is rapidly excreted via urine, mainly as a glucurono-conjugated form, within the first 48 hours of application (Pfaffl *et al*., 2003).

An increased risk for testicular tumours was observed in studies of mice exposed to DES (Newbold *et al*., 1985, 1987; Giusti *et al*., 1995), and an increased incidence of cryptorchidism, a strong risk factor for testicular cancer, has been observed in DES sons (Gill *et al*., 1979; Giusti *et al*., 1995). In a study done by Klip *et al*. (2002), it was indicated that the sons of mothers

that were exposed to DES *in utero*, were more likely to suffer from hypospadias (Evans *et al*., 2011). Various other studies suggested that cryptorchidism, increased incidence of genital abnormalities, possibly increased risk of prostate and testicular cancer may well be due to DES exposure (Veurink *et al*., 2005; Woodruff *et al*., 2008).

2.9.2.4 Methyltestosterone (MT)

MT is an exogenous, synthetic, aromatizable androgen and potent AR agonist (Selzam *et al*., 2004; Grote *et al*., 2004). In a study done by Grote *et al*. (2004) on the effects of organotin compounds on pubertal male rats, they found that the body weight was significantly reduced in the MT group. Studies have indicated that exposure to estrogenic substances during puberty reduced body weight by affecting the central nervous system (CNS) region that regulates appetite and delayed pubertal maturation in the male rat (Grote *et al*., 2004). High doses of testosterone have been shown to induce a loss in body weight and it has been proposed that these inhibitory effects may be due to aromatization of testosterone to estradiol (Hervey and Hutchinson 1973; Gentry and Wade, 1976; Mooradian *et al*., 1987; Grote *et al*., 2004).

A study using fathead minnow found that 1µg/L 17α-MT induced a concentration-response induction of plasma vitellogenin, likely due to its conversion into 17α-methylestradiol (Pawlowski *et al*., 2004). MT produces both androgenic and estrogenic effects in juvenile and reproductively-active adult minnows (Ankley *et al*, 2001; Parrott and Wood, 2002; Zerulla e*t al*., 2002; Hornung e*t al*., 2004). The suite of effects observed in males included ovipositor development, vitellogenin production and decreased plasma testosterone and 11-ketotestosterone concentrations (Pawlowski *et al*., 2004). In the study done by Hornung *et al*. (2004) they suggested that the simultaneous production of estrogenic and androgenic effects following exposure of fish to MT could be due to CYP19, the cytochrome P450 enzyme that is responsible for the conversion of endogenous androgens to estrogens (Callard e*t al*., 1978; Simpson e*t al*., 1994). This might contribute to the estrogenic effects via the conversion of MT to 17α-methylestradiol, which occurred at 20 and 200 µg/L concentrations (Hornung *et al.*, 2004).

In tilapia culture the production of all-male populations through treatment of fry with 17α-MT-impregnated food has become the most popular procedure. Contreras-Sanchez *et al*. (2001) demonstrated that there was a significant "leakage" of MT into water and sediment in tilapia ponds/hatcheries probably from uneaten or unmetabolized food (McElwee *et al*., 2002). This leakage may pose an unintended risk of exposure to hatchery workers as well as fish or other non-target aquatic organisms to anabolic steroids if MT persists in the environment after treatment of tilapia fry. MT treatment studies done in laboratories showed that MT can be detected in water and eventually accumulates and remains in the sediment of model pond for up to eight weeks (Fitzpatrick *et al*., 1999; Contreras-Sanchez *et al*., 2001; McElwee *et al*., 2002).

A study done by Wason *et al*. (2003) indicated that the T_4 levels were slightly increased and the sperm count was lowered at 200 and 40mg/Kg in male rats. In terms of general toxicity there was a decrease in body weight in males at 200mg/kg. Selzam *et al*. (2005) used Japanese quail to investigate the effects of MT on spermatogenesis and found that there was a significant reduction in spermatid number at the 50 and 110ppm dose, possibly due to adverse impact on the HPG axis. Studies by Ankley *et al*. (2001) demonstrated that 17α-MT at > 100µg/L induced hypertrophy of the testes, and the appearance of degenerative or necrotic spermatogonia. Therefore 17α-MT shows both androgenic and estrogenic properties (Grote *et al*., 2004).

2.9.2.5 17β-Trenbolone

Trenbolone acetate is a highly potent anabolic androgenic steroid which is primarily used legally as a VGS agent in livestock production. It is used either alone or in combination with 17β-estradiol (European Commission, 1999; Wilson *et al*., 2002; Durhan *et al*., 2006).

17β-trenbolone-acetate is rapidly converted to the biological active steroid 17β-trenbolone. 17β-trenbolone is structurally similar to testosterone but does not undergo 5α reduction due to the presence of a 3-oxotriene structure, which prevents A ring reduction (Pottier *et al*., 1981; Doyle, 2000; Yarrow *et*

al., 2010). 17β-trenbolone undergoes biotransformation to less biologically active androgens (Benten e*t al*. 1999; Wilson e*t al*., 2002) similar to other anabolic androgenic steroids, such as 19-nortestosterone (Sundaram e*t al*., 1995; Yarrow e*t al*., 2010). Despite its inability to undergo 5α reduction 17β-trenbolone remains highly anabolic (Yarrow e*t al*., 2010). 17β-trenbolone administration has shown to reduce prostate mass in growing male rodents (Mantovani, 1992; Yarrow e*t al*., 2010).

It has also been reported that 17β-trenbolone is not a substrate for the aromatase enzyme and is relatively non-estrogenic (Le Guevel and Pakdel, 2001; Hemmer e*t al*., 2008). 17β-trenbolone reduces serum testosterone (Renaville e*t al*., 1988; Fabry e*t al*., 1983; Silcox e*t al*., 1986) and dihydrotestosterone (DHT) possibly through the pituitary or hypothalamic feedback inhibition (Yarrow e*t al*., 2010). Indirect evidence indicated that disruptions of the HPG axis was in livestock which experienced reduced testicular circumference and weight (O'Lamhna and Roche, 1983; Silcox e*t al*., 1986) and delayed puberty (Moran e*t al*., 1990), following administration of 17β-trenbolone. This may be due to a direct hypothalamic feedback inhibition, as evidenced by reduced GnRH transcription in brains of fish models (Zhang e*t al*., 2008) or perhaps through direct effects on testicular steroid biosynthesis, as demonstrated by down-regulated expression of testicular CYP 17 (Zhang e*t al*., 2008).

Literature indicates that 17β-trenbolone can interfere with the endocrine and reproductive system of fish. It inhibits hepatic estrogen receptor and vitellogenin transcripts and consequently impaired fecundity in fathead minnow (Ankley e*t al*., 2002, 2003) and Japanese medaka (Zhang e*t al*., 2008). It has been postulated that exposure to exogenous androgens such as 17β-trenbolone leads to the compensatory response of decreased endogenous androgen (testosterone and 11-ketotestosterone) production and, in turn, the decreased production of estradiol production since estradiol is converted from testosterone by CYP 19 aromatase in vertebrates (Zhang e*t al*., 2008).

Excreted 17β-trenbolone is comparatively stable in animal waste, suggesting the potential for exposure to aquatic animals via direct discharge, runoff or both (Schiffer e*t al*., 2001; Ankley e*t al*., 2003; Durhan e*t al*., 2006). An *in vitro* competitive binding study with fathead minnow androgen receptor demonstrated that 17β-trenbolone had a higher affinity for the receptor than that of the endogenous ligand, testosterone (Ankley e*t al*., 2003). Trenbolone acetate is administered to livestock via controlled release implants, upon entry to the blood; the acetate is rapidly hydrolysed to 17β-trenbolone, a potent androgen receptor agonist (Ankley e*t al*., 2003). It has been reported that the half-life for degradation of 17α- and 17β-trenbolone in liquid cattle manure were in the order of 260 days. Both metabolites bind with high affinity to androgen receptors in mammals and fish (Wilson e*t al*., 2002, 2004), and they are potent androgens in vivo (Durhan e*t al*., 2006).

Carcinogenicity studies of trenbolone in rodents indicated that 10 or 100ppm trenbolone in the diet was associated with an increase in liver cancer in mice and 50 ppm may have enhanced pancreatic cancer in rats (Doyle, 2000). Deleterious effects of trenbolone acetate exposure were reported in the reproduction of both male and female mammals of various species (European Commission, 1999). In the adult male, trenbolone acetate administered by ingestion, injection or implants induces a decrease in testis, seminal vesicle and prostate weights and alterations in spermatogenesis. In the adult female, virilization and alteration or suppression of ovarian cycles occurs. Some data in rodents indicate that administration of trenbolone acetate during the intrauterine or/and perinatal period alters the reproductive function in adults. In a multi-generation study, it has been shown that trenbolone acetate, administered to female rats at dietary concentrations of 3 and 18 ppm between 2 weeks before mating and 3 weeks after birth of young exerts effects on reproductive performance which are more marked in F2 pups than in F1 pups of a comparable age. The female F1 pups from F1-treated parents showed signs of virilization, a delay in the mean vaginal opening and the presence of occlusive strands in the vagina or incomplete vaginal opening. Male pups showed a delay in testicular descent and a decrease in seminal vesicles, prostate, testes and epididymis weights. In addition in the F2

generation, the adrenal weight is also decreased in both sexes (European Commission, 1999).

2.10 Studies of veterinary growth stimulant effects in other countries

Initial studies focused on estrogen discharge from sewage treatment works contaminating the aquatic environment (Jobling e*t al*., 2002a, b; Hotchkiss e*t al*., 2008). It has been suggested that estrogens excreted directly by livestock or applied in slurry could persist long enough in soils to provide a source of contamination for surface waters (Khan e*t al*., 2008).

Studies done by Soto *et al.* (2004) and Orlando *et al*. (2004), both suggested that estrogenic activity in surface waters near intensive livestock farms may be high enough in some places to cause endocrine disruption in some aquatic organisms. The study done by Matthiessen *et al*. (2006) suggested that estrogenic contamination of headwater streams associated with livestock farms are widespread in England and Wales.

In a study done by Irwin *et al*. (2001) estrogenic activity was detected in ponds below feedlots housing cattle. In the study done by Orlando *et al*. (2004) they examined whether endocrine activity could be detected in natural stream/ river systems below feedlots. This was determined by studying the reproductive endocrinology and secondary sex characteristics of the wild fish population. They discovered that fish collected below a feedlot exhibited altered reproductive biology, including decreased testosterone synthesis, altered head morphometrics and smaller testis size (Orlando e*t al*., 2004).

Chapter 3

3. Materials and Methods

3.1 Extraction procedure for water samples

Water samples were collected at three different feedlots at a particular point by the Food and Drug Assurance Laboratories (Pty) LTD. Water samples were collected induplicate, in 1L glass bottles. For the extraction and concentration of potential estrogen and androgen-like compounds a solid phase extraction (SPE) was performed. The water sample (1L) was first passed through a glass wool filter (Macherey-Nagel, Cat. No. 000904), then through a 0.45 micron, 47mm sterile filter (MicroSep, Osmonics Cat. No. E04WG047S1) to remove particles. The samples were subsequently extracted onto a pre-conditioned Chromabond C18ec SPE cartridge (Chromabond Macherey-Nagel, Cat. No. 730 014), at a flow rate of 10 ml/min (Routledge *et al*., 1998; Aneck-Hahn, 2003). In order to assess the extraction process for possible estrogenic and androgenic contamination from the SPE cartridge, control water (water from the Millipore Milli-Q synthesis ultrapure water system) was included with each extraction batch. The samples were then eluted from the cartridge with 3.5ml methanol (Cat. No. HPLC 34860) into sterile glass tubes (Pierce amber reacti-vials™, 5ml). The extract was evaporated under a gentle stream of nitrogen using a Pierce Reacti-Vap and a Reacti-Therm heating and stirring module. The sample residue was reconstituted with 1 ml ethanol (Cat. No. HPLC 34870) and placed in sterile amber bottles 4 ml (Chromatography research supplies, Cat. No. 154515), and stored at -20°C prior to analysis (Aneck-Hahn, 2003; Aneck-Hahn *et al.*, 2008, 2009). These extracts are suitable for the assessment of estrogenic and androgenic activity in the selected bioassays.

3.2 Phase 1: Bio-assays

The bio-assays were conducted at the Department of Urology, at the University of Pretoria to detect EDC activity (estrogenic or androgenic). The following three assays were conducted:

1) Recombinant yeast screen assay for estrogenic activity
2) T47D-KBluc reporter gene assay for estrogenic and anti-estrogenic activity

3) MDA-kb2 reporter gene assay for androgenic and anti-androgenic activity

3.2.1 Recombinant yeast screen assay (YES) for estrogenic activity

Yeast does not contain endogenous steroid hormones as in mammals. Mammalian steroid receptors introduced into yeast function as they do in mammalian cells as steroid-dependent transcriptional activators. When a steroid responsive receptor gene is introduced into yeast along with the steroid receptor, chemical interaction with that receptor can be determined by measuring the receptor gene product.

A recombinant yeast strain using *Saccharomyces cerevisiea* was developed in the Genetics Department at Glaxo to identify compounds that can interact with the human estrogen receptor (hER). This yeast was transfected with the human ERα gene, and a plasmid containing an estrogen response element (ERE)-linked *lac-Z* gene encoding the enzyme *β*-galactosidase. Active ligands bind to the receptor and induce *β*-galactosidase expression in a dose dependent manner (which is used to measure the receptors' activity) (Routledge and Sumpter, 1996). *β*-galactosidase metabolizes the chromogenic substrate, chlorophenol red-b-D-galactopyranoside (CPRG), which is normally yellow, into a red product that can be measured by absorbance at 540 nm.

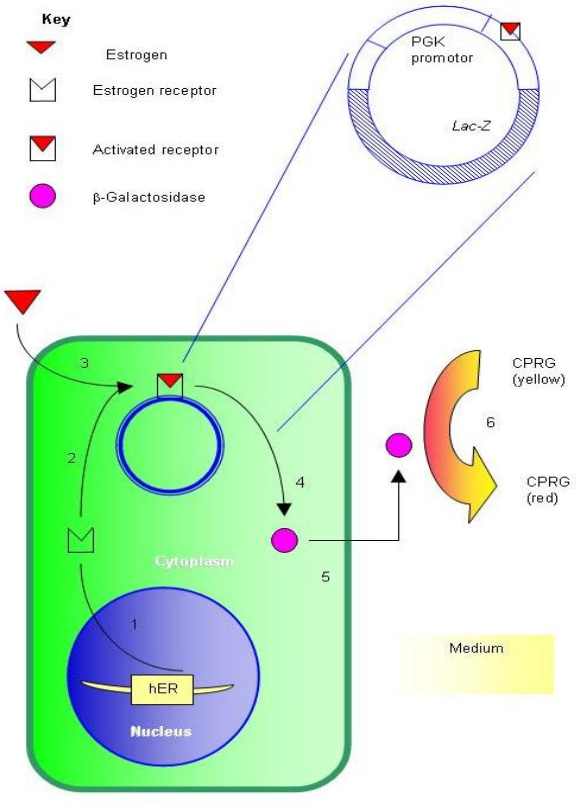

Figure 3.1: Schematic of the estrogen-inducible expression system in yeast.

The human estrogen receptor gene is integrated into the main genome and is expressed (1) in a form capable of binding to estrogen response elements (ERE) within a hybrid promoter on the expression plasmid (2). Activation of the receptor (3), by binding of ligand, causes expression of the reporter gene *Lac-Z* (4) which produces the enzyme β-galactosidase. This enzyme is secreted into the medium (5) and metabolizes the chromogenic substrate CPRG (normally yellow) into a red product (6), which can be measured by absorbance. (Routledge and Sumpter (1996) with minor adjustments by Aneck-Hahn (2003).

The YES was performed according to the method described by Routledge and Sumpter (1996) with minor adjustments by Aneck-Hahn (2003).The positive control was E_2 and the negative control was the solvent ethanol (Aneck-Hahn, 2003; Bornman *et al*., 2007; Aneck-Hahn *et al*., 2005, 2008, 2009).

