# Effects of *in utero*-, lactational- and direct exposure to selected endocrine disrupting chemicals on the rat male reproductive system

**BY**

**SEAN MARK PATRICK**

**B.Sc (Wits), B.Sc (Hons) (Pret), M.Sc (Pret)**

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| **Supervisor:** | **Prof. C. de Jager** |
| **Co-Supervisor:** | **Prof. M.S. Bornman** |
| **Co-Supervisor:** | **Prof. A.M. Joubert** |
| **Date:** | **September 2015** |

I, **SEAN MARK PATRICK** declare that the thesis titled "**Effects of *in utero*-, lactational- and direct exposure to selected endocrine disrupting chemicals on the rat male reproductive system**" which I hereby submit for the degree *Doctor of Philosophy in Environmental Health* at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university.

_____ 30 September 2015

Sean Mark Patrick Date

## Summary

### Effects of *in utero*-, lactational- and direct exposure to selected endocrine disrupting chemicals on the male rat reproductive system

Endocrine disrupting chemicals (EDCs) are ubiquitous natural or synthetic substances, present in the environment, that possess hormonal activity. EDCs have the ability to disrupt hormonally dependent processes and potentially elicit adverse health effects in both animals and humans. Possible adverse effects on fertility and reproductive parameters following acute and chronic exposure to these chemicals have been reported in the scientific literature. However, the association between exposure to EDCs present in a malaria area and impaired male reproductive health remains inconsistent. In South Africa (SA), malaria remains a public health threat and various programs are in place in an effort to prevent malaria transmission. EDCs in a malaria endemic area in the Limpopo Province, SA were identified as: (i) the organochlorine pesticide, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT); (ii) the persistent metabolite of DDT, 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (DDE); (iii) the active substance in insecticide-treated nets (ITNs) and used for indoor residual spraying (IRS), deltamethrin (DM); (iv) the anti-oxidant used in the preparation of lubricating oil additives, resins, plasticizers and found in polyvinyl chloride (PVC), para-nonylphenol (*p*-NP); (v) and phytoestrogens (coumestrol, genistein, zearalenone), which form part of a normal diet.

The aim of the study was to investigate the effects of *in utero*-, lactational- and direct exposure to selected concentrations of previously studied EDCs found in the environment on male reproductive health using the rat model. The objectives were to (1) determine the impact of *in utero*-, lactational- and direct exposure to EDCs on male-specific endocrine sensitive endpoints (anogenital distance and gonadosomatic index), male accessory glands (prostate, seminal vesicles), epididymis and liver (2) determine the effects of EDC exposure on epididymal sperm count and testosterone levels, (3) assess and compare the testicular histology and spermatogenesis cycle using the spermatogenesis staging program STAGES and the Johnsen Scoring system, (4)

determine the possible relationship between exposure to selected EDCs and the increase incidence of testicular apoptosis.

We adapted the Organization for Economic Cooperation and Development (OECD) 415 reproductive toxicity protocol to include one control and three experimental groups, a longer prenatal exposure period, and several additional endpoints. Male Sprague-Dawley rats were exposed in utero- for 2 weeks, indirectly during lactation for 3 weeks and directly for 10 weeks to cottonseed oil [control - group 1; n=24]; DDT (35mg/kg) [group 2; n=11]; DDE (35mg/kg) [group 3; n=27]; and a mixture of DDT (35mg/kg), DM (0.5mg/kg), p-NP (2.5µg/kg), genistein (2.5µg/kg), coumestrol (2.5µg/kg) and zearalenone (2.5µg/kg) [group 4; n=15]. Following exposure and at 13 weeks of age, changes in organ weights, epididymal sperm counts, histological assessments, staging of the spermatogenic cycle and testicular apoptosis were assessed.

Treatment effects were found for male reproductive tract development as evidenced by anogenital distance (AGD) in newborns and in liver characteristics. Compared with AGD in the control group (group 1; 17.54 +/- 0.65mm), AGD was significantly shorter in the mixture group (group 4; 15.20 +/- 0.16mm; $P = 0.005$), indicating possible feminization. In comparison with the control group mean liver mass (group 1; 17.36 +/- 2.16 g), was significantly higher in all three experimental groups: DDT (group 2; 21.16 +/- 1.29g; $P < 0.001$), DDE (group 3; 20.65 +/- 5.06g; $P = 0.003$) and the mixture (group 4; 19.45 +/- 2.00g; $P = 0.031$). Since enlargement of the liver is a marker of liver toxicity, the increase in liver mass observed in this study indicates that exposure to these selected EDCs had a significant effect on the liver of male rats. Lipid droplet formation and hepatic disorganization were present in the liver of the DDT, DDE and mixture groups suggesting that the liver may be a primary target. The changes in liver function may therefore be involved in the reproductive effects observed in this study.

When animals had reached adulthood at the end of the study, the effects of EDC exposures were found for a number of endpoints. Prostate mass in the control group (group 1; 0.83 +/-0.24g) was significantly higher in the DDT group (group 2; 1.02 +/-

0.19g; *P* = 0.018). Prostate mass was not, however, correlated with testosterone levels which were significantly higher in the DDE and mixture groups. Testicular histology revealed marked effects in all groups including dilated tubular lumens, detachment of the seminiferous epithelium, necrosis in the interstitium, disorganization of the seminiferous epithelium with few germ cells present, reduced seminiferous tubule diameter with no lumen, absent seminiferous tubules and decreased layers of germ cells. Although these changes were not seen in all tubules, treatment was associated with decreased mean seminiferous tubule diameters, decreased epithelial thickness, and smaller luminal diameters. Application of the Johnsen scoring system showed that the treatment effects manifested primarily as a Johnsen Score of 9 tubules (a Johnsen Score of 9 is defined as a seminiferous tubules with many spermatozoa present, but germinal epithelium disorganization with marked sloughing or obliteration of the lumen). Specifically, controls had, on average, 19% abnormal tubules, compared 46%, 25% and 56% in the DDT, DDE and mixture groups, respectively. Surprisingly, however, the lesions in histology did not translate into changes in epididymal sperm counts. This suggests that spermatogenesis proceeded normally in a proportion of tubules, resulting in sperm production sufficient to maintain apparently normal epididymal sperm stores.

The results of this study indicate that *in utero*-, lactational- and direct exposure to mixture of EDCs found in a malaria area, at the levels used here, has negative impacts on normal genital development after *in utero* exposure and on spermatogenesis in adulthood after combined prenatal, lactational and postnatal exposure. These findings raise concerns to EDC exposures to mothers living in malaria-areas and the reproductive health of their male offspring. Significant differences were found in the endocrine-sensitive endpoints: AGD, testosterone, testicular STAGES and Johnsen score.

This study shows that *in utero*-, lactational- and direct exposure to EDCs present in a malaria-area negatively affects male reproductive parameters in rats. These findings raise concerns to EDC-exposures to mothers living in malaria-areas and the reproductive health of their male offspring. Since this reproductive toxicology study

constitutes *in utero*-, lactational and direct lifespan exposure to environmentally relevant concentrations of EDCs present in a currently malaria-vector control area, these results might be considered indicative of the effects following similar human exposures. Safer alternatives should be sought particularly in malaria vector-control programs – where adverse reproductive health effects have been reported following chronic exposure to these potentially harmful chemicals.

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| | |
|---|---|
| °C | Degrees Celsius |
| %Bo | Percentage Binding |
| α-ZEN | Alpha Zearalenone |
| Aal | Aaligned |
| Ab | Antibody |
| ABP | Androgen Binding Protein |
| Ag | Antigen |
| AGD | Anogenital Distance |
| Aiso | Aisolated |
| APAF | Apoptosis Activating Factor |
| APEOs | Alkylphenol polyethoxylates |
| Apr | Apaired |
| β-ZEN | Beta Zearalenone |
| BF | Bouins' Fluid |
| Bo | Total Binding |
| BPA | Bisphenol A |
| Caspases | Cysteine Aspartyl-Specific Proteases |
| CDC | Centre for Disease Control and Prevention |
| CIS | Carcinoma *in situ* |
| CPM | Counts Per Minute |
| DAB | 3,3'-diaminobenzidine |
| DDA | bis(*p*-chlorophenyl) acetic acid |
| DDD | 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane |
| DDE | 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene |

| | |
|---|---|
| DDMU | 1-chloro-2,2-bis(*p*-chlorophenyl)ethane |
| DDT | 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane |
| DES | Diethylstilbestrol |
| DFF | DNA Fragmentation Factor |
| DHT | Dihydrotestosterone |
| Di | Diplotene Cells |
| DM | Deltamethrin |
| DNA | Deoxyribonucleic Acid |
| EDCs | Endocrine Disrupting Chemicals |
| ERs | Estrogen Receptors |
| ERα | Estrogen Receptor Alpha |
| ERβ | Estrogen Receptor Beta |
| EtOH | Ethanol |
| F1 | First Generation |
| FAO | Food and Agriculture Organization |
| FasL | Fas Ligand |
| FSH | Follicle Stimulating Hormone |
| g | Grams |
| GSI | Gonadosomatic Index |
| H & E | Haematoxylin and Eosin |
| HPG Axis | Hypothalamic-Pituitary-Gonadal Axis |
| Hrs | Hours |
| HSDs | Hydroxysteroid Dehydrogenase |
| HSI | Hepatosomatic Index |
| IAP | Inhibitors of Apoptosis |

| | |
|---|---|
| IHC | Immunohistochemistry |
| ICSI | Intra Cytoplasmic Sperm Injection |
| In | Intermediate |
| IPCS | International Programme for Chemical Safety |
| IRS | Indoor Residual Spraying |
| ITNs | Insecticide-treated Nets |
| IVF | *In Vitro* Fertilization |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives |
| kg | Kilogram |
| Lc | Leptone Cells |
| l | Liter |
| LH | Luteinizing Hormone |
| M-I | Meiosis 1 |
| M-II | Meiosis 2 |
| min | Minutes |
| ml | Milliliter |
| mm | Millimeter |
| MOMP | Mitochondrial Outer Membrane Permeabilization |
| mRNA | messenger RNA |
| NBF | 10% Neutrally Buffered Formalin |
| NOAEL | No Observed Adverse Effect Level |
| NSB | Non-specific Binding |
| OECD | Organization for Economic Cooperation and Development |
| P1 | Pregnant Female |
| p450arom | p450 aromatase |

| | |
|---|---|
| PARP | Poly-(ADP-ribose) Polymerase |
| PBS | Phosphate Buffered Saline |
| PCBs | Polychlorinated Biphenyls |
| PCD | Programmed Cell Death |
| PI | Preleptotene Spermatocytes |
| *p*-NP | para-Nonylphenol |
| PS | Phosphotidylserine |
| PVC | Polyvinyl Chloride |
| RIA | Radioimmunoassay |
| RNA | Ribonucleic Acid |
| RT | Room Temperature |
| SA | South Africa |
| SD | Standard Deviation |
| SHBG | Steroid Hormone Binding Globulin |
| TIAR | T-cell Restricted Intracellular Antigen Related Protein |
| TDS | Testicular Dysgenesis Syndrome |
| TGCTs | Testicular Germ Cell Tumors |
| TNF | Tumor Necrosis Factor |
| TNFR1 | TNF Receptor 1 |
| TRAIL DR4/5 | TNF-Related Apoptosis-inducing Ligand Death Receptor 4 and 5 |
| TUNEL | Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-End Labeling |
| UDPGT | Uridine Diphosphate Glucuronyltransferase |
| µl | Microliter |
| µm | Micrometer |

| | |
|---|---|
| UNEP | United Nations Environment Program |
| UPBRC | University of Pretoria Biomedical Research Centre |
| UPR | Unfolded Protein Response |
| USA | United States of America |
| VLDL | Very Low Density Lipids |
| WHO | World Health Organization |
| Z | Zygotene Cells |
| ZEN | Zearalenone |
| %Bo | Percentage Binding |

# Chapter 1: Introduction

## 1.1. Background

Environmental pollution has been a controversial topic and is at the heart of scientific driven research to address the associated public health impacts, in particular the global burden of disease. Scientists have highlighted that the concern was not simply the pollution of the environment, but also the effect that chemical pollutants are having on human and animal health (1, 2). Endocrine disrupting chemicals (EDCs) are ubiquitous in the environment and have the ability to interfere with, amongst others, hormone-dependent physiological processes through the interaction with hormone receptors (3). The potential of EDCs to disrupt normal hormone-dependent processes both pre- and post-natally in humans and wildlife is of great concern. The 2012 'State of the science of endocrine disrupting chemicals' report (3) detailed various aspects of monitoring and investigation of possible solutions to reduce the adverse health effects caused by exposure to EDCs. Recently journal the *Endocrinology* devoted the October 2015 issue to 'Prenatal Programming and Endocrinology', discussing topics on developmental origins of health and disease and the fetal basis of adult disease (4). Numerous studies focused on the effects that both acute and chronic exposure to EDCs may have on humans and wildlife. These studies have shown that exposure to EDCs potentially impact growth and development of various bodily organs, bodily processes and fertility (5-12). Exposures to various EDCs have been reported to influence embryonic development, especially at the androgen-sensitive sex-determining programing windows during early gestation (13). Changes in endogenous hormone regulation during embryonic development may result in impaired functioning of bodily systems, such as the male urogenital system (14, 15). The impact of embryonic exposure may be identifiable at birth, as in the case of urogenital abnormalities, or later in adult life, such as testicular cancer in young men, or poor semen quality after puberty. However, effects manifesting in adulthood are difficult to attribute to pre- and postnatal developmental exposure without reliable exposure data occurring during either pre- or postnatal periods.

Known EDCs include various alkylphenols, dioxins and furans, pharmaceuticals, phthalates, phytoestrogens and polychlorinated biphenyls (PCBs) and organochlorine pesticides (3, 16).

The use of pesticides has given humankind a weapon to fight both the onslaught of insect-derived crop damage and vector-borne diseases. The organochlorine pesticide 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) was successfully used across the world until its ban in most countries, including the United States of America (USA) in 1972, following reports of adverse health effects in wildlife (17). In South Africa (SA), the use of DDT was banned for agricultural use in 1976, but not for malaria vector control. In the Limpopo Province, South Africa, DDT has been sprayed continually since the 1940s (7). In 2002 SA ratified the Stockholm Convention and is therefore permitted to use DDT for malaria vector control (18) through indoor residual spraying (IRS) programs (19). The major concern with IRS programs stem from incorrect storage, application and contamination of the surrounding areas (20) which poses a health concern for both animals and humans.

Technical-grade DDT consisting of 65–80% of the active insecticidal ingredient *p,p'*-DDT and 15–21% of the less insecticidal *o,p'*-DDT (17, 21) is used for IRS. The *o,p'*-DDT and to a lesser extent *p,p'*-DDT component, both have estrogenic properties (21). Dietary and environmental exposures to *p,p'*-DDT and its metabolites result in bio-accumulation of these chemicals in adipose tissue and serum in the human body (21-24). The DDT from the circulation is metabolized into 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE) which is the persistent metabolite that bio-accumulates in fatty tissue. DDT and *p,p'*-DDE have the ability to cross the placenta with concentrations in cord blood being similar to concentrations in maternal blood (21). *p,p'*-DDE is a potent inhibitor of androgen binding to the androgen receptor (25), androgen-induced transcriptional activity and androgen action in males during development and in adulthood (26). This suggests that abnormalities in male sex development induced by *p,p'*-DDE may be mediated at the level of the androgen receptor (26).

In malaria vector programs the synthetic pyrethroid deltamethrin (DM) is used as an alternative to DDT. DM is specifically used on painted surfaces in homes and is the

active substance in insecticide-treated nets (ITNs) and IRS. Furthermore, DM has been reported to have EDC properties. Decreased semen quality and reproductive function impairment has been observed in male rats following exposure to DM (27).

In a malaria area in the Vhembe district, Limpopo Province, SA, one potential EDCs mixture of exposure was identified. In addition to DDT, DDE and DM (8, 28), *para*-nonylphenol (*p*-NP) and phytoestrogens (coumestrol, genistein and zearalenone) (8, 29-31) were identified. In SA, *p*-NP was found in drinking water and sediment and in high levels of fatty tissue from both eland and fish (29, 32). *p*-NP belongs to the alkylphenol polyethoxylate group of chemicals and is used in the food packaging industry and is a constituent of detergents, pesticides, herbicides, cosmetics and in paints (33-35). *p*-NP has the ability to act as a weak estrogen and mimics the action of natural estrogens in the body (33). Animal exposure studies using *p*-NP show a decrease in testicular mass and lower sperm counts in rats (36, 37).

The dietary intake of the VhaVenda people in this malaria area in Limpopo Province, South Africa, is primarily plant based (38, 39). Phytoestrogens are plant-derived compounds that have hormonal properties. Exposure to varying concentrations of phytoestrogens affects male reproductive health (40). Coumesterol is found in sunflower seeds and soybeans and has the ability to bind to the estrogen receptors (ERs) with high affinity (41). Reproductive toxicity studies show that exposure to coumestrol causes a dose-dependent impairment of normal sperm production, with an increase in abnormal head and tail shapes (42). Genistein is a phytoestrogen found in soybeans and legumes. Although it is non-steroidal, it binds to the ERs and indirectly affects estrogenic activity (10). Reproductive toxicity studies have shown that genistein exposure inhibits testicular cell line growth and proliferation (43). Furthermore, genistein induces structural changes in the urethroprostatic complex of rats (44) and alters the regulation of steroid hormone binding globulin (SHBG) levels (10). The mycotoxin zearalenone is a weak estrogen that is able to bind to the ERs and is found in maize, wheat, barley and rye (45). Tropical areas, such as the Vhembe district, provide the ideal conditions for zearalenone to invade crops (31). Reproductive toxicity studies have

shown that, amongst others, zearalenone exposure induces testicular apoptosis in male rats (46).

Exposure to these EDCs, especially during the critical developmental windows, interferes with embryonic development. Incorrect embryonic patterning due to growth inhibition, failure to regulate adequate signal transduction and hormonal levels has been shown to induce apoptosis (47). Exposure to these estrogenic- or anti-androgenic EDCs have the potential to disrupt the development, maturation and functioning of the male reproductive system.

## 1.2. Problem statement

EDCs are ubiquitous in the environment and have the ability to interfere with physiological processes through the interaction of hormone receptors (3). Possible adverse effects on fertility and reproductive parameters, following acute and chronic exposure to these relevant chemicals have been reported in the scientific literature (6, 8, 28, 29, 37, 48-53).

Studies have focused on the effects that both chronic and acute exposure to EDCs may have on humans (6), wildlife and in a laboratory setting (36). A reproductive toxicology study by Kilian *et al* (2007) investigated the effects of DM in isolation and in conjunction with DDT and phytoestrogens (37). The co-exposure of DM, DDT and phytoestrogens showed a significant adverse effect on various reproductive parameters. The most alarming aspect of these studies was the fact that the experimental doses used were within the range of human exposures. These reports suggest that exposures to complex mixtures may be additive or synergistic, thus future studies should assess the impact of exposure to “real world” mixtures and not only single chemical exposure. When exposure occurs to a single chemical, in some instances no adverse health effects are observed, exposure to a mixture of chemicals may, however, illicit an adverse effect (54). The “something from nothing” principle proposes that exposure to a single

chemical may have no observed effect (55). However, exposure to several of those chemicals in a mixture, due to synergistic or additive effects, may be significant.

An important aspect of exposure studies which is often overlooked in scientific literature is the timing and duration of exposure (56, 57). During development, exposure to chemicals during the critical androgen-sensitive windows may result in altered embryonic patterning and/or a negative impact on the reproductive system development (58, 59).

This study was designed to investigate the potential effects of *in utero*-, lactational- and direct exposure to selected EDCs, found in a malaria area, in South Africa, on male reproductive tract development, hormonal and testicular parameters in adulthood, in Sprague-Dawley rats.

**Hypothesis**

Exposure to *in utero*-, lactational- and direct exposure to selected EDCs, present in a malaria area, negatively affects reproductive tract development at birth and adult testis structure and function in adult male rats.

# Chapter 2: Literature review

## 2.1. Male reproduction

Reproduction is defined as the perpetuation of a species, ensuring transfer of genetic material thereby creating viable offspring (60). The role of the male in reproduction is to provide one half of the genetic material through sperm, which then fuse with the ovum ultimately resulting in fertilization (1). The role of the male is more complex than providing sperm. The male reproductive system is a finely regulated hormonally controlled system that produces timely and adequate sperm through spermatogenesis (61). The male reproductive system is comprised of the testes (the site for spermatozoa and male sex hormone formation), the ductal system (where mature spermatozoa are released the accessory glands) and the male sex organ (the penis) (1, 60).

## 2.2. Testes

The testes have a dual function in reproduction namely, androgen synthesis and spermatogenesis (61). These two functions are executed in separate compartments of the testes. The androgen synthesis takes place in the interstitium, whilst spermatogenesis takes place in the seminiferous tubules of the testes (Figure 1) (1). The seminiferous tubules comprise 80% of the testis and empties into a ductal system which leads into the epididymis (61). The epididymis promotes sperm maturation, facilitates the transport of spermatozoa along the duct and stores spermatozoa (62).

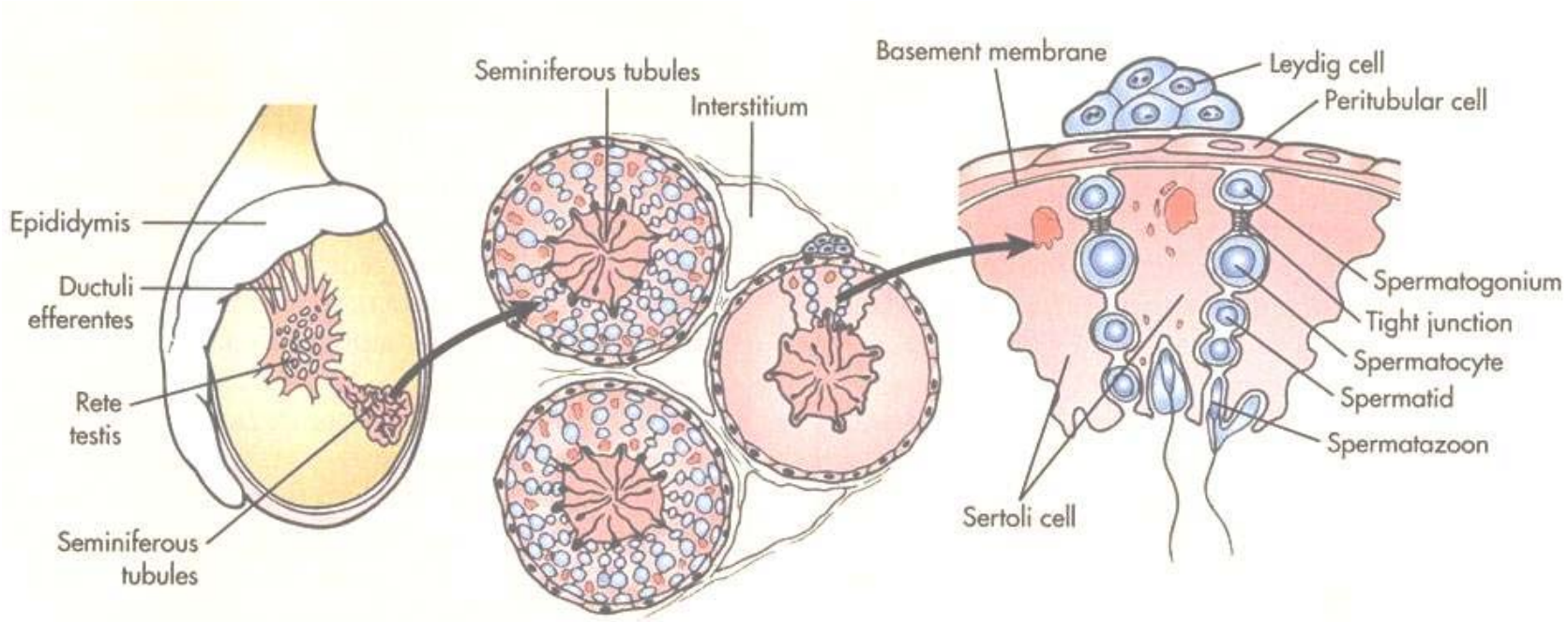


Figure 1: The architecture of the testis (1).

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## 2.3. Sertoli and Leydig cells

Spermatogenic progression and survival of germ cells are dependent on the Sertoli and Leydig cells (1). The Sertoli cells are located within the germinal epithelium of the testes on the basal membrane and extend to the lumen of the seminiferous tubules (Figure 1 and Figure 2). The Sertoli cells have follicle-stimulating hormone (FSH) receptors on their membranes. Additionally, the Sertoli cells support the structure of the germinal epithelium and they form the blood-testis-barrier (63). The Sertoli cells produce and secrete cytokines, growth factors, steroids, prostaglandins and modulators of cell division. The Sertoli cells are known as nurse cells as they nourish germ cells through these secretory products and participate in germ cell movement and spermiation (64).

Sertoli cells are responsible for the final testicular volume and sperm production in adult mammals. An individual Sertoli cell is in morphological and functional contact with a defined number of germ cells (63). The number of germ cells per Sertoli cell differs among various species - in human males there are 10 germ cells to 1 Sertoli cell whereas in rats there are 50 germ cells to 1 Sertoli cell (63). This suggests that in certain species the higher number of Sertoli cells the greater the sperm production, provided all Sertoli cells are functioning adequately (64, 65).

The Leydig cells are usually located in clusters between the seminiferous tubules of the testes (Figure 1) (1). The Leydig cells have the capacity for steroidogenesis and contain a rich smooth endoplasmic reticulum and mitochondria with tubular cristae. These characteristics are similar to other steriodogenic cells such as the cells in the adrenal gland and the ovary (64). Luteinizing hormone (LH) binds to receptors and stimulates the Leydig cells to produce testosterone (Figure 2). Spermatogenesis requires both FSH and very high levels of testosterone to promote germ cell maturation (64, 66).

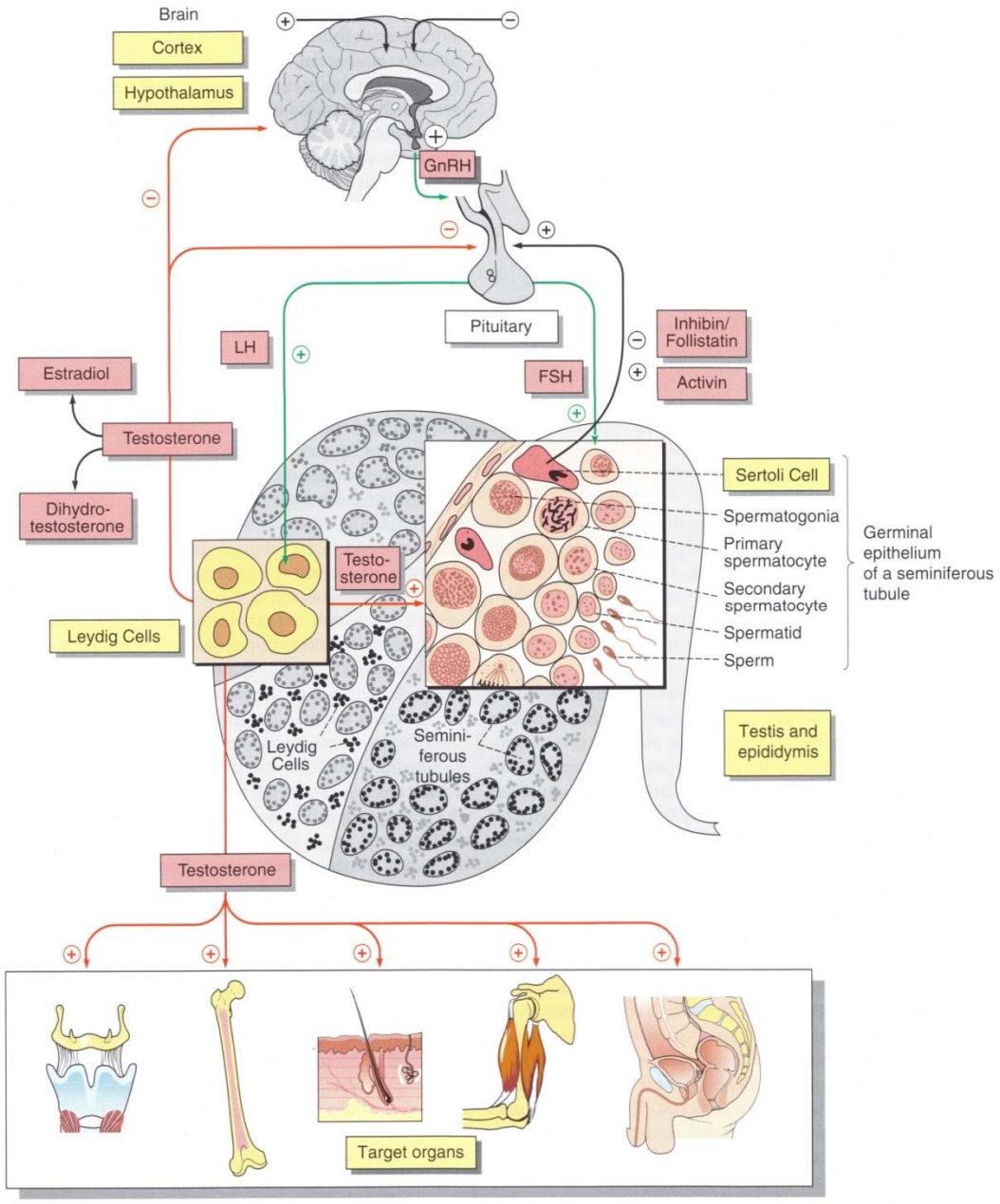


Figure 2: The hypothalamic-pituitary-gonadal axis indicating the hormone regulation of the testis and other target organs including positive and negative feedback loops (64).

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## 2.4. Spermatogenesis

Spermatogenesis is defined as a process in which germ cells undergo differentiation and metamorphosis, maturing into functional spermatozoa (67). The process is characterized by precise timing and synchronized development of the germ cells. Spermatogenesis occurs continually during the reproductive lifetime where the germ cells undergo stages of mitotic division, differentiation and ultimately meiosis (68).

The stages of the rat spermatogenic cycle (Figure 3) may be divided into three phases (66), namely the:

I. *Proliferative phase* - where the spermatogonia undergo rapid and successive divisions
II. *Meiotic phase* - where genetic material of the spermatocytes are recombined and then segregated
III. *Spermiogenic phase* - where the spermatids are mature and equipped for fertilization of the ovum

### 2.4.1. Proliferative phase

Spermatogonia are immature germ cells that undergo numerous mitosis steps to increase the number of spermatogonia. Thereafter the spermatogonia undergo meiosis to ensure genetic differentiation (66). Three types of spermatogonia exist, namely:

- Stem cell spermatogonia - $A_{isolated}$ ($A_{iso}$) spermatogonia
- Proliferative spermatogonia - Type A spermatogonia are proliferative ($A_{paired}$ - $A_{pr}$, $A_{aligned}$ – $A_{al}$).
- Differentiating spermatogonia - Type $A_1$, $A_2$, $A_3$, $A_4$, Type B and Intermediate (In).

The latter two subtypes are most sensitive to EDCs due to their high mitotic rate and rapid division (69, 70), especially during the androgen dependent stages.

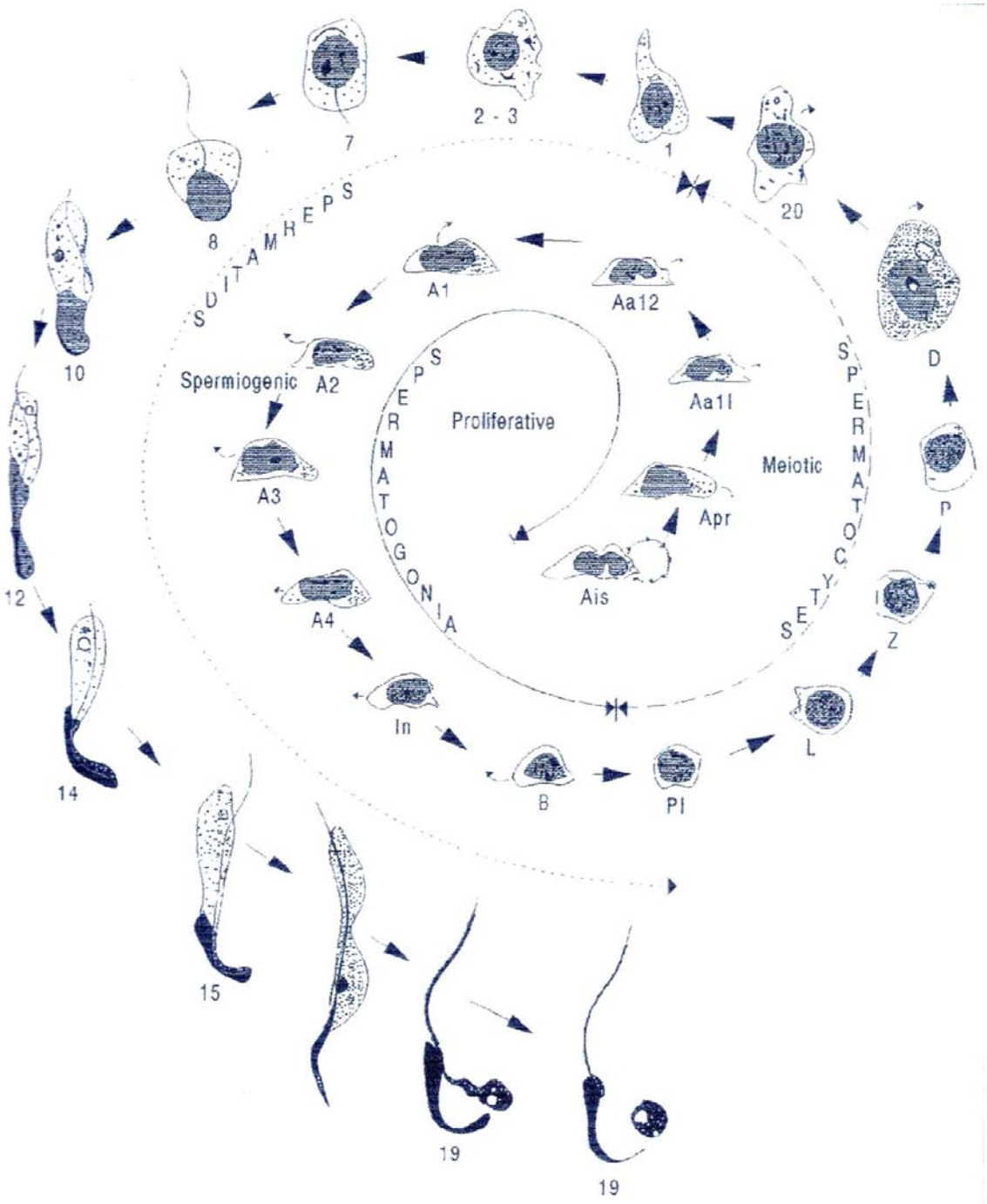


Figure 3: The proliferative, meiotic and spermiogenic phases of the rat spermatogenic cycle showing the development and maturation of the spermatogonia, spermatocytes and spermatids (66).

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### 2.4.2. Meiotic phase

Type B spermatogonia are the most mature spermatogonia. They divide after the differentiating phase to form young primary spermatocytes. These androgen-dependent young primary spermatocytes are referred to as preleptotene (PI) spermatocytes (66). The PI differ from the Type B cells in that they are smaller and have less chromatin along the nuclear envelope. During the meiotic divisions, chromosomes are recombined and genetic material is halved in each cell, resulting in haploid spermatid formation (1). The transition from PI to leptone cells (Lc) is a gradual process in which the nuclei lose their peripheral chromatin, forming thin chromatin threads. The prophase of the first meiotic phase, initiated by the presence of Lc cells, is long and results in a gradual transition between the various phases within the prophase stage (66). Homologous chromosomes pairings occur in zygotene cells (Z), while in the pachytene cells of the rat the chromosomes become fully paired. The brief diplotene phases are characterized by diplotene cells (Di) which are the largest primary spermatocytes as and the largest germ cells (1). The first meiotic division is referred to as Meiosis I (M-I) and the cells formed are the secondary spermatocytes. The second meiotic division Meiosis II (M-II) follows M-I and produce spermatids (66).

### 2.4.3. Spermiogenic phase

The spermiogenic phase in the rat is 21 days long and is essential for young spermatids to evolve into spermatozoa. The spermiogenic phase is intriguing in that no cell division occurs during the cell transformation process of spermatid to spermatozoa (64, 66). The spermiogenic phase has five specific endpoints, namely:

1. Development of the flagellum

   In the youngest spermatids the presence of the flagellum can be noted. The two centriole pairs migrate to the cell surface and forms a structure containing microtubules referred to as an axoneme (66). The axoneme formation enables the spermatid plasma to protrude from the cell. The flagellum is comprised of a

middle, principal and end piece, and its function is to impart sperm motility. The flagellum development is a continuous process starting at the onset of spermatogenesis and culminates in spermiation (66). Whilst in the testis, the sperm are effectively immotile. Motility is only developed when the sperm are in the epididymis. Once the sperm enter the female reproductive tract, they become vigorously motile (71).

2. Development of the acrosome

Acrosome development is a gradual process that is initiated at the onset of spermatogenesis and is completed in the late stages of the spermatogenic cycle (66). Immature rat spermatids do not contain an acrosome but rather display a peri-nuclear Golgi apparatus (72). The Golgi apparatus produces pro-acrosomal vesicles that contain pro-acrosomal granules. The pro-acrosomal granules coalesce within a large membrane bound vesicle that contains a single granule, called the arosomal vesicle. The acrosomal vesicle is rounded at first but then indents the nucleus, which is under androgen control. The Golgi apparatus contributes to the continual development of the acrosome until the acrosome density increases (72). During the last 14 days prior to spermiation, the shape of the spermatid head and the acrosome change through an unknown mechanism. The ventral acrosome separates from the main acrosome during spermiogenesis. This is a feature unique to the rat species, making classification of rat spermiogenesis possible by assigning steps and using these steps to classify cell associations into stages (66).

3. Nuclear shaping and condensation

The nucleus of the spermatid remains roughly spherical up to a certain point during spermiogenesis. Thereafter the rat sperm head becomes falciform (sickle-shaped). The cytoskeletal complex formed around the nucleus by a microtubule sleeve, the manchette, may attribute to the changes seen in the nuclear shape. It is these nuclear shape changes that are useful in the secondary classification of spermiogenesis into stages (66).

4. Cytoplasmic elimination

The elimination of cytoplasm enables the spermatid to reduce in size and become streamlined. A large portion of the cytoplasm is relocated to the region between the sperm head and the basement membrane (72). Prior to release, the spermatid volume is reduced by approximately 25% of its original size, thus enhancing propulsion through the fluid environment (66). The reduction of spermatid size occurs in three phases with the first phase being elimination of water from the nucleus and the cytoplasm during spermatid elongation. In the second phase just before sperm release, some cytoplasm is eliminated by minute structures referred to as tubulobulbar complexes. The third and final phase is characterized by separation of a cytoplasmic package, referred to as a residual body; which accounts for a 25% reduction in spermatid volume. The residual bodies contain packed ribonucleic acid (RNA) and other organelles and are phagocytized by the Sertoli cells and then transported to the base of the tubule where they are digested by the Sertoli cells (72). After cytoplasmic elimination a small amount of cytoplasm, the cytoplasmic droplet, is found around the neck of the spermatid (66).

5. Spermiation

The spermiation phase is initiated when the elongated spermatids move towards the seminiferous tubule lumen. The spermatids are positioned so that they can be released into the lumen. At the beginning of the spermiation phase, the general shape of the acrosome head and flagellum has been completed and only minor modifications occur prior to sperm release (66). The number of stages comprising spermatogenesis varies depending on the species. Spermatogenesis in the rat is comprised of 14 stages (I – XIV) whereas in the human male there are only 6 stages (I – VI) (73). A spermatogenic cycle is defined as the succession of stages through a period of time and in the human male that is 16 days. At least four spermatogenic cycles are required for the complete development from a Type A spermatogonium into mature sperm. In the rat, the duration of spermatogenesis is 51-53 days compared to the human male where the duration is a minimum of 64 days (66). Spermatogenesis is a highly hormone-dependent process that is

reliant on precise and correctly timed delivery of hormones, to ensure correct and timely maturation of spermatogonium into functional sperm cells (68).

## 2.5. Endocrine system

The role of the endocrine system is to maintain homeostasis by enabling the organism to adapt to changes in both the internal and external environments (1). It is a communication system that consists of cellular receptors and glands which secrete hormones into the organism's bloodstream (61). The endocrine system consists of closed-loop feedback mechanisms, where both positive and negative hormonal influences enable the maintenance of homeostasis (1).

Hormones play a pivotal role in the body as they affect, amongst others, cellular synthesis, secretion and mitosis, reproduction, embryonic differentiation and growth (61). Hormones are secreted by endocrine cells and can reach target cells in the same location by directly diffusing through the interstitial fluid that separates them (paracrine function). Hormones can also act on their cells and control their own secretion (autocrine function). Endocrine cells are found primarily in the pituitary, thyroid, adrenal and parathyroid glands, the pancreatic islets and the gonads (1).

The endocrine system ensures correct delivery of hormones to their target organs (14). These hormones can exert their action at low concentrations ($10^{-9}$ or $10^{-12}$ g/ml) in the bloodstream and bind to specific hormone receptors at target organ sites. This receptor bound state results in the initiation of a cascade of events that influence developmental, growth, regulatory, and homeostatic mechanisms throughout the body (74).

The endocrine system may function independently or in conjunction with the nervous system (60). The two signaling systems both have neurons and endocrine cells that act as hormones or as neurotransmitters. The endocrine system largely responds to a chemical stimulus and the nervous system to a physical stimulus, however there is potentially an overlap in the stimuli (14). A component of the endocrine system, the hypothalamic-pituitary-gonadal (HPG) axis (64), is the main regulator of spermatogenesis and reproduction (61).

The sensitivity of an organism to the action of hormones is influenced by the changes in the number of receptors, the receptor binding affinity, hormone degradation rate and receptor competition with natural and synthetic agonists and/or antagonists. Humans and rats have the same basic physiology and share similar hormonal control mechanisms (75, 76). Thus, rats are an ideal model to study the effects of exposure to compounds that may interfere with the action of endogenous hormones. There are differences in steroidogenesis between rats and humans, but in general the processes underlying male development are remarkably similar in both species.

### 2.5.1. Testosterone

Testosterone is a steroid hormone secreted primarily from the Leydig cells (Figure 4) in the testes (14). Testosterone is bound to the androgen-binding protein (ABP) that is produced by the Sertoli cells. The release of FSH enables the testosterone-ABP complex to cross the blood testis barrier (64).

Prenatally, testosterone is essential for genital virilization, midline fusion, scrotal thinning, phallic enlargement and prostate and seminal vesicle development in week 4-6 of human gestation. In the second trimester of pregnancy, testosterone is essential for gender identity in which the feminization or masculinization of the fetus is affected (77). In the testis, testosterone plays a vital physiological role and is essential for normal spermatogenesis. Testosterone therefore promotes the differentiation of spermatogonia (Figure 4) by stimulating genes within the Sertoli cells. Testosterone is mediated by the action of FSH (1, 61) through FSH receptors found within the Sertoli cells. FSH therefore influences androgen action indirectly through production of the Sertoli cell factors.

In addition, testosterone induces the formation of the peritubular cells (Figure 4) which express androgen receptors and upon testosterone stimulation produce the peritubular modifying substance that influences Sertoli cell factor secretion. Testosterone therefore directly targets the Sertoli cells, as well as directly modulating Leydig cell function and Leydig cell androgen receptor expression (Figure 4) (64, 78).

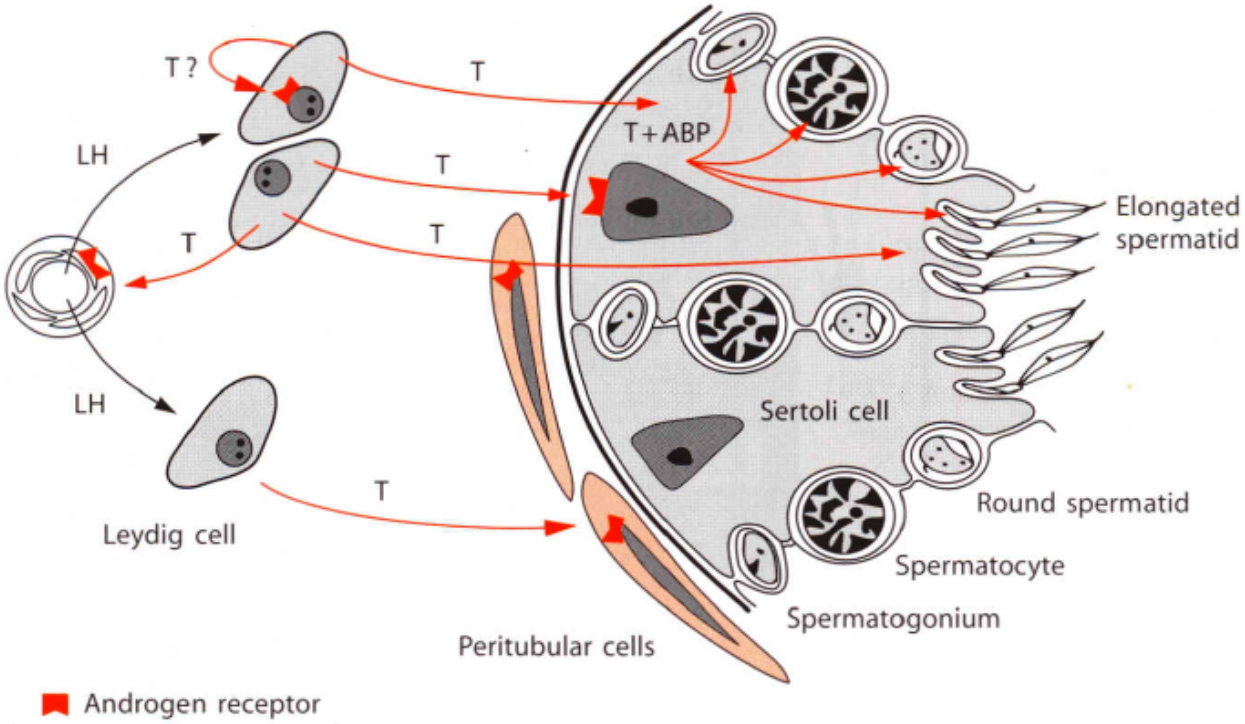


Figure 4: The interaction of testosterone with androgen receptors located on the Sertoli cells, in the Leydig cells and in the peritubular cells in the testis (64).

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### 2.5.2. Aromatization of testosterone

In the testes a small amount of testosterone is converted into the potent androgen dihydrotestosterone (DHT) by the enzyme 5α-reductase (Figure 5) (1, 78). Although testosterone is crucial for male reproduction, an essential step in the synthesis of steroid hormones in the testis is the aromatization of testosterone (71). Conversion of androgens to estrogens (specifically, conversion of androstenedione to estrone and testosterone to estradiol) in the last step of estrogen biosynthesis is mediated by aromatase (Figure 5) (1).

Aromatase is a microsomal enzymatic complex composed of the glycoprotein cytochrome p450 aromatase (p450arom) and a ubiquitous reductase. In the mammalian testis aromatase is mainly localized in the Leydig cells (68). In the rat testis p450arom has been immunolocalized in the Leydig cells, the germ cells and especially in the elongated spermatids (79). There is a 2-4 fold greater aromatase activity in the spermatozoa compared to the younger germ cells (80).

Aromatase is regarded as a potential EDC target since variation in its expression and thus its function can disrupt the estrogen production rate. This leads to changes in estrogen levels resulting in disruption of estrogen-affected processes (81). Aromatase and ERs localization during various stages of murine germ cell development suggest that there is a possibility of direct estrogenic action on the germ cells (67, 82). Chemical disruption of any part of the process of steroidogenesis, either by androgen inhibition or estrogenic stimulation, may compromise the progression of hormonal dependent processes such as spermatogenesis (67, 83).

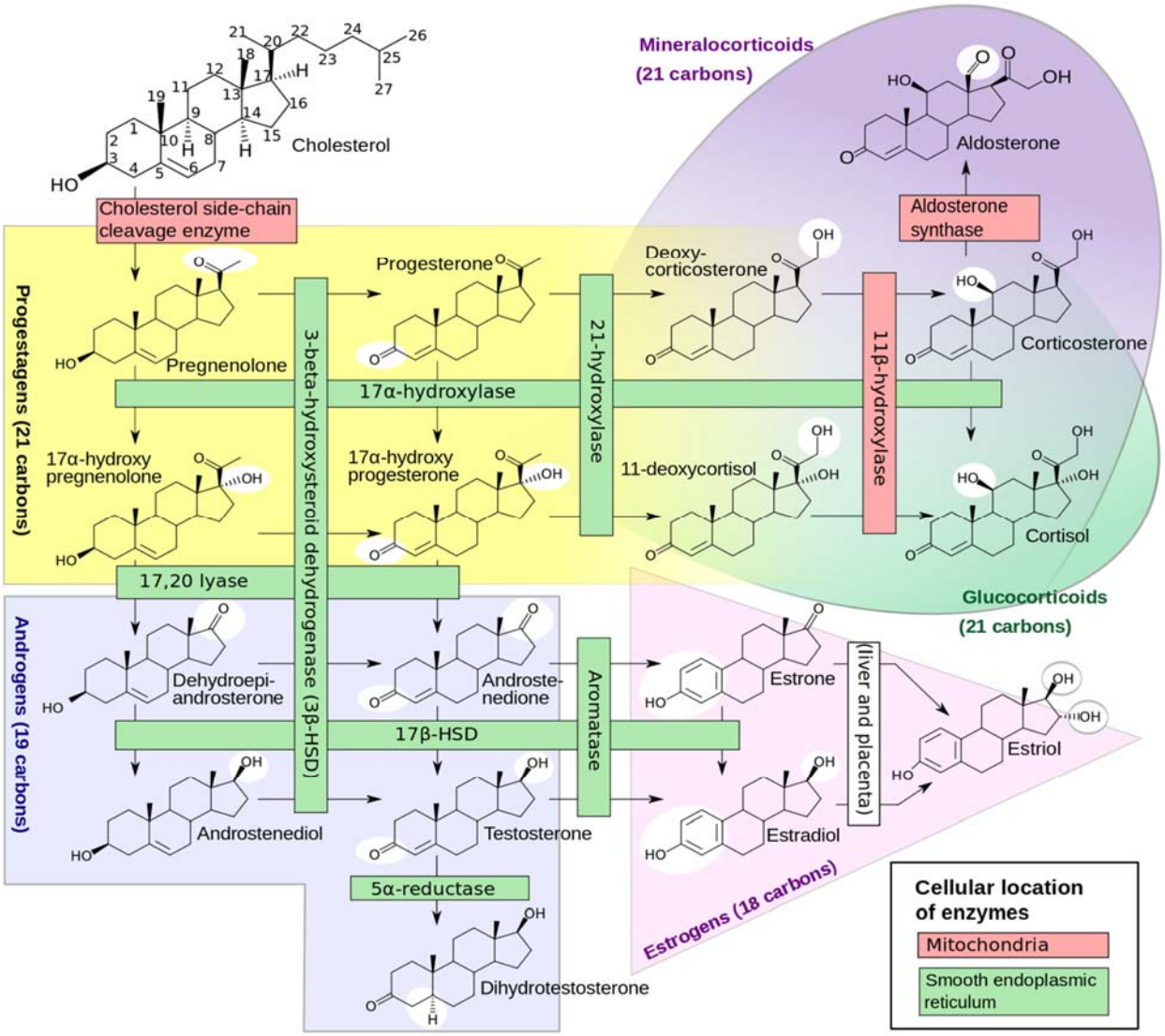


Figure 5: The steroid hormone biogenesis pathways indicating the main steroid hormone classes, the individual steroids, the enzymatic pathways and the cellular location of these enzymes (78).

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### 2.5.3. Estrogen

The aromatization of testosterone produces the hormone estrogen (Figure 5) (1, 74). The steroidal hormone estrogen is involved in the control of reproductive processes including sexual differentiation, maturation, control of the cell cycle and proliferation. Estrogens have been associated with the regulation of female bodily functions, but they are also produced in male vertebrates. ER activity is expressed throughout the HPG axis and the testes of a variety of vertebrate species (61).

There are two types of estrogen receptors, namely Estrogen Receptor Alpha (ERα) and Estrogen Receptor Beta (ERβ). The presence of both subtypes has been isolated in immature human mammalian germ cells and in ejaculated spermatozoa (82). In the rodent model, ERα and ERβ were localized in the fetal and adult Leydig cells. This suggests that estrogens take part in the regulation of testicular function (68, 74, 84).

Studies have demonstrated that germ cells are an important source of estrogens as the germ cells are also a site for aromatization of testosterone (68, 85). The number of gonocytes, as well as the maturation of spermatids have been shown to be under the control of estrogens in the rat (82). Administration of estrogen to neonatal rats increases the number of spermatogonia at 16 days of age (85). Estrogens also play a vital role in the final phase of spermatid differentiation. In the adult monkeys, treatment with an aromatase inhibitor impaired spermatid differentiation, showing that estrogen is essential for spermatid differentiation (86).

The role of estrogen hormone involvement in male reproductive tract development and function has been intensively investigated (85, 87-89). These include localization of aromatase and estrogen target sites in the reproductive tract, analysis of testicular phenotypes in aromatase and ERα and ERβ transgenic deficient mice, and investigation of the effect environmental chemicals have on male reproduction.

## 2.6. Endocrine disrupting chemicals

The International Programme for Chemical Safety (IPCS) in 2002 (90) defined an endocrine disrupting chemical as:

"*An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub) populations."*

Compounds known as EDCs exhibit the potential to interfere with the normal functioning of the endocrine system by mimicking, inhibiting or enhancing the actions of endogenous hormones, resulting in adverse health effects (15, 91). The term adverse health effect is defined by the Federal Institute for Risk Assessment (92) as:

"*A change in morphology, physiology, growth, reproduction, development or lifespan of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increased susceptibility to the harmful effects of other environmental influences*"

In 2012, the World Health Organization/United Nations Environmental Program (WHO/UNEP) report, endorsed the 2002 IPCS definition of EDCs for use in their evaluation (3). EDCs promote adverse health effects by potentially interfering with hormone synthesis, subsequent hormonal secretion, storage, release, transport, binding, efficacy and gene expression (9, 10, 15, 93-105). Natural estrogens bind to ER with a high affinity and specificity, while EDCs that mimic hormones, bind to the ER with a lower affinity (106). Natural androgens, such as testosterone, bind to ABP. This complex is then transported in the blood bound with a high affinity to SHBG.

However, exogenous estrogens do not bind to SHBG or ABP, but rather circulate in the unbound form, thus enabling a greater hormonal response (107). The interaction of these exogenous estrogens with androgen receptors may result in the antagonization of endogenous androgens whilst interaction with estrogen receptors may result in mediation of cellular actions of hormones. This mimicking potential enables the compound to interfere with the synthesis, secretion and the action of estrogen (92).

Known EDCs include various alkylphenols, dioxins and furans, organochlorine pesticides, pharmaceuticals, phthalates, phytoestrogens and polychlorinated biphenyls (PCBs) (3, 16).

### 2.6.1. Organochlorine pesticides

#### 2.6.1.1. DDT and metabolites

The organochlorine pesticide DDT was widely used as an insecticide for agricultural purposes and is currently used for IRS in malaria vector control programs (22, 108). Technical grade DDT consisting of 65–80% of the active insecticidal ingredient *p,p'*-DDT and 15–21% of the less insecticidal *o,p'*-DDT (17, 21) is used for IRS. The *o,p'*-DDT, and to a lesser extent *p,p'*-DDT component, have estrogenic properties (21).

The parent compound, DDT, is metabolized in the liver into two direct metabolites, namely 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (*p'p*-DDE) and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane (*p'p*-DDD) (Figure 6). The metabolite DDD is further metabolized by dechlorination to 1-chloro-2,2-bis(*p*-chlorophenyl)ethane (DDMU). DDMU is metabolized by dechlorination and oxidation to form bis(*p*-chlorophenyl) acetic acid (DDA) (Figure 6) (22). The rate of DDT metabolism in rodents is higher than that in humans. DDT and DDE have half-lives of 5 years (109) and 8.6 years (110) respectively in humans. In rodents the half-lives of both DDT and DDE are 120 days (111). Regardless of the difference in the rate of metabolism the metabolic pathways of DDT are expected to be similar in both humans and rodents with DDT being cleared more rapidly than DDE (22).

The metabolism of DDT primarily occurs in the liver as higher concentrations of DDD are found in the liver compared to other tissues in the body (112). The metabolism of DDD is rapid and it is dechlorinated to produce DDMU and DDA. The metabolite DDA is sufficiently soluble in water and is excreted in the urine (113).The metabolism of DDE is slower resulting in a longer half-life than DDT, therefore increasing the potential to elicit a hormonal effect with long term exposure.

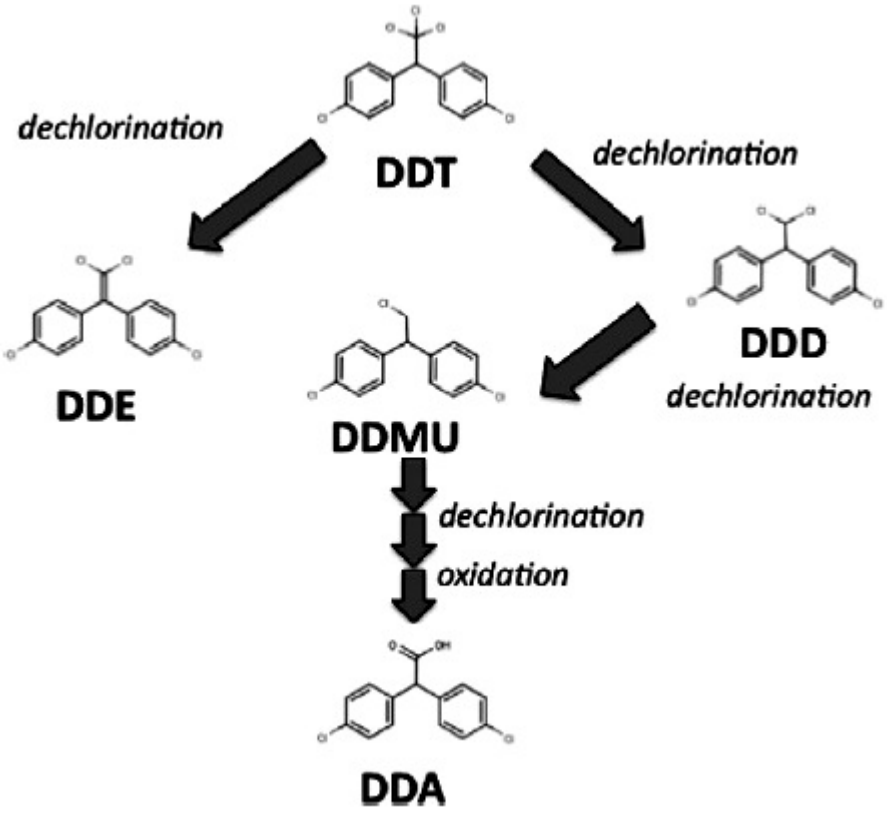


Figure 6: The metabolic pathway of DDT in the liver. The main metabolites of DDT, DDE and DDD are formed after dechlorination. DDD is further metabolized by dechlorination to form DDMU. DDMU is metabolized by dechlorination and oxidation to form DDA (22).