3.2.2 The T47D-KBluc reporter gene assay for estrogenic and anti-estrogenic activity

The US EPA developed an estrogen-dependent stable cell line. The T47D human breast cancer cells, which contain both endogenous ER-α and -β, were transfected with an ERE luciferase reporter gene construct. This provides an *in vitro* system that can be used to evaluate the ability of chemicals to modulate the activity of estrogen-dependent gene transcription. The cell line has the potential to be used both for screening chemicals and as an aid in defining the mechanism of action of chemicals with estrogenic and anti-estrogenic activity. This is valuable for a first-pass type *in vitro* assay, as a ligand for either receptor could drive the luciferase reporter gene, thereby eliminating the need for a separate assay for ERα and ERβ (Bornman *et al*., 2007; Wilson *et al*., 2004).

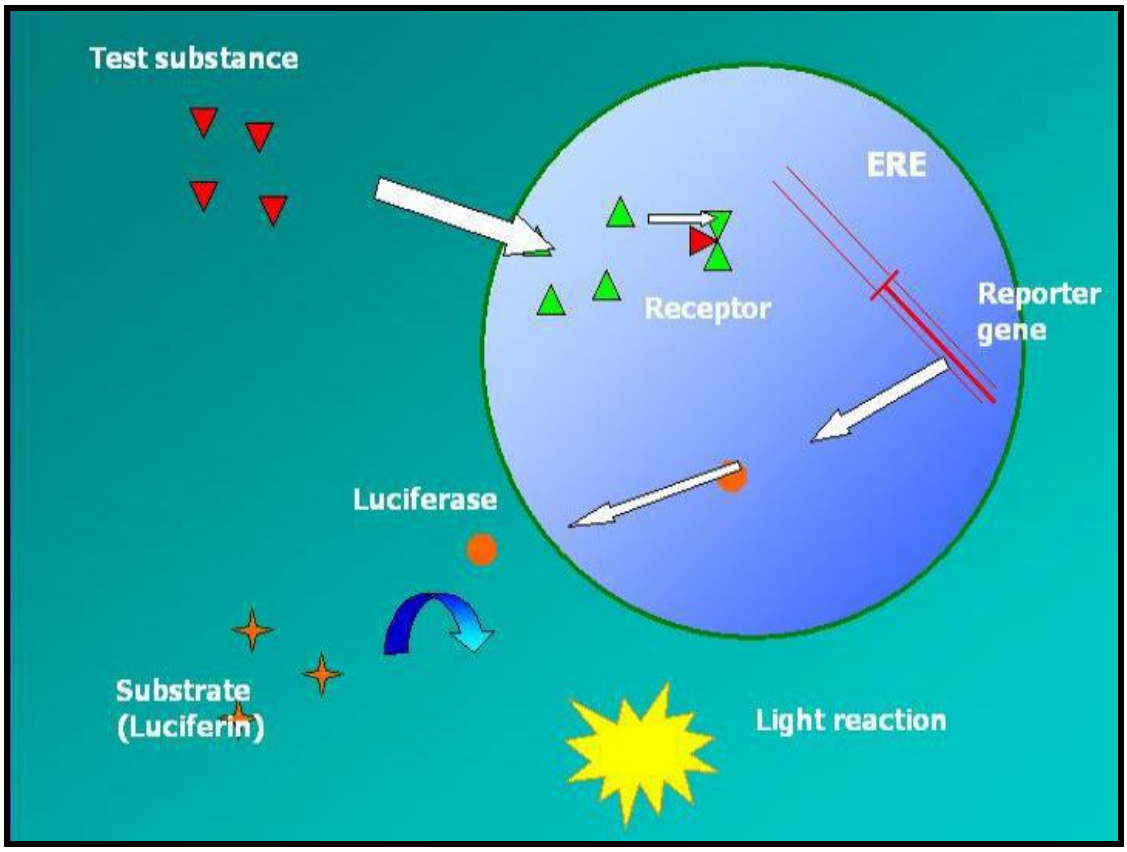

Figure 3.2: Schematic representation of the estrogen-inducible reporter-gene system in the T47D-KBluc cell line (Aneck-Hahn *et al*., 2006).

In principle, compounds enter the cell; estrogen receptor ligands bind to the ER; two ligand-bound receptors dimerize and bind coactivators; then the dimer binds to the ERE on the reporter gene construct and activates the luciferase reporter gene. The presence of the luciferase enzyme can then be assayed by measuring the light produced when the enzyme substrate, luciferin, and appropriate cofactors are added. The amount of light produced is relative to the degree of estrogenic activity of the test chemical. When testing chemicals using the T47D-KBluc cells, an estrogen is defined as a chemical that induced dose dependent luciferase activity, which could be specifically inhibited by the anti-estrogen ICI 182 780. Agonists stimulate luciferase expression and are compared to the vehicle control (media plus ethanol) or to the E_2 control. Anti-estrogens block the E_2-induced luciferase expression, which is compared to the E_2 control (Bornman *et al*., 2007; Wilson *et al*., 2004). The agonist is E_2, the antagonist is ICI 182 780 and a solvent as the negative control (Bornman *et al*., 2007; Aneck-Hahn *et al*., 2005).

3.2.3 MDA-kb2 receptor gene assay for androgenic and anti-androgenic activity

The MDA-kb2 assay was developed to screen androgen agonists and antagonists and to characterize its specificity and sensitivity to EDC's. The MDA-MB-453 breast cancer cell is stably transformed with the mouse mammary tumour virus (MMTV) luciferase.neo reporter gene construct. Both the glucocorticoid receptor (GR) and AR are present in the cells and both receptors act through MMTV promoter, compounds that act through either AR or GR receptors activate the MMTV luciferase reporter. The presence of the luciferase enzyme can then be assayed by measuring the light produced when the enzyme substrate, luciferin and appropriate co-factors are added. The amount of light produced is relative to the degree of androgenic activity of the test chemical flutamide was used as an antagonist, inhibiting AR but not GR mediated responses whilst DHT was used as an agonist (positive control) and the solvent ethanol was used as a negative control (Bornman *et al*., 2007; Wilson *et al*., 2002; Wilson and Lambright *et al*., 2002).

3.3 Phase 2: Animal studies: reproductive and thyroid toxicology.

3.3.1Test system

Project H031-07 was approved by the Ethics Committee of the University of Pretoria. The study was conducted according to the guidelines of the "National Code of Animal use in Research, Education and Testing of Drugs and Related Substances in South Africa".

The OECD 415 protocol, which outlines the guidelines for testing of chemicals for a one-generation reproduction toxicity study, was modified to accommodate one control and three experimental groups (OECD, 1983).

Twenty, seven day pregnant, female (P1) Sprague-Dawley rats were obtained from the University of KwaZulu-Natal – Biomedical Resource Unit. The rats were randomly divided into a control group (Group 1) and three experimental groups (Groups 2, 3 and 4) each containing five rats, respectively. All animals were housed in standard polycarbonated cages, in rooms with constant temperatures (22°C ± 2ºC), constant humidity (55 ± 10%) and 12 hour day/night cycles. Rats were maintained on a stock pellet diet (Epol®: Epol mice cubes, lot nr. 30101, Pretoria, SA) and had free access to food and tap water.

3.3.2 Experimental design

The study design allowed for the endocrine disrupting effects of maternal exposure (P1) to selected growth stimulants as possible EDCs on the fertility and reproductive parameters in the lifetime exposure F1 males (offspring of P1 females) to be investigated (Figure 3.4).

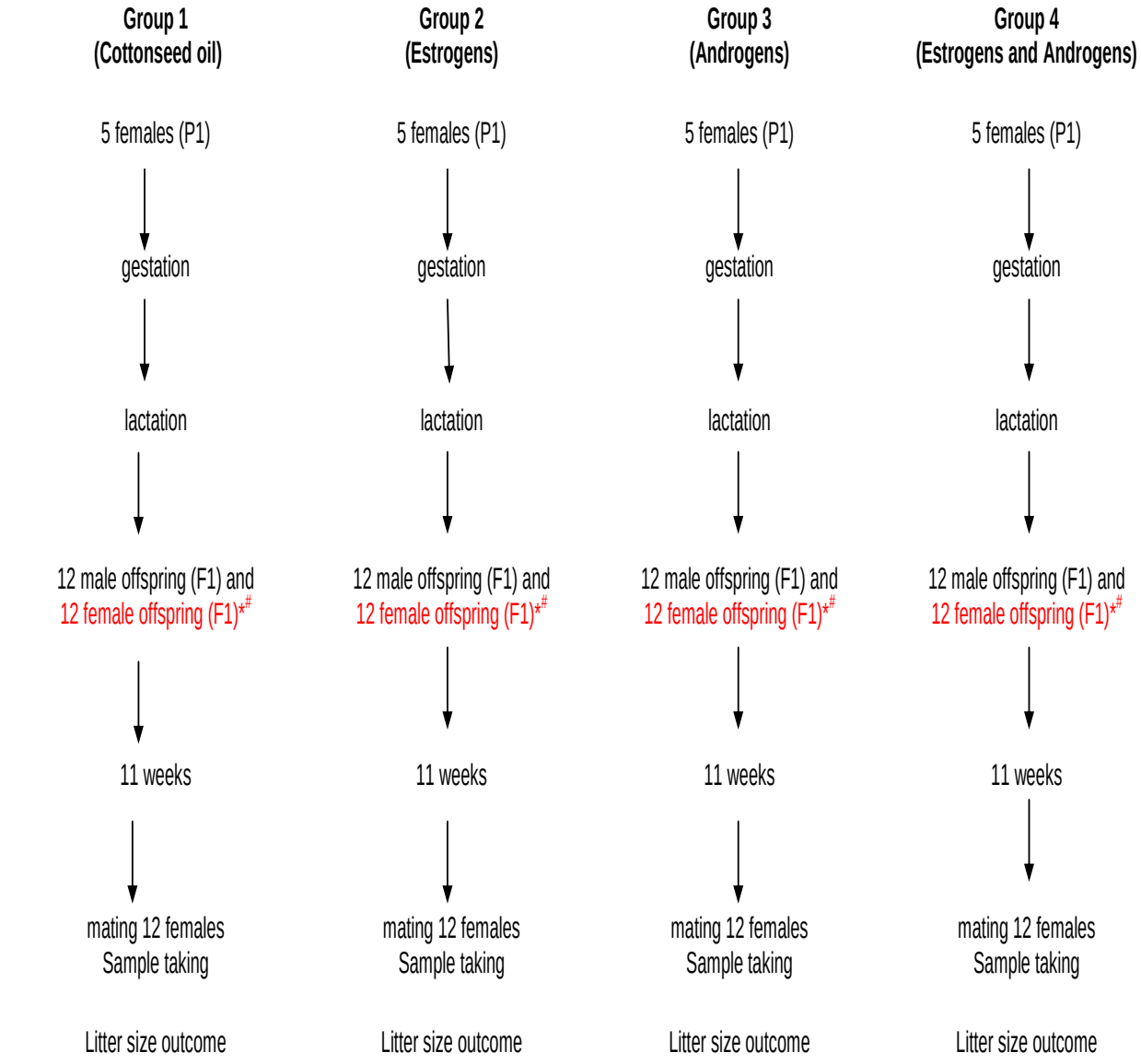

Figure 3.4: Schematic representation of the experimental design to assess the possible EDC effects of veterinary growth stimulants on the reproductive outcome of F1 males

3.3.2.1 Maternal exposure (P1 females)

Seven day pregnant P1 females were orally gavaged, on a daily basis for a three week period and during lactation till weaning of the F1 male pups (a subsequent three week period). The total duration of exposure of the P1 females to the test chemicals was 6 weeks.

3.3.2.2 Direct exposure (F1 males)

After weaning twelve male offspring (F1) from each experimental and control group were subsequently dosed till 11 weeks of age. The individual animals were kept in separate cages and were directly exposed daily to the substances by oral gavage for the 11-week period at which time the F1 males were terminated for sample collection. All the males in the F1 generation were mated with the females (F1) of the same age (from the same groups) before termination in order to determine litter size outcome.

3.4 Oral dosing

The following compounds used for this study were based on the compounds identified by the The Onderstepoort Veterinary Institute and the subsequent biological activity as indicated by the bio-assays; zilpaterol, DES, α-zearalanol, β-trenbolone, and MT. The dosing concentrations were selected according the detection limit of the compounds identified by the FDA with an additional 20% added, in order to make the dosage environmentally relevant. The (P1) females were divided into 4 groups of 5 animals each. The control group (Group 1) was dosed with the vehicle only (cottonseed oil: Cat no 053K0077 Sigma-Aldrich, Steinheim, Germany). The following three groups were dosed with different mixtures of growth stimulants (as identified by the FDA) till weaning (Table 3.1). Group 2 was dosed with a β-agonist, zilpaterol (supplied to FDA laboratories by the feedlot) and two known estrogens, DES (Sigma, 99%) and α-zearalanol (Sigma, 97%). Group 3 was dosed with two known androgens β-trenbolone (Dr Ehrenstorfer GmbH, 95%) and MT (Dr Ehrenstorfer GmbH), and Group 4 was dosed with a combination of growth stimulants used in Groups 2 and 3 [zilpaterol, DES, α-zearalanol, β-trenbolone, MT, (Table 3.1)]. All chemical mixtures were administered intra-

gastrically and the dose rate for all groups, including the control group, was 1 ml/kg adjusted according to body mass of each animal.

Table 3.1: The selected veterinary compound mixtures allocated to the three experimental groups at environmentally relevant concentrations

Group 1 (Control)	Group 2 (Estrogens)	Group 3 (Androgens)	Group 4 (Estrogens and Androgens)
Cottonseed oil only	0.12 µg/kg zilpaterol 0.24 µg/kg diethylstilbestrol (DES) 2.4 µg/kg α-zearalanol	12 µg/kg β-trenbolone 6 µg/kg methyltestosterone	0.12 µg/kg zilpaterol 0.24 µg/kg DES 2.4 µg/kg α-zearalanol 12 µg/kg β-trenbolone 6 µg/kg methyltestosterone

3.5 Observations

Throughout the experiment, all the animals (P1 and F1) were observed daily for clinical health, behavioral changes, signs of toxicity and mortality. They were weighed and dosed at the same time every day to exclude any external variables.

3.6 Sample and data collection

Samples were taken at each specimen collection point for histological analysis, as histology plays a pivotal role in the assessment of chemical exposure

3.6.1 F1 males

With the termination of the F1 males the following information and biological samples were collected:

- blood
- body weight
- anogenital distance
- total testis mass
- total epididymal mass
- total seminal vesicular mass
- prostate mass
- liver mass
- epididymal sperm count

F1 males were weighed before termination and blood was collected from the heart. The left testis was placed in Bouin's solution for histological evaluation, with special reference to spermatogenesis (De Jager *et al*., 1999).

3.6.2 Females (F1)

The following was noted:

- number of offspring

3.6.3 F2 pups

The following was noted:

- presence of congenital defects.

- male/female ratio per litter.
- number of stillbirths (if present).
- weight at weaning.

3.7. Procedures

3.7.1 Fixative

Bouin's fixative was prepared by mixing 375ml saturated Picric acid (Cat no. 295544M) with 125 ml Formaldehyde (Merck (PTY) LTD, Cat nr. SAAR2436020LP) and 25 ml of Acetic acid (Merck (PTY) LTD, Cat no. AB00063.2.5.)

3.7.2 F1 males

The weights of the F1 males were recorded before they were anaesthetised with Isofor provided by University of Pretoria Biomedical Research Centre (UPBRC). Blood was collected by cardiac puncture spun down and the serum used for T_3 and T_4 estimation.

3.7.3 Anogenital distance

The AGD was assessed by measuring the length of the perineum from the base of the genital tubercle to the center of the anus when the skin was naturally extended, without stretching using a ruler and recorded in millimetres (Kilian *et al*., 2007)

3.7.4 Testes and epididymis

The left and right testes and epididymides were removed, separated, cleaned and weighed individually. The left testis was placed in Bouin's solution and was used for histology, with special reference to spermatogenesis (de Jager I & II *et al*., 1999).