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In exposure based studies the DDA measurement is non-specific as DDD exposure is not generally expected (22).

In addition to possessing hormonal properties, both DDT and DDE are lipid soluble. This characteristic allows DDT and DDE to be deposited in the fat tissues of organisms (22). Bio-concentration occurs over the period of exposure. The DDT and DDE from the surrounding media are stored in the fat of the organism at higher levels than found in the surrounding environment. During this period, the potential exists for DDT to elicit its hormonal mimicking properties. This occurs either directly or indirectly through its breakdown into metabolites (21). Steroidogenesis requires the breakdown of fat stores in order for hormonal synthesis to take place (65). During fat breakdown DDT and its metabolites are released into the blood stream.

DDT is estrogenic and possesses the ability to interfere with the natural binding of estradiol to the ERs. In contrast, DDE has anti-androgenic properties; thereby inhibiting the action of natural androgens and enhancing the effects of estrogen. The altered ERs binding affinity alters endocrine system functioning. This results in adverse effects such as developmental defects and possible cancers in the exposed organism (114).

The Pine River Statement on DDT, which reviewed 494 studies published between 2003 and 2008, concluded that ”DDT and its breakdown product DDE may be associated with adverse health outcomes such as breast cancer, diabetes, decreased semen quality, spontaneous abortion and impaired neurodevelopment” (52). Male rats exposed to 50 and 100mg/kg/day body weight DDT for 10 consecutive days led to a dose-dependent reduction in testicular weight, as well as percentage motile spermatozoa in the epididymis (115). Dosing male rats with a range of DDE concentrations (5, 16, 50 and 160mg/kg/day) for a period of 10 days showed a dose-dependent increase in liver and kidney weights (116).

Exposure to DDT and DDE has been shown to have adverse reproductive health effects. A greater concern is that exposure is still on-going, as there are areas with current use of DDT for malaria vector control (117). A concerted effort should be made to ensure safe use of the pesticide as well as continue to find safer, yet effective alternatives. Alternatives currently being investigated are

chemoprophylaxis, ecosystem compatible predators, bacterial larvicides, mosquito nets, screens and wall linings (118).

### 2.6.2. Pyrethrins and pyrethroids

Pyrethrins are semi-synthetic compounds derived from chrysanthemumic acid of the flowers of *Chrysanthemum cinerariifolium.* Pyrethrins have an anti-parasitic effect against larval and mature forms of numerous parasites (119). Pyrethroids were chosen for parasitic control as they have low toxicity in humans, even in an occupationally-exposed setting (120). Since the 1970s the use of documented pyrethroid insecticides has been increasing and there was speculation that pyrethroid insecticides could replace organophosphorous insecticides (117). Pyrethroids are divided into two subtypes. Type I, also known as T-syndrome pyrethroids which characteristically lack the alphacyano substituent. Type II, also known as CS-syndrome pyrethroids, contains an alphacyanophenoxybenzyl substituent.

Pyrethroid insecticides are suspected EDCs; however in most instances the mechanism of action remains unclear. Some pyrethroids have reported estrogenic activity (121) while others have anti-estrogenic (122) and anti-androgenic activities (123).

#### 2.6.2.1. Deltamethrin

Deltamethrin (DM) is currently in use as the active substance in ITNs (124, 125) for use in malaria vector control. The use of pyrethroids as part of the IRS program has been supported by the WHO as an alternative to the use of pesticides such as DDT for malaria vector control. The use of DM also spans into agriculture, as well as public health preservation programs for farm animals (125-127). DM has been extensively used as an ecto-parasiticide in animals and has also been utilized as an

insecticide in crop production (128). There are reports of resistance to pyrethroids, which is great concern for malaria vector control programs (129).

Researchers using animal models investigated the effect that acute and chronic DM exposure may have on the overall and specifically the reproductive health of the animal (37, 119). DM has been reported to have weak estrogenic activity in bioassays. After oral administration, DM is rapidly absorbed and excreted within 24hrs of initial dosing in rats, with an average of 33% in urine and 44% in feces. Rapid and extensive metabolism in rats results in cleavage of the ester bond and oxidation of the $4^{th}$ C bond of the phenoxy ring of the alcohol moiety. It is these moieties that are further metabolized and the residues are then stored in the skin, stomach and fat tissue. DM is suggested to be less toxic in aqueous solution as opposed to oil based solutions (130).

Mice, rats and dogs exposed to DM for 13 weeks showed exposure-related clinical signs such as salivation, tremors, impaired locomotor activity and changes in bodyweight were noted. The lowest no observed adverse effect level (NOAEL) of short-term DM exposure was 1mg/kg bw/day; these were found to be similar in both rats and dogs (130, 131). Pregnant female rats treated with DM from day 6 to day 15 of pregnancy resulted in growth retardation, increase placental weight, dilation of the renal pelvis, as well as hypoplasia of the lungs of the female rats (132). Adult male rats treated for 3 days a week for 6 weeks to 0.6mg/kg DM showed a lower body and testes weights, as well as decreases in sperm counts and motility (119). These results suggest that the *in utero* exposure to DM induces changes in the male offspring and adult male reproductive physiology.

### 2.6.3. Alkylphenols polyethoxylates

Alkylphenol polyethoxylates (APEOs) are non-ionic surfactants that consist of a branched-chain alkylphenol reacted with ethylene oxide resulting in an ethoxylate chain. After its introduction in the early 1940's APEOs have become common constituents in the formulation of a variety of paints, lubricants, resins, detergents

and pesticides (133). As of 2009, approximately 500000 tons of APEOs was produced annually (134).

#### 2.6.3.1. Nonylphenol

A degradation product of APEO, *para*-nonylphenol (*p*-NP) is widely used in industry as a constituent of resins, paints, cosmetics, lubrication oil, plasticizers, detergents and as a carrier of insecticides. In the food processing and packing industry, *p*-NP is found in PVC in varying concentrations (133). *p*-NP has a 3 times higher estrogenic activity than DDT and has been associated with wildlife reproductive abnormalities, as well as deformities. *p*-NP is approximately 7000 times less potent then the estrogenic hormone 17β-estradiol (135, 136). The main source of *p*-NP exposure is commonly linked to effluent from sewage treatment plants being in close proximity to urban or industrialized areas. There have been numerous reports worldwide of *p*-NP found in rivers in the USA (137), Europe (138), Asia (139) and South Africa (29).

The estrogenicity of *p*-NP has been documented since the 1930s. However, the health and environmental implications were only realized in the 1990s when Soto *et al* (1991) demonstrated that *p*-NP elicits an effect on cultured human breast cells (35). Human breast cells proliferate in the presence of estrogen and exposure to *p*-NP produced a similar effect. Bioaccumulation of *p*-NP was shown to be higher in aquatic organisms in surface waters, particularly in close vicinity to industrial effluent plants (134). Fish from these polluted rivers had concentrations of alkylphenolic compounds within their organs that were significantly higher than that of animals in the surrounding environment (140, 141). Male rainbow trout exposed to 30µg/L *p*-NP showed a marked reduction in testicular weight in one study (140), whilst another study found male fish producing the female egg yolk protein, vitellogenin, at the same exposure levels of *p*-NP (141).

Adult male rats exposed to various concentrations of *p*-NP caused impaired seminiferous tubule histology, epididymal toxicity, impaired testicular mass and sperm count, particularly at the higher concentrations of *p*-NP (36). Maternal exposure of rats to *p*-NP resulted in an overall lowered sperm count and a decrease

in testicular and epididymal mass with increasing *p*-NP concentrations. These findings indicate that the testes and epididymis being the target sites for *p*-NP exposure (142).

### 2.6.4. Phytoestrogens

Plant-derived chemicals that are present in the environment such as mycotoxins and phytoestrogens have the ability to mimic estrogen (143). There are three main classes of phytoestrogens, namely: coumentans, isoflavones and ligans. Although phytoestrogens are weaker than natural estrogens, at least 20 have been identified in approximately 300 plants ranging from vegetables, fruits, herbs and coffee (10, 144). In the Western diet, soy-based food does not constitute a large proportion of the staple diet as in the Asian diet. Where a typical Western diet comprises 0.15-3mg/day, a typical Asian diet is comprised of 20-50mg/day. In SA, in the Vhembe district, the diet is high in plant based foods, such as maize and beans (30). In addition to their diets, one also has to account for genetic, environmental and lifestyle variables. Epidemiological studies have found the phytoestrogen levels in Asian woman to be much higher than those of women in western population, although some studies have also found no proven protective relationship between a high soy-based diet and a reduction in breast cancer risk in Chinese, Japanese and various multi-ethnic American women investigated (145).

#### 2.6.4.1. Coumesterol

Coumesterol forms part of the Coumestan family of phytoestrogens and is generally found in soybean sprouts, sunflower oil and alfalfa (41). Compared to isoflavones, coumestrol has a 30-100 times higher potency level and a greater binding affinity for both ERα and ERβ (146). Researchers investigating the estrogenic activities of coumestrol in competition binding assays with human estrogen receptor α or β protein, found that coumestrol binds as strongly as 17β-estradiol to both ERα and ERβ (147). Using a human cervical cancer cell model, exposure to 20 - 120nM coumestrol resulted in inhibition of cell cycle growth and induction of cell death, leading to cytotoxcity; a similar result observed with 17β-estradiol exposure (148).

Similarly, in a recent study assessing the effects of coumestrol administration to maternal mice during pregnancy and lactation, a dose of 200µg/kg body weight showed no effect on body weight gains, in either male or female neonatal mice (146).

#### 2.6.4.2. Genistein

Genistein (4',5,7-trihydoxyisoflavone) is a naturally occurring phytoestrogen which is found in a wide variety of plant-derived foods, particularly in soy-beans and soy-based foods (143). Genistein belongs to the class of phytoestrogens known as isoflavones, which even though found mainly in soybeans, are also bioavailable in legumes and alfalfa sprouts (149).

Given its biphenolic nature, genistein binds to the ERs and may mimic or alter the endogenous action of estrogens. Genistein may act as an ER agonist or antagonist eliciting a hormonal response. Genistein has a greater binding affinity for the ERβ compared to ERα, with a 20-30 fold stronger affinity for ERβ. The biological activity of genistein is therefore attributed to its competitive binding to ERs (150).

Genistein inhibits cell growth and proliferation pathways thereby effecting multiple organ systems (151). Genistein inhibits the activity of protein tyrosine kinases in numerous tissues including breast cancer cells by catalyzing the phosphorylation of growth factors involved in tumor cell proliferation (143). Exposure to genistein concentrations between 0.001-10µM stimulates growth of breast tumor cells, suggesting a proliferative effect of genistein. However, at concentrations greater than 10µM, inhibition of the breast tumor cell growth are reported, indicating a potential chemo-preventative effect (149). Administration of genistein (0, 20, 150, 1000mg/kg/day) to pregnant female rats from day 6 of pregnancy to day 12 resulted in a decrease in body weight was noted as well as increased pup mortality (150). A recent study investigated the effects of genistein on the prostate cancer cell epigenome and showed that genistein has an effect on the androgen signaling pathway (152).

### 2.6.4.3. Zearalenone

Zearalenone (ZEN) is a non-steroidal mycotoxin that is commonly found in maize, wheat, barley and rye. It is produced by various *Fusarium* species such as *F. germinearum and F. verticilliodes* and is considered a non-steroidal estrogenic mycotoxin (153). The Fusarium species generally invade and grow on crops in moist cool field conditions but have also been shown to affect crops in poor storage conditions (154). Tropical areas, such as in the Vhembe district in SA, provides the ideal conditions for ZEN to invade crops. The maize in the Vhembe district is poorly stored due to improper infrastructure and lack of adequate farming knowledge (31).

Studies have shown that ZEN is not easily degraded by common food processing procedures (155). ZEN is rapidly absorbed following oral administration and is metabolized in the intestinal cells (156). In the metabolism of ZEN both biliary and entero-hepatic cycling are important processes. ZEN is excreted in large amounts in the bile, it enters the liver and ultimately the systemic circulation via the portal blood supply (45, 157).

Two ZEN biotransformation pathways have been hypothesized in animals: hydroxylation and conjugation. Hydroxylation results in the formation of ZEN metabolites alpha-Zen (α-ZEN) and beta-ZEN (β-ZEN) which are thought to be catalyzed by the enzymes 3α - and 3β -hydroxysteroid dehydrogenase (HSDs). Conjugation of ZEN and its metabolites, with the aid of glucuronic acid, is catalyzed by the enzyme uridine diphosphate glucuronyltransferase (UDPGT) (158). The metabolites α-ZEN and β-ZEN have the ability to adopt a conformation that resembles that of 17β-estradiol. The similarity is only in structure but with a limited binding affinity to SHBG. This resemblance allows direct access to estrogenic receptors on target cells and estrogenic agonist action. The metabolites have been found to exhibit a 50 times higher potential estrogen potency than their actual concentrations suggest (159, 160).

In 2000, the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) established a provisional maximum tolerable intake of 0.5μg/kg bodyweight ZEN (156, 161). However, it has been shown that ZEN exposure causes

hepatocellular adenomas, decreased sperm counts, increased embryo resorptions, abnormal fetal growth and reduced litter size (45, 161, 162).

Rats fed ZEN-contaminated food produced the estrogenic syndrome with clinical hyper-estrogenism and infertility (162). The same pattern of adverse reproductive effects were observed when ZEN was administrated to mature gilts (163), particularly the effect on the development of the embryo. Female Sprague-Dawley rats exposed to ZEN at the pre-prepubertal development stage resulted in early vaginal opening, irregular estrous cycles and anovulatory ovaries (164).

## 2.7. Environmental mixtures

Humans and wildlife are typically exposed to multi-component chemical mixtures, present in the surrounding environmental media (water, air, soil), in food or in consumer products (165). The Center for Disease Control and prevention (CDC) in the USA released a comprehensive report of 212 chemicals present in the environment. These chemicals included lead, phenols and organic compounds, which were measured in blood and urine collected from study participants (166). Exposure to a single chemical may have no observed effects, but exposure to several of those chemicals in a mixture, due to synergistic or additive effects, may be significant (55). A chemical mixture is defined as any set of multiple identifiable or non-identifiable chemicals, regardless of source, potentially contributing to joint toxicity in a target population (167). Even though the chemical burden varies among individuals, the combination of chemicals makes toxicity assessment difficult.

Few reproductive toxicology studies have been conducted using mixtures of chemicals with diverse modes of action (168). These studies indicated that the adverse health effects caused by exposure to such mixtures is greater than those of the individual chemicals (55). Consensus has been reached that customary chemical-by-chemical approach to risk assessment, in the field of mixture toxicology, may underestimate the risk that these chemicals may have on human and environment health (165).

Chemical mixtures in the environment are comprised of multiple components from numerous sources, often with varying modes of action. Studies assessing the effect

of such mixtures are scarce in the scientific literature (167, 169). Thus, evidence is needed on the action and effects of exposure to mixtures of compounds found in the environment that are within the range of human exposure. Whether exposure occurs to an isolated chemical or to a complex mixture of chemicals, studies mentioned above indicate not only general adverse health effects, but also adverse reproductive health effects due to the hormonal impact these EDCs have on the body.

## 2.8. Effects of EDC exposure

### 2.8.1. Male reproductive health

The effects of exposure to EDCs on male reproductive health is of great concern, as reproductive disorders are becoming increasingly frequent, resulting in individuals with compromised reproductive potential (13). *In utero* exposure to EDCs, particularly exposure during the male programming window affects fetal growth. It is during the male programming window that the fetal testis first forms and initiates testosterone production. The action of androgens, such as testosterone, during this programming window is essential for the development of the both reproductive tract and the external genitalia (170). There are a host of adverse male reproductive health disorders and conditions that have been attributed to altered hormonal balance. Amongst others, reproductive disorders such as cryptorchidism, hypospadias, reduced sperm counts and testicular germ cell tumors, or collectively known as the testicular dysgenesis syndrome (TDS) (Figure 7) will be discussed below (13, 171).

#### 2.8.1.1. Testicular dysgenesis syndrome

Hypospadias, cryptorchidism, poor semen quality and germ cell tumors are usually investigated in unison instead of in isolation. It is suggested that these four disorders share a common etiological origin and collectively they constitute the syndrome termed TDS (92). The TDS hypothesis (Figure 7) suggests that diminished androgen action during fetal life negatively impacts the Sertoli and Leydig cells. Thus, altering the support of the germ cells and the androgen synthesis required for

spermatogenesis. The TDS hypothesis proposes that environmental components and chemical exposures are major etiological factors (171).

Chemical exposure has been linked to lowered testosterone levels. Lower testosterone levels interfere with steroid hormone uptake into the fetal Leydig cell, impeding steroid synthesis (172). Lowered testosterone levels decrease the production of DHT, which is essential for male reproductive tract development as the hormone DHT (Figure 5). In addition, in male rats, DHT is required for perineum growth in order to produce a normal AGD. AGD is a sensitive marker for prenatal disruption of the development of the male reproductive system (92, 173). The AGD is the distance from the anus to the testes in males and the distance from the vaginal opening to the anus in females. In males the AGD is longer than in females (92).

Fetal rats exposed to anti-androgen chemicals, dibutyl-di-(2-ethylhexyl) and butyl benzyl phthalates, show a decrease in the AGD of males rats due to reduced DHT levels as result suppressed testosterone levels (174). AGD is therefore an important biomarker of diminished androgen action and is proven useful in animal reproductive toxicity studies (37, 150, 175). The balance in action between androgens and estrogens may be of crucial in determining normal and/or abnormal male reproductive tract development

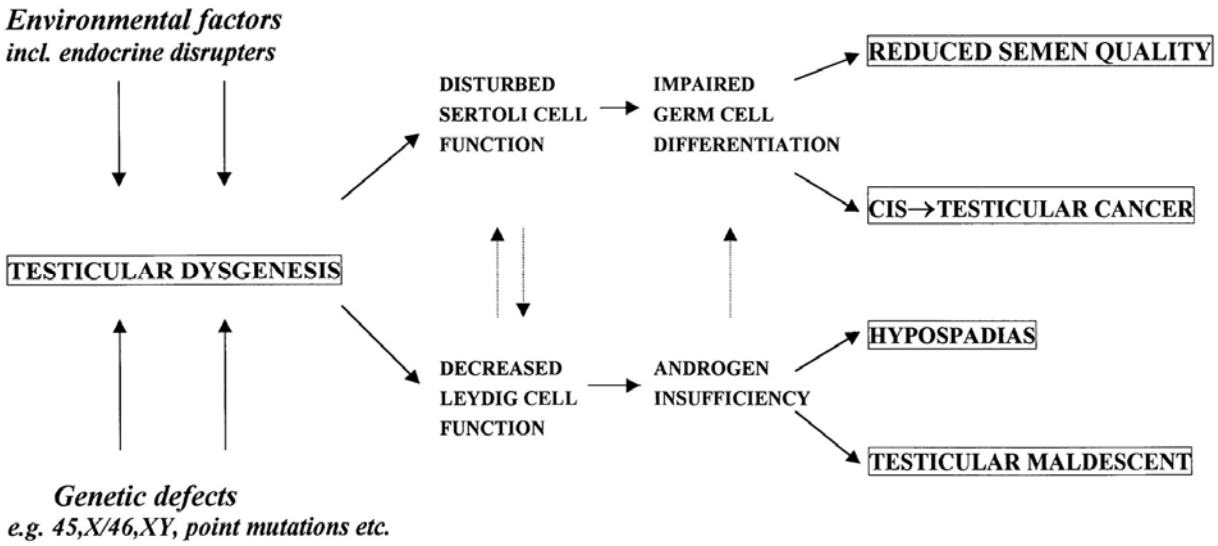


Figure 7: The environmental and genetic factors contributing to the testicular dysgenesis syndrome (TDS) and the resultant clinical representation manifesting as reduced semen quality, carcinoma *in situ*, hypospadias and cryptorchidism (171).

Skakebæk NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: an increasingly common development disorder with environmental aspects. Human Reproduction, 2001;16(5):974, by permission of Oxford University Press.

#### 2.8.1.2. Hypospadias

The term hypospadias refers to the abnormal positioning of the urethral opening. Androgen action during fetal life is vital to ensure the correct location and opening of the urethra at the tip of the penis. The alteration in the action of androgen results in the urethral opening occurring on the underside of the glans penis, i.e. mild (glandular hypospadias) or in severe cases the urethral opening is positioned on the penile shaft or even in close proximity to the scrotal sac (92). Hypospadias are one of the most common congenital malformations occurring in male fetuses between gestational weeks 8–16 (176) and are considered a complex disorder involving both genetic and environmental contributors (177). The relationship between chemical exposure and hypospadias has been investigated. EDCs have been suggested to disrupt the hormonal balance of the fetus and thereby disturb sexual differentiation either by an estrogenic or an anti-androgenic effect (2). Intrauterine growth restriction is a major risk factor for hypospadias suggesting this association may be due to androgen deficiency in early pregnancy (57). However, limited evidence suggests that there is a slight increased risk of hypospadias associated with exposure to mixtures of EDCs (3). In a case–control study of serum samples collected in early pregnancy among 237 women giving birth to boys with hypospadias and 237 controls, it was demonstrated that high serum levels of *p,p*'-DDE) (>1.0ng/ml) had an OR of 1.69 for hypospadias (178).

#### 2.8.1.3. Cryptorchidism

One of the most common congenital malformations in male babies at birth is cryptorchidism (176, 179). There are two phases to testicular descent, with the first being the trans-abdominal phase. In this phase, during early gestation, the fetal testis migrates to the pelvis from its point of origin near the kidneys. The second phase is the trans-inguinal phase. This phase occurs towards the end of gestation and the testis descends from the pelvis into the scrotum. The trans-inguinal phase is androgen-dependent and thus disruption of this phase is the common cause of cryptorchidism at birth (92).

Although the etiology of cryptorchidism is partly unknown, genetic and environmental factors that act as endocrine disruptors of testicular descent, may contribute to the incidence of the birth defect, in the recent years (180).

The relationship between chemical exposure and cryptorchidism has been investigated. In mothers who have experienced high DDT exposures, relationships between maternal serum levels of DDT/DDE and cryptorchidism in their male children have been found (181). A correlation between mothers with high DDE level in their breast milk and cryptorchidism in their male children have also been found (182).

#### 2.8.1.4. Reduced semen quality

Semen quality is determined by sperm counts, concentration, motility and ejaculation volume (183). These parameters are known to be variable and are affected by abstinence, season, clothing, drug abuse and changes in testosterone levels (92, 184-188). During fetal life androgen action is essential for Sertoli cell proliferation. Only a given number of Sertoli cells can sustain a limited number of germ cells - critically determining sperm counts. Lowered androgen action through endocrine disruption impeded testosterone action, which negatively impacts semen quality in adulthood (189).

A study in SA found reduced seminal parameters in healthy males in an area that is currently sprayed with DDT for malaria vector control. The study sampled n = 311 healthy males (18-40 yrs of age) and found a significant positive association between lipid adjusted levels of *p,p*'-DDT and *p,p*'-DDE with the participants presenting with asthenozoopermia (reduced sperm motility - 32% of total n) (6). Ejaculate volumes (mean 1.9 ± 1.33ml) were found to be lower than the WHO recommended volume of ≥ 2.0ml (190).

Countries with men exhibiting poor semen quality also exhibit a higher prevalence of cryptorchidism and hypospadias (191). These three reproductive endpoints, poor semen quality, cryptorchidism and hypospadias all correlate with testicular germ cell tumors (TGCTs) incidence. In fact the three reproductive disorders are risk factors for each other and they are all predicative risks for germ cell cancers (92).

#### 2.8.1.5. Testicular germ cell tumors

There has been a worldwide progressive increase in the incidence of TGCTs over the last six decades particularly in European countries (192). The most frequent malignancies found in young adults and adolescents are TGCTs. In young men, TGCTs arise from precursor cells referred to as carcinoma *in situ* (CIS) cells, which originate during fetal life (193). Additionally, the endocrine-regulated timing of puberty is thought to be under the influence of EDCs. The early onset of puberty in boys has been related to the subsequent risk of testicular cancer (194, 195).

The increase in the incidence of TGCTs has been attributed to several risk factors including cryptorchidism, environmental toxins, lifestyle changes, familial predisposition and the microenvironment of the testis (13, 196). Geographical differences and ethnic differences account for the major variation in TCGT incidence patterns. The difference seen among ethnic groups in a North American study suggests that there are genetic factors that contribute to TCGT incidence particularly in similar lifestyle conditions (197).

Environmental toxicants such as the DDT metabolite *p,p'*-DDE and PVC (of which *p*-NP is a constituent) have been classified as intermediate contributors towards TCGT development (198). The incidence of TCGTs is more prevalent in Western and Northern Europe in comparison with Asian and African Countries (199). In a small case-control Norwegian study (n = 49 cases; 51 controls) it was found that the risk of developing TCGTs weakly correlated with plasma levels of *p,p'*-DDE (200), whereas a larger study (n = 754 cases; 928 controls) indicated that men with TCGTs presented with higher plasma levels of *p,p'*-DDE (201). Suggesting a possible association of *p,p'*-DDE with TCGTs incidence.

#### 2.8.1.6. Prostate cancer

The prostate is an exocrine gland of the male reproductive system. The prostate plays a pivotal role in reproduction by secreting an alkaline fluid that constitutes approximately 30% of semen volume along with the spermatozoa and seminal vesicle fluid (202). The development of the prostate is dependent on the action of

androgen, but is modulated by estrogen (203). In humans, during the third trimester of pregnancy the androgen levels decline and the maternal estrogen levels rise, inducing stromal and the epithelial cell differentiation (204). This differentiation is directly influenced by both estrogens and androgens. Thus, it is plausible that exposure to both endogenous and exogenous estrogenic and/or anti-androgenic compounds could interfere with prostate growth.

Chronically elevated estrogen levels in men have been associated with increased prostate cancer risk (205). In rats, it has been suggested that estrogens, in combination with androgens, induce prostate cancer (206). The prostate gland may be affected by exposure to EDCs through anti-androgenic pathways. Since prostate cancer is an androgen-dependent disease, chemicals possessing EDC activity may contribute to prostate cancer development.

Exposure to Bisphenol A (BPA) has been associated with prostate cancer (207) and prostate hyperplasia (24) in the rodent model. The link between prostate cancer and environmental factors, excluding dietary exposures, arises from the association of increased prostate cancer rates in occupationally exposed farm workers (227). Diethylstilbestrol (DES) exposure to the fetus during gestation has been shown to induce prostatic squamous metaplasia is human male offspring (243). However, no association between in utero DES exposure and development of prostate cancer has been demonstrated (245). In the rat model, DES exposure during in utero has shown prostatic hyperplasia and increased susceptibility to develop cancer (248). Low-dose fetal exposure to BPA or DES therefore resulted in prostatic hyperplasia in adulthood, which is an effect associated with increased levels of prostatic ARs. Although exposure to DDT and DDE has been shown to negatively affect the male reproductive system, no association has been made between DDT and DDE exposure and prostate cancer risk (277). Moreover, the additive or synergistic effects of exposure to complex mixtures of EDCs and prostate cancer risk and development are unknown (91).

Globally, prostate cancer is one of the most common male cancers. Prostate cancer incidence is the highest in Scandinavian countries (22 cases per 100,000 people) and the lowest in Asia (5 per 100,000 people). However, little information exists on prostate cancer in Africa (208). In SA, according to pathology based data from the

National Cancer Registry (1986-2006) and mortality data from Statistics SA (1997-2009), prostate cancer was the most common male cancer in all the SA population groups (208).

#### 2.8.1.7. Cellular death

The process of apoptosis is a natural mechanism that maintains the optimal cell number and cell proliferative rates throughout the body. Apoptosis plays a pivotal role in the developing human fetus, including the removal of inter-digital webs during limb development (209). This tightly regulated process eliminates extraneous or damaged cells ensuring optimal cell viability (210).

The term apoptosis is derived from the Greek words *apo* meaning “apart” and *ptosis* meaning “fallen” which is used to describe the shedding of leaves from a tree (211). Apoptosis is a form of programmed cell death (PCD) whereby the cell activates an inherent suicide mechanism that ultimately eliminates the cell in an ordered fashion (212). Apoptosis is marked by cellular shrinking, condensation and blebbing of the plasma membrane with the cell eventually breaking up forming apoptotic bodies. These apoptotic bodies pinch off from the cell, in a similar fashion as leaves shedding from a tree (211, 213).

In addition to apoptosis there are other forms of cellular death (Table 1), namely oncosis, autophagy and mitotic catastrophe (211, 214). Apoptosis differs from oncosis, autophagy and mitotic catastrophe (Table 1). Apoptosis is an active process where the cell nucleus remains intact, allowing the cell to expend energy and maintain control of its demise (47). Oncosis is characterized by cellular swelling, which leads to nuclear and cellular breakdown. This results in inflammation and leakage of the cytosol into the areas surrounding the cell leading to necrosis. Oncosis is therefore a passive process where the organelles of the cell are completely ruptured (213). Autophagy (Table 1) is regulated by environmental cues and is characterized by bulk degradation or recycling of damaged or dysfunctional cellular components (215). The cellular components are then encapsulated in autophagic vesicles. These vesicles fuse with lysosomes and/or other vacuoles and the contents of the vesicle are degraded (216). Mitotic Catastrophe (Table 1) is a conserved stress response mechanism, which is initiated by dysfunctional

progression of mitosis. The cells are eradicated during or close to metaphase (217).

Table 1: Comparison of various cell death types (218)

| | **Apoptosis** | **Oncosis** | **Autophagy** | **Mitotic Catastrophe** |
|---|---|---|---|---|
| **Stimulus** | Intracellular, Cytotoxic agents | Extracellular, Reactive oxygen species | Starvation, cellular remodeling | Aberrant mitosis, DNA damage |
| **Nucleus** | Condensation, pyknosis | Intact | Blebbing, segregation possible pyknosis | Intact |
| **Cell Membrane** | Intact, blebbing | Swelling, rupture | Possible blebbing | Intact |
| **Cytoplasm** | Intact, condensed | Rupture, spillage | Autophagic vacuoles | Intact |
| **Caspase Activity** | Dependent | Independent | Independent | Independent |
| **Mitochondria** | Intact | Ruptured | Possible dilatation | Possible dilatation |
| **Result** | Apoptotic bodies | Inflammation | Autophagic vesicles | Giant multinucleate cells |

#### **2.8.1.7.1.** Apoptotic cell death

**I.** Initiation Phase

Cells may undergo apoptosis through the activation of two major signaling pathways, namely the extrinsic pathway and the intrinsic pathway. The extrinsic pathway, referred to as the death receptor pathway, is activated by ligand-bound death receptors, belonging to the Tumor Necrosis Factor (TNF) superfamily of receptors (219):

- TNF receptor 1 (TNFR1)
- TNF-related apoptosis-inducing ligand death receptor 4 and 5 (TRAIL-DR4/DR5)
- Fas

The intrinsic pathway is activated by intrinsic signals such as chemical induced DNA damage, oxidative stress and growth factor deprivation. The resultant effect is the activation of apoptosis through the involvement of the mitochondria or the endoplasmic reticulum (220). The mitochondria are the most important cells in the process of intrinsic activated apoptosis, even though they do not directly trigger apoptosis. The mitochondrial outer membrane permeabilization (MOMP) results in the release of proteins into the cytoplasm and constitutes a “point of no return” in PCD (47). The members of the Bcl-2 family, a regulatory family of proteins housed in the mitochondria, control the MOMP. Upon apoptotic signal initiation, the pro Bcl-2 proteins such as Bax are activated and cause MOMP. After initiation of MOMP, cell death is initiated through the release of apoptotic molecules (47, 221).

The endoplasmic reticulum also participates in the intrinsic process of apoptosis. The endoplasmic reticulum is the most important cellular stress sensor and can withhold protein synthesis and metabolism in order to first restore cellular homeostasis. The endoplasmic reticulum stress activates the unfolded protein response (UPR) (221, 222). The UPR activates caspase 12 mediated through the Bcl-2 family, resulting in downstream caspases being triggered (222). The endoplasmic reticulum stress also induces MOMP and activates apoptosis via the mitochondrial pathway. The Bcl-2 family of proteins coordinates the crosstalk between the mitochondria and the ER

initiating caspase-dependent intrinsic-mediated apoptosis (47, 223).

Crosstalk also exists between the intrinsic and the extrinsic pathways, both at the initiation and execution levels. This occurs despite the difference in pathway activation, as both pathways converge at the activation of the executioner caspase 3 (224). DNA damage results in intrinsic apoptotic molecules such as Bax to activate the mitochondrial pathway, as well as the up-regulation of genes such as FAS Ligand (FasL), in the extrinsic pathway. During the initiation phase of apoptosis, the initiator caspases, phosphotidylserine and the mitochondria play important roles.

a) Initiator Caspases

Cellular enzymes that degrade proteins, biologically known as proteases, are synthesized as inactive pro-enzymes in cells. Triggers within the specific cell activate these proteases and upon activation, they elicit their actions (225). **C**ysteine **asp**artyl-specific prote**ases** (Caspases) are a family of cellular proteases that are present in all animal cells in their inactive form (226). The initiator caspases are caspase 8, 9, and 10 and they start to degrade the cytoskeletal proteins (227). Degradation of the cytoskeletal proteins result in the loss of cellular rigidity that leads to cellular shrinkage (211). This degradation results in the formation of membrane blebs (Figure 8). Blebs are defined as fluid-filled structures that are devoid of organelles (225). In addition to bleb formation, initiator caspases initiate the translocation of phosphatidylserine and the release of cytochrome c (228).

b) Phosphatidylserine

The plasma membrane of a cell is comprised mainly of phospholipids. These phospholipids play an important role in both the construction and functioning of the cell membrane (1). The phospholipid phosphatidylserine (PS) is normally confined to the inner layer of the plasma membrane (see Figure 8, which shows the PS flip). The activated initiator caspases induces the action of the enzyme scramblase (229) and inactivation of the enzyme translocase (47). Scramblase activation mediates the outward flip of PS to the outer membrane (230). Translocase, which normally inhibits the spontaneous PS flip to the outer membrane, is thus inhibited. The PS flip to the outer membrane serves as a cell-cell recognition signal. The PS flip alerts the surrounding cells and macrophages that the cell is undergoing death (229).

c) Mitochondria

The mitochondria play an integral role in cellular death by releasing signals, which either trigger or inhibit cellular death. The mitochondria are stimulated by the initiator caspases to release apoptosis activating factor (APAF) and cytochrome c (Figure 8) (227). Within the mitochondrion, the gene bax promotes release of molecules while the gene bcl-2 inhibits the release of molecules (224) (Figure 8).

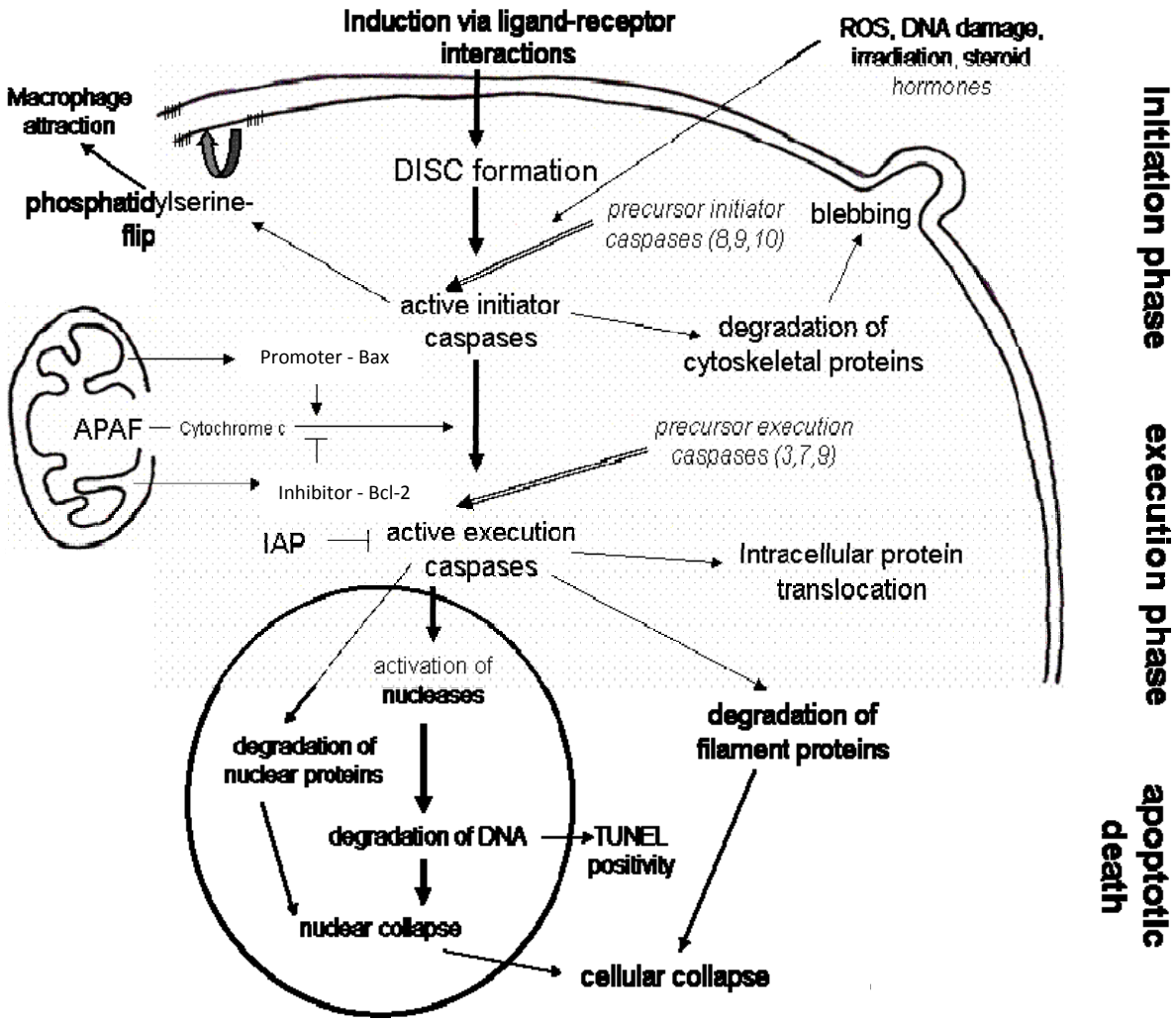


Figure 8: The caspase mediated apoptotic cascade (220). (Adapted with kind permission from Springer Science + Business Media: Journal of Anatomy and Embryology, The apoptosis cascade – morphological and immunohistochemical methods for its visualization, issue 200, 1999, pg 2, Huppertz B, Frank H-G, Kaufmann F, Figureure 1).

## II. Execution Phase

The inhibitors of apoptosis (IAP) (Figure 8) inhibit the activation of execution caspases, by binding directly to the activated caspases or by blocking caspase activation (231). If no inhibition occurs, the execution caspases are then activated and the cell has irrevocably committed itself to undergo death (216).

a) Execution caspases

Activation of execution caspases (caspase 3, 7, and 9), in particular caspase 3, initiates a host of cascades (Figure 9), including the translocation of proteins from the nucleus to the cytoplasm (226). In addition to protein translocation, proteins critical to cell survival are cleaved through activation of proteases or by the executionary caspases (225). Degradation of cytoskeletal proteins are responsible for the apoptotic characteristics, such as blebbing, nuclear and cellular collapse (47).

b) Protein translocation

Protein translocation serves as a useful marker for determination of the execution stages of apoptosis. Ordinarily the protein T-cell restricted intracellular antigen related protein (TIAR) is restricted to the nucleus, where it acts as a nuclease, cleaving DNA (232). During the activation of execution caspases, TIAR is gradually translocated from the nuclease to the cytoplasm (Figure 9) (220). The enzyme transglutaminase II is evenly distributed throughout the cytoplasm in normal non-apoptotic cells. During later stages of apoptosis, transglutaminase II is activated and translocated to beneath the plasma membrane. In this new sub-plasmalemmal location of transglutaminase II forms crosslinks between the cytoplasmic proteins (220). These crosslinks result in formation of extensive protein scaffolds, which inhibit the apoptotic cell from releasing its cytoplasmic contents into the interstitium, thus preventing inflammation (233).

c) Nuclear protein degradation

Active execution caspases target DNA maintenance and repair proteins, such as Poly-(ADP-ribose) polymerase (PARP), topoisomerase IIα and lamins (Figure 9). PARP assists in the repair of DNA damage and is vital for maintaining DNA integrity in normal cells (234). Thus, PARP is a target for execution caspases, which, after

cleavage, reduces DNA repair and amplified chromatin damage. Seeing as the protein topoisomerase IIα binds chromatin in the normal cell, it is an execution caspases target (235). PARP and topoisomerase IIα are rendered inactive by a combination of caspase-dependent activated endonucleases, leading to single strand breaks of the DNA (234, 235). Lamins are found along the inner surface of the nuclear membrane. They are the major structural proteins of the nuclear envelope and are involved in maintaining the structure of the nucleus (218, 220). Apoptotic degradation of the lamins leads to structural changes within the nucleus, resulting in nuclear collapse and fragmentation (236).

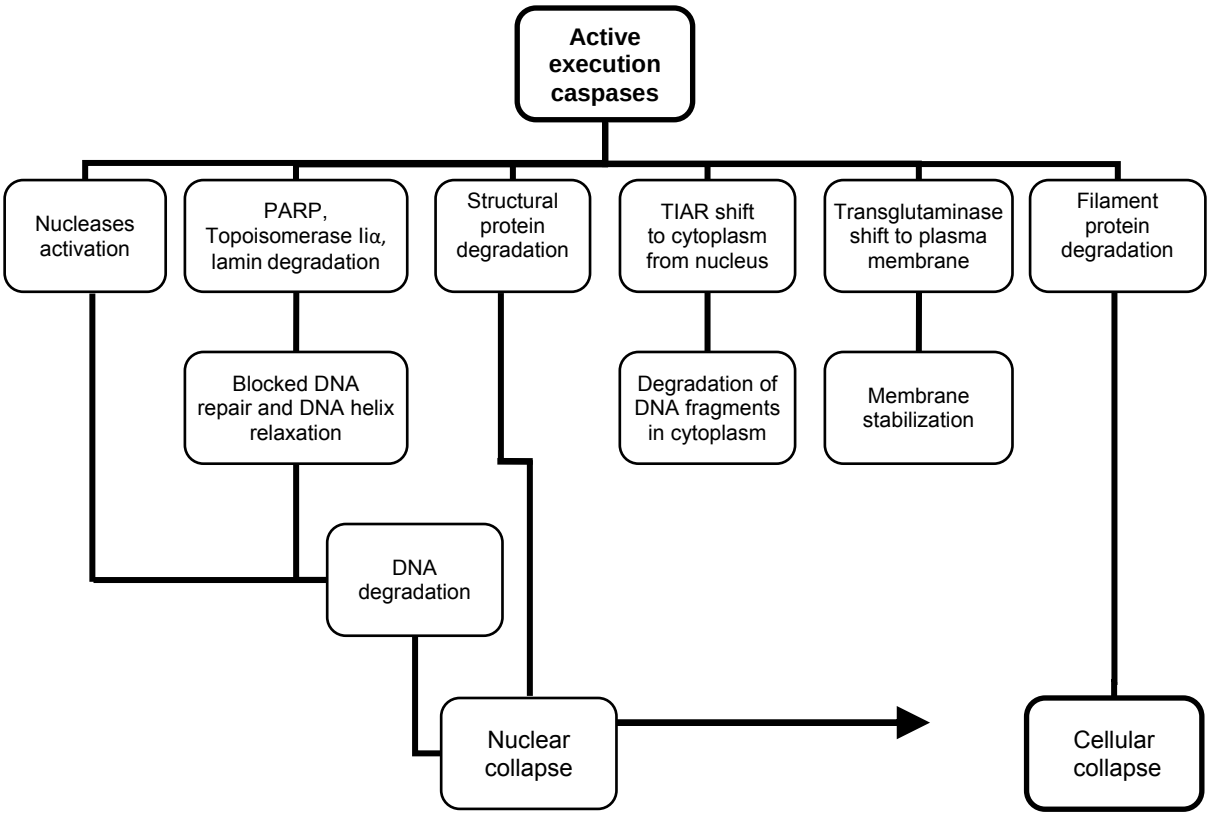


Figure 9: Schematic representation of the action of execution caspases.

## III. Apoptotic death

a) DNA degradation

The role of DNA fragmentation in apoptotic death has been a contentious issue (233). In 1972 it was suggested that endonucleolytic DNA degradation was a component of apoptosis (237). No mitochondrial DNA fragmentation is noted in apoptosis, indicating that DNA fragmentation is a specific apoptotic event (47). DNA fragmentation has been linked to endonuclease activity and has subsequently been used as the biochemical marker of apoptosis (233).

Activation of endonucleases, such as DNA Fragmentation Factor (DFF), lead to the discovery that DNA fragmentation is a component of cellular death (220, 235). This stemmed from the observation that in almost all situations of morphologically characterized apoptosis, the biochemical event of internucleosomal DNA fragmentation is prominent, leading to its utility as the maker of apoptosis (238). Biochemically, DNA is fragmented by endonucleases in the linker regions of between histones on the chromosomes, in 180-200 base pairs (239). Series of nucleosomal chains of 180-200 base pairs multiples are characteristic to apoptotic cell death and seen as the ‘apoptotic ladder’, which is used as a marker in biochemical assays (238).

b) Apoptotic Bodies

Following nuclear degradation the cell emits processes and budding occurs. These buds contain condensed nuclear fragments (240) which detach from the cells and are referred to as apoptotic bodies. These apoptotic bodies are then phagocytized by aggregated macrophages or surrounding cells, which have been attracted by the PS externalization (241).

Testicular apoptosis has been studied in both *in vitro* studies and *in vivo* toxicological studies. These studies investigated the effects that exposure to various EDCs may have on reproductive health, focusing on testicular apoptosis. Male rats exposed to *p,p’*-DDE at varying doses (20, 60 and 100mg/kg body weight) showed a significant induction of apoptotic cell death at concentrations greater than 20mg/kg per body weight (242). The rise in apoptotic cell death induction has been attributed to an increase in caspase activity and apoptosis associated genes.

Exposure to 1mg/kg DM for 21 days induces testicular apoptosis, detected by DNA fragmentation, in male rats (126) which is a characteristic of apoptosis. Similarly, a study by Assinder *et al* (2007) found that after 24 days exposure to a high mixtures of phytoestrogens (between 53.5 and 225µg/g genistein) resulted in apoptotic cell death in the spermatocytes and round spermatids (40). Following a single dose of 5mg/kg ZEN administered to adult male rats, apoptosis was observed in sperm cells as well as DNA fragmentation following histological investigation (243). Although apoptosis has been detected in the abovementioned studies, realistic concentrations found in the environment are typically lower than the experimental doses used. Therefore, a gap exists in the literature regarding the assessment of apoptosis using a mixture of chemicals, particularly in a malaria area.

An often unappreciated and sometimes overlooked aspect of cellular death is that cells within a population may begin apoptosis at various times after a cell death signal has been initiated (244). Thus, the duration of apoptosis varies from cell to cell and from species to species. This makes the accurate detection of apoptosis and the interpretation of an immunohistochemical (IHC) assay crucial for the accuracy of a particular study (245). The field of IHC has been proved to be a valuable assessment tool in cellular research. The specificity, durability and the utility of the reaction products in fixed tissue sections makes IHC methods highly effective tools (246). Due to the antibody (Ab) antigen (Ag) complexes formed, numerous target Ab kits are produced to detect Ag presence in various tissues. Caspase-mediated apoptosis follows two main steps in apoptotic cell death, cells first express caspase activity and then fragment their DNA (225). Thus an immunohistochemical technique to assess the expression of caspase 3, the main execution caspase, serves as a valuable tool to assess the initiation of apoptosis. The caspase 3 assay has an application in the field of scientific research as it is considered to be the main executioner (225).

A study examined the effect that exposure to *p*-NP potentially has on male offspring dosed throughout gestation and lactation. Following exposure to a concentration of 100mg/kg *p*-NP, positive caspase 3 labeling was found in the spermatogonia and spermatocytes, suggesting that *p*-NP has the ability to initiate apoptotic cell death (247). Frigo *et al* (2005) demonstrated the use of a caspase 3/7 assay in the identification of apoptosis inducted through apoptotic ligand activation by DDT (248). Pro-caspase 3 is present in its inactive form in normal cells. It is only through the

initiator caspases 8, 9 and 10 are cleaved and activated, thus an immunohistochemical reaction can be visualized (225).

Following caspase activation, the cell undergoes the apoptotic cascade, resulting in DNA fragmentation (220). The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay has become the most widely used *in situ* hybridization method to detect DNA fragments of apoptotic cells (238, 249). It would therefore be possible to assess the effect of EDC exposure on apoptosis in the testes of male rats using immunohistochemical and *in situ* hybridization assays.

From the literature review, limited evidence exists on the possible health effects on people **currently** living in malaria area. The effects of exposure to a relevant mixture of EDCs at concentrations within in the human exposure range in a suitable model may provide valuable information. In the Limpopo Province, South Africa, the VhaVenda people are exposed to a unique mixture of EDCs that may pose a threat in general and especially in male reproductive health. Sprague-Dawley rats are the most suitable animal model for various human health effects, including reproductive health. Critical stages during embryogenesis and early development are under hormonal control and any perturbations during these sensitive stages may have detrimental effects later in life. Combining *in utero*-, lactational- followed by direct exposure represents the exposure from gestation to reproductive maturity that is comparable to the human scenario. Using standard and non-standard endocrine sensitive endpoints, this study aims to address the identified gaps in literature and add to the body of evidence regarding human health exposure to EDCs.

# Chapter 3: Aim and Objectives

## Aim

Using an internationally-accepted reproductive toxicity test protocol in rats, the potential effects on the male reproductive system of combined *in utero*-, lactational- and direct exposure to DDT and DDE, pesticides used for Malaria control in the Limpopo Province of South Africa, in comparison with a mixture of DDT, DDE and other natural and manmade EDCs, selected based exposures commonly encountered by residents of this Province, were investigated.

## Objectives

1. To determine the impact of *in utero*-, lactational- and direct exposure of EDC exposure on male-specific endocrine disruptive endpoints (anogenital distance and gonadosomatic index), male accessory glands (prostate and seminal vesicles), epididymis and liver of male rats.

2. To determine the effects of EDC exposure on epididymal sperm count and total testosterone levels of male rats exposed during *in utero*-, lactation- and directly to selected EDCs.

3. To assess and compare the testicular histology and spermatogenesis cycle of male rats exposed during *in utero*-, lactation- and directly to selected EDCs, using the spermatogenesis staging program STAGES.

4. To better characterize effects on the testes by evaluating the testicular histology using the Johnsen scoring system and evaluation of apoptosis to provide insights on cellular mechanisms and targets in the testes.

# Chapter 4: Materials and Methods

## 4.1. Experimental animal model

Sprague-Dawley rats were used as experimental species as they are routinely used for reproductive toxicology studies (54, 70, 250). The study was performed at the University of Pretoria Biomedical Research Centre (UPBRC) using twenty-four pregnant female Sprague-Dawley rats. Animals were housed according to standard procedures with 12-h-day/night cycles, constant temperature (21 ± 2°C) and humidity (45% ± 10%) in standard poly-carbonate Euro-standard type III cages, as per standard operating procedures. The animals were maintained on a diet of rodent pellets (Epol rodent cubes, Pretoria, South Africa) and high-pure water. Animals had free access to both food and water. Ethical clearance was obtained by the Animal Use and Care Committee of the University of Pretoria prior to the commencement of the project (Project number: H010/11) and in accordance with the South African code for the use and care of animals in research (SANS 10386) (251).

## 4.2. Study design

The study design was based on the Organization for Economic Cooperation and Development (OECD) One-generation reproductive toxicity study 415 protocol (252). The original protocol was modified to include longer fetal exposure duration and additional non-standard endocrine-sensitive endpoints (Figure 10). Pregnant females (P1) were randomly allocated to the four experimental groups and dosed with either cottonseed oil, DDT, DDE or a mixture of EDCs throughout the duration of their gestation and lactation period.

### 4.2.1. Maternal exposure – P1

Dosing started at day 7 of the gestation cycle to avoid interference with blastocyst implantation and subsequent embryonic growth. On day 7, the 24 P1 females were divided into four experimental groups, containing 6 P1 females in each group (Figure 10). The P1 females in each group were continually dosed with either cottonseed oil, DDT, DDE or a mixture of EDCs throughout the pregnancy. Following birth, the P1

females were dosed during the lactation period of 3 weeks. Thus, the pups (F generation) were indirectly exposed to the EDCs during lactation.

### 4.2.2. Direct exposure – F1

Following the lactation period, the male (F1) pups from each of the experimental groups were kept in their respective groups. The F1 pups were directly dosed daily for 10 weeks, until reaching sexual maturity at 13 weeks of age (Figure 10).

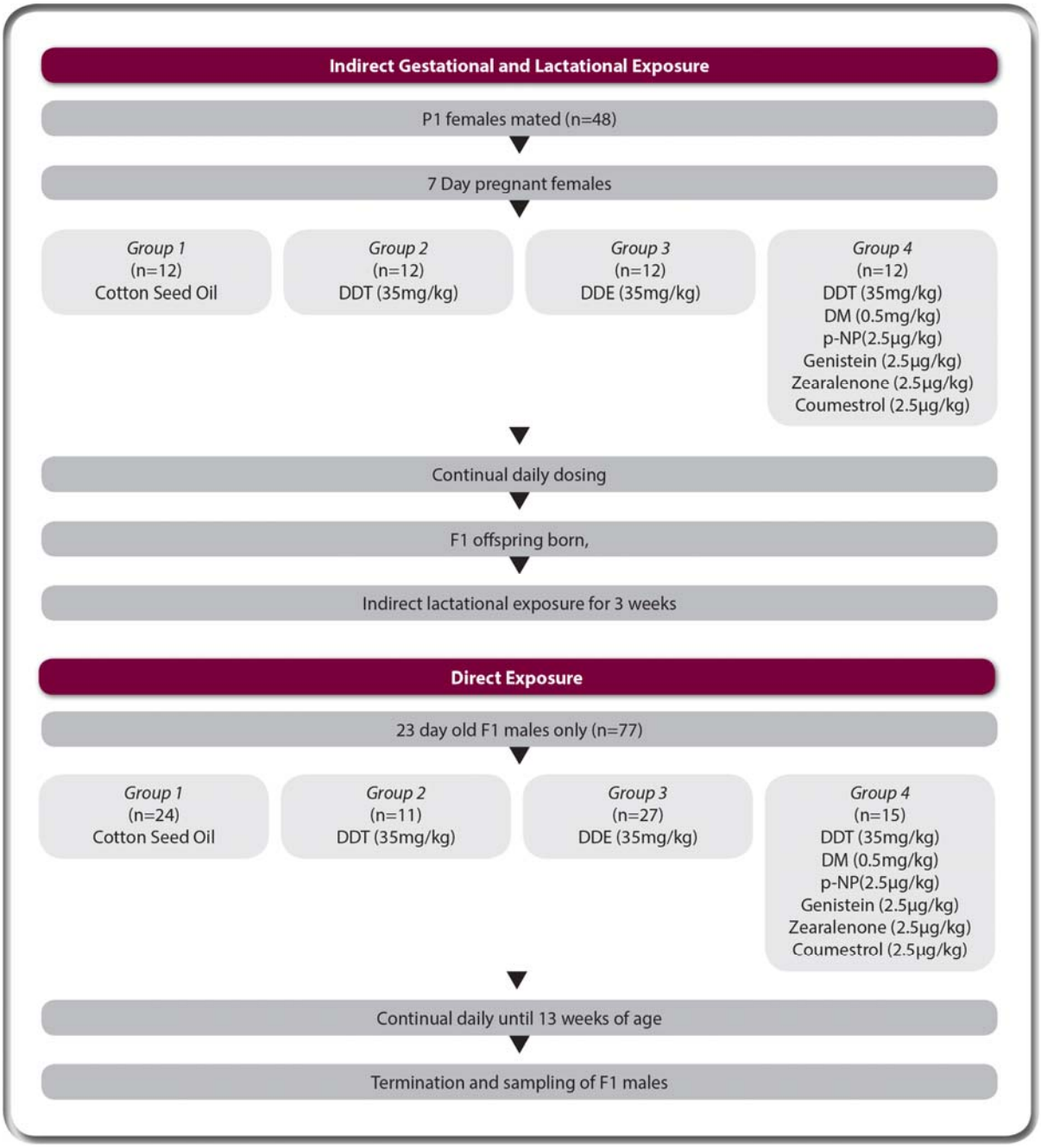


Figure 10: Experimental design describing the *in utero*-, lactational- and direct exposure to either cottonseed oil, DDT, DDE or a mixture of EDCs. Experimental groups in both P1 and F1 generation, including the sample size, chemical doses, dosing duration and termination endpoints are indicated.

*Oral dosing and dosing procedure*

Four experimental groups were used in this study:

**Group 1**: Control group – Cottonseed oil as a vehicle [Sigma-Aldrich, Steinheim, Germany; catalogue number: C7767, CAS Number: 8001-29-4].

**Group 2**: DDT group – 35mg/kg 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) [Sigma-Aldrich, Steinheim, Germany; catalogue number: 50-29-3].

**Group 3**: DDE group – 35mg/kg 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) [Sigma-Aldrich, Steinheim, Germany; catalogue number: 123897, CAS Number 72-55-9].

**Group 4**: mixture group – 35mg/kg DDT [Sigma-Aldrich, Steinheim, Germany; catalogue number: 50-29-3], 0.5mg/kg deltamethrin (DM) [Chem Service, West Chester, PA, USA; catalogue number: PS-2071], 2.5µg/kg para-nonylphenol (*p*-NP) [Sigma-Aldrich, Steinheim, Germany; catalogue number: 290858, CAS Number 84852-15-3], 2.5µg/kg coumestrol [Sigma-Aldrich, Steinheim, Germany; catalogue number: 27885, CAS Number 479-13-0], 2.5µg/kg genistein [Sigma-Aldrich, Steinheim, Germany; catalogue number: G6776, CAS Number 446-72-0] and 2.5µg/kg zearalenone [Sigma-Aldrich, Steinheim, Germany; catalogue number: Z2125, CAS Number 17924-92-4].

Doses were determined from concentrations of previously studied chemicals present in a malaria area in South African, (6, 8, 20, 37, 48, 51). The chemicals were selected to represent a possible real-life exposure scenario that men, living in a malaria area, may encounter. All chemical substances were administered by oral gavage at a volume of 1ml/kg, which was calculated daily and adjusted for body weight.