3.7.5 Cauda epididymal sperm count

The left cauda epididymis was separated from the caput-corpus and placed in 2 mℓ of phosphate buffered saline (PBS) medium (Cat no. P4417, Sigma) in a Petri dish. The cauda epididymis was cut open to free the sperm. The PBS with sperm was transferred to a Falcon tube. The Neubauer method (WHO,

1999) was used to determine the sperm concentration, expressed as million/ml (WHO, 1999).

3.7.6 Thyroid function

Blood was collected by cardiac puncture and was allowed to clot. The clotted blood was then spun down at 3000 rpm for 15 minutes to collect serum for TSH, T_3 and T_4 estimations. Tests were conducted at the Faculty of Veterinary Science, Department of Production Animal studies, Reproduction Section. The following kits were used: Coat-A-Count Canine TSH IRMA (PIIK9T-5, 2006-12-29; Cat no IK 9T1), Coat-A-Count Total T_3 (PITKT3-5, 2006-12-29; Cat no TKT31) and Coat-A-Count Canine T_4 (PITKC4-5, 2006-12-29, Cat no TKC41). Coat-A-Count Canine TSH IRMA is an immunoradiometric assay designed to quantitatively measure canine stimulating hormone (canine thyrotropin, cTSH) in serum. Coat-A-Count Total T_3 is a solid-phase radioimmunoassay designed for the qualitative measurement of total circulating triiodothyronine (T_3) in serum or plasma. Coat-A-Count Canine T_4 is a solid-phase radioimmunoassay designed for the qualitative measurement of total thyroxine (T_4) in canine serum.

3.7.7 F2 pups

All the male and female pups were weighed at birth and again at weaning, but only weight at weaning were used. There was no further sample collection for this group. The litter size and sex ratio were also determined.

3.8. Analyses

3.8.1 Histology (stages)

Testes were fixed for three days in Bouin's fixative, after which the testicular samples were then washed with running tap water, 30% ethanol, 50% ethanol and then 70% ethanol to remove the fixative (Carson, 1992; Van Dyk, 2008). Testes were embedded in paraffin wax and the testicular tissue was dehydrated in a graded series of ethanol. Thin sections, 3 µm, were cut on a microtome and stained with a modified Periodic Acid-Schiff's reaction (PAS) and counterstained with hematoxylin. The histological preparations were made at the Department of Anatomical Pathology at Onderstepoort, Pretoria.

Staging of spermatogenesis was done using an Olympus BX 41 microscope and Altra 20 Olympus camera with 10x, 20x and 100x objectives. Spermatogenesis was assessed using the computer package, STAGES 2.1 (Vangaurd Media Inc., Illinois, UDA), together with Russell's histological atlas (Russell *et al*., 1990). In each of the 47 F1 male rats, thirty randomly selected seminiferous tubules were evaluated to identify and classify the 14 stages of spermatogenesis. The tubular diameter, seminiferous epithelium and lumen diameter for all thirty tubules were measured horizontally and vertically. The mean values of the horizontal and vertical measurements for each parameter were used for the statistical analyses of the mentioned variables.

3.8.2 Statistical analyses

For between group comparisons of all variables one-way ANOVA (Analysis of Variance) was performed using ranks followed by pair-wise comparisons with Fisher's LSD (least significant difference method). Pair-wise comparisons between the control group and the treatment groups were performed at the Bonferroni adjusted level of significance (0.05/4 = 0.012) with the Wilcoxon Rank Sum test.

For male F1 data the Kruskal-Wallis All-Pairwise Comparisons Test was also performed but appears to be more stringent for between-group comparisons. Therefore the ANOVA results on ranked data should be reported, followed by the Wilcoxon Rank Sum test for pair-wise comparisons with control group. Only significant findings and findings >0.012 and <0.05 are reported.

Chapter 4

4. Results

4.1 Phase1: Bio-assays

4.1.1 YES assay

The results of the feedlot water samples using the YES assay are tabulated in Table 4.1.

Table 4.1: Estrogenic activity in water samples from selected feedlots using the YES assay

Sample	Estrogenic activity		Cytotoxicity
	Result	EEq (ng/l)	
1	<dl		Detected in 0.25x concentrated sample and higher concentrations (up to 1000x)
3	<dl		Detected in 6x concentrated sample and higher concentrations (up to 1000x)
4	Positive	0.38 ± 0.15	Detected in 25x concentrated sample and higher concentrations (up to 1000x)
8	n/q		Detected in highest concentrated sample (50x)
13	<dl		Detected in highest concentrated sample (50x)
14	n/q		Detected in 3x concentrated sample and higher concentrations (up to 1000x)
20	n/q		Detected in 1.5x concentrated sample and higher concentrations (up to 1000x)
39	<dl		-
41	n/q		-
45	<dl		-
46	<dl		-

<dl: Below detection limit of the assay plate

n/q: EEq not quantifiable, for less than 3 point above the dl were obtained

- : No cytotoxicity observed

An EEq (estradiol equivalents) value could only be calculated for sample 4 (Water management dam from feedlot: 0.38 ± 0.15 ng/l). Samples 8 (Water from feeding cradles), sample 14 (borehole water - used in feedlot), sample 20 (water downstream from feedlot) and sample 41 (borehole - downstream) had points above the detection limit of the assay, but not enough points to be able to calculate an EEq value. Cytotoxicity was observed in most of the samples and could be responsible for masking of estrogenic activity if such activity was present in the samples.

4.1.2 The T47D-KBluc reporter gene assay

The results of the feedlot water samples using the T47D-KBluc assay are tabulated in Table 4.2

Table 4.2: Estrogenic and anti-estrogenic activity in water samples from selected feedlots using the T47D-KBluc reporter gene assay

Sample	Estrogenic activity		Anti-estrogenic activity/Cytotoxicity
	Result	EEq (ng/l)	
1	<dl		Detected in 0.1x concentrated sample and higher concentrations (up to 1000x)
3	Positive	2.57 ± 0.39	-
4	Positive	0.32 ± 0.04	Detected in unconcentrated sample and higher concentrations (up to 1000x)
8	Positive	0.02 ± 0.004	-
13	Positive	0.13 ± 0.03	-
14	Positive	0.14 ± 0.02	-
20	Positive	0.94 ± 0.67	Detected in 3x concentrated sample and higher concentrations (up to 1000x)
39	<dl		-
41	Positive	0.47 ± 0.01	-
45	Positive	0.25 ± 0.14	-
46	Positive	0.04 ± 0.007	-

<dl: Below detection limit of the assay plate

- : No cytotoxicity observed

Nine of the 11 feedlot water samples tested positive for estrogenic activity. The EEq values ranged from 0.02ng/l to 2.57ng/l. Sample 3 was the only sample to (Run-off water from feedlot gathered in settling dam), exceeded the Predicted-No-Effect-Concentration (1 ng/l) for E_2 in water. When different sample concentrations of sample 1 (Ground water - upstream from feedlot), sample 4 (Water management dam from feedlot - inloop) and sample 20 (Water downstream from feedlot) were co-incubated with 100 pM (27 ng/l) E_2 (positive control), the E_2 activity was reduced at higher concentrations of the sample extracts, indicating possible anti-estrogenic activity or cytotoxicity of the sample. In order to distinguish between an anti-estrogenic or cytotoxic reaction in the T47D-KBluc assay, a test for cytotoxicity [e.g. Methythioazol Tetrazolium assay (MTT)] would be required, which was not in the scope of this study. Sample 4 and sample 20 tested positive for estrogenic activity, but also reduced 17β-estradiol activity at higher concentrations. The EEq values for these two samples are therefore probably underestimated due to the concurrent presence of a cytotoxic or anti-estrogenic substance in the sample mixture.

4.1.3 The MDA-Kb2 reporter gene assay

No androgenic activity was observed in any of the feedlot water samples, as none of the samples produced a two-fold induction in comparison to the vehicle control. When different sample concentrations of sample 1 and sample 41 were co-incubated with 1 nM (290 ng/l) DHT (positive control), the DHT activity was reduced at higher concentrations of the sample extracts, indicating possible anti-androgenic activity or cytotoxicity of the sample. In order to distinguish between an anti-androgenic or cytotoxic reaction, a test for cytotoxicity (e.g. MTT test) would be required.

4.2 Phase2: Animal studies

4.2.1 P1 females - maternal weight

The maternal weight gain of the P1 females is summarized in Table 4.3. The weight gained in the experimental groups compared to the Control group was not statistically significant (Figure 4.1).

Table 4.3: Mean maternal weight gain (g) between groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean maternal weight gain (g)	SD	p-value
1 2	5 5	87.200 82.800	16.162 23.048	1.0000
1 3	5 5	87.200 106.00	16.162 10.801	0.2963
1 4	5 5	87.200 78.750	16.162 22.530	0.2963

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

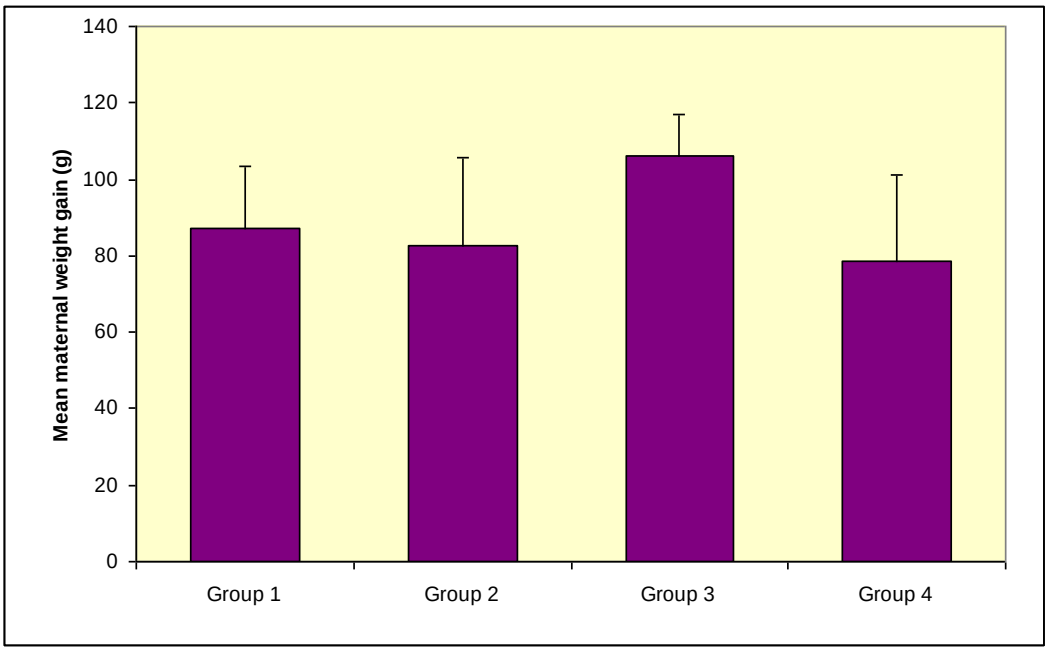

Figure 4.1: Mean and SD of the maternal weight gain by the P1 females in the control and experimental groups

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

4.2.2 Litter size

The mean litter size for the different veterinary growth stimulants groups are summarized in Table 4.4. There were no statistically significant differences indicated between the Control group and the experimental Groups 2 - 4 using the Wilcoxon Rank Sum test (Figure 4.2).

Table 4.4: Mean litter size between the control group and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean litter size	SD	p-value
1 2	5 5	11 10	2.5100 2.7019	0.1736
1 3	5 5	11 11	2.5100 3.1623	0.3705
1 4	5 5	11 8	2.5100 5.9414	0.2516

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

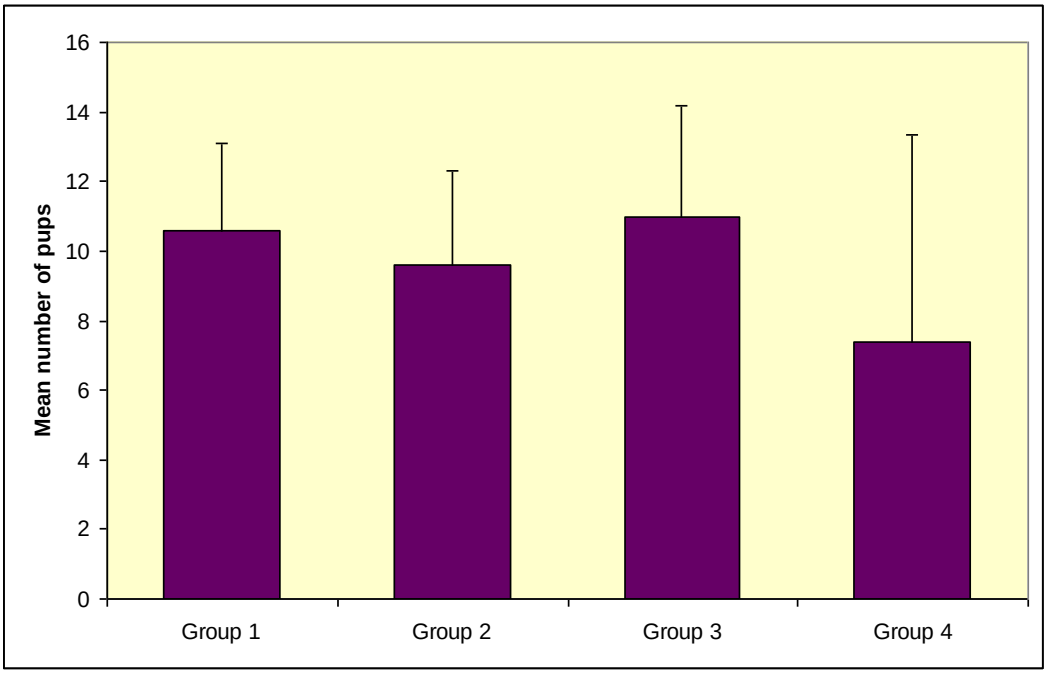

Figure 4.2: Mean and SD of the litter size outcome of the P1 females in the control and experimental groups

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

4.3 F1 males

4.3.1 Mean body weight

The mean body masses of the F1 males at 11 weeks of age are summarized in Table 4.5. There were no statistically significant differences seen between the Control group and the experimental groups (Figure 4.3).

Table 4.5: The mean body mass (g) between the control group and the experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean body weight (g)	SD	p-value
1	11	374.76	42.672	0.5588
2	12	374.00	15.731	
1	11	374.76	42.672	0.1481
3	12	360.34	20.815	
1	11	374.76	42.672	0.1316
4	12	358.04	26.576	

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

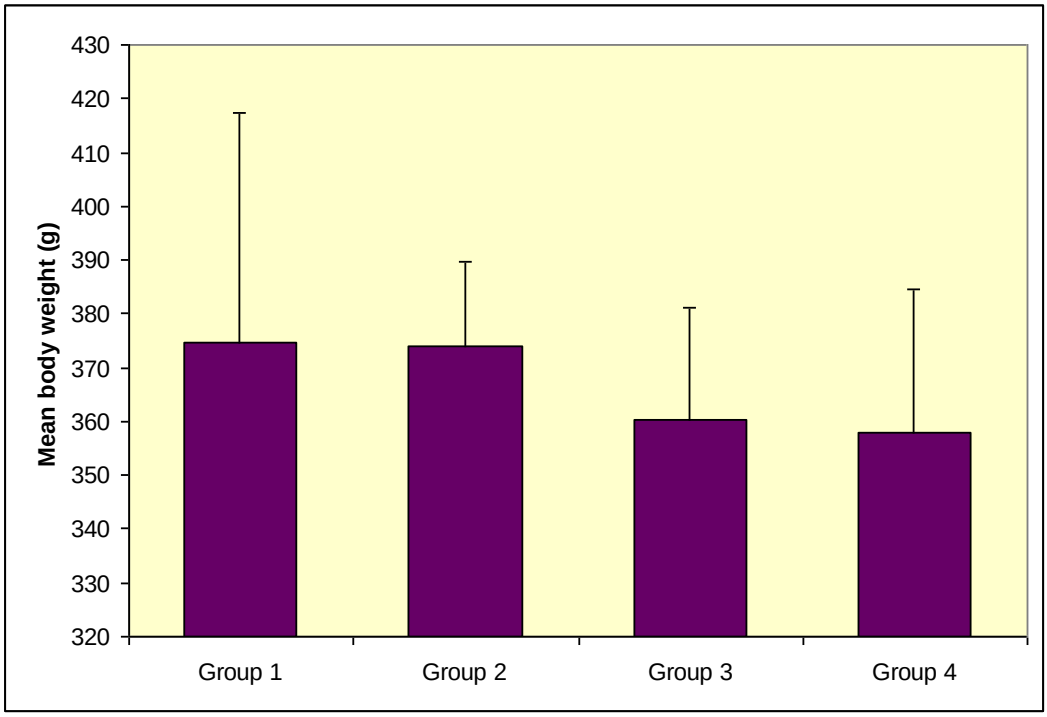

Figure 4.3: Mean and SD of the body weight of the F1 males in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

4.3.2 Mean anogenital distance

The mean anogenital distance is summarized in Table 4.6. The anogenital distance of experimental Group 3 is shorter compared to the Control group. The mean anogenital distance was statistically significantly shorter in Group 3 ($p = 0.0117$), compared to the Control group (Figure 4.4). Although not statistically significant there is also a trend in Group 2 ($p = 0.0670$) to be shorter than the Control group.