### 4.2.3. Sampling method

After dosing and at 13 weeks of age, blood was drawn from the adult F1 males. Following the blood collection, animals were euthanized with an overdose of isoflurane by insufflation (Isofor®, Safeline Pharmaceutical [Pty] Ltd., SA) under controlled conditions.

The anogenital distance, which is the length of the perineum from the base of the genital tubercle to the center of the anus (253), was measured and recorded. Body weight was recorded and the testes, seminal vesicles, the right epididymis, prostate and the liver were removed and weighed. Any macroscopic abnormalities were recorded before organs were fixed in the relevant fixatives for further analysis. The left epididymis was used for determining the epididymal sperm count. Blood was used to determine total testosterone.

### 4.3. Fixatives

Two different fixatives were used to fix the collected organs namely Bouin's' Fluid (BF) and 10% Neutrally Buffered Formalin (NBF). For routine histological analysis, BF is used as it produces sections with a high clarity and superb cellular preservation (254). Briefly, the tissues were fixed in BF for 24hrs at room temperature (RT). After 24hrs, samples were washed in tap water for 1hr and then dehydrated stepwise in 30% ethanol (EtOH) for 1hr, in 50% EtOH for 1hr and finally stored in 70% ethanol until further tissue processing. Since BF is a stringent fixative, it is not recommended for IHC as there is a high frequency of antigen masking resulting in insufficient antibody binding (255, 256).

However, NBF is not as stringent a fixative as BF as the frequency of antigen masking is far less than in BF fixed tissues. NBF is therefore recommended for IHC. Briefly, the two buffer salts, sodium phosphate mono- and dibasic, were dissolved in 900ml distilled water (solution A). Thereafter the pH of solution A was adjusted to 7.3. Then 100ml 40% formalin was added to solution A. The tissues were fixed for 24hrs in NBF at RT. After 24hrs, samples were washed in tap water for 1hr and then dehydrated stepwise in 30% EtOH for 1hr, in 50% EtOH for 1hr and finally stored in 70% ethanol until further tissue processing (254, 256).

### 4.4. Cauda epididymal sperm count

Sperm count was determined by cauda epididymal sperm extraction according to WHO (1999) guidelines. The left cauda epididymis was separated from the caput-corpus and was placed in 2ml phosphate buffered saline (PBS) medium in a petri

dish. The cauda epididymis was macerated to expel the sperm into the medium. The PBS containing the sperm was placed in a Falcon tube. The Neubauer method (190) was used to count the sperm and the counts were expressed in millions per ml.

The coverslip was applied onto the Neubauer chamber by horizontal sliding. Correct coverslip position was confirmed by the presence of Newton's rings on both sides of the Neubauer chamber. A positive displacement pipette (Gilson Microman; Anachem, Luton, United Kingdom) was used for the transfer of semen, at a standard dilution of 1:20, to the Neubauer chamber. Each side of the Neubauer chamber was carefully loaded until it was full, ensuring not to overfill the chamber. The Neubauer chamber was placed in a humidified chamber for 10min to allow for cells to sediment before counting.

The Neubauer chamber is divided into nine large squares (Figure 11 A). All the sperm heads in the large center square (Figure 11 B) were counted. Only sperm heads on the left and top lines of each square (Figure 11 C) were counted, as per the WHO (1999) protocol. The total number of the perm counted in the large center square is multiplied by $10^6$ to give the concentration per ml of semen (190).

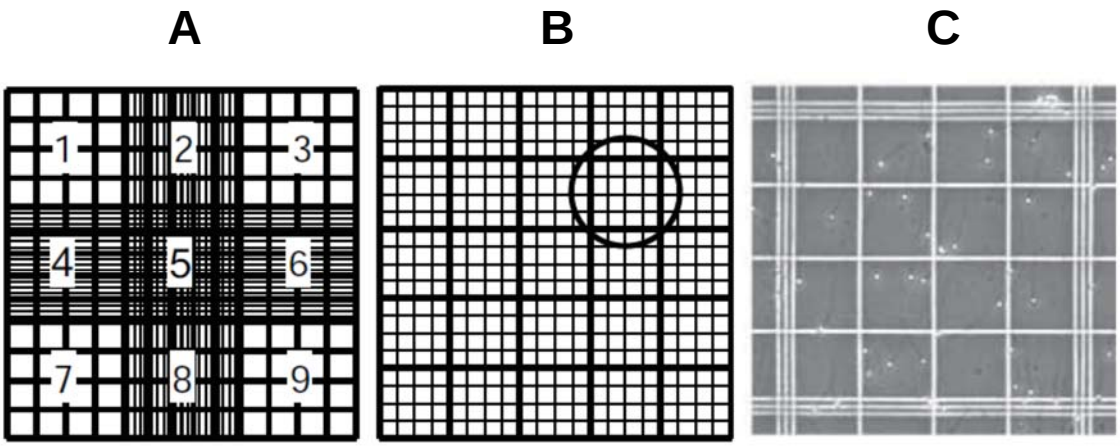


Figure 11: Neubauer chamber used for the quantification of sperm, showing all nine grids (A); the central grid (number 5) which is comprised of 25 square (B); one of the 25 squares of the central grid (the circled square in B) is bound by triple lines (C) (185).

### 4.5. Total testosterone

On the day of termination, blood was collected between 8am and 10am by heart puncture in additive-free tubes, centrifuged and plasma was stored at -80°C. Levels of serum testosterone were determined using the radioimmunoassay (RIA) method, according to the manufacturer's instructions (Immunotech, Marseille, France: Cat number – IM1087). A volume of 50µl of the sample was added to the tubes, and then 500µl of the tracer was added to the mixture. An additional two tubes were used to obtain the total cpm, in which only 500µl of the tracer was added. Tubes were covered and incubated for 60min at 37°C in a temperature controlled waterbath.

#### 4.5.1. Interpretation of radioimmunoassay counts

Following incubation, the supernatant of the tubes containing the tracer was aspirated and a gamma-counter was used to count the total counts per minute (CPM) of the pellet. The average non-specific binding (NSB) counts were subtracted from each average count (except the total counts).

The percentage of tracer bound was calculated as follows:

(Total binding counts/total counts) X 100

The percentage of total binding (%B/Bo) for each standard and sample was calculated as follows:

%B/Bo = (sample or standard/total binding) X 100

The %B/Bo for each standard was plotted on the y-axis and the known concentration of the standard on the x-axis and a reference curve was made. The concentration of specified hormone was determined in the unknown samples and controls by interpolation of the reference curve in GraphPad Prism (257).

## 4.6. Testicular assessments

### 4.6.1. Histology

The haematoxylin and eosin (H & E) staining technique was used to stain the different cells that constitute the testes and to aid in identifying abnormalities.

#### 4.6.1.1. Haematoxylin and eosin staining technique

Haematoxylin is a chemical base that stains acids. It binds to DNA and stains the nucleus blue. Eosin is an acidic substance that binds to bases, staining the protein-rich cytoplasm pink (256). The Department of Pathology at the Onderstepoort Veterinary Institute embedded blocks of testes in paraffin wax, made sections of 4µm thick which were subsequently collected on Superfrost slides (Menzel-Glaser, Germany).

The slides were deparaffinised with xylene and rehydrated through three washes of 100% EtOH, one wash of 95% EtOH and one wash of 80% EtOH in distilled water; each, for 3min per wash. Slides were then rinsed in one wash of deionized water for 5min. Excess water was blotted from the slides and slides were immersed in haematoxylin for 5min. Thereafter, slides were rinsed with deionized water and immersed in tap water for 5min, to allow the stain to develop. Slides were washed in acid ethanol to destain, rinsed for two washes in tap water for 1min each then rinsed for one wash in deionized water for 2min. Slides were immersed in eosin for 30 seconds and then dehydrated in 95% EtOH in distilled water and one wash of 100% EtOH; each for 5min. Finally, slides were cleared in three washes of xylene and mounted with Entellan (Merck, Darmstadt, Germany).

#### 4.6.1.2. Microscopic evaluation

Testicular tissue sections of the F1 male rats were viewed at 10x and 40x magnification, using a Nikon BH-2 microscope fitted with a CC-2 digital camera, coupled to a computer with analysis Imaging Processing software (Soft Imaging System, Münster, Germany). A qualitative examination of the testes was made taking into account the tubular stages of the spermatogenic cycle. The examination was conducted to identify exposure-related effects including absent germ cell types or layers, spermatid retention and sloughing of spermatogenic cells into the lumen. Furthermore, any cell- or stage-specific effect observed in the testis was noted.

### 4.6.2. Staging of the testes

Spermatogenic cycle stages were evaluated at a 10x and 40x magnification, using a Nikon Optiphot microscope. The computer software program, STAGES™ 2.1 (Vanguard Media Inc. IL, USA) was used to identify the various stages of the rat spermatogenic cycle (250). The spermatogenic process is characterized by precise timing and synchronized development of germ cells that occur within the seminiferous tubules (66). Each of the 14 stages of the spermatogenic cycle characterizes development and maturation of germ cell into a mature sperm (73). In each rat testis, 30 randomly selected seminiferous tubules were selected and the 14 stages of the rat spermatogenic cycle were identified and classified according to the criteria set out in Russell et al (1990). The seminiferous tubule diameter, seminiferous epithelial thickness and lumen diameter were measured (Figure 12) and reported.

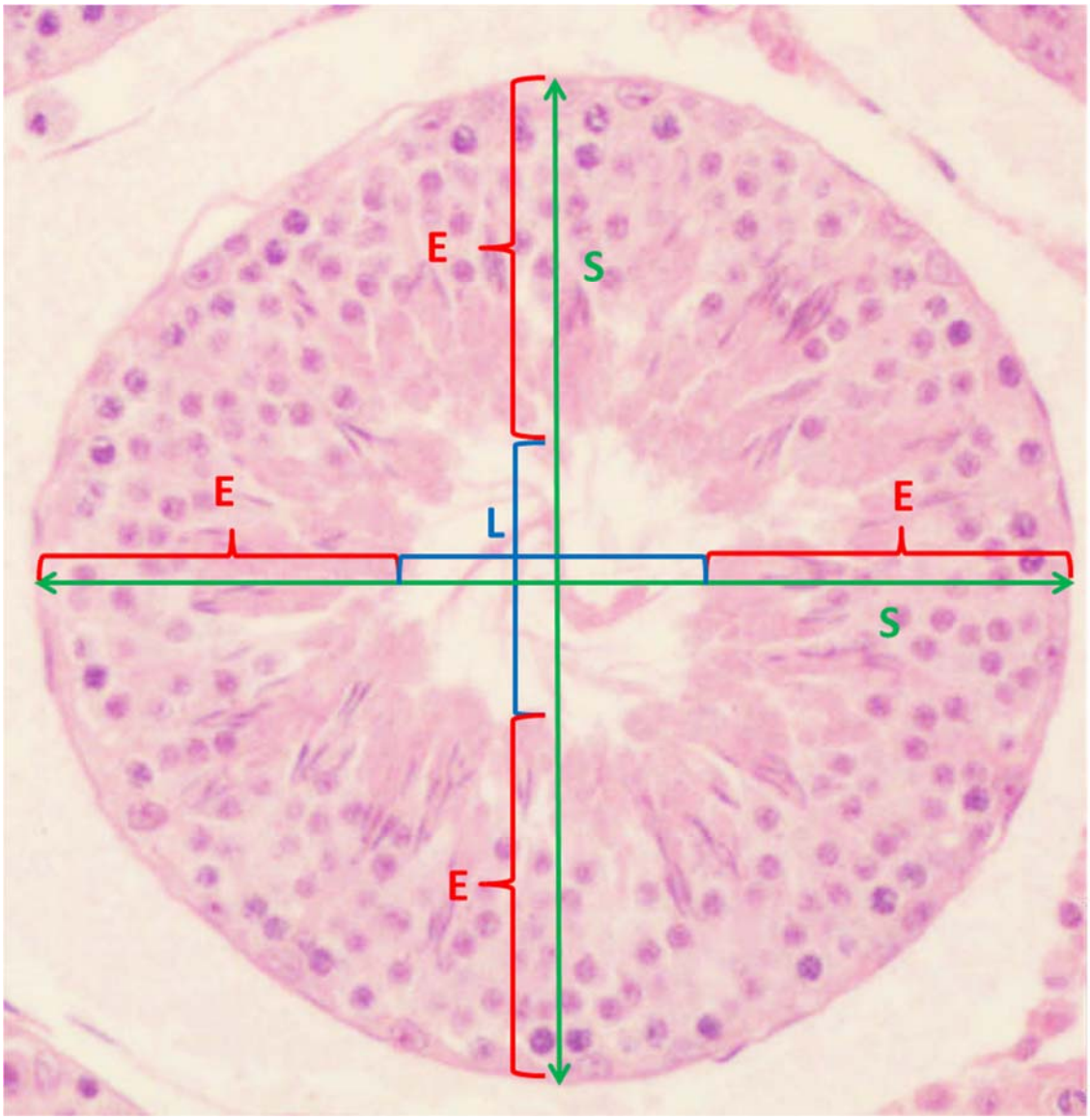


Figure 12: Measurements of the seminiferous tubules. S: Seminiferous tubule diameter, E: Seminiferous epithelium thickness, L: Lumen diameter.

### 4.6.3. Johnsen Score

The testicular histology slides were used to score the seminiferous tubules according to the criteria set out in the Johnsen method of evaluation of spermatogenesis (258). The Johnsen method was initially developed for assessment of human testes, but has successfully been used to assess rat testes in surgical intervention studies (259, 260) but not in a reproductive toxicology study. 100 random seminiferous tubules per slide (total n=77) in each of the experimental groups was evaluated, using a 10x objective lens. The seminiferous tubule was given a score from 1 – 10 according to the criteria set out by Johnsen, detailed below in Table 2. Traditionally, the mean Johnsen score has been used in humans, however this gives an unrealistic representation of the testicular section. However, the cut-off point of seemingly normal seminiferous tubules and hence spermatogenesis could possibly be identified by the difference in the normal (Johnsen score = 10) and abnormal (Johnsen score – sum of 9 to 1) seminiferous tubules.

Table 2: Johnsen score and accompanying criteria for the evaluation of seminiferous tubules

| Score | Description |
|---|---|
| 10 | Complete spermatogenesis with many spermatozoa (spermatozoa are defined as cells having achieved the small head form of the spermatozoon). Germinal epithelium organized in a regular thickness leaving an open lumen. |
| 9 | Many spermatozoa present but germinal epithelium disorganized with marked sloughing or obliteration of lumen. |
| 8 | Only few spermatozoa (< 5 – 10) present in section. |
| 7 | No spermatozoa, but many spermatids present. |
| 6 | No spermatozoa and only few spermatids (< 5 - 10) present. |
| 5 | No spermatozoa, no spermatids, but several or many spermatocytes present. |
| 4 | Only few spermatocytes (< 5) and no spermatids or spermatozoa present. |
| 3 | Spermatogonia are the only germ cells present. |
| 2 | No germ cells, but Sertoli cells are present. |
| 1 | No cells in tubular lumen. |

## 4.7. Apoptosis - Immunohistochemical evaluation

The principle of IHC is based on the affinity between an Ag and an Ab (246). An Ag is a substance which, upon induction by a specific stimulus, has the ability of stimulating the immune system to produce a response solely directed at the initiating specific stimulus. In response to Ag production, amongst other responses, the immune system forms Ab. Therefore the Ag has a specific affinity for the Ab whose formation it caused (254). It is this affinity that is the attractive force between the Ag and Ab, enabling them to form a bond. For the purpose of scientific research Ab are produced to enable phagocytes to eradicate foreign substances from the body (261). The field of IHC has been proved to be a valuable assessment tool in cellular research. The specificity, durability as well as the utility of the reaction products in fixed tissue sections makes IHC methods highly effective tools (246). Due to the complex formed between the Ab and Ag, numerous target antibody kits are produced to detect antigen presence in various tissues.

### 4.7.1. Sample preparation procedure

Sections of 4µm thick testicular tissue were floated on the surface of a distilled water bath (45°C) and collected on Superfrost slides (Menzel-Glaser, Germany). The slides were then prepared for the caspase 3 IHC assay.

### 4.7.2. Cleaved caspase 3 immunohistochemistry

Slides were deparaffinised with xylene and then rehydrated through two washes in 100% EtOH and 95% EtOH in distilled water; each, for 10min per wash. Epitopes masked by fixation were revealed by an antigen revival procedure. Antigen revival involved heating the slides immersed in an Antigen Unmasking Solution (Vectorlabs, Burlingame, CA, USA) in a domestic microwave at 100% power until boiling, and then at 30% power for 10min. The slides were then cooled for 30min at RT.

Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in distilled water in darkness for 10min. Following a rinse in distilled water, slides were

placed in 0.1% (v/v) Tween 20 (Sigma-Aldrich, Steinheim, Germany) in phosphate buffered saline (PBS-A: 137 mM NaCl, 29 mM $NaH_2PO_4 \cdot H_2O$, 9 mM $Na_2HPO_4$, pH 7.4) for 5min. Slides were then incubated with blocking solution (5% normal goat serum in 0.1% Tween 20-PBS-A) for 1hr at RT. Slides were then incubated (4°C) overnight with a 1:200 dilution of cleaved caspase-3 antibody (Asp175) (Cell Signaling Technology, Beverly, MA, USA) at 4°C.The following day the slides were washed three times in of 0.1% Tween 20/PBS-A for 5min each. The secondary antibody (biotinylated antirabbit IgG) was added to each of the slides, ensuring that the entire section is covered, and incubated at RT for 30min. Following three PBS-A washes for 5min each, slides were incubated with the Vectastain avidin-biotin-complex (Vectorlabs, Burlingame, CA, USA) for a further 30min at RT after which slides were washed three times in PBS-A for 5min each.

Immunoreactions were detected by incubating the slides with 3, 3´-diaminobenzidine (DAB) (Vectorlabs, Burlingame, CA, USA). A DAB solution was prepared (2500µl glass distilled water, 24µl buffer stock solution, 50µl DAB stock solution, 40µl hydrogen peroxide solution) and the slides were incubated with 150µl DAB solution was for 1-2min in darkness. Thereafter, slides were rinsed in glass-distilled water for 5min, and counterstained with Haematoxylin QS (Vectorlabs, Burlingame, CA, USA), a modification of Mayer's Haematoxylin, specifically developed for immunohistochemistry, with less than 45 seconds staining time. After counterstaining, slides were finally rinsed in running tap water until water was colorless. Slides were dehydrated in 3 washes of 100% butanol, cleared in 3 washes of 100% xylene, and mounted with Entellan (Merck, Darmstadt, Germany).

### 4.8. Apoptosis - *In situ* hybridization

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay has become the most widely used *in situ* hybridization method to DNA fragments of apoptotic cells (238, 249). The TUNEL assay labels the 3'OH ends of the DNA with TdT using dUTP.

### 4.8.1. Sample preparation procedure

Sections of 4µm thick testicular tissue were floated in a distilled water bath (45°C) and collected on Superfrost slides (Menzel-Glaser, Germany). Slides were prepared for TUNEL *in situ* hybridization.

### 4.8.2. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay

Slides were deparaffinized with xylene and then rehydrated through two washes in 100% EtOH in distilled water for 3 minutes each; one wash in 95% EtOH in distilled water for 5min and one wash in 75% EtOH for 5min. Permeabilization of the tissue was achieved by incubating the slides in 0.5% triton X-100 for 10min at RT.

Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in distilled water in darkness for 10min. Quenching was followed by two washes in phosphate buffered saline (PBS-B: 50mM sodium phosphate, pH 7.4; 200mM NaCl). Subsequent steps for TUNEL staining were carried out using the ApopTag-Peroxidase Kit according to the supplier's instructions (Chemicon, Temecula, CA). DNA fragmentation was detected by incubating the slides with the TdT-mediated dUTP nick end-labeling reaction mixture, in a humidified chamber at 37°C for 60min. Following incubation, slides were treated with the antidigoxigenin-peroxidase complex for 30min at RT and then washed in three PBS-B washes for 5min each.

DNA fragments were detected by incubating the slides with DAB (Vectorlabs, Burlingame, CA, USA). A DAB solution was prepared (2500µl glass distilled water, 24µl buffer stock solution, 50µl DAB stock solution, 40µl hydrogen peroxide solution) and the slides were incubated with 150µl DAB solution was for 1-2min in darkness. Slides were rinsed in glass-distilled water for 5min and counterstained with Haematoxylin QS (Vectorlabs, Burlingame, CA, USA), a modification of Mayer's Haematoxylin. After staining, slides were rinsed in running tap water until the water was colorless, dehydrated in 3 washes of 100% butanol, cleared in 3 washes of 100% xylene, and mounted with Entellan (Merck, Darmstadt, Germany).

### 4.9. Statistical analyses

As F1 males share a common mother, a P1 female, the Survey command in STATA 12 was used to create survey sets. A total of 16 survey sets (clusters representing a litter from each female) were created with the F1 males allocated to their respective clusters. The data was then analyzed using the Survey Linear Regression for ranked data. The experimental groups were compared to the control group, correcting for clusters, with a significance level of $P < 0.05$. Furthermore, differences between the experimental groups were conducted using the adjusted Wald Test with a significance value of $P < 0.05$. All statistical analyses were conducted using STATA 12 (StataCorp, TX, USA) (262) in collaboration with Prof PJ Becker from the Faculty of Health Sciences, University of Pretoria, South Africa.

# Chapter 5: Results

## 5.1. Anogenital distance

The statistical analysis of the mean AGD (mm) of the F1 males is summarized in Table 3. Compared to the control group (group1; 17.54mm), a statistically significantly shorter AGD was observed in the mixture group (group 4; 15.20mm; *P* = 0.005). Although, not statistically significant, compared to the control group, the DDT group (group 2; 18.55mm; *P* = 0.863) had a longer mean AGD; whilst the DDE group (group 3; 17.33mm; *P* = 0.360) had a shorter mean AGD (Figure 13).

Table 3: Statistical analysis of the difference in the mean anogenital distance (mm) between the control (group 1) and DDT, DDE and mixture groups (groups 2-4), using survey linear regression analysis.

| Experimental groups | n | Mean (mm) | Standard Deviation | *P*-value | 95% Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 17.54 | 0.65 | 0.863 | (-0.15 ; 0.61) |
| DDT | 11 | 18.55 | 0.17 | | |
| Control | 24 | 17.54 | 0.65 | 0.360 | (-0.97 ; 0.37) |
| DDE | 27 | 17.33 | 0.41 | | |
| Control | 24 | 17.54 | 0.65 | **0.005** | (-1.69 ; -0.36) |
| Mixture | 15 | 15.20 | 0.16 | | |

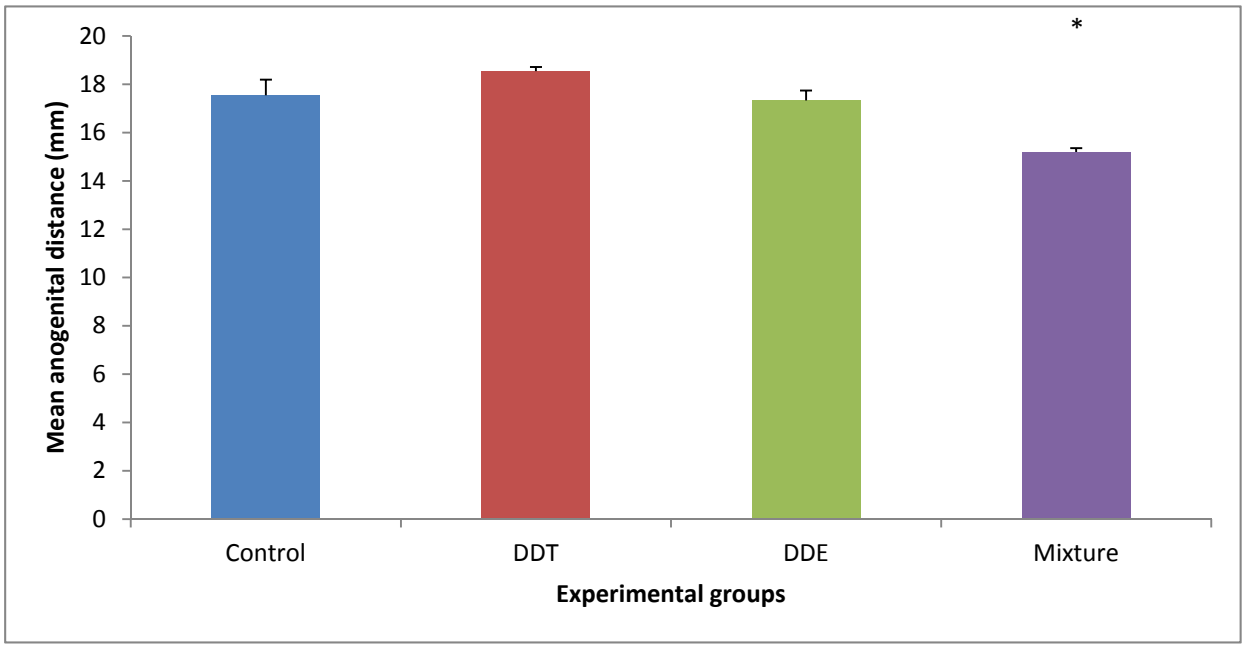


Figure 13: Mean and standard deviation (SD) of the anogenital distance (mm) of the F1 males in the control (group 1) and DDT, DDE and mixture groups (groups 2-4).

Mixture group (group 4) = DDT + DM + *p*-NP + phytoestrogens

* Significant: $P < 0.05$ when compared to the control group

## 5.2. Body mass

The statistical analysis of the mean body mass (g) at termination of the F1 males is summarized in Table 4. Compared to the control group (group 1; 430.34g), no statistically significant differences were observed between the body mass of the DDT (group 2; 437.54g; *P* = 0.561), DDE (group 3; 414.91g; *P* = 0.317) and the mixture group (group 4; 419.08g; *P* = 0.499) (Figure 14).

Table 4: Statistical analysis of the difference in mean body mass (g) between the control (group 1) and experimental (groups 2-4) groups, using survey linear regression analysis.

| Experimental groups | n | Mean (g) | Standard Deviation | *P*-value | 95% Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 430.34 | 34.92 | 0.561 | (-18.63 ; 33.03) |
| DDT | 11 | 437.54 | 23.94 | | |
| Control | 24 | 430.34 | 34.92 | 0.317 | (-47.18 ; 16.33) |
| DDE | 27 | 414.91 | 32.15 | | |
| Control | 24 | 430.34 | 34.92 | 0.499 | (-45.87 ; 23.35) |
| Mixture | 15 | 419.08 | 32.74 | | |

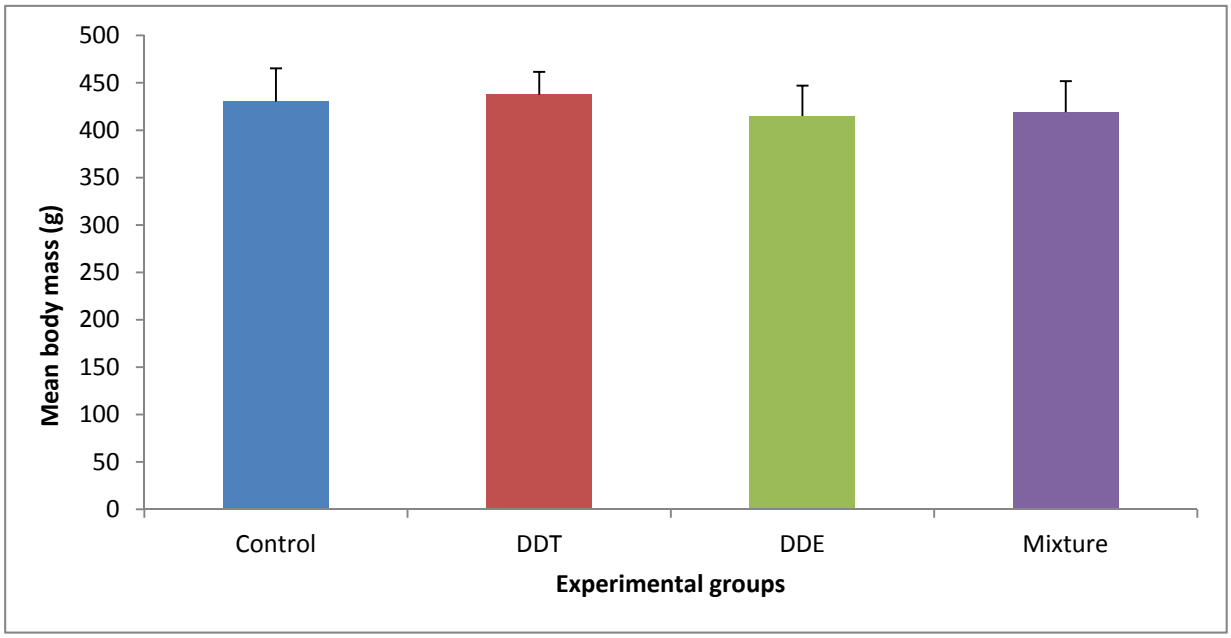


Figure 14: Mean and SD of the body mass (g) of the F1 males in the control (group 1) and experimental groups (groups 2-4).

Mixture group (group 4) = DDT + DM + *p*-NP + phytoestrogens

### 5.3. Liver

The statistical analysis of the mean liver mass (g) of the F1 males is summarized in Table 5. Compared to the control group (group 1; 17.36g), statistically significant differences were observed between the mean liver mass of the DDT (group 2; 21.16g; $P < 0.001$), the DDE (group 3; 20.65g; $P = 0.003$) and the mixture group (group 4; 19.45g; $P = 0.03$) (Figure 15).

Table 5: Statistical analysis of the difference in the mean liver mass (g) between the control (group 1) and experimental (groups 2-4) groups, using survey linear regression analysis.

| Experimental groups | n | Mean (g) | Standard Deviation | *P*-value | 95% Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 17.36 | 2.16 | **< 0.001** | (2.25 ; 5.18) |
| DDT | 11 | 21.16 | 1.29 | | |
| Control | 24 | 17.36 | 2.16 | **0.003** | (1.32 ; 5.26) |
| DDE | 27 | 20.65 | 5.06 | | |
| Control | 24 | 17.36 | 2.16 | **0.031** | (0.23 ; 3.96) |
| Mixture | 15 | 19.45 | 2.00 | | |

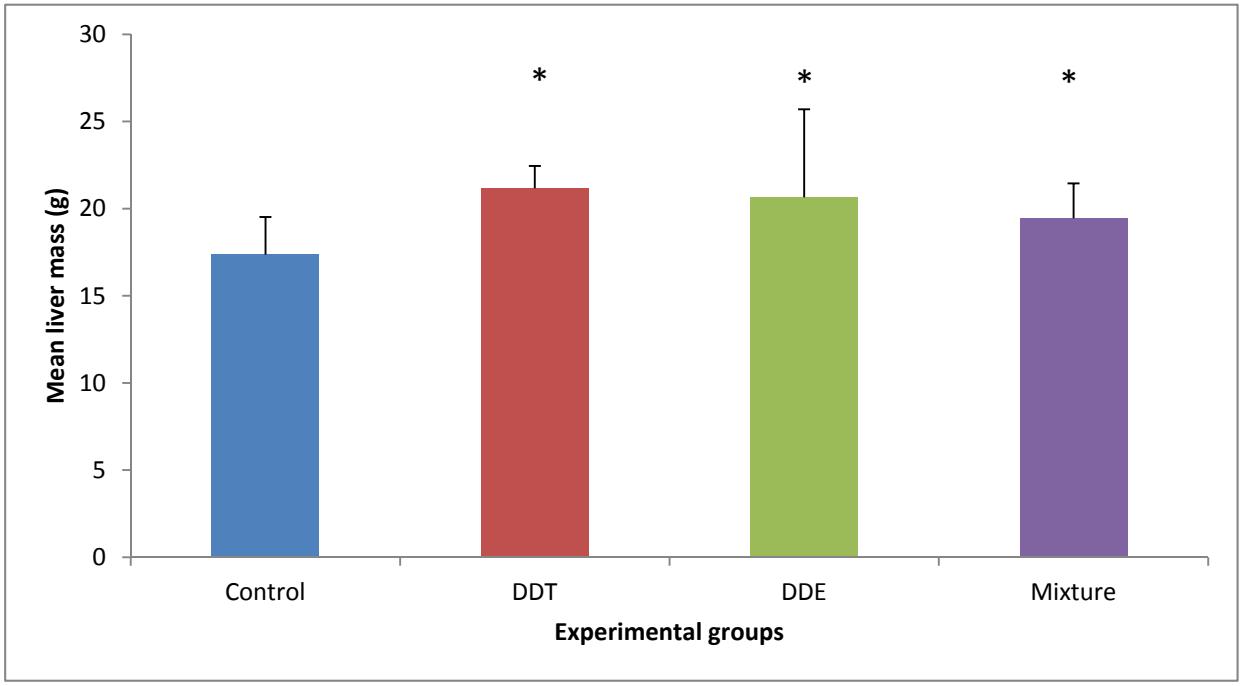


Figure 15: Mean and SD of the liver mass (g) of the F1 males in the control (group 1) and experimental groups (groups 2-4).

Mixture group (group 4) = DDT + DM + *p*-NP + phytoestrogens

* Significant: $P < 0.05$ when compared to the control group

### 5.4. Hepatosomatic index

The statistical analysis of the mean hepatosomatic index (HSI) of the F1 males is summarized in Table 6. Compared to the control group (group 1; 4.028), a statistically significantly higher HSI was observed in the DDT (group 2; 4.837, $P$ <0.001), DDE (group 3; 4.962, $P$ < 0.001) and mixture groups (group 4; 4.642, $P$ = 0.001) (Figure 16).

Table 6: Statistical analysis of the difference in the mean hepatosomatic index (HSI) between the control (group 1) and experimental (groups 2-4) groups, using survey linear regression analysis.

| Experimental groups | n | Mean | Standard Deviation | *P*-value | 95% Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 4.028 | 0.309 | **<0.001** | (0.664 ; 0.954) |
| DDT | 11 | 4.837 | 0.138 | | |
| Control | 24 | 4.028 | 0.309 | **<0.001** | (0.691 ; 1.176) |
| DDE | 27 | 4.962 | 1.083 | | |
| Control | 24 | 4.028 | 0.309 | **0.001** | (0.313 ; 0.915) |
| Mixture | 15 | 4.642 | 0.334 | | |

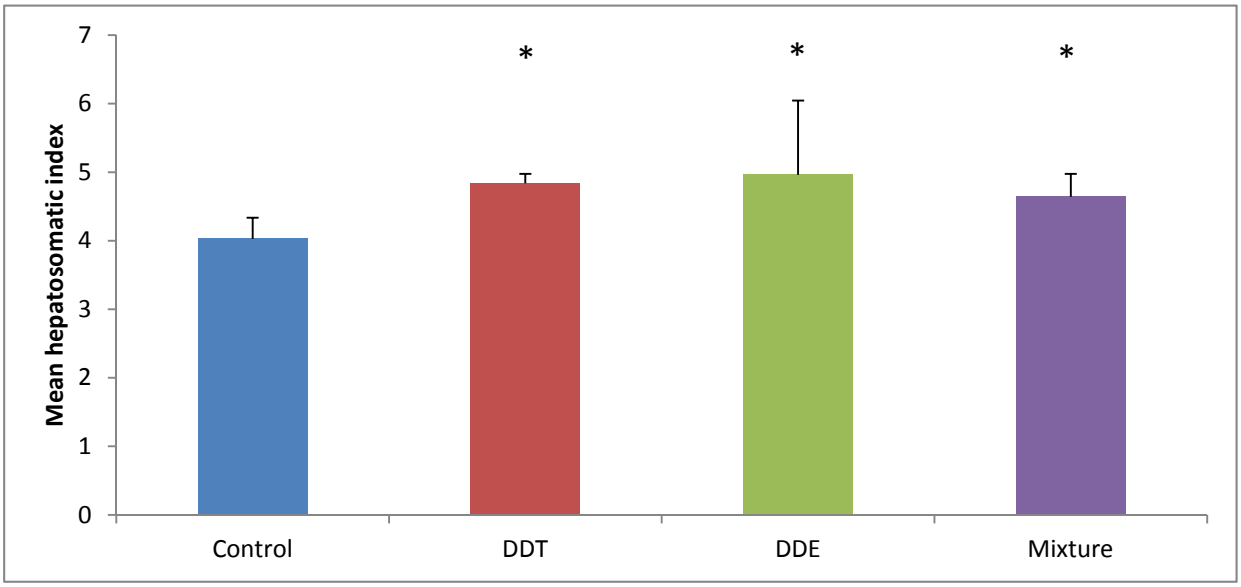


Figure 16: Mean and SD of hepatosomatic index (HSI) of the F1 males in the control and experimental groups

Mixture group = DDT + DM + *p*-NP + phytoestrogens

* Significant: $P < 0.05$ when compared to group 1

## 5.5. Liver histology

The histology of the liver in the control group revealed no abnormal tissue morphology or abnormal cellular distribution (Figure 17). Lipid droplet formation was observed in the liver tissue in the DDT, DDE and mixture groups. In addition to lipid droplets, hepatocyte disorganization was noted in the DDE group.

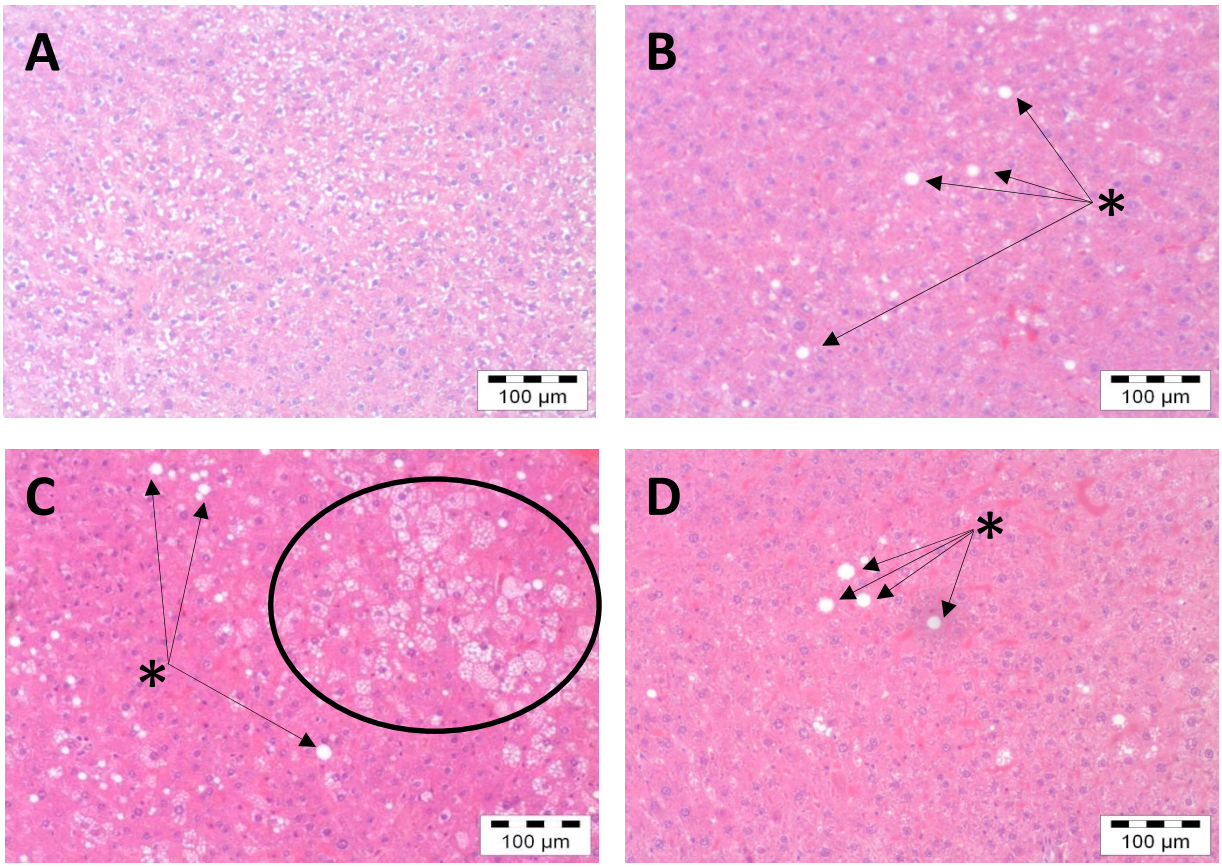


Figure 17: Histology of the liver showing lipid droplets in the liver of rats in the experimental groups. A: Control group (group 1; cottonseed oil); B: DDT group (group 2) showing the presence of lipid droplets (*); C: DDE group (group 3) showing abnormal liver histology indicated by the black ring and lipid droplets (*); D: mixture group (group 4) with the presence of lipid droplets (*).

### 5.6. Prostate

The statistical analysis of the mean prostate mass (g) of the F1 males is summarized in Table 7. Compared to the control group (group 1; 0.83g), a statistically significantly larger prostate mass was found in the DDT group (group 2; 1.02g; $P$ = 0.018). However, compared to the control group, there were no statistically significant differences between the prostate mass of the DDE (group 3; 0.82g; $P$ = 0.858) and the mixture (group 4; 0.83g, $P$ = 0.981) groups (Figure 18).

Table 7: Statistical analysis of the difference in the mean prostate mass (g) between the control (group 1) and experimental (groups 2-4) groups, using survey linear regression.

| Experimental groups | n | Mean (g) | Standard Deviation | *P*-value | 95% Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 0.83 | 0.24 | **0.018** | (0.04 ; 0.34) |
| DDT | 11 | 1.02 | 0.19 | | |
| Control | 24 | 0.83 | 0.24 | 0.858 | (-0.14 ; 0.12) |
| DDE | 27 | 0.82 | 0.23 | | |
| Control | 24 | 0.83 | 0.24 | 0.981 | (-0.17 ; 0.17) |
| Mixture | 15 | 0.83 | 0.21 | | |

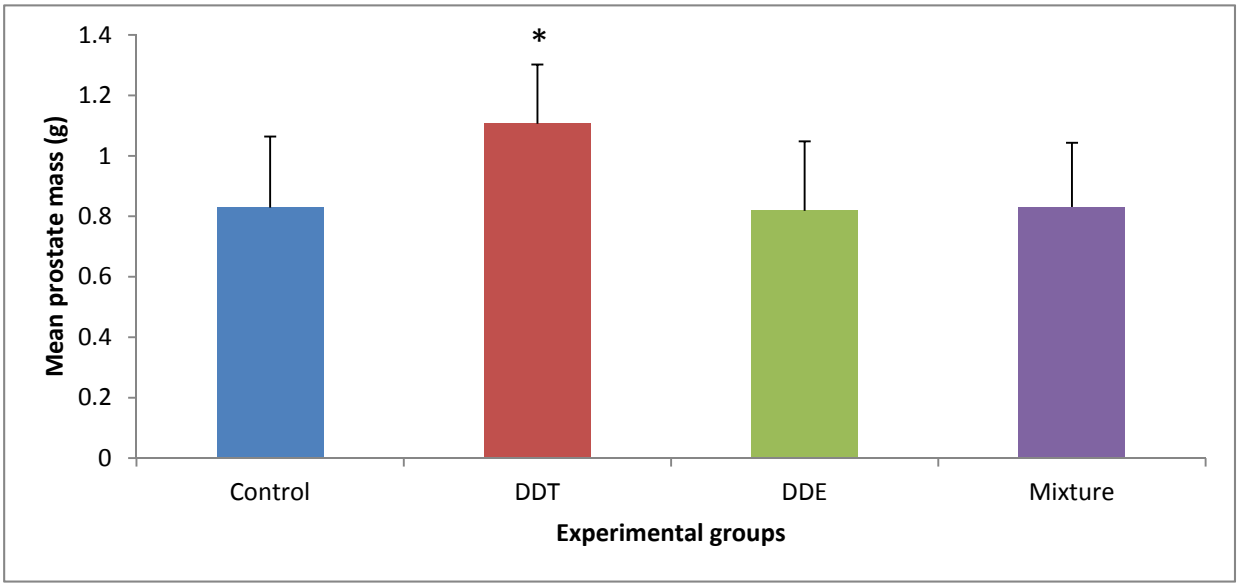


Figure 18: Mean and SD of the prostate mass (g) of the F1 males in the control and experimental groups.

Mixture group = DDT + DM + *p*-NP + phytoestrogens

* Significant: $P < 0.05$ when compared to the control group

### 5.7. Seminal vesicles

The statistical analysis of the mean seminal vesicle mass (g) of the F1 males is summarized in Table 8. Compared to the control group (group 1; 1.46g), no statistically significant differences were observed between the mean seminal vesicle mass of the DDT (group 2; 1.60g, $P$ = 0.294), DDE (group 3; 1.57g, $P$ = 0.430) and the mixture groups (group 4; 1.58g, $P$ = 0.494) (Figure 19).

Table 8: Statistical analysis of the difference in the mean seminal vesicle mass (g) between the control (group 1) and experimental (groups 2-4) groups, using survey linear regression analysis.

| Experimental groups | n | Mean (g) | Standard Deviation | *P*-value | 95% Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 1.46 | 0.38 | 0.294 | (-0.14 ; 0.42) |
| DDT | 11 | 1.60 | 0.43 | | |
| Control | 24 | 1.46 | 0.37 | 0.430 | (-0.17 ; 0.39) |
| DDE | 27 | 1.57 | 0.47 | | |
| Control | 24 | 1.46 | 0.37 | 0.494 | (-0.24 ; 0.48) |
| Mixture | 15 | 1.58 | 0.35 | | |

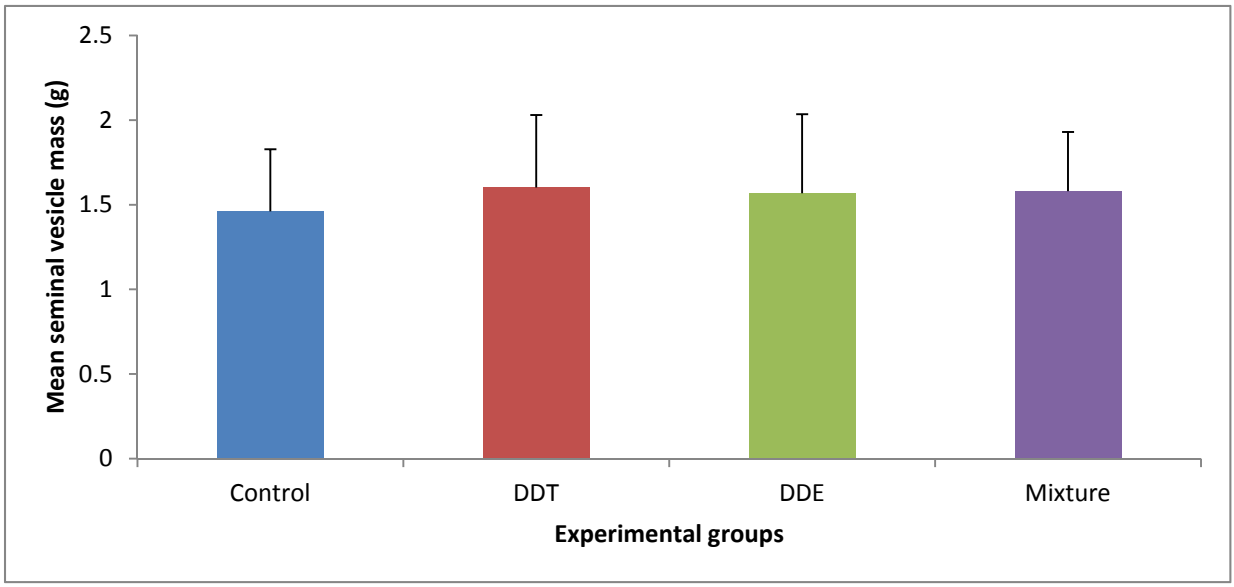


Figure 19: Mean and SD of the seminal vesicle mass (g) of the F1 males in the control and experimental groups.

Mixture group = DDT + DM + *p*-NP + phytoestrogens

### 5.8. Epididymis

The statistical analysis of the mean epididymal mass (g) of the F1 males is summarized in Table 9. Compared to the control group (group 1; 1.47g), no statistically significant differences were observed between the mean seminal vesicle mass of the DDT (group 2; 1.59g, $P$ = 0.227), DDE (group 3; 1.42g, $P$ = 0.530) and the mixture groups (group 4; 1.44g, $P$ = 0.721) (Figure 20).

Table 9: Statistical analysis of the difference in the mean epididymal mass (g) between the control (group 1) and experimental (groups 2-4) groups, using survey linear regression analysis.

| Experimental groups | n | Mean (g) | Standard Deviation | *P*-value | 95% Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 1.47 | 0.26 | 0.227 | (-0.81 ; 0.32) |
| DDT | 11 | 1.59 | 0.25 | | |
| Control | 24 | 1.47 | 0.26 | 0.530 | (-0.21 ; 0.11) |
| DDE | 27 | 1.42 | 0.30 | | |
| Control | 24 | 1.47 | 0.26 | 0.721 | (-0.16 ; 0.11) |
| Mixture | 15 | 1.44 | 0.19 | | |

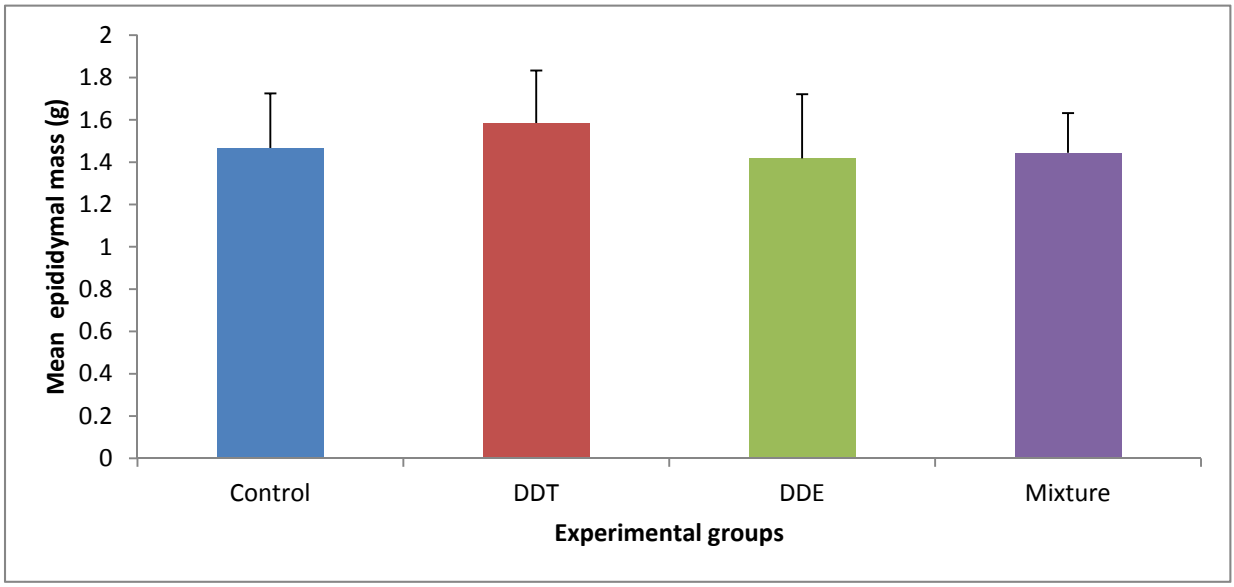


Figure 20: Mean and SD of the epididymal mass (g) of the F1 males in the control and experimental groups.

Mixture group = DDT + DM + *p*-NP + phytoestrogens

### 5.9. Total cauda epididymal sperm count

The statistical analysis of the mean total cauda epididymal sperm count (x$10^6$/ml) of the F1 males is summarized in Table 10. Compared to the control group (group 1; 48.46 x$10^6$/ml), no statistically significant differences were observed between the total cauda epididymal sperm count of the DDT (group 2; 60.13 x$10^6$/ml, $P$ = 0.063), DDE (group 3; 50.70 x$10^6$/ml, $P$ = 0.685) and the mixture group (group 4; 38.72 x$10^6$/ml, $P$ = 0.090) (Figure 21).

Table 10: Statistical analysis of the difference in the mean cauda epididymal sperm count (x$10^6$/ml) between the control (group 1) and experimental (groups 2-4) groups, using survey regression analysis.

| Experimental groups | n | Mean (x$10^6$/ml) | Standard Deviation | *P*-value | 95% Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 48.46 | 14.36 | 0.063 | (-0.74 ; 24.08) |
| DDT | 11 | 60.13 | 17.50 | | |
| Control | 24 | 48.46 | 14.36 | 0.685 | (-9.28 ; 13.74) |
| DDE | 27 | 50.70 | 16.47 | | |
| Control | 24 | 48.46 | 14.36 | 0.090 | (-21.20 ; 1.72) |
| Mixture | 15 | 38.72 | 12.34 | | |

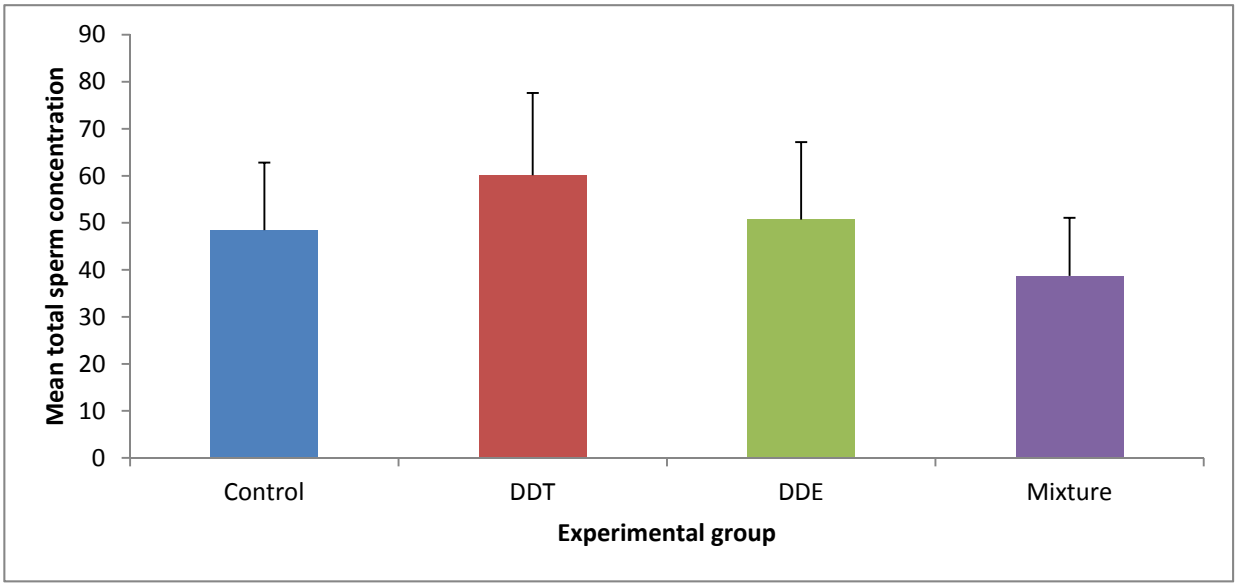


Figure 21: Mean and SD of the caudal epididymal sperm concentration (x$10^6$/ml) of the F1 males in the control and experimental groups.

Mixture group = DDT + DM + *p*-NP + phytoestrogens

### 5.10. Total testosterone

The statistical analysis of the mean testosterone concentration (nmol/L) of the F1 males is summarized in Table 11. Compared to the control group (group 1; 21.33nmol/L), statistically significantly higher testosterone concentrations were observed in the DDE (group 3; 28.12nmol/L; $P$ = 0.038) and in the mixture group (group 4; 28.612nmol/L; $P$ = 0.023), but not in the DDT group (group 2; 23.06nmol/L; $P$ = 0.392) (Figure 22).

Table 11: Statistical analysis of the difference in the mean total testosterone levels (nmol/L) between the control (group 1) and experimental (groups 2-4) groups, using survey linear regression analysis.

| Experimental groups | n | Mean (nmol/L) | Standard Deviation | *P* -value | 95% Confidence Interval |
|---|---|---|---|---|---|
| Control | 23 | 21.33 | 1.74 | 0.392 | (-6.58 ; 15.85) |
| DDT | 10 | 23.06 | 3.01 | | |
| Control | 23 | 21.33 | 1.74 | **0.038** | (0.89 ; 26.21) |
| DDE | 26 | 28.12 | 3.53 | | |
| Control | 23 | 21.33 | 1.74 | **0.023** | (2.15 ; 25.23) |
| Mixture | 14 | 28.62 | 2.96 | | |

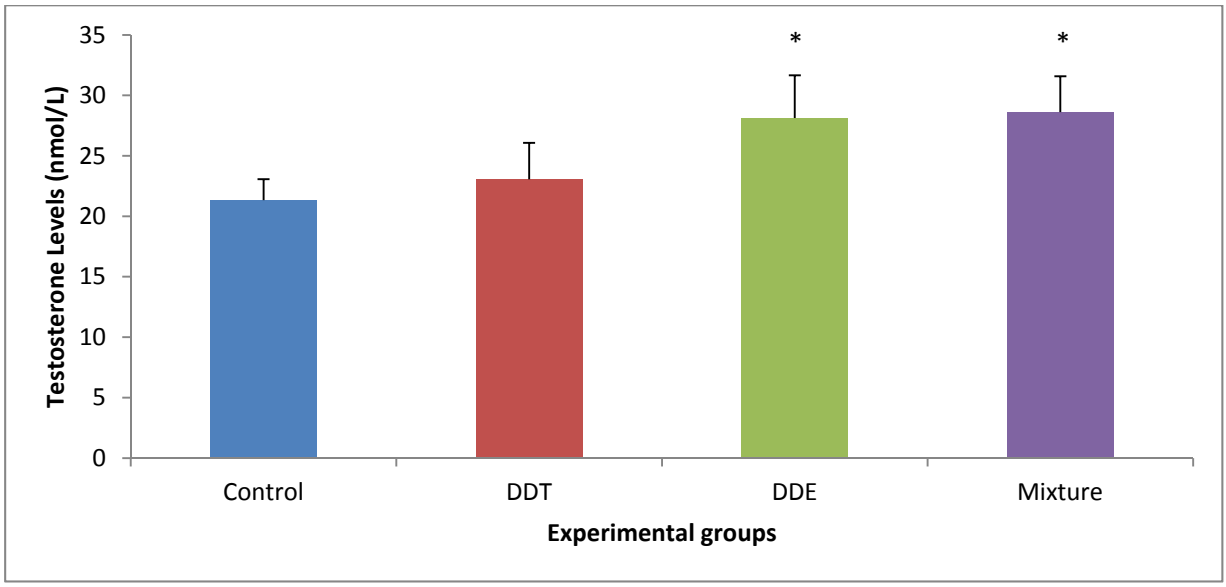


Figure 22: Mean and SD of the testosterone levels (nmol/ml) of the F1 males in the control and experimental groups.