Table 4.6: Mean anogenital distance (mm) between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean anogenital distance (mm)	SD	p-value
1 2	10 12	40.900 38.833	2.0248 3.1286	0.0670
1 3	10 12	40.900 38.167	2.0248 2.1249	**0.0117***
1 4	10 12	40.900 39.417	2.0248 1.5050	0.0865

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

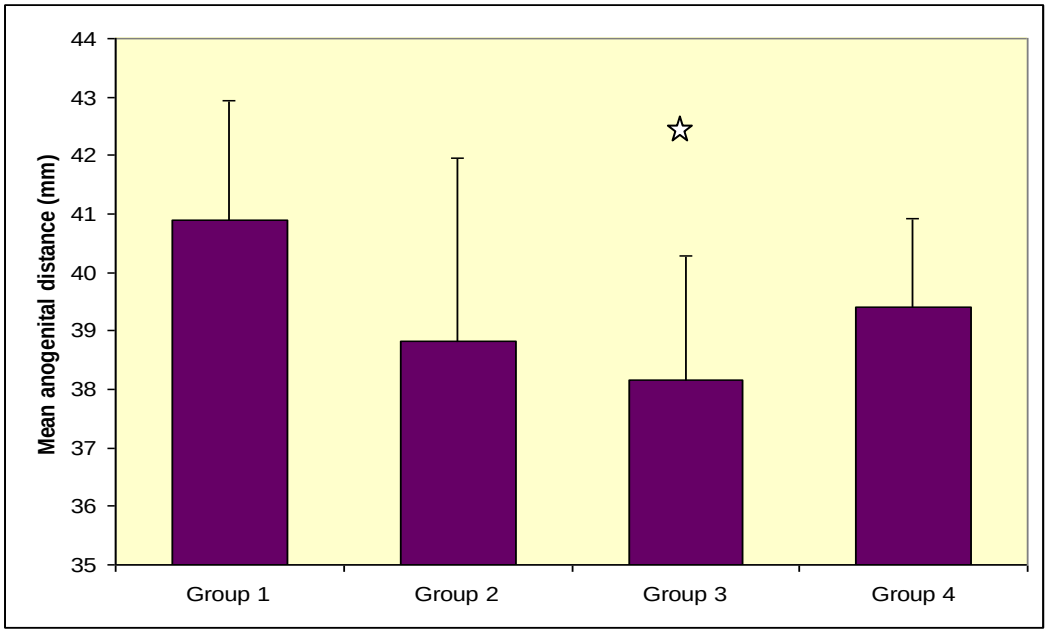

Figure 4.4: Mean and SD of the anogenital distance of the F1 males in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

* significant: $p < 0.05$ when compared to Group 1

4.3.3 Mean prostate mass

The mean prostate mass is summarized in Table 4.7. The Wilcoxon Rank Sum Test indicated a statistically significant difference between Group 4 ($p = 0.0151$) and the Control group (Figure 4.5). There were no statistically significant differences found between the Control group and Group 2 ($p = 0.5181$) and the Control group and Group 3 ($p = 0.2549$).

Table 4.7: Mean prostate mass between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean prostate mass (g)	SD	p-value
1	11	0.9277	0.1520	0.5181
2	12	0.8778	0.1272	
1	11	0.9277	0.1520	0.2549
3	12	0.8600	0.2004	
1	11	0.9277	0.1520	**0.0151***
4	12	0.7789	0.1269	

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

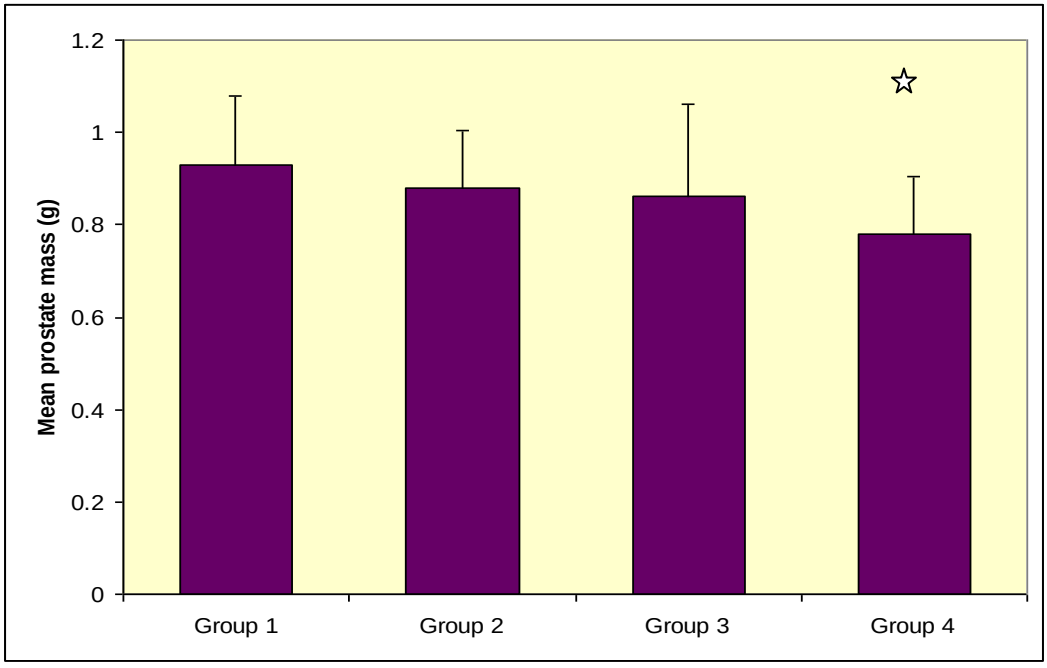

Figure 4.5: Mean and SD of the prostate mass of the F1 males in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

* significant: $p < 0.05$ when compared to Group 1

4.3.4 Mean seminal vesicle mass

The mean seminal vesicle mass for the different veterinary growth stimulants is summarized in Table 4.8. The Wilcoxon Rank Sum Test indicated that the mean seminal vesicle mass of Group 4 ($p = 0.0074$) is statistically significantly lower than the Control group (Figure 4.6). There were no statistically significant differences between the Control group and Group 2 ($p = 0.2815$) and Control group and Group 3 ($p = 0.2301$).

Table 4.8: Mean seminal vesicle mass between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean seminal vesicle mass (g)	SD	p-value
1 2	11 12	0.7488 0.7916	0.0936 0.1267	0.2815
1 3	11 12	0.7488 0.6862	0.0936 0.1178	0.2301
1 4	11 12	0.7488 0.6271	0.0936 0.0741	**0.0074***

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

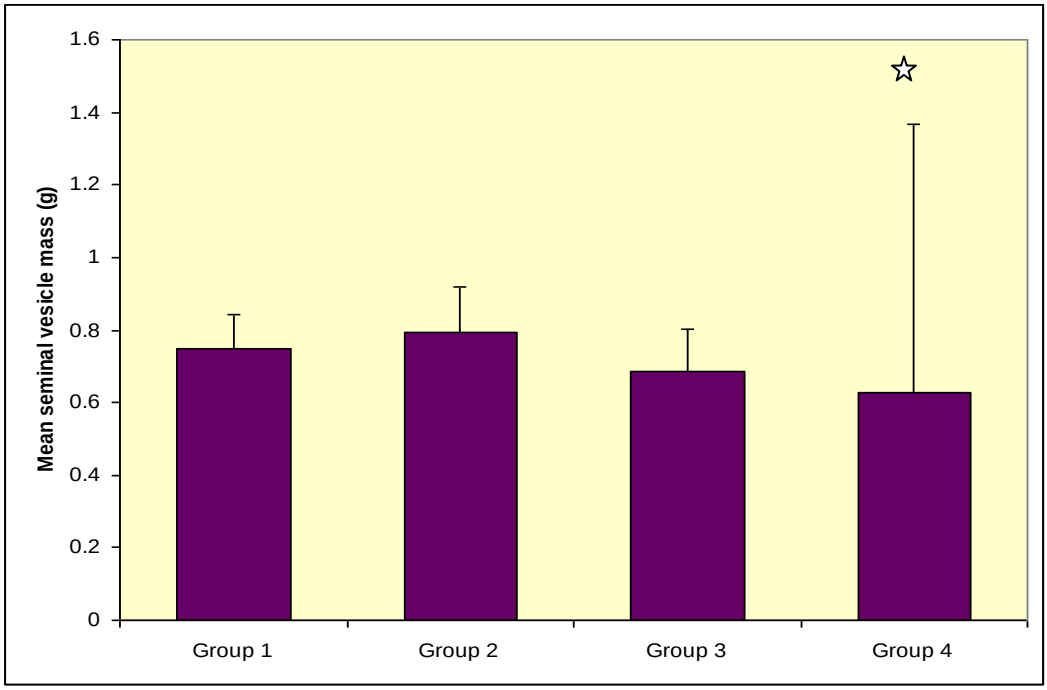

Figure 4.6: Mean and SD of the seminal vesicle mass of the F1 males in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

* significant: $p < 0.05$ when compared to Group 1

4.3.5 Mean epididymal mass

The mean epididymal mass for the different veterinary growth stimulants is summarized in Table 4.9. There were no statistically significant differences between the Control group and the experimental groups (Figure 4.7).

Table 4.9: Mean epididymal mass between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean epididymal mass (g)	SD	p-value
1 2	11 12	0.5616 0.5687	0.0417 0.0476	0.4060
1 3	11 12	0.5616 0.5835	0.0417 0.0369	0.3401
1 4	11 12	0.5616 0.5964	0.0417 0.0589	0.2301

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

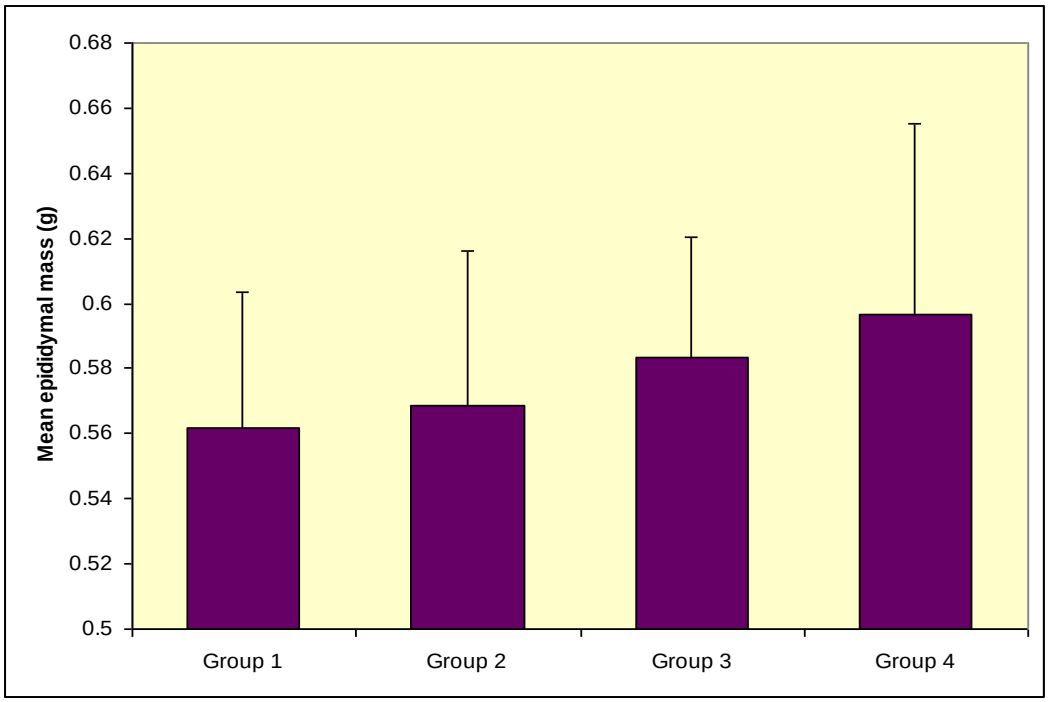

Figure 4.7: Mean and SD of the epididymal mass of the F1 males in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

4.3.6 Mean testicular mass

The mean testicular mass masses are summarized in Table 4.10. There were no statistically significant differences between the Control group and the experimental groups (Figure 4.8).

Table 4.10: Mean testicular mass (g) between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean testicular mass (g)	SD	p-value
1 2	11 12	1.7807 1.8253	0.1790 0.1865	0.6891
1 3	11 12	1.7807 1.7904	0.1790 0.1675	0.6009
1 4	11 12	1.7807 1.8623	0.1790 0.1033	0.2815

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

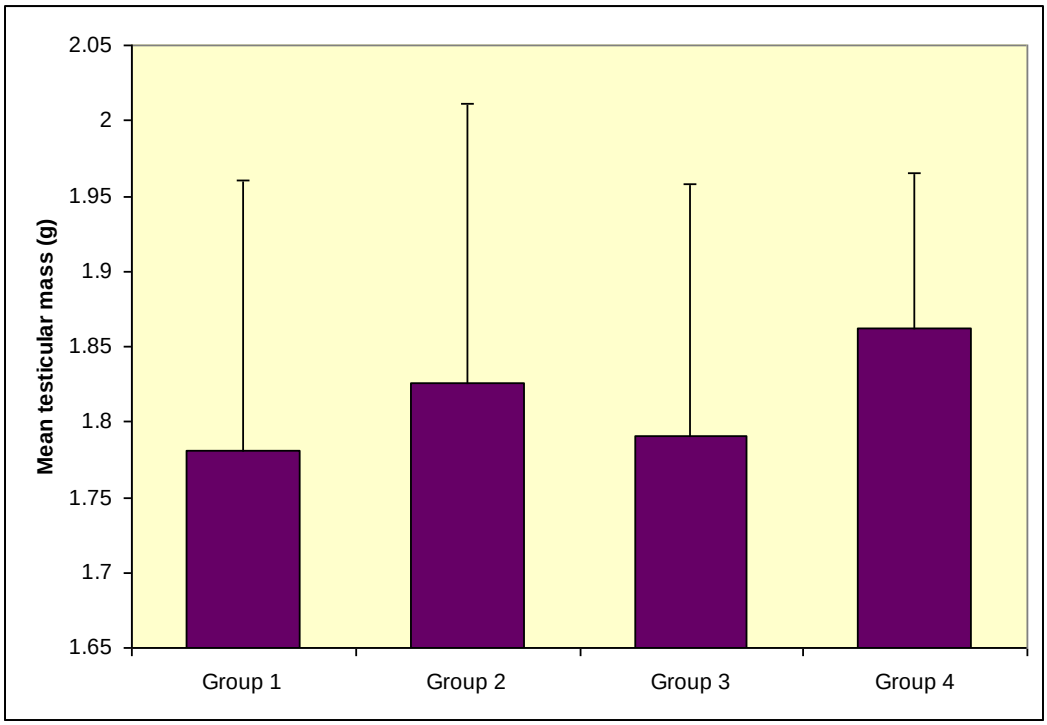

Figure 4.8: Mean and SD of the testicular mass of the F1 males in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

4.3.7 Testicular histology

4.3.7.1 Stages of spermatogenesis

In all three experimental groups spermatogenesis appeared normal and all 14 stages were present. However there were signs of apical sloughing found in Group 3 and 4.

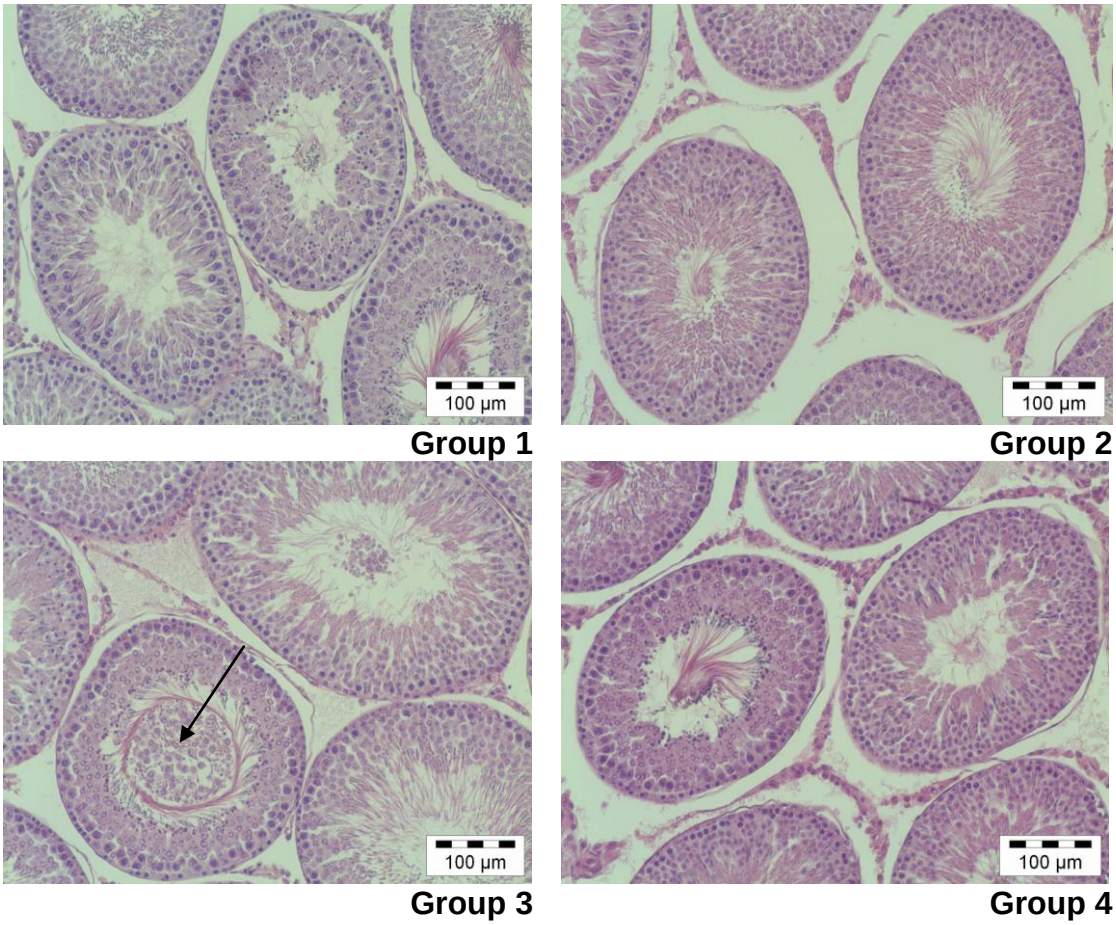

Figure 4.9: Testicular histology.

A normal seminiferous tubule is represented by Group 1 (Control). Note the apical sloughing in the experimental Group 3, as indicated by the arrow. The mean lumen diameter in Groups 3 and 4 were significantly larger than in Group1.

4.3.7.2 Mean seminiferous tubule diameter

The statistical analysis for the seminiferous tubule diameter is summarized in Table 4.11. The Wilcoxon Rank Sum test showed that there were no statistically significant differences between the tubule diameter of the Control group and the experimental groups (Figure 4.10).

Table 4.11: Mean seminiferous tubule diameter between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean seminiferous tubule diameter (µm)	SD	p-value
1 2	11 12	306.83 312.57	15.944 16.220	0.5181
1 3	11 12	306.83 316.83	15.944 19.027	0.3722
1 4	11 12	306.83 314.78	15.944 7.6758	0.2301

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

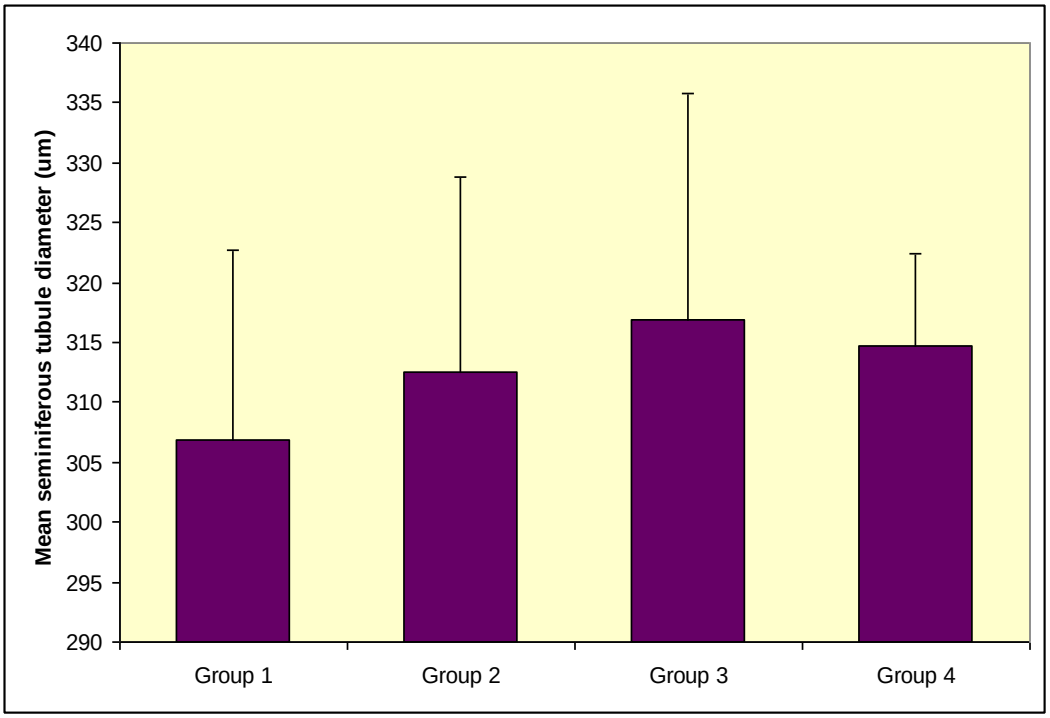

Figure 4.10: Mean and SD of the seminiferous tubule diameter of the F1 males in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

4.3.7.3 Mean seminiferous epithelium thickness

The statistical analysis for the seminiferous epithelium thickness is summarized in Table 4.12. The Wilcoxon Rank Sum test showed that there were no statistically significant differences between the seminiferous epithelium thickness of the Control group and the experimental groups (Figure 4.11).

Table 4.12: Mean seminiferous epithelium thickness (µm) between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean seminiferous epithelium thickness (µm)	SD	p-value
1	10	100.19	5.6242	0.4483
2	12	101.70	4.6496	
1	10	100.19	5.6242	0.3390
3	12	103.68	6.6864	
1	10	100.19	5.6242	0.4098
4	12	102.56	5.5921	

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

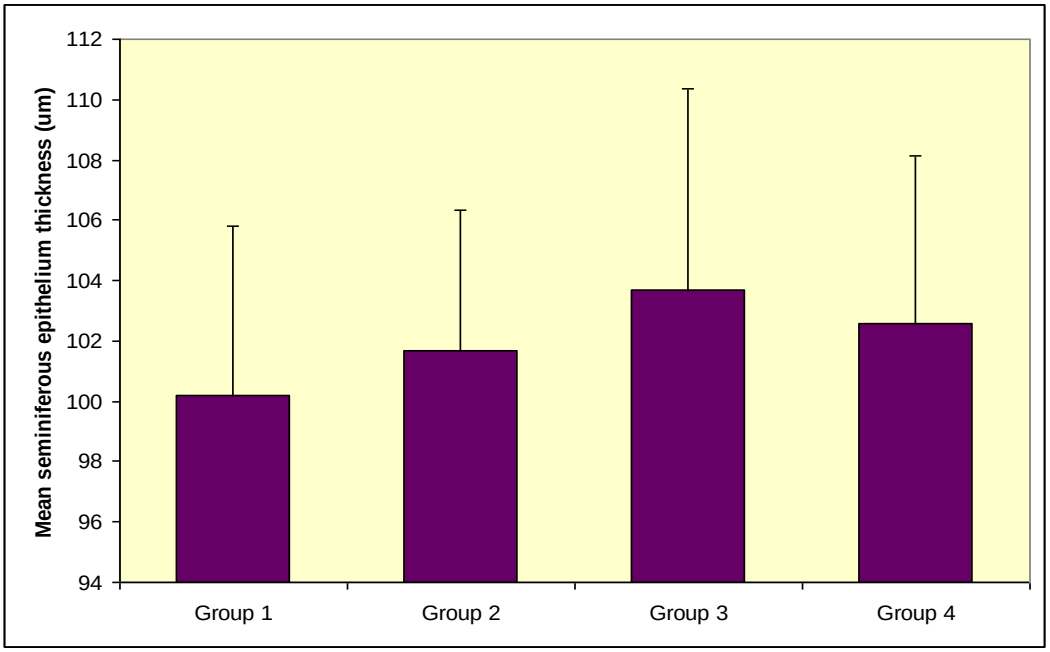

Figure 4.11: Mean and SD of the seminiferous epithelium thickness of the F1 males in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

4.2.7.4 Mean lumen diameter

The statistical analysis for the lumen diameter is summarized in Table 4.13. The Wilcoxon Rank Sum test showed that lumen diameter was statistically significantly increased between the control group and Group 3 ($p = 0.455$) and the Control group and Group 4 ($p = 0.0289$) (Figure 4.12).

Table 4.13: Mean lumen diameter (µm) between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean lumen diameter (µm)	SD	p-value
1 2	11 12	114.04 120.60	11.049 10.736	0.1150
1 3	11 12	114.04 121.69	11.049 8.9078	**0.0455***
1 4	11 12	114.04 121.56	11.049 5.5110	**0.0289***

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

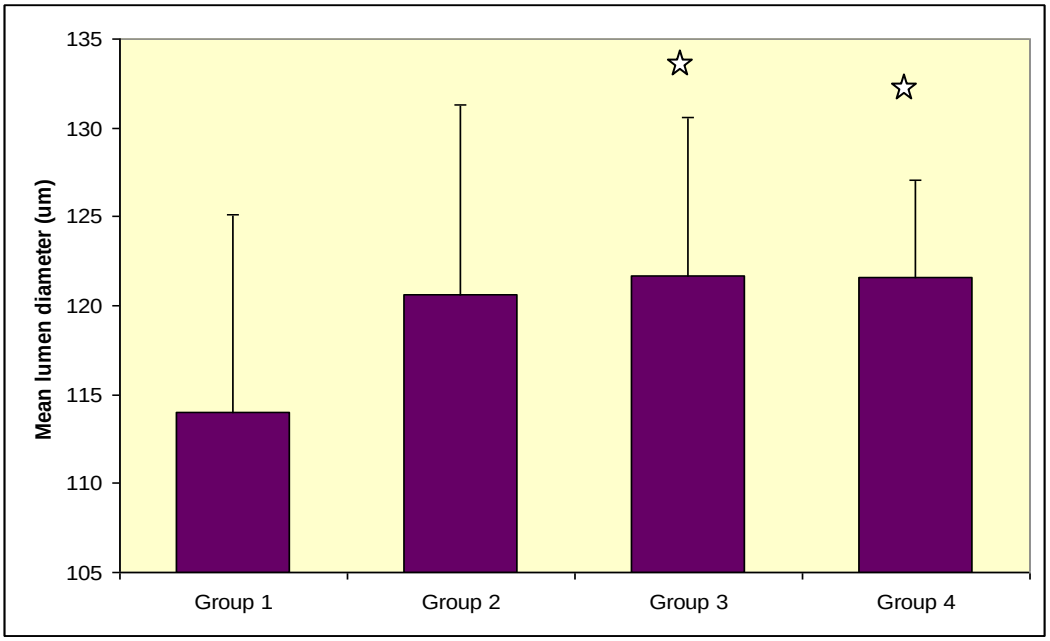

Figure 4.12: Mean and SD of lumen diameter of the F1 males in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

* significant: $p < 0.05$ when compared to Group 1

4.3.8 Mean total cauda epididymal sperm count

The total cauda epididymal sperm counts are summarized in Table 4.14. The sperm counts in Group 2, 3 and 4 were lower than the Control group. The Wilcoxon Rank Sum test only indicated a statistically significant difference between the Control group and Group 3 (Figure 4.13).

Table 4.14: Mean total cauda epididymal sperm count between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean sperm count (x106)	SD	p-value
1	11	57.036	18.005	0.1756
2	12	46.300	12.887	
1	11	57.036	18.005	0.0337*
3	12	41.083	11.817	
1	11	57.036	18.005	0.1568
4	12	47.033	15.711	

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

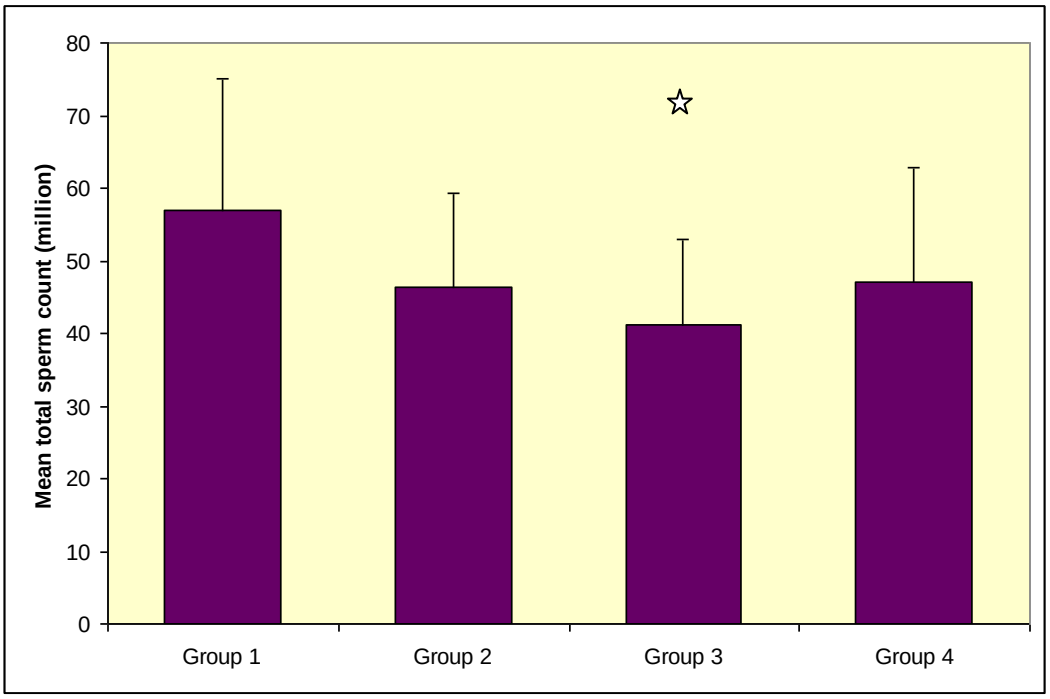

Figure 4.13: Mean and SD of the total cauda epididymal sperm count of the F1 males in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

* significant: $p < 0.05$ when compared to Group 1

4.3.9 Mean liver mass

The mean liver mass is summarized in Table 4.15. The Wilcoxon Rank Sum test indicated that there were no statistically significant differences between the Control group and the experimental groups (Figure 4.14).