Mixture group = DDT + DM + *p*-NP + phytoestrogens

* Significant: $P < 0.05$ when compared to the control group

### 5.11. Testes

The statistical analysis of the mean testes mass (g) of the F1 males is summarized in Table 12. Compared to the control group (group 1; 3.684g) statistically significant higher testicular masses were found the DDT (group 2; 3.88g, $P = 0.019$), DDE (group 3; 3.95g, $P = 0.047$) and the mixture group (group 4; 4.02g, $P < 0.001$) (Figure 23).

Table 12: Statistical analysis of the difference in the mean testes mass (g) between the control (group 1) and experimental (groups 2-4) groups, using survey linear regression analysis.

| Experimental groups | n | Mean (g) | Standard Deviation | *P*-value | 95% Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 3.68 | 0.22 | **0.019** | (0.04 ; 0.35) |
| DDT | 11 | 3.88 | 0.16 | | |
| Control | 24 | 3.68 | 0.22 | **0.047** | (0.004 ; 0.54) |
| DDE | 27 | 3.95 | 0.32 | | |
| Control | 24 | 3.68 | 0.22 | **<0.001** | (0.18 ; 0.50) |
| Mixture | 15 | 4.02 | 0.31 | | |

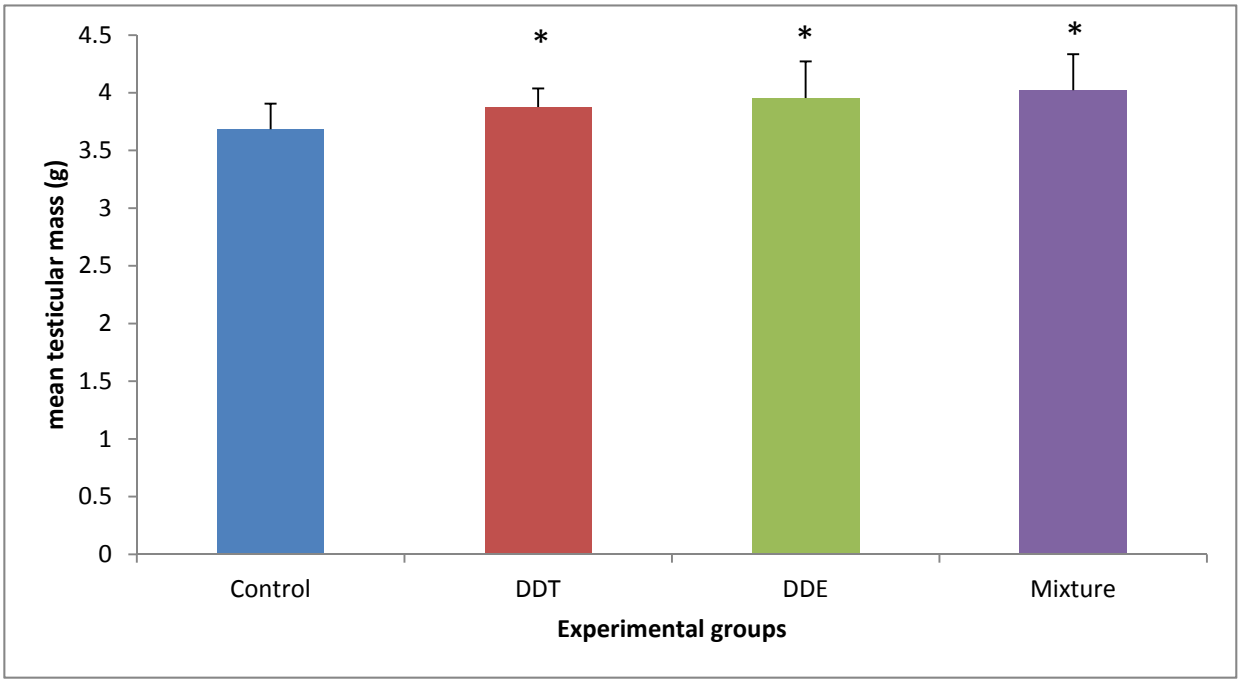


Figure 23: Mean and SD of the testes mass (g) of the F1 males in the control and experimental groups.

Mixture group = DDT + DM + *p*-NP + phytoestrogens

* Significant: $P < 0.05$ when compared to the control group

## 5.12. Gonadosomatic index

The statistical analysis of the mean gonadosomatic index (GSI) of the F1 males is summarized in Table 13. Compared to the control group (group 1; 0.86), statistically significantly higher GSI were found in the DDE (group 3; 0.96; *P* = 0.036) and in the mixture groups (group 4; 0.97; *P* = 0.016), but not in the DDT group (group 2; 0.89; *P* = 0.435) (Figure 24).

Table 13: Statistical analysis of the difference between the mean gonadosomatic index between the control (group 1) and experimental (groups 2-4) groups, using survey linear analysis.

| Experimental groups | n | Mean | Standard Deviation | *P*-value | 95% Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 0.86 | 0.08 | 0.435 | (-0.05 ; 0.10) |
| DDT | 11 | 0.89 | 0.06 | | |
| Control | 24 | 0.86 | 0.08 | **0.036** | (0.01 ; 0.18) |
| DDE | 27 | 0.96 | 0.08 | | |
| Control | 24 | 0.86 | 0.08 | **0.016** | (0.02 ; 0.19) |
| Mixture | 15 | 0.97 | 0.10 | | |

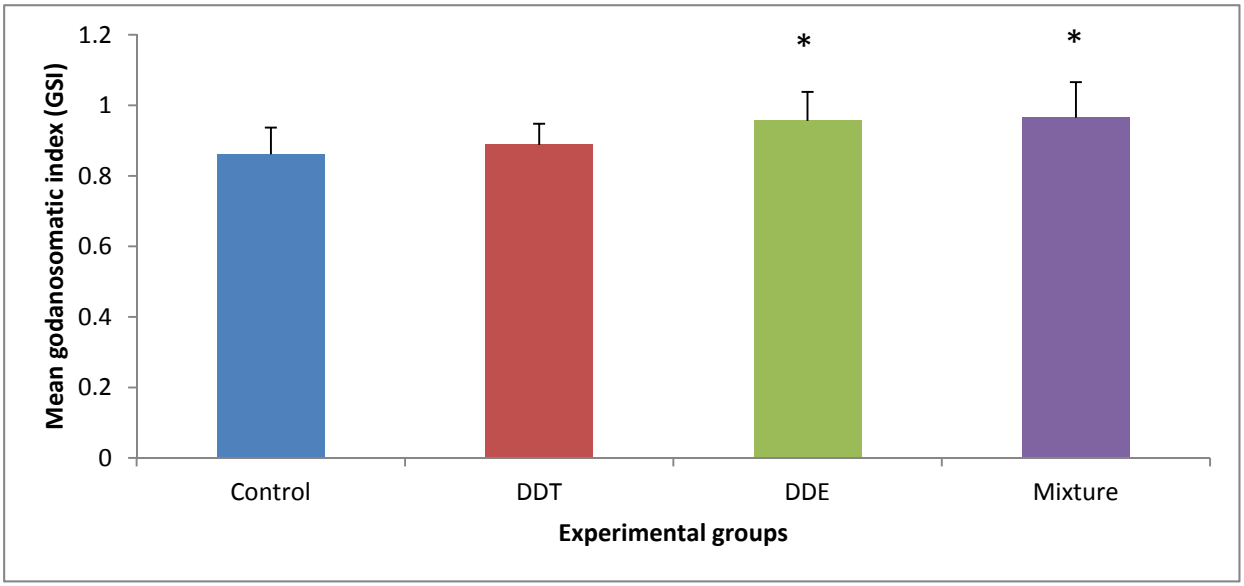


Figure 24: Mean and SD of the gonadosomatic index (GSI) of the F1 males in the control and experimental groups.

Mixture group = DDT + DM + *p*-NP + phytoestrogens

* Significant: $P < 0.05$ when compared to the control group

## 5.13. Testicular histology

Since the seminiferous tubules at the edge of the testicular section are large and irregular in shape, these tubules were not evaluated. The seminiferous tubules in the center of the testes are of similar size and shape, however. Starting in the center of the testicular section, 30 tubules were selected and examined and the spermatogenic stage of the particular tubule was recorded.

Normal spermatogenesis, with all 14 spermatogenic stages, was observed in the testicular histology slides of the DDT, DDE and mixture groups. In the DDT, DDE and mixture groups dilated tubular lumens, detachment of the seminiferous tubule, necrosis in the interstitium, disorganization of the seminiferous epithelium with few germ cells present, reduced seminiferous tubule diameter with no lumen, absent seminiferous tubules and decreased cellularity of the seminiferous epithelium was observed (Figure 25).

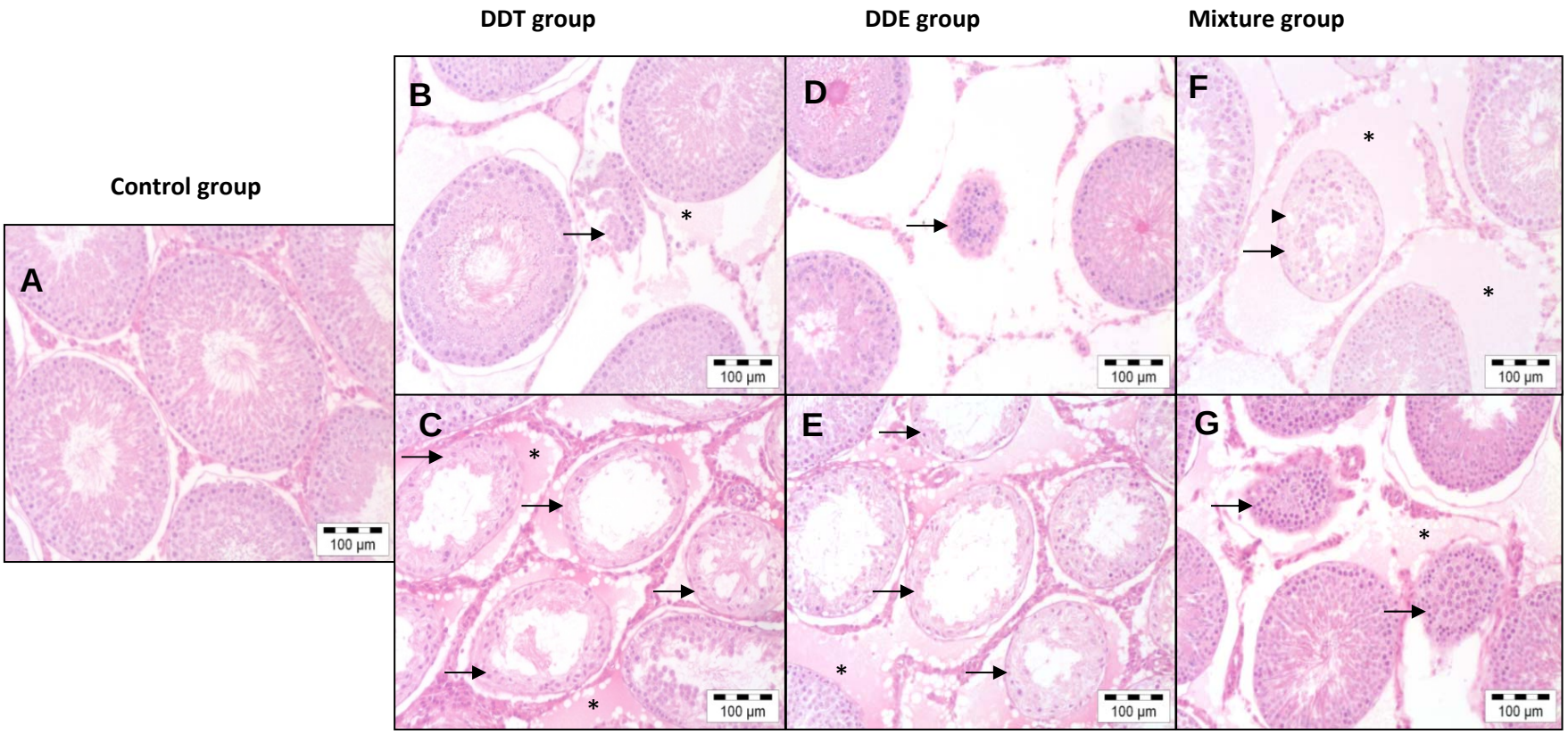


Figure 25: Testicular histology - Normal testicular histology in the control group (group 1; A), abnormal testicular histology in DDT group (group 2; B-C), DDE (group 3; D-E) and in the mixture group (group 4; F-G); necrosis in the interstitium (*), vacuolization of the seminiferous epithelium (arrows heads) and seminiferous tubule disorganization with reduced germ cell layers (arrows).

### 5.14. Histological measurements

The seminiferous tubule diameter, seminiferous epithelium thickness and lumen diameter was measured in 30 randomly selected seminiferous tubules, of the testicular sections of the F1 males.

### 5.14.1. Seminiferous tubule diameter

The statistical analysis of the mean seminiferous tubule diameter (µm) of the F1 males is summarized in Table 14. Compared to the control group (group 1; 295.42µm), statistically significantly smaller mean seminiferous tubule diameters were found in the DDT (group 2; 260.65µm, $P < 0.001$), DDE (group 3; 260.00µm, $P < 0.001$) and in the mixture group (group 4; 257.78µm, $P < 0.001$) (Figure 26).

Table 14: Statistical analysis of the difference between the mean seminiferous tubule diameter (µm) between the control (group 1) and experimental (groups 2-4) groups, using survey regression analysis.

| Experimental groups | n | Mean (µm) | Standard Deviation | *P*-value | 95 % Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 295.42 | 19.25 | **<0.001** | (-35.94 ; -18.30) |
| DDT | 11 | 260.65 | 17.98 | | |
| Control | 24 | 295.42 | 19.25 | **<0.001** | (-39.13 ; -17.75) |
| DDE | 26 | 260.00 | 14.53 | | |
| Control | 24 | 295.42 | 19.25 | **<0.001** | (-36.50 ; -19.74) |
| Mixture | 11 | 257.78 | 9.36 | | |

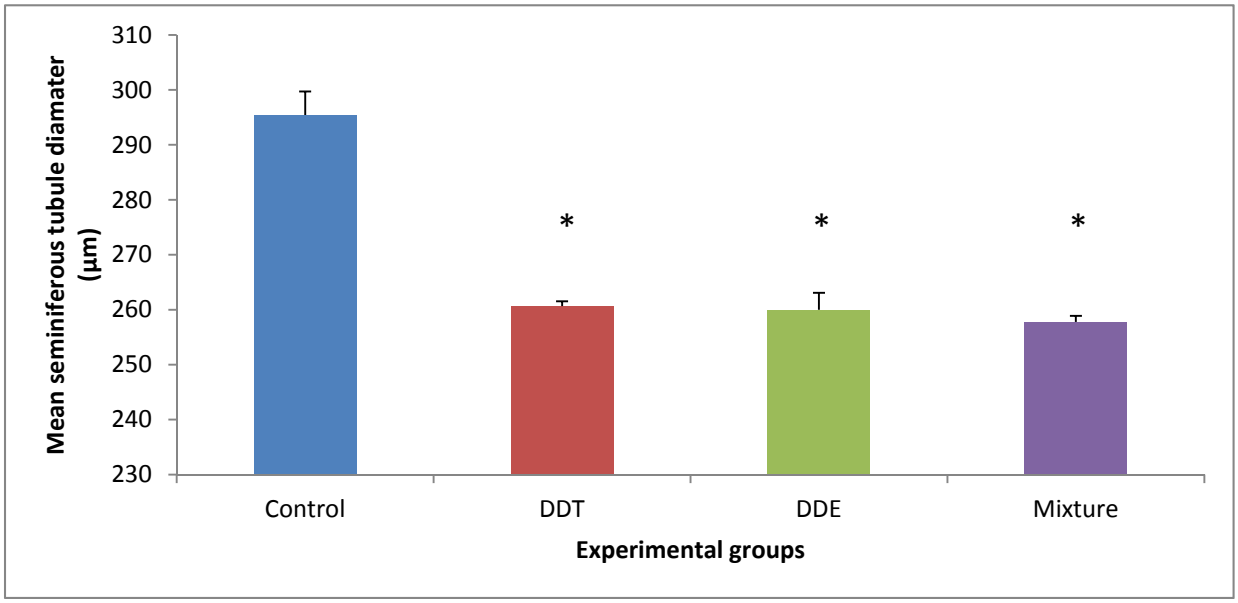


Figure 26: Mean and SD of mean seminiferous tubule diameter (µm) of the F1 males in the control and experimental groups.

Mixture group = DDT + DM + *p*-NP + phytoestrogens

* Significant: $P < 0.05$ when compared to the control group

### 5.14.2. Seminiferous epithelium thickness

The statistical analysis of the mean seminiferous epithelium thickness (µm) of the F1 males is summarized in Table 15. Compared to the control group (group 1; 100.40µm), statistically significantly smaller mean seminiferous epithelium thickness were found in the DDT (group 2; 84.77µm, $P < 0.001$), DDE (group 3; 86.33µm, $P < 0.001$) and in the mixture groups (group 4; 82.40µm, $P < 0.001$) (Figure 27).

Table 15: Statistical analysis of the difference between the mean seminiferous epithelium thickness (µm) between the control (group 1) and experimental (groups 2-4) groups, using survey regression analysis.

| Experimental groups | n | Mean (µm) | Standard Deviation | *P*-value | 95% Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 100.40 | 8.58 | **<0.001** | (-19.16 ; -12.11) |
| DDT | 11 | 84.77 | 3.45 | | |
| Control | 24 | 100.40 | 8.58 | **<0.001** | (-18.03 ; -10.12) |
| DDE | 26 | 86.33 | 4.10 | | |
| Control | 24 | 100.40 | 8.58 | **<0.001** | (-22.23 ; -13.77) |
| Mixture | 11 | 82.40 | 8.45 | | |

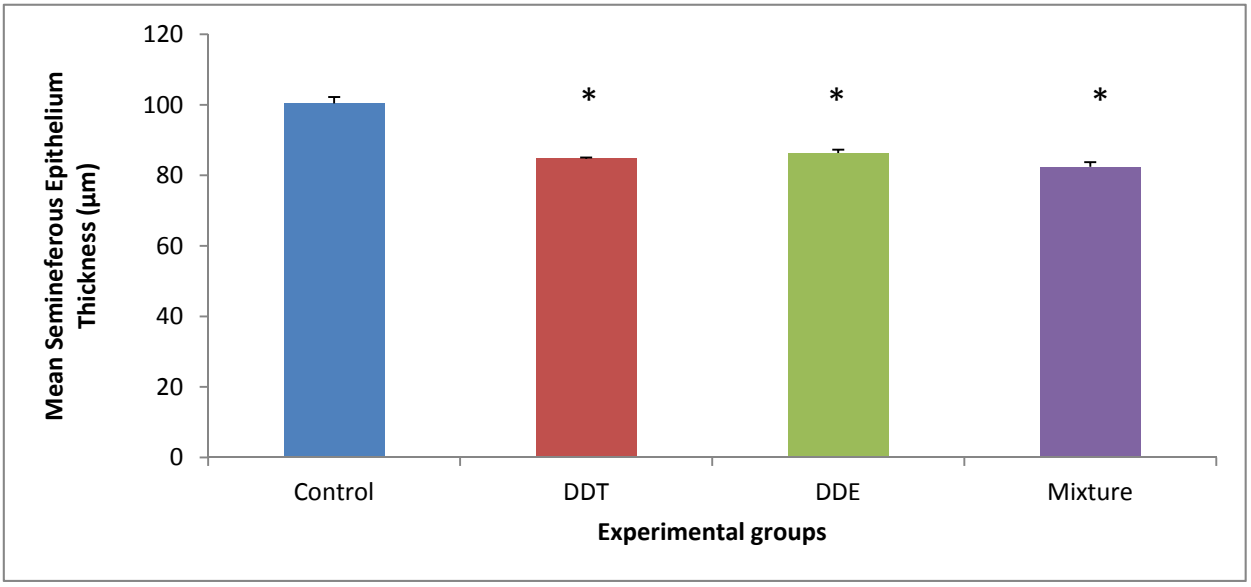


Figure 27: Mean and SD of mean seminiferous epithelium thickness (µm) of the F1 males in the control and experimental groups.

Mixture group = DDT + DM + *p*-NP + phytoestrogens

* Significant: $P < 0.05$ when compared to the control group

### 5.14.3. Lumen diameter

The statistical analysis of the mean lumen diameter (µm) of the F1 males is summarized in Table 16. Compared to the control group (group 1; 106.84 µm), statistically significantly smaller lumen diameters were found in the DDT (group 2; 87.62µm, $P < 0.001$), DDE (group 3; 80.15µm, $P < 0.001$) and in the mixture (group 4; 96.34µm, $P < 0.001$) groups (Figure 28).

Table 16: Statistical analysis of the difference between the mean lumen diameter (µm) between the control (group 1) and experimental (groups 2-4) groups, using survey regression analysis.

| Experimental groups | n | Mean (µm) | Standard Deviation | *P*-value | 95% Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 106.84 | 20.38 | **<0.001** | (-25.55 ; -13.59) |
| DDT | 11 | 87.62 | 12.40 | | |
| Control | 24 | 106.84 | 20.38 | **<0.001** | (-38.01 ; -28.10) |
| DDE | 26 | 80.15 | 8.08 | | |
| Control | 24 | 106.84 | 20.38 | **<0.001** | (-16.87 ; -6.46) |
| Mixture | 11 | 96.34 | 19.48 | | |

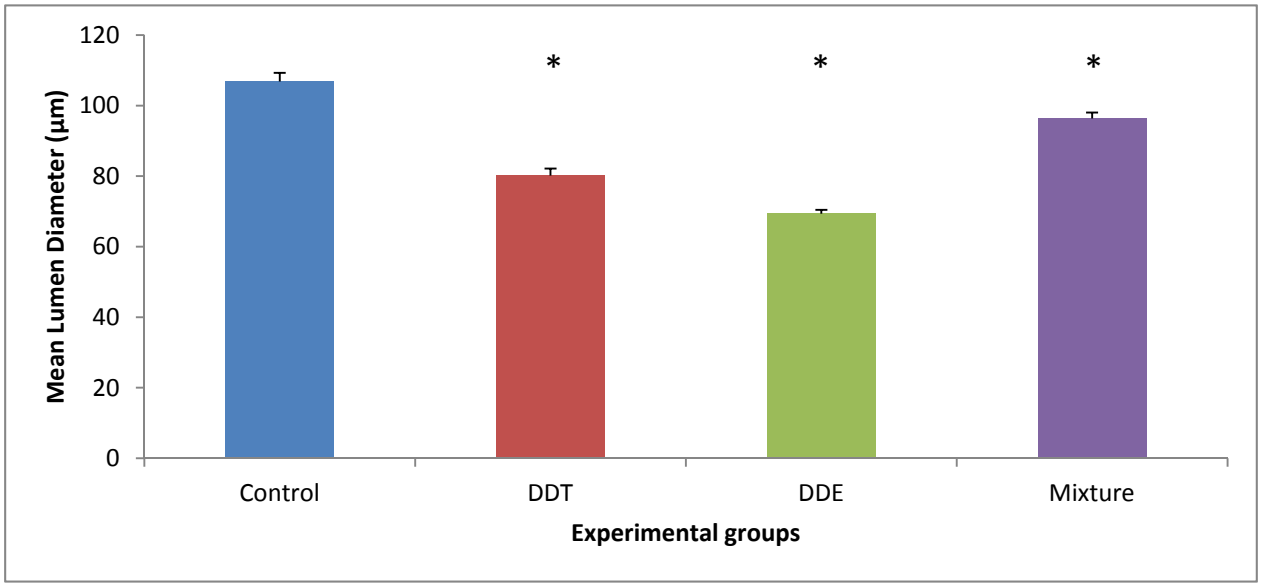


Figure 28: Mean and SD of mean lumen diameter (µm) of the F1 males in the control and experimental groups.

Mixture group = DDT + DM + *p*-NP + phytoestrogens

* Significant: $P < 0.05$ when compared to the control group

## 5.15. Testicular scoring

Traditional evaluation of the testes has limitations due to lack of standardization. The use of a scoring system, such as the Johnsen score (258), with defined criteria is essential for the assessment of complete and/or incomplete spermatogenesis.

### 5.15.1. Johnsen Score

Using the criteria set out by Johnsen, 100 random seminiferous tubules of each sample in each group was evaluated and given a score from 1 – 10 (Table 17). The experimental groups, DDT, DDE and mixture group had lower scores of 10 and higher scores of 9 in comparison to the control group. However, the cut-off point of seemingly normal seminiferous tubules and hence spermatogenesis could possibly be identified by the difference in the normal (Johnsen score = 10) and abnormal (Johnsen score – sum 1 to 9) seminiferous tubules as illustrated in Figure 29.

Table 17: Johnsen scores of the seminiferous tubules of the F1 males exposed to selected endocrine disrupting chemicals.

| Group | 10 | 9 | 8 | 7 | 6 | 5 | 4 | 3 | 2 | 1 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Control** | 81 | 17 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **DDT** | 54 | 41 | 1 | 0 | 0 | 1 | 0 | 3 | 0 | 0 |
| **DDE** | 75 | 23 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| **Mixture** | 45 | 54 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

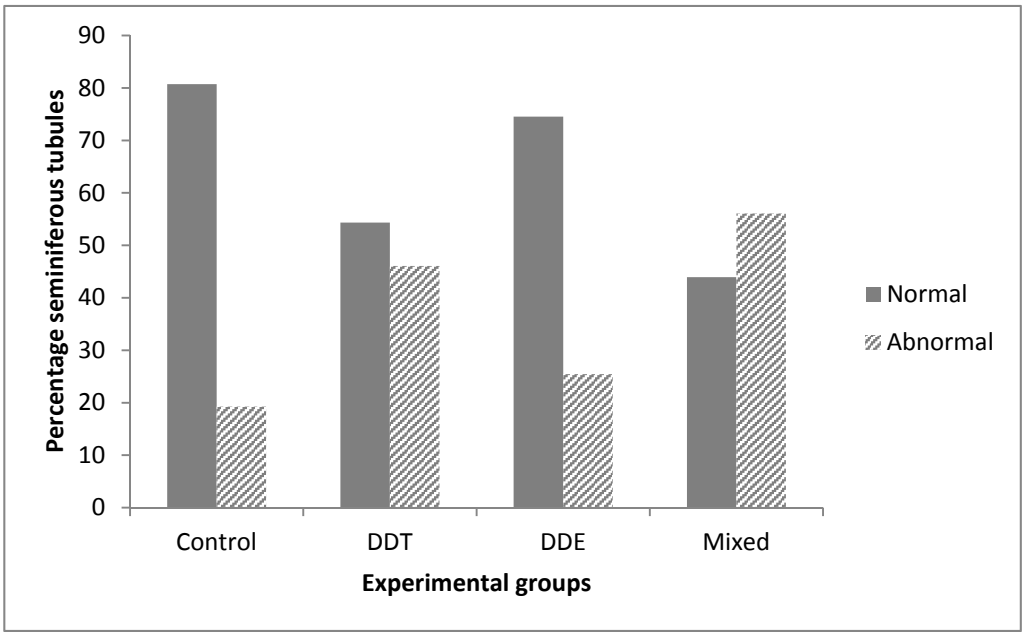


Figure 29: Johnsen scores of the seminiferous tubules of the F1 males exposed to selected endocrine disrupting chemicals showing normal (score = 10) and abnormal (sum scores 1 to 9).

### 5.16. Percentage caspase 3 positive seminiferous tubules

The statistical analysis of the mean percentage caspase-3 positive seminiferous tubules (%) of the F1 males is summarized in Table 18. Compared to the control group (29.13%), no statistically significant differences were observed between the mean percentage caspase 3 positive seminiferous tubules of the DDT (26.01%, $P$ = 0.270), DDE (25.51%, $P$ = 0.204) and the mixture groups (27.19%, $P$ = 0.401) (Figure 30). The caspase 3 positive stained sections show the staining pattern (clusters of positive cells) in the experimental groups (Figure 31).

Table 18: Statistical analysis of the difference in the mean percentage positive caspase 3 seminiferous tubule (%) between the control (group 1) and experimental (groups 2-4) groups, using survey regression analysis.

| Experimental groups | n | Mean (%) | Standard Deviation | *P*-value | 95 % Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 29.13 | 11.93 | 0.270 | (-8.93 ; 2.69) |
| DDT | 11 | 26.01 | 12.26 | | |
| Control | 24 | 29.13 | 11.93 | 0.204 | (-9.44 ; 2.19) |
| DDE | 27 | 25.51 | 9.85 | | |
| Control | 24 | 29.13 | 11.93 | 0.401 | (-6.72 ; 2.84) |
| Mixture | 14 | 27.19 | 9.73 | | |

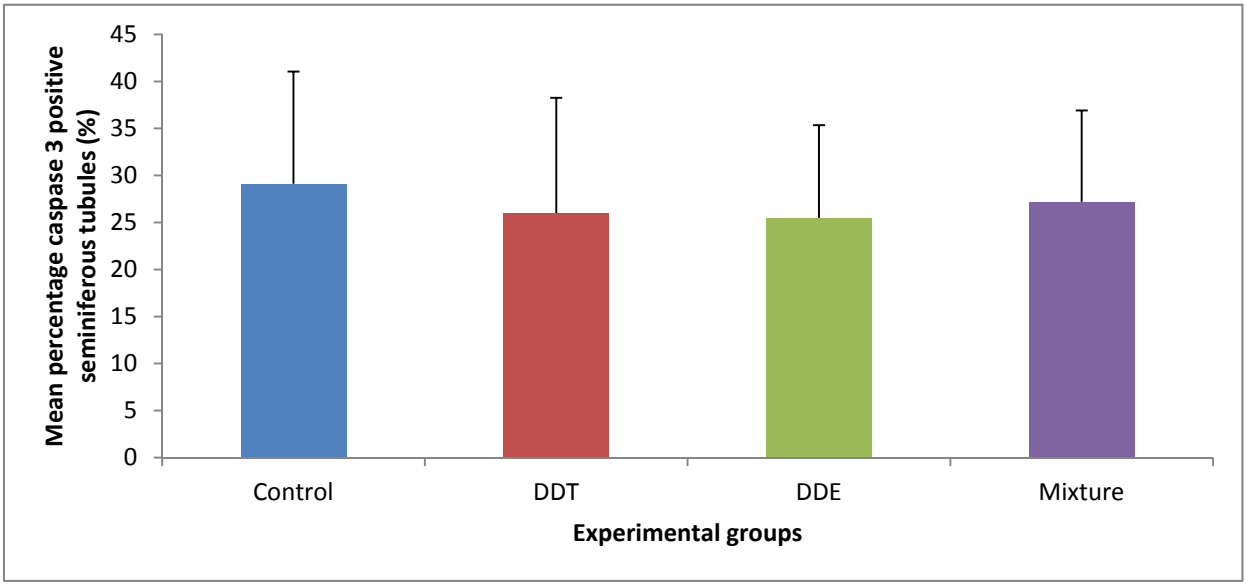


Figure 30: Mean and SD of the percentage positive caspase 3 seminiferous tubules (%) of the F1 males in the control and experimental groups.