Table 4.15: Mean liver mass (g) between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean liver mass (g)	SD	p-value
1	11	18.132	2.8593	0.7818
2	12	17.364	1.1399	
1	11	18.132	2.8593	0.1316
3	12	16.074	1.2492	
1	11	18.132	2.8593	0.0905
4	12	16.362	1.6465	

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

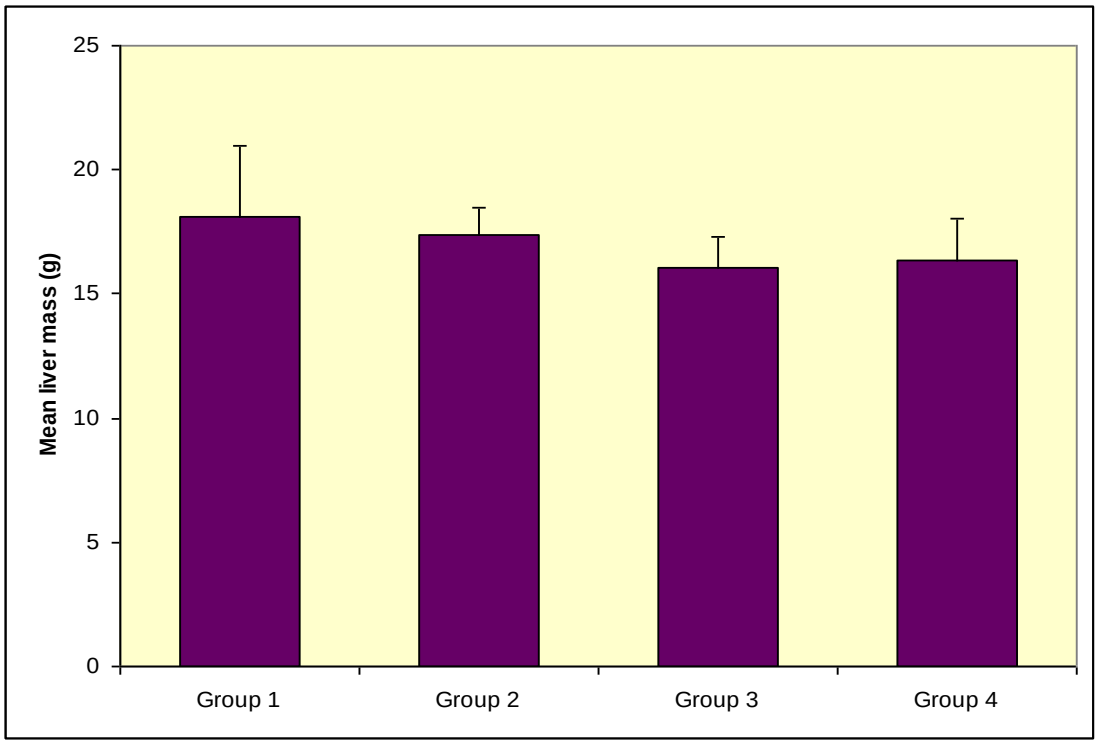

Figure 4.14: Mean and SD of the liver mass of the F1 males in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

4.3.10 Thyroid function: T_4

The T_4 for the different groups is summarized in Table 4.16. The Wilcoxon Rank Sum test indicated that the T_4 values between the Control group and Groups 2 and 3 were statistically significantly higher than the Control group (Figure 4.15).

Table 4.16: Mean T_4 values between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean T_4 nmol/L	SD	p-value
1	11	64.395	8.2583	**0.0089***
2	12	74.190	12.426	
1	11	64.395	8.2583	**0.0210***
3	12	74.464	11.263	
1	11	64.395	8.2583	0.4060
4	12	64.793	22.148	

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

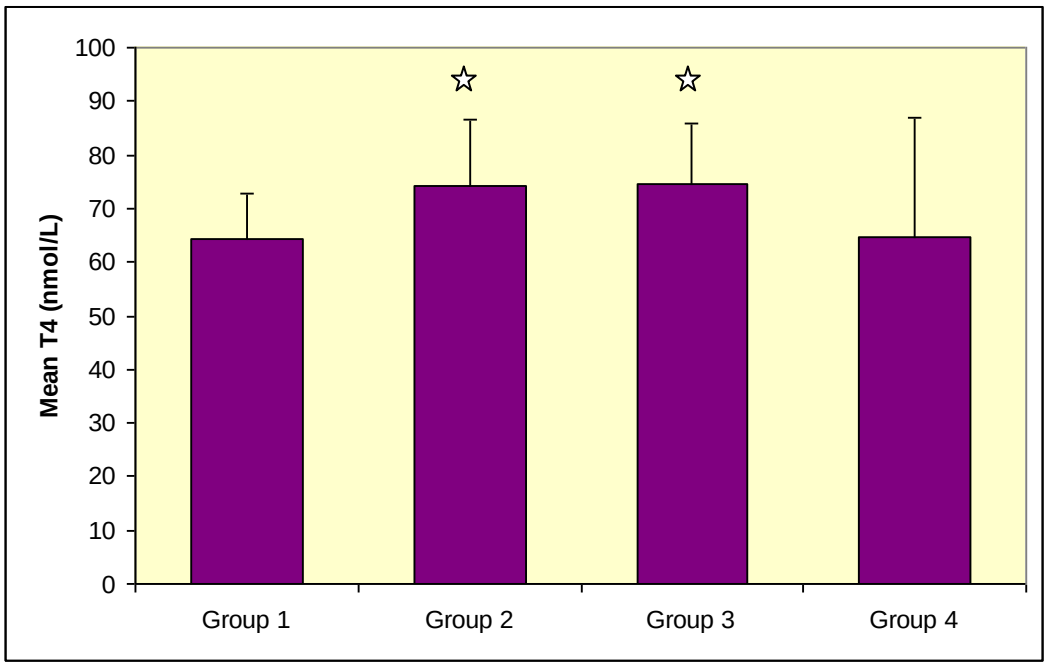

Figure 4.15: Mean and SD of the T_4 values of the F1 males in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

* significant: $p < 0.05$ when compared to Group 1

4.3.11 Thyroid function: T_3

The T_3 values for the different groups are summarized in Table 4.17. The Wilcoxon Rank Sum test indicated no statistically significant differences for the mean T_3 values (Figure 4.16).

Table 4.17: Mean T_3 values between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean T_3 (nmol/L)	SD	p-value
1	11	1.2127	0.1188	0.5793
2	12	1.2717	0.2500	
1	11	1.2127	0.1188	0.7578
3	12	1.2367	0.1339	
1	11	1.2127	0.1188	0.3396
4	12	1.2958	0.1970	

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

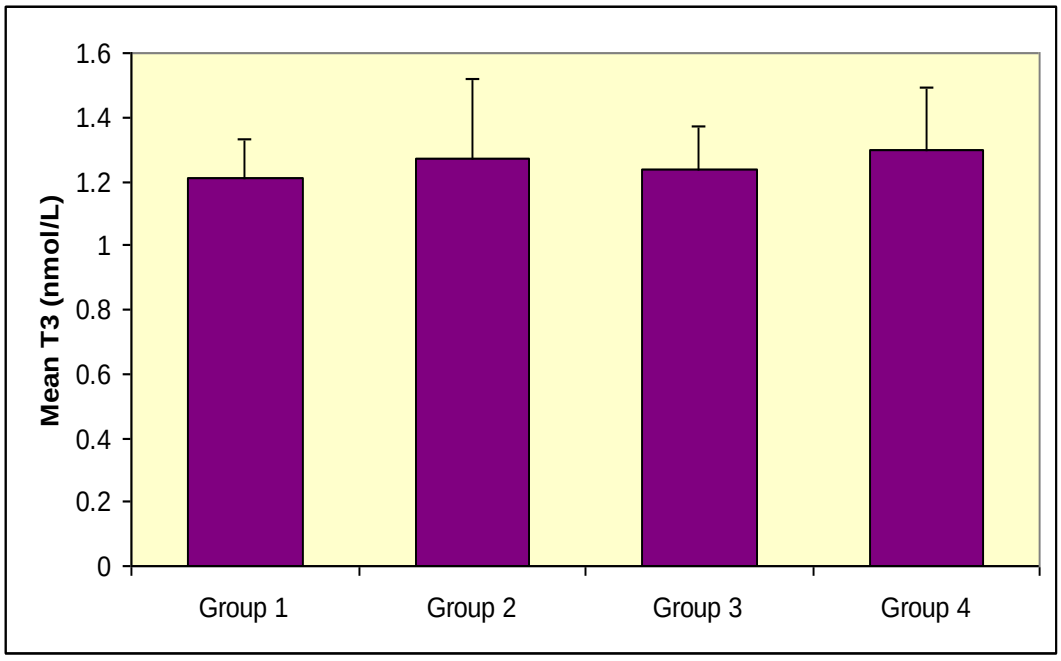

Figure 4.16: Mean and SD of the T_3 values of the F1 males in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

4.4 F2 pups

4.4.1 F2 males

The mean body mass between the control and experimental groups is summarized in Table 4.18. The Wilcoxon Rank Sum test indicated a statistically significant decrease in the body mass between the Control group and Group 2 ($p = 0.0000$) (Figure 4.17).

Table 4.18: F2 males: mean body mass between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	F2 males: mean body mass (g)	SD	p-value
1	67	53.567	8.2356	**0.0000***
2	67	47.657	5.4677	
1	67	53.567	8.2356	0.1458
3	53	51.943	9.1891	
1	67	53.567	8.2356	0.8902
4	57	53.386	5.1712	

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

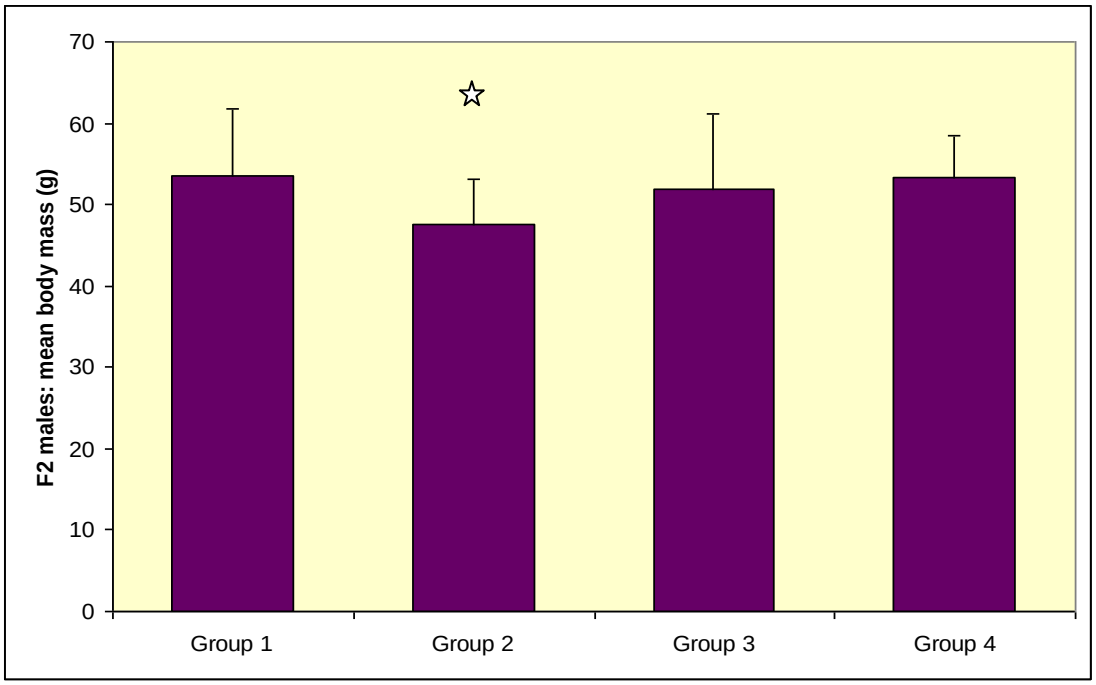

Figure 4.17: Mean and SD of the body mass of the F2 male pups in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

* significant: $p < 0.05$ when compared to Group 1

4.4.3 F2 females: mean body mass

The mean body mass between the Control and experimental groups is summarized in Table 4.19. The Wilcoxon Rank Sum test indicated a statistically significant decrease in the body mass between the Control group and Group 2 ($p = 0.0000$), the Control group and Group 3 ($p = 0.0189$) and the Control and Group 4 ($p = 0.0119$) (Figure 4.18). All the experimental groups' body masses were decreased compared to Control group.

Table 4.19: F2 females - mean body mass between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	F2 females: mean body mass (g)	SD	p-value
1	55	53.473	8.1624	**0.0000***
2	57	47.246	5.6388	
1	55	53.473	8.1624	**0.0189***
3	66	50.167	8.2436	
1	55	53.473	8.1624	**0.0119***
4	65	49.908	4.3100	

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

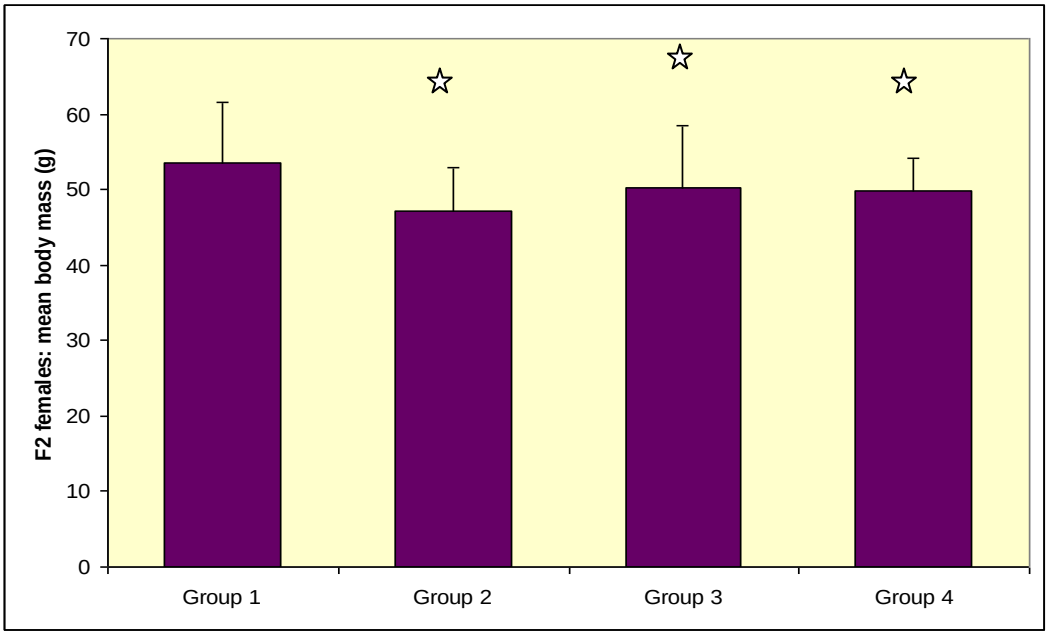

Figure 4.18: Mean and SD of the body mass of the F2 female pups in the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

* significant: $p < 0.05$ when compared to Group 1

4.4.5 F2 pups male:female ratio

For the F2 pups - male:female ratio, between the Control and experimental groups, the Wilcoxon Rank Sum test indicated no statistically significant differences (Figure 4.19). The ratio between males and females is summarized in Table 4.20.

Table 4.20: F2 pups - male:female ratio between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	F2 Pups male:female mean ratio	SD	p-value
1	11	1.4202	0.7231	0.6207
2	10	1.4664	1.0575	
1	11	1.4202	0.7231	0.0523
3	10	0.8504	0.3545	
1	11	1.4202	0.7231	0.1233
4	12	1.1480	1.1522	

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

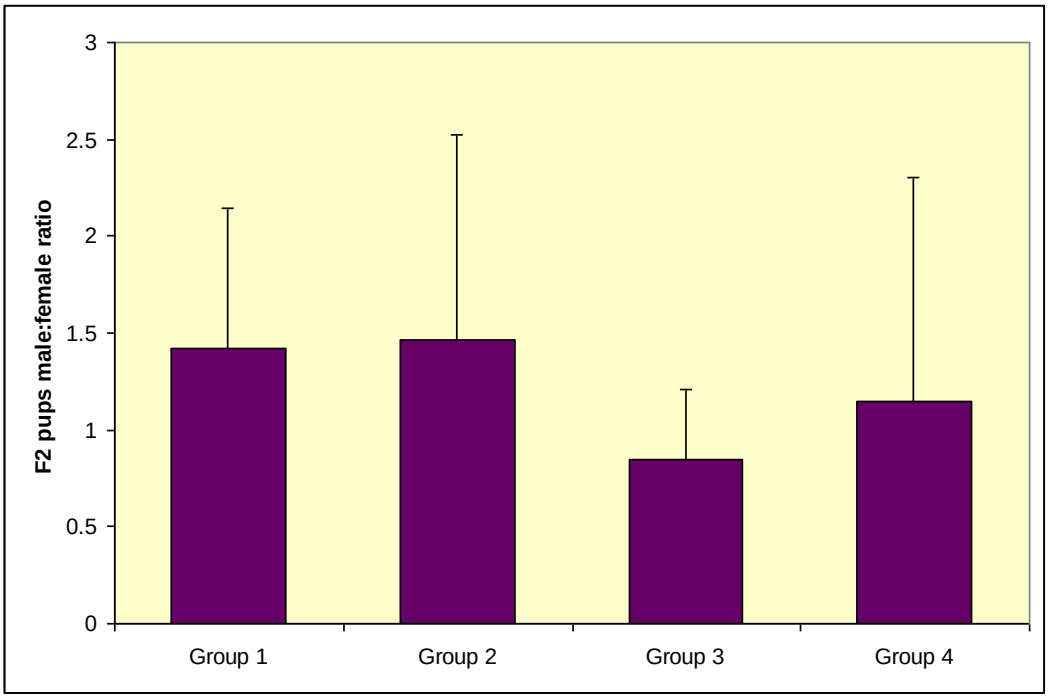

Figure 4.19: Mean and SD of the ratio of males: females of the F2 pups between the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

4.4.6 F2 pups: litter size

For the litter size between the Control group and the experimental groups the Wilcoxon Rank Sum indicated no statistically significant differences (Figure 4.20). The litter sizes are summarized in Table 4.21.

Table 4.21: F2 pups litter size between the control and experimental groups, according to the Wilcoxon Rank Sum test

Experimental groups	n	Mean litter size	SD	p-value
1	11	11.091	1.8141	0.1736
2	10	12.400	1.9551	
1	11	11.091	1.8141	0.3705
3	10	11.900	2.5582	
1	11	11.091	1.8141	0.2516
4	12	10.167	1.3371	

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

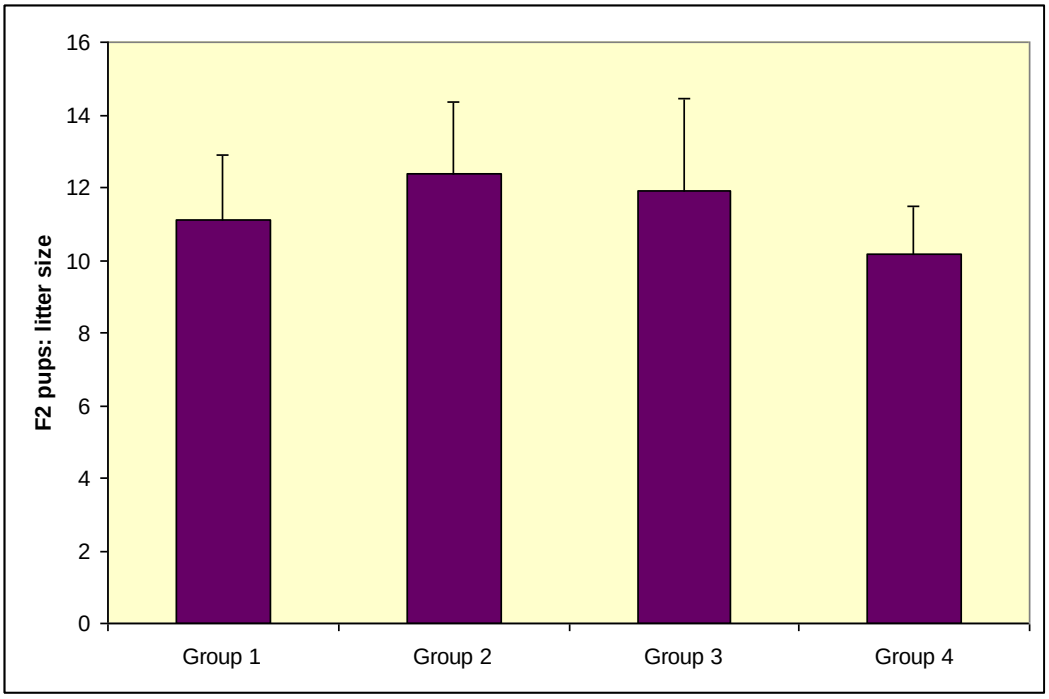

Figure 4.20: Mean and SD of the litter size of the F2 pups in between the control and experimental groups.

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

Chapter 5

5. Discussion

5.1 Phase1: Bio-assays

The bio-assay results indicate that water samples analysed from selected feedlots contained compounds with estrogenic activity. This activity may not be solely attributed to the VGS because EDCs commonly occur as mixtures in the environment. Other sources of estrogenic contamination (in the forms of natural hormones and ground pollution) may be present in the sampling point areas. The bio-assay results are summarized in Table 5.1.

Table 5.1: Summary of the bio-assay results

Estrogenic activity YES			Estrogenic activity T47D-KBluc	
Samples	**Result**	**Cytotoxicity**	**Result**	**Cytotoxicity**
1	<dl	✓	<dl	✓
3	<dl	✓	Positive	-
4	Positive	✓	Positive	✓
8	n/q	✓	Positive	-
13*	<dl	✓	Positive	-
14	n/q	✓	Positive	-
20	n/q	✓	Positive	✓
39*	<dl	-	<dl	-
41	n/q	-	Positive	-
45	<dl	-	Positive	-
46	<dl	-	Positive	-

<dl: Below the detection limit of assay; n/q: positive but not quantifiable; ✓: cytotoxicity present;
* Same sample site different sampling period

The YES screen is a suitable screening tool for the determination of the overall estrogenic activity in complex samples taken from aquatic environments (Beck *et al*., 2006; Aneck-Hahn *et al*., 2008, 2009). In a study by Matthiessen *et al*. (2006) investigating possible livestock contamination (in streams up and below livestock farms in the United Kingdom) found estrogenic activity in the aquatic environment Similarly this study found estrogenic activity in ground water in and around cattle feedlots (Matthiessen *et al*., 2006). Beck *et al*. (2006) found that the inhibition of yeast growth possibly caused by acute toxic constituents was the reason for the masking of an estrogenic response in more highly concentrated extracts, this was also found to be the case in a study by Bornman *et al*. (2007). Similarly in this study, the toxicity found in the YES assay could be masking the estrogenic activity in the samples (1, 3, 4, 8, 13, 14, and 20) and therefore give false negative results, as the estrogenic response may lie in the toxic range. This is confirmed by the estrogenic activity measured for five sample sites (3, 8, 13, 14 and 20) in the T47D-KBluc assay.

Some of the samples that were below the detection limit and in the toxic range of the YES assay tested positive for estrogenic activity in the T47D-KBluc assay (Table 5.1). The explanation for this can be due to the fact that the thick cell wall of the yeast prevents compounds form entering the cells (Dhooge *et al*., 2006; WRC Report 1505/1/07, 2007) or because the yeast cells contain only the ERα and the T47D-KBluc cells contain both the endogenous ERα and ERβ. The T47D-KBluc assay is therefore a more sensitive assay for estrogenic activity compared to the YES assay (Wilson *et al*., 2004; Wehmas *et al*., 2011).

Out of the three feedlots with 47 samples points 27 samples points had detectable levels of zilpaterol with values that range form <10 – 23703ng/l. From this Veterinary Report WRC K5-1686 zilpaterol appears to have estrogenic activity at high concentrations (de Jager *et al*., 2009).

Although only sample 3 (run off water from the feedlot gathered in a settling dam) exceeded the Predicted-No-Effect-Concentration (1 ng/l) for E_2 in water

it must be noted that EEq values could be under estimations as the pH of the water was not adjusted (pH 3) prior to extraction and the chemical analysis should also be taken into consideration. It should be kept in mind that water and sediment samples from feedlots consist of a complex mixture of chemicals with possible (anti)androgenic and (anti)estrogenic activity, as well as other chemicals with possible EDC effects that were not measured. This could also affect the outcome of the assay. It should therefore be noted that EEq's are only rough estimates. The complexity of the sample, pH, extraction procedure and the nature of the assay (i.e. a biological system) might all have an influence on the results and may possibly lead to an under-estimation of the results or even to false negatives (Khan *et al*., 2008).

The absence of androgenic activity measured in the MDA assay may be attributed to the complexity of the samples and the fact that the pH was not adjusted (pH 3) prior to extraction. Another possible reason could be that androgens present were also below the assay detection limit. A third reason could be as Blake *et al*. (2010) concluded that the steroidal estrogen estradiol could bind to the androgen receptor in MDA-kb2 cells. Estradiol shows both agonism and antagonism in MDA-kb2 cell line, but only at high concentrations. Steroidal estrogens therefore potentially interfere with the response of the cells to androgens (Blake *et al*., 2010).

In summary, VGS have the potential to reach rivers and ground water due to there persistence in the environment. Findings of the bio-assays provide evidence that there is estrogenic activity present in the feedlot environment. These compounds may contribute to the EDC burden in the aquatic environment.

5.2 Phase2: Animal studies

5.2.1 P1 females

The P1 maternal weight gain and litter size outcome were not affected by the combinations of the compounds administrated. This is an indication that the combinations of veterinary growth stimulants did not have a biological significant effect at the dose levels tested.

5.2.2 F1 males

The significant statistical analyses of the reproductive parameters are summarized in Table 5.2

Table 5.2: A summary of the reproductive parameter results of the F1 males from the different treatment groups, using the Wilcoxon Rank Sum test

Variables	Group 1	Group 2	1:2	Group 3	1:3	Group 4	1:4
	Mean	Mean	p-value	Mean	p-value	Mean	p-value
Body mass (g)	374.76	374.00	ns	360.64	ns	358.04	ns
Ano-genital distance (mm)	40.900	38.830	0.0670	38.170	**0.0117***	39.420	0.0865
Seminal vesicle mass (g)	0.7488	0.7916	ns	0.6862	ns	0.6271	**0.0074***
Epididymal mass (g)	0.5616	0.5687	ns	0.5830	ns	0.5964	ns
Testicular mass (g)	1.7807	1.8253	ns	1.7904	ns	1.8623	ns
Seminiferous tubule diameter (µm)	306.83	312.57	ns	316.83	ns	314.78	ns
Seminiferous epithelium thickness (µm)	100.19	101.70	ns	103.68	ns	102.56	ns
Lumen diameter (µm)	114.04	120.60	ns	121.69	**0.0455***	121.56	**0.0289***
Total sperm count ($x10^6$)	57.036	46.300	ns	41.083	**0.0337***	47.033	ns
Prostate (g)	0.9277	0.8778	ns	0.8600	ns	0.7789	**0.0151***
Liver (g)	18.132	17.364	ns	16.074	ns	16.362	ns
T_3 nmol/ℓ	1.2127	1.2717	ns	1.2367	ns	1.2958	Ns
T_4 nmol/ℓ	64.395	74.190	**0.0089***	74.464	**0.0210***	64.793	Ns

Group 1 = Control; Group 2 = zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-zearalanol (2.4 µg/kg); Group 3 = β-trenbolone (12 µg/kg), MT (6 µg/kg); Group 4 = zilpaterol, DES, α-zearalanol; β-trenbolone, MT

*significant: $p < 0.05$ when compared to Group 1

In this study no statistically significant differences were found between the mean body mass of the Control group and the experimental groups. However the mean masses of androgen (Group3) and combination group (Group4) were lower than the Control group. Similar results were found by Wason *et al*. (2003) in study looking at 17α-methyltestosterone at higher concentrations (10, 40 and 200 mg/kg), where the male rat's weights decreased by 23% in the 200 mg/kg group (Wason *et al.*, 2003). In the NADA approval document for Finaplix® (1986), Hunter found that trenbolone also lowered the body weight gain (NADA, 1986). Studies done by Yamamoto *et al*. (2003) and Odum *et al*. (2002), concluded that maternal and direct exposure to DES decreased male offspring body mass (Yamamoto *et al*., 2003; Odum *et al*., 2002). Goyal *et al*. (2003) also concluded that levels as high as 10ug of DES lowered the body mass by almost 7% compared to the control.

The AGD is a sexually dimorphic measure of the perineal length and is therefore an established reproductive endpoint for rodent studies examining the effects of exposure to EDCs (Phillips and Foster, 2008; Hsieh *et al*., 2008). In multiple mammalian species the AGD in males is usually twice the distance of females (Hsieh *et al*., 2008; Rhees *et al*., 2008). This suggests that the AGD is under hormonal influence (Hsieh *et al*., 2008). In mammals the masculinisation of the external male genitalia and longer ano-genital distance is regulated by DHT (Phillips and Foster, 2008; Clark *et al*., 1990). Mammals seem to possess a single AR that mediates the effects of endogenous androgens including, testosterone, androstenedione and DHT (Phillips *et al*., 2008). As a result chemicals with either androgen or antiandrogen activity may interact with the AR, preventing endogenous hormonal action (Phillips *et al*., 2008). In Group 3 (androgen group) the mean ADG was statistically significantly shorter than the Control group. Although not statistically significant, the AGD of Group 2 (estrogen) and 4 (combination) were also shorter than the Control group. Reduced male AGD is an indication of feminisation and has been observed after treatment with estrogenic compounds (Gray *et al*., 1994; Kelce *et al*., 1994; Kilian *et al*., 2007). Hornung *et al*. (2004) found that aromatisation of MT to 17α-methylestradiol had estrogenic effects after exposure (Hornung *et al*., 2004). The shorter AGD in

this study could be due to the fact that MT which converted to 17α-methylestradiol (a potent estrogen) may have contributed the effect of the mixture (Hornung *et al*., 2004; Kishner and Svec, 2008).

The prostate is androgen dependant and requires testosterone for its growth, development, differentiation and function (García-Flórez M *et al*., 2005; Huang *et al.,* 2004). The circulating androgens are testosterone and DHT. Circulating testosterone is converted to estrogen by the enzyme aromatase in several tissues. Estrogen can also influence the physiology and pathology of the prostate although it is an androgen-dependant organ. Furthermore estrogen receptors α and β are expressed in the prostate stroma and epithelium respectively. Aromatase has been identified in the human prostate, and therefore suggests that the prostate is a site for aromatization and a possible source of estrogen. (García-Flórez *et al*., 2005; Prins and Korach, 2008). Elevated levels of endogenous estradiol (maternal or excess local production.) or exogenous estradiol (DES or potential environmental estrogens) have been shown to induce permanent disturbances in prostate growth during prostate morphogenesis (Santti *et al.,* 1994; Huang L *et al.,* 2004).

Long term treatment of adult rats with estrogens causes prostatic involution and ultimately leads to epithelial squamous metaplasia, but exposure to high-dose estradiol neonatally, results in a permanent reduction in prostatic growth (Prins *et al.,* 1993; Santti *et al.,* 1994; Prins and Birch, 1997; Huang L *et al.,* 2004). Similarly in this study it was found that the prostate mass was reduced in the combination group (Group 4). The effect could be attributed to a synergistic estrogenic activity in the combination group as a result of the conversion of MT to 17α-methylestradiol.

Prins *et al*. (2001) and Goyal *et al*. (2001) found that the seminal vesicle weight in DES exposed rats also had a lower seminal vesicle mass. Similarly in this study the mean seminal vesicle mass in Group 4 (combination group) was statistically significantly lower compared to the Control group. The effect could be attributed to a synergistic estrogenic activity in Group 4 as a result of the conversion of MT to 17α-methylestradiol.

The mean epididymal mass of the experimental groups did not differ statistically significantly from the Control group, although at histological level there may well be effects but this was not investigated.

The mean testicular mass across the experimental groups was higher than the Control group, although not statistically significantly. Dilatation of the tubular lumen may be a diffuse change that may not be immediately obvious by microscopic examination. But such a change is generally reflected by an increase in testicular weight. This can be an important indicator of fluid accumulation (Creasy, 2001). Fluid secretion in the testis serves as an important function in the transport of oxygen and nutrients from blood to tubule via the interstitial fluid and of sperm and proteins from the rete testis via the seminiferous tubule fluid (Creasy, 2001).

During fetal life the Sertoli cells in the testis are the first somatic element to differentiate and are androgen dependant. Exposure to environmental chemicals can induce a reduction in Sertoli cell numbers (Auharek *et al*., 2010) due to the fact that the Sertoli cells are one of the most targeted cells for toxicity. This reduction may result from testosterone suppression. The Sertoli cell also produces the seminiferous tubule fluid that is androgen dependant. This fluid is mostly absorbed by the rete testis, efferent ducts and epididymal epithelium (Creasy, 2001). The fluid also contains androgen-binding protein and inhibin that are important secretory products of the Sertoli cell. Furthermore, the seminiferous tubule fluid serves as the transport medium for sperm. The volume of the tubular fluid is a function of the rate of secretion, the rate of transport from the tubule, and the rate of reabsorption in the rete and epididymis (Creasy, 2001). Alteration in any of these functions may be reflected by the tubular lumen dilatation or contraction. A tubular lumen dilatation may be the result of obstruction of the fluid outflow (Creasy, 2001). In this study assessing stages, tubular lumen dilatation was observed in Group 3 (androgen) and 4 (combination). In the androgen group (Group 3), one rat showed apical sloughing of the immature germ cells which is also a sign of Sertoli cell toxicity. Studies done by Atanassova *et el*. (1999) and Assinder *et al*. (2007) found similar results where neonatal exposure to

estrogens resulted in an increased seminiferous tubule lumen possibly due to a decreased fluid resorption in the efferent duct (Atanassova *et al*., 1999; Assinder *et al*., 2007; Walczak-Jędrzejowska *et al*., 2007).

Various studies over the past decades have suggested that there is a decline in semen quality (Damstra *et al*., 2002; Sharpe and Skakkeback, 2003; Safe 2005; Phillips and Tanphaichitr, 2008). It has been proposed that the decline is due to the increased exposure to environmental chemicals (Damstra *et al* 2002; Sharpe and Skakkeback, 2003; Safe 2005; Phillips and Tanphaichitr, 2008). Studies done by De Jager *et al*. (1999) concluded that *in utero* exposure followed by direct exposure had more profound effects than direct exposure during adult life. (De Jager *et al*., 1999 II; Sharpe and Skakkebaek, 1993). The Sertoli cells are the first somatic element to differentiate in the fetal testis. The Sertoli cells provide the environment and support (including the phases of development, physical support and nutrition of germ cells) for germ cells during spermatogenesis in adult life and are therefore the ultimate determinant of sperm-producing capacity and sperm count in adulthood (Dalgaard, 2001; Phillips and Tanphaichitr, 2008; Auharek *et al*., 2010). The Sertoli cell maturation is dependant on FSH. (Dalgaard *et al*., 2001; de Jager *et al.,* 1999 II). In the adult testis both FSH and LH are required for the maintenance of spermatogenesis. However, estrogen has the ability to suppress GnRH (in the hypothalamus), FSH and LH (in the pituitary) secretion. This results in a decreased Sertoli cell multiplication due to negative-feedback suppression. (Satem, 1995; Dalgaard, 2001). Studies done by Atanassova *et al*, (1999) indicated that neonatal exposure to estrogens like DES increased apoptosis at all stages of spermatogenesis in rats (Atanassova *et al*, 1999; Phillips and Tanphaichitr, 2008). DES induced apoptosis due to the suppression of gonadal testosterone (Assinder *et al*., 2007). In this study the total sperm count was lowered across the groups but was statistically significantly lower in Group 3 (androgen group). This can be due to the fact that the xenobiotic androgen receptor agonist and estrogenic compounds can cause a reduction in testosterone production from the testis, together with a reduced release of gonadotropins, LH and FSH from the

pituitary. Estradiol also exerts a negative feedback on FSH secretion. This can result in depressed spermatogenesis (Wason *et al.,* 2003; Kilian *et al* 2007).

The mean liver mass of the experimental groups did not statistically significant differ from the Control group. But there was a trend in all experimental groups to be lower than the Control group. The concentrations in this study may be too low for macroscopic effects in the liver and may be effects at microscopic level, but this was not included in the study.

The regulation of growth and differentiation of various tissues and organs, energy homeostasis and metabolic pathways is dependant on the thyroid hormone (Jahnke *et al*., 2004; Jugan *et al.,* 2010). The critical thyroid hormones (TH) for these processes are T_4 and T_3. Furthermore the perinatal development of the central nervous system is also influenced by the TH (Christian and Trenton, 2003; Jugan *et al*., 2010). Disruption of the TH axis can result in severe impairment of brain maturation, resulting in mental retardation and neurological defects (Zoeller *et al*., 2002; Jahnke *et al*., 2004; Colborn, 2004; Jugan et al 2010). In this study the T_3 between the Control group and experimental groups did not differ. But the T_4 in estrogen (Group 2) and the androgen group (Group 3) were statistically significantly increased from the Control group. Yamamoto *et al*. (2003) found that the T_4 in male rats exposed to 1.5ug/kg/day of DES increased. In an OECD Environmental Health and Safety Publication, it was established that MT exposure results in an increased T_4 (OECD, 2006). This may be well due to the fact that MT can be aromatized to methyl-E_2 that is a potent estrogen (Wason *et al*., 2003). Furthermore why an estrogen rather than androgen/ anti-androgen involvement is suspected is due to the fact that that T_4 is generally transported by a protein TBG (Yamamoto *et al*., 2003; Bisschop, 2006). TBG synthesis is increased by estrogen administration and decreased by androgen and glucocorticoid (Yamamoto *et al*., 2003; Bisschop, 2006). TBG and T_4 have parallel dynamics therefore an increase in TBG is associated with an increase in T_4 (Emerson *et al*., 1990; Yamamoto *et al*., 2003; Bisschop 2006).

5.2.3 F2 generation

The F2 generation was used to investigate the F1 male fertility outcome after a lifetime exposure to selected VGS. In this study the F1 females and F2 generation pups were not directly exposed to VGS. Therefore, the decrease in body mass may be due to parental exposure. EDCs are able to cross the blood testis barrier and there are two ways through which the F1 males can transfer VGS to their offspring (Pryor *et al*., 2000; Kilian, 2004; Newbold *et al*., 2006). Firstly, via the seminal fluid and secondly via sperm, DNA damage caused by environmental contaminants might be transferred by spermatozoa to the developing embryo (Pryor *et al.,* 2000; Kilian, 2004). The proportion of the volume of semen contributed by spermatozoa and epididymal secretions is < 15% in most animals. Most of the seminal volume is derived from the ductal system of the accessory glands (Pryor *et al.,* 2000; Kilian, 2004). The presence of exogenous chemicals in these secretions may result in exposure of the female partner during mating. The presence of substances in the seminal fluid might also affect the completion of meiosis in the oocyte or male pronuclear formation in the fertilised zygote (Pryor *et al.,* 2000; Kilian, 2004).

5.2.3.1 F2: Litter size and male: female mean ratio

Results from this study indicated that VGS had no effect on the litter size and male to female ratio in the F2 generation.

In summary, alteration of sexual differentiation and thyroid function may result from *in utero* and lactation exposure to VGS that disrupt endocrine function by acting as an estrogen, an anti-androgen or by acting as a fetal germ cell toxicant. The findings from this study provide evidence that exposure of rats to certain VGS mixtures during gestation, lactation and direct exposure up to 11 weeks has a negative influence on male reproductive and thyroid parameters, specific testicular histology and sperm concentration. Therefore humans may also ultimately be affected through exposure to VGS resulting in adverse reproductive and general health effects.

Chapter 6

6. Conclusion

6.1 Phase1: Bio-assays

Over the past years the environment, especially the aquatic environment has been overloaded with EDCs. Instrumental analyses are used to measure concentrations of individual chemicals, but complex mixtures can be difficult to assess using analytical chemistry due to difficulty in detecting low concentrations of potential potent chemicals and/or the presence of unsuspected EDCs (Blake *et al*., 2010). Therefore, to help address shortcomings of instrumental analyses and/or *in vivo* tests in dealing with mixtures, *in vitro* assays have been developed to sensitively detect chemicals with a specific endocrine mechanism of action (Blake *et al*., 2010; Svobodova and Cajthanl, 2010).

Samples received for feedlots clearly indicated estrogenic activity. But when comparing the results from the two different assays it is clear that there is a need for more than one assay.

For complex environmental samples, the YES is a suitable method for the determination of the overall estrogenic activity in samples taken from an aquatic environment. Although acute toxic constituents can mask or suppress the estrogenic response in highly concentrated extracts. In this study the T47D- KBluc measured estrogenic activity in water samples where cytotoxicity was present in the YES. The possible “false” negative result found in the YES may be due to compounds in the water samples that only react / bind to the ERβ or alternatively may be toxic to the yeast cells. Another reason may be due to the impermeability of the yeast membrane to some chemicals. The T47D-KBluc may be considered a more sensitive method than the YES as the T47D-KBluc cells contain both endogenous ERα and ERβ. However, the 3 day incubation period in the YES assay allows for slower acting chemical’s activity to be assessed.

Results from this study strengthen the argument for the use of a battery of screening bio-assays which can complement each other and give a more comprehensive assessment on the estrogenic activity in environmental samples. In the MDA-Kb2 assay no androgenic activity was found, therefore an alternative assay may be recommended which is more sensitive for environmental samples (Hartig *et al*., 2002; Bornman *et al*., 2007). As indicated by Blake *et al*., (2010) steroidal estrogens could bind to androgen receptors in MDA-Kb2 cells, but only at high concentrations, thereby potentially interfering with response to the cells to androgens.

6.2 Phase2: Animal studies

In literature animal research studies have mostly focused on adverse affects of a single chemical, in reality wildlife and humans are daily exposed to a mixture of chemicals in air, water and food. A single agent or chemical may be weakly estrogenic and have a low threshold of activity but in combination may produce noticeable effects (de Jager *et al*., 1999; Kilian *et al*., 2007). Low doses of EDCs may exert more potent effects than higher doses, particularly if exposure occurs during a critical developmental window (Woodruff *et al*., 2008; Diamanti-Kandarakis *et al*., 2009).

Current evidence suggests that mammals are more susceptible to EDCs during fetal and post-natal life than in adulthood (Sweeney, 2002; Woodruff *et al*., 2008), due to a critical window of susceptibility. A critical window of susceptibility is a time-sensitive interval during development when exposure to environmental contaminants can disrupt or interfere with the physiology of a cell, tissue or organ (Morfford *et al*., 2004; Woodruff *et al*., 2008). It is also a period characterized by marked cellular proliferation and development and numerous changing metabolic capabilities in the developing organism (Woodruff *et al*., 2008). During this period many normal homeostatic endocrine feedback mechanisms and immune systems are not fully developed and there is the potential that low dose exposure could have adverse effects on the developing fetus (Crisp *et al*., 1998; Sweeney, 2002).

The reproductive system is one of the systems most affected by the critical window of susceptibility, because it can cause disruption of the embryonal programming and gonadal development. In 2001, Skakkebaek *et al*. suggested the concept of TDS. It implied that poor semen quality, testis cancer, cryptorchidism and hypospadia might all be symptoms of an underlying testicular dysgenesis (Jorgensen *et al*., 2010).

The thyroid is another important system that is affected by EDC exposure. The thyroid affects the adult body's major systems and is crucial to fetal development (Brown, 2003). The thyroid can also influence proper gonadal development. Therefore, environmental contaminants that alter thyroid hormone signaling, particularly during the critical neonatal period, could have permanent effects on testicular development (Cooke *et al*., 2004).

Estrogenic activity found in water samples collected at feedlots are confirmed by effects found in exposed animals in this study to the relevant VGS. The data from this study indicates that VGS had effects on reproductive and thyroid parameters in maternally and direct exposed male rats. This is cause for concern as essentially these compound mixtures end up in the aquatic system.

Recommendations

From this study it seems that the MDA-Kb2 assay for androgenic activity is not sensitive enough to measure androgenic activity in environmental samples. An alternative or complementary assay should be considered for measuring activity in the environmental samples. The EPA is currently investigating a more sensitive assay which uses cells modified to contain the AR and multiple reporter genes to amplify androgenic responses. If the above-mentioned assay proves to be efficient in detecting androgenic activity in environmental samples, it is recommended that the cell line be included in future projects.

At the time of analysis, the available TSH kit was not able measure the TSH levels accurately enough. For future reference a specie specific kit would be more appropriate for accurate detection.

Exposure to EDCs from aquatic sources can result from both recreational use and ingestion from drinking water from either contaminated surface or ground water. Human exposure to EDCs from these sources may therefore pose health risks. The effects caused by low concentrations of EDCs are difficult to assess, especially in situations where mixtures may be causing additive, synergistic and/or antagonistic effects. In this study there was evidence of estrogenic activity in water from boreholes and surface water. It must be considered that this activity may not soley be attributed to VGS, other EDCs from other sources may therefore contribute to the activity. Regular monitoring should therefore be implemented in the ecosystem in proximity to feedlots, including when flooding and spills occur. The role of sediment and soil as a source of contamination should also be considered in future.

There is evidence in scientific literature indicating EDC effects occurring as a result of *in utero* exposure in relation to male reproductive and general health. Establishing causal links between reproductive endpoints across the lifespan is important in relation to diseases developed in adulthood. It is often difficult to establish a link between exposure and health outcomes in human populations. Rats are model species used to study the mechanism or tissues as many endpoints in rats can be used to predict adverse outcomes in humans. This study has shown a number of significant reproductive effects for example the decreased AGD, increased lumen diameter and lower sperm count. Thyroid effects were also observed in the both the estrogen and androgen group. Future studies investigating the effects of VGS should include the effects of the metabolites of the parent compounds as they may have a different mechanism of action as is the case of MT (androgen) which converts methylestradiol (estrogen) during exposure.

Understanding the mechanisms of action of EDCs on reproductive and general health, preventing/limiting exposure to these chemicals to humans and wildlife is important. These results have implications as far as public health is concerned, especially regarding the education of the public about the risks of exposure to EDCs including VGS. In order to do this, scientific and

epidemiologic studies are needed to translate the data to human health and risk assessment.

Evidence and observations from this study suggest the need for further investigation and monitoring of cattle feedlots regarding the use of EDCs and other VGSs. There is reason to suspect an impact on surface and ground water following the current findings. Currently no environmental impact studies are done for registration of VGS. It is suggested that monitoring should become part of the licencing process of these potentially harmfull compounds.

Chapter 7

7. References

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