Mixture group = DDT + DM + *p*-NP + phytoestrogens

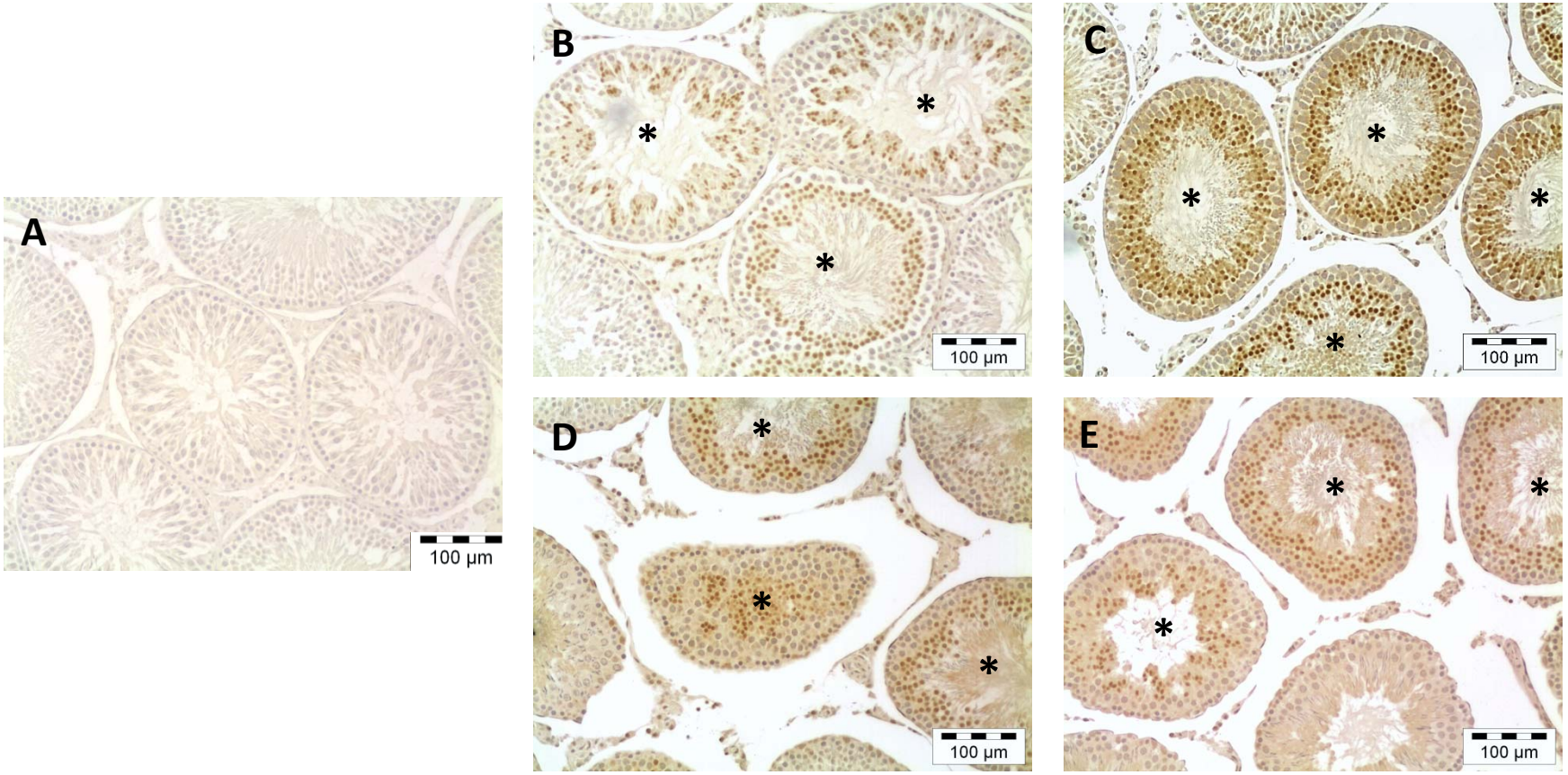


Figure 31: Caspase 3 IHC of F1 male rats following exposure to selected EDCs. The brown stain indicates positive caspase 3 labeled germ cells in the seminiferous tubules (*), with the intensity of the stain indicating a stronger positive reaction. A: Negative Control; B: Control group (group 1; Cottonseed oil); C: DDT group (group 2); D: DDE group (group 3); E: Mixture group (group 4; DDT, DM, *p*-NP, phytoestrogens).

### 5.17. Percentage terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling positive seminiferous tubules

The statistical analysis of the mean percentage TUNEL positive seminiferous tubules (%) of the F1 males is summarized in Table 19. Compared to the control group (group 1; 3.68%), no statistically significant differences were observed between the mean percentage TUNEL positive seminiferous tubules of the DDT (group 2; 3.72%, $P = 0.338$), DDE (group 3; 3.59%, $P = 0.116$) and mixture group (group 4; 3.57%, $P = 0.231$) (Figure 32). The TUNEL-positive stained sections show the staining pattern in the experimental groups (Figure 33).

Table 19: Statistical analysis of the difference between the mean percentage positive TUNEL seminiferous tubule (%) between the control (group 1) and experimental (groups 2-4) groups, using survey regression analysis.

| Experimental groups | n | Mean (%) | Standard Deviation | *P*-value | 95 % Confidence Interval |
|---|---|---|---|---|---|
| Control | 24 | 3.68 | 0.32 | 0.338 | (-0.10 ; 0.25) |
| DDT | 11 | 3.72 | 0.19 | | |
| Control | 24 | 3.68 | 0.32 | 0.116 | (-0.08 ; 0.46) |
| DDE | 27 | 3.59 | 0.32 | | |
| Control | 24 | 3.68 | 0.32 | 0.231 | (-0.19 ; 0.21) |
| Mixture | 14 | 3.57 | 0.21 | | |

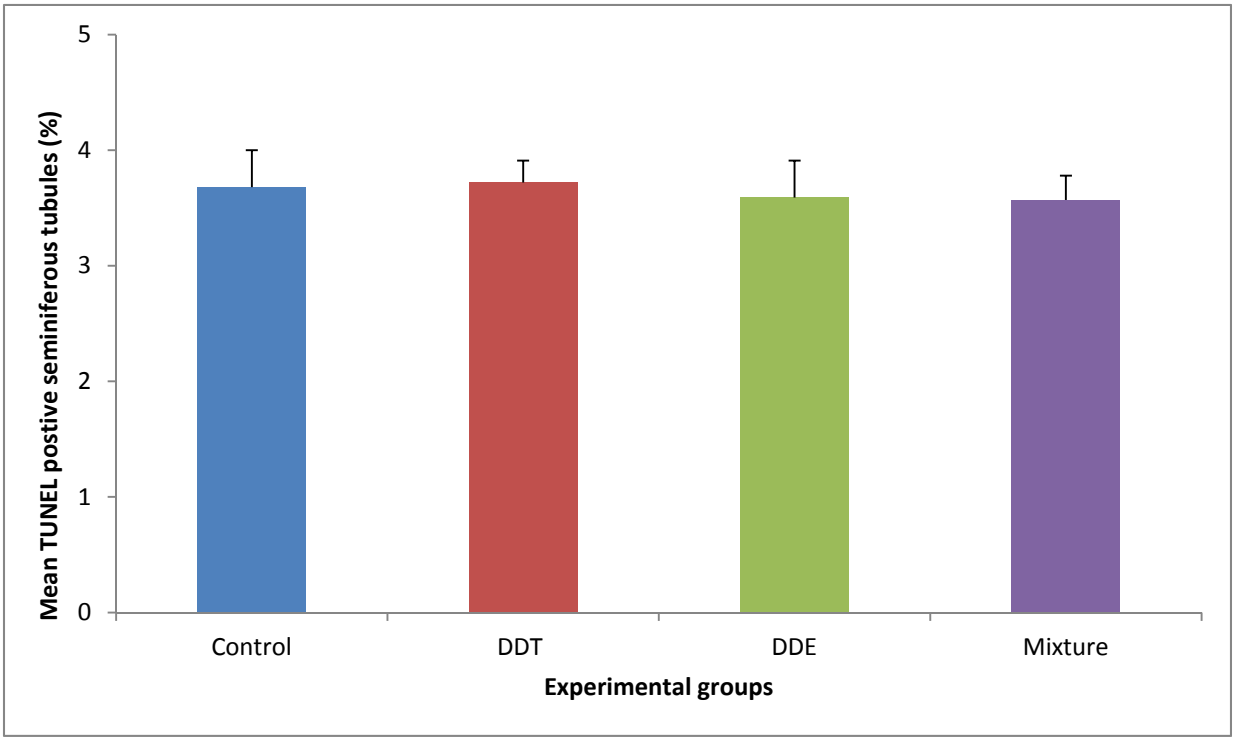


Figure 32: Mean and SD of the percentage positive TUNEL seminiferous tubules (%) of the F1 males in the control and experimental groups.

Mixture group = DDT + DM + *p*-NP + phytoestrogens

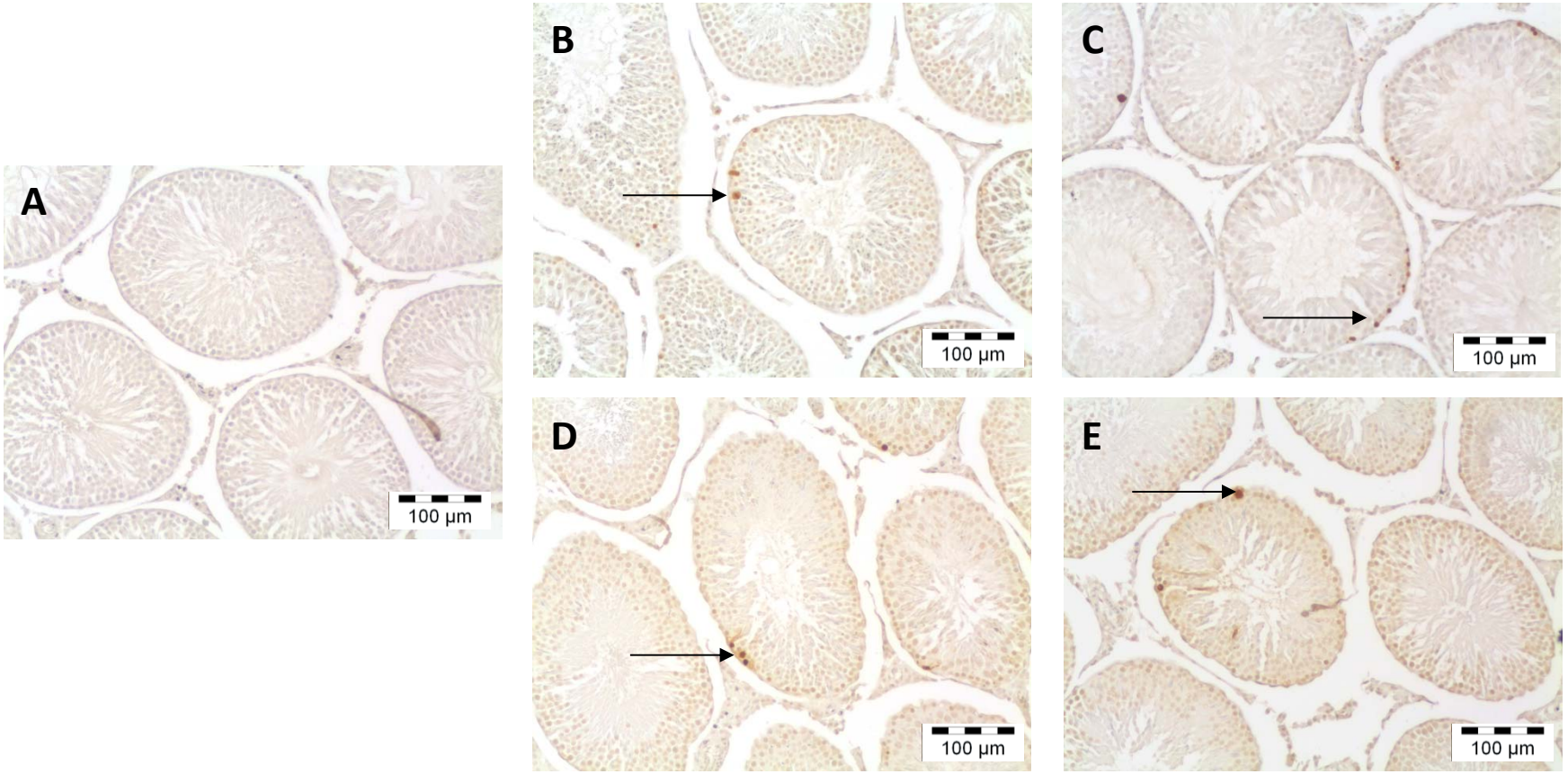


Figure 33: TUNEL *in situ* hybridization of F1 male rats following exposure to selected EDCs. TUNEL-positive germ cells in the seminiferous tubules (arrows) A: Negative control; B: Control group (group 1; Cottonseed oil); C: DDT group (group 2); D: DDE group (group 3); E: Mixture group (group 4; DDT, DM, *p*-NP, phytoestrogen).

# Chapter 6: Discussion

This study investigated the effects of *in utero*-, lactational- and direct exposure to concentrations of previously identified chemicals present in a malaria area, using Sprague Dawley rats. The original protocol for reproductive toxicity studies of the Organization for Economic Cooperation and Development (OECD) 415 (252) was modified to include endocrine sensitive endpoints as well as a deeper analysis of histology and measures of apoptosis.

The findings of this study are summarized in Table 20. Level of significant differences of the control group (group 1) compared to the DDT (group 2), DDE (group 3) and mixture (group 4) groups are noted.

Table 20: Summary of results, including level of significances between groups, after male rats have been during exposed *in utero*-, lactational- and directly to selected EDCs

| Variable | Group 1 | Group 2 | 1:2 | Group 3 | 1:3 | Group 4 | 1:4 |
|---|---|---|---|---|---|---|---|
| | mean | mean | *P*-value | mean | *P*-value | mean | *P*-value |
| **Anogenital distance (mm)** | 17.54 | 18.55 | 0.863 | 17.33 | 0.360 | 15.20 | **0.005*** |
| **Body mass (g)** | 430.34 | 437.54 | 0.561 | 414.91 | 0.317 | 419.08 | 0.499 |
| **Liver mass (g)** | 17.36 | 21.16 | **<0.001*** | 20.65 | **0.003*** | 19.45 | **0.031*** |
| **HSI** | 4.028 | 4.837 | **<0.001*** | 4.962 | **<0.001*** | 4.642 | **0.001*** |
| **Prostate mass (g)** | 0.83 | 1.02 | **0.018 *** | 0.82 | 0.858 | 0.83 | 0.981 |
| **Seminal vesicles mass (g)** | 1.46 | 1.60 | 0.294 | 1.57 | 0.430 | 1.58 | 0.494 |
| **Epididymal mass (g)** | 1.47 | 1.59 | 0.227 | 1.42 | 0.530 | 1.44 | 0.721 |
| **Total sperm count ($x10^6$)** | 48.46 | 60.13 | 0.063 | 50.69 | 0.685 | 38.72 | 0.090 |
| **Testosterone (nmol/L)** | 21.33 | 23.06 | 0.392 | 28.12 | **0.038*** | 28.62 | **0.023*** |
| **Testicular mass (g)** | 3.68 | 3.88 | **0.019 *** | 3.95 | **0.047*** | 4.02 | **<0.001*** |
| **GSI** | 0.86 | 0.89 | 0.435 | 0.96 | **0.036*** | 0.97 | **0.016*** |
| **Seminiferous tubule diameter (µm)** | 295.42 | 260.65 | **<0.001*** | 260.00 | **<0.001*** | 257.78 | **<0.001*** |
| **Seminiferous epithelium thickness (µm)** | 100.40 | 84.77 | **<0.001*** | 86.33 | **<0.001*** | 82.40 | **<0.001*** |
| **Lumen diameter (µm)** | 106.84 | 87.62 | **<0.001*** | 80.15 | **<0.001*** | 96.34 | **<0.001*** |
| **Caspase-3 positive seminiferous tubules (%)** | 29.13 | 26.01 | 0.270 | 25.57 | 0.204 | 27.19 | 0.401 |
| **TUNEL positive seminiferous tubules (%)** | 3.68 | 3.72 | 0.338 | 3.59 | 0.116 | 3.57 | 0.231 |

Group 1 = Cottonseed oil (Controls); Group 2 = 35mg/kg DDT; Group 3 = 35mg/kg DDE; Group 4 = 35mg/kg DDT + 0.5mg/kg DM + 2.5µg/kg p-NP + 2.5µg/kg coumestrol, 2.5µg/kg genistein, 2.5µg/kg zearalenone

**Bolded** and * = significant (P <0.05)

The mean **AGD** was significantly shorter in the mixture group (*P* = 0.005) and not significantly in the DDE group (*P* = 0.360) compared to the controls. The AGD was longer in the DDT group, but not significantly (*P* = 0.863). AGD is a sensitive marker of prenatal disruption of the development of the male reproductive system (173). The AGD in males is longer than in females – generally double the distance in females measured in multiple mammalian species, thereby suggesting that the AGD is under hormonal influence (180). The mixture group (group 4) received technical grade DDT, DM, *p*-NP and phytoestrogens, all of which have estrogenic properties. Synergistic activity between chemicals (55) could have further enhanced the total additive estrogenicity, resulting in a shorter AGD of the mixture group (group 4).

After prenatal exposure to the anti-androgen DDE, the mean AGD was also shorter, but not significantly. A shorter anogenital distance in males signifies feminizing changes (263), which might be related to lower/impaired androgen function during the hormone-sensitive male programming window (13). However, not only androgen plays an important role during masculinization, but an optimal androgen-estrogen balance is also involved. Maintaining the appropriate androgen–estrogen balance is crucial for normal development of the structure and function of the male reproductive tract. Disruption of the balance during early foetal development may lead to abnormal development of the male reproductive tract (57, 264). Also, Rivas et al (2002) demonstrated in a rat model that reduced androgen action sensitizes the reproductive tract to estrogen action; therefore this would seem a likely explanation for the shorter AGD after estrogenic exposure. Although the shorter AGD may seem a 'minor' phenotypic variant of normal male, the longer term implications may be more serious. In humans AGD measures have been related to prenatal exposure to endocrine disruptors (173, 266-270) or prostate cancer (271) and with semen quality and other reproductive outcomes (272-275).

The comparison of organ mass between exposed and unexposed groups of rats in reproductive toxicological studies has conventionally been used to evaluate the toxic effect of the test chemical or mixture of chemicals (276). Significant changes in body and organ weights are sensitive indicators of chemically induced effects on the body

and/or organs (276). There were no statistically significant differences in the mean body mass of the control group compared to the DDT or to any of the other exposed groups. The mean body weight of the DDE and the mixture group were lower than in the controls (DDT, $P = 0.561$; DDE, $P = 0.317$; mixture, $P = 0.499$) indicating non-significant (subtle) changes.

The **liver** plays and important role in the transformation, detoxification and excretion of chemicals from the body, therefore the liver is particularly susceptible to chemical toxicity (61). Enlargement of the liver is a marker of liver toxicity in toxicology studies (277). The mean liver mass in all treatment groups were significantly higher compared to the control group (DDT, $P<0.001$; DDE, $P<0.001$; mixture group, $P<0.001$). The mean hepatosomatic index (HSI) were, likewise, significantly higher in all exposure groups (DDT, $P<0.001$; DDE, $P<0.001$; mixture, $P<0.001$). These results resembled the finding from a study where adult male rats exposed for 7 days to 100mg/kg DDE, had a 13% higher liver mass (278). In this study, the liver mass was 19% higher than in controls after lifelong (*in utero*, lactational and direct) exposure to 35mg/kg DDE suggesting that chronic, low dose exposure had a significant effect on the liver of male rats.

Hepatocyte hypertrophy following chemical exposure is the most common cause of increases in absolute and relative liver mass (279). Singh et al (2014) suggested that exposure to chemicals and their metabolites may produce necrosis, degeneration and hepatic accumulation of fatty acids in the liver. In the present study mild steatosis was also observed in the liver tissue of the exposure groups (DDT, DDE and mixture groups). Mild steatosis develops when chemicals interfere with lipid mobilization during the formation of very low density lipids (VLDL). The VLDLs are synthesized in the liver and transport endogenous triglycerides back into the circulation (281). However, a decrease in VLDL synthesis results in fatty acid accumulation in the liver (282). This is due to endogenous factors, such as inhibition of lipid utilization in the hepatocytes (resulting in decreased beta-oxidation), increase in lipid synthesis in the hepatocytes (resulting in an increase in fatty acid and triglyceride formation) and a decrease in lipid export (resulting in a decrease in VLDL secretion) (283). *p,p'*-DDE was detected in VLDL of occupationally-exposed males (284) suggesting that lipoprotein production is

an DDT target. Thus, *in utero*-, lactational- and direct exposure to EDCs may increase lipid synthesis in the hepatocytes and lead to steatosis (fatty liver, build-up of fats in the liver). Furthermore, hepatic steatosis is a risk for steatohepatitis (an inflammatory response associated with steatosis) and cirrhosis (late stage fibrosis due to chronic liver damage) of the liver (Choi and Ginsberg, 2011).

The mean **prostate** mass was significantly higher in the DDT group (group 2; 1.02g, $P$ = 0.018), compared to the control group (group 1; 0.83g). Technical grade DDT has estrogenic properties mainly due to the *o,p'*–DDT isomer (285). Estrogens have direct effects on the adult prostate gland and have been implicated in the etiology of prostatic disease (89). ERα and ERβ are expressed in the prostate stroma and epithelium respectively (286). In humans, during the third trimester of pregnancy the androgen levels decline and the maternal estrogen levels rise, inducing stromal and epithelial cell differentiation. This differentiation is directly influenced by both estrogens and androgens (287).

It seems plausible that exposure to both endogenous and exogenous estrogenic and/or anti-androgenic compounds could interfere with prostate growth (288). A permanent disturbance in prostate growth has been shown in the presence of elevated endogenous or exogenous estrogenic compounds (289, 290). Chronic elevated estrogen levels in men have been associated with increased prostate cancer risk (205). In this study, although not significant, the prostate mass in the DDE group was lower compared to the control group. The anti-androgenic effect of DDE in lowering the prostate mass is similar to other reports (287, 291). The prostate mass in the mixture group (group 4) was similar to the control group (group 1), a finding that is inconsistent with reports in scientific literature (288, 292, 293). In this study, the compounds in the mixture group (group 4) all had varying degrees of estrogenic properties. During the development of the prostate estrogen influences the programming of the stromal cells and directs early morphogenic events. However, if estrogenic exposure should rise during these critical development periods, permanent changes in the cellular differentiation and size of the prostate will occur (294). Exposure to a mixture of

chemicals may exert an additive or a synergistic effect, thus effects may exist on a histological level, which was not studied.

The mean masses of the **seminal vesicles** and the **epididymides** showed no significant differences compared to the control group. Both organs are androgen-dependent and were not histologically examined or functional biological markers measured. Since the seminal vesicles contribute the fluid component to the ejaculate (295) and epididymal function includes sperm maturation and acquisition of motility (296), the adverse effects could have been on a level not assessed by organ mass.

There were also no significant differences between the mean **epididymal sperm counts** of the control and exposed groups. This finding was inconsistent with reproductive toxicology studies with exposure to DDT and DDE (6, 297), DM (37) and *p*-NP (142) which all showed significant decreases in the sperm counts. The duration of exposure in the present study was different from these studies and could account for some variance. The epididymis serves as a sperm reserve, thus over time, a decrease in sperm production may not translate to a decrease in sperm reserves.

In the testes, testosterone plays a vital physiological role and is essential for normal spermatogenesis (71) as it promotes the differentiation of spermatogonia by stimulating genes within the Sertoli cells (63). The mean total testosterone concentrations were significantly higher in the DDE (group 3; 28.12nmol/L, $P = 0.038$) and mixture (group 4; 28.62nmol/L, $P = 0.023$ compared to the controls. These findings confirmed the previous findings of higher testosterone concentrations following exposure to 300mg/kg p,p'-DDE for 15 days (298). In a study investigating hormonal changes associated with DDT uptake in men (299), exposure to estrogenic- and anti-androgenic compounds increased steroid hormone binding globulin (SHBG), but it was not measured in the present study. Blood samples for the present study was collected between 08:00am and 10:00am to allow for standardization in terms of the testosterone circadian rhythm (300), which added value to the findings. Several reproductive toxicology studies did not mention the time of the blood draw (298, 301, 302).

Compared to the control group (group 1; 3.68g), the **mean testicular mass** was statistically significantly higher in the exposure groups (DDT, *P* = 0.019; DDE, *P* = 0.047; mixture *P*<0.001). Increases and decreases in testicular mass have been reported following exposure to various EDCs at various exposure durations and doses (36, 292). Testicular mass may be affected by chemical induced changes such as fluid accumulation or impaired cellular proliferation and/or differentiation (292). Thus, an increase in testicular mass has been attributed to dilatation of the tubular lumen (69). Sertoli cells are androgen dependent and some EDCs are Sertoli cell toxicants. The Sertoli cell produces the seminiferous tubule fluid (63) which is absorbed by the rete testis, efferent ducts and the epididymal epithelium (69, 296). The tubular fluid volume is a function of the secretion and reabsorption rates in the rete testis and epididymis. Changes in these functions may **manifest as either contracted or dilated tubular lumens** (69).

In this study, histological examination of the testes showed dilated tubular lumens, detachment of the seminiferous tubule, necrosis in the interstitium, seminiferous epithelium disorganization with few germ cells layers present, reduced seminiferous tubule diameter with no lumen, absent seminiferous tubules, epithelial sloughing and seminiferous epithelial vacuolization in the exposed groups. The seminiferous tubule diameters and seminiferous epithelium thickness were also reduced in the exposed groups, particularly in the DDE (group 3) and mixture groups (group 4). Although in the exposed groups the seminiferous tubule diameter and the epithelium thickness were smaller, the striking difference was that the relative luminal size was larger, which is in accordance with testicular histology findings of reproductive toxicology studies investigating the effects of EDC exposures (36, 37). These findings suggest that the chemicals used in this study, had toxic effects on Sertoli cells, which might account, at least in part, for the abnormal seminiferous tubules observed.

When assessing the **spermatogenic stages**, all 14 stages of the rat spermatogenic cycle (73) were present in the testicular sections in this study. However, when analyzing the seminiferous tubule diameter, epithelium thickness and lumen diameter per spermatogenic cycle stage, clear differences could be seen in structure of the abnormal

tubules in the exposed groups compared to the control. The seminiferous tubule diameter, epithelium thickness and lumen diameter in the control group followed the normal size changes throughout the spermatogenic cycle as described in the scientific literature (73), However, the seminiferous tubule diameter, epithelium thickness and lumen diameter in the exposed groups did not follow the same trends as in the control groups. The changes in the size of the seminiferous tubule diameter, epithelium thickness and lumen diameter during spermiation and the meiotic divisions differed greatly in comparison with the control. This highlights the fact that the spermatogenic stages and the quantitative measurements of the seminiferous tubules should not be evaluated in isolation, but that a staggered approach should be taken to assess the impact exposure to EDCs might have on the testis. These results indicated that there were subtle changes in the natural progression of spermatogenesis, which might negatively affected the production of sperm and ultimately possibly fertility.

A closer evaluation the testicular histology using the **Johnsen scoring system** (258) was used for the first time, to the best of my knowledge, in a reproductive toxicology study in rats to assess the effects of *in utero*-, lactational- and direct exposure to environmentally relevant concentrations of EDCs. The Johnsen score enables classification of the seminiferous tubules into normal complete spermatogenesis and abnormal incomplete spermatogenesis following strict assessment criteria (258). Using the Johnsen Scoring system, this study reported compromised spermatogenesis in treatment groups. The DDT, DDE and mixture groups (groups 2-4) had higher numbers of abnormal seminiferous tubules with incomplete spermatogenesis in comparison to the control group (group 1). These findings were in accordance with the observation that exposure to various EDCs have a negative effect on the testicular histology and, therefore, result in incomplete spermatogenesis (57, 303). Testicular histology of rats exposed to varying doses of *p*-NP displayed abnormalities, including apical sloughing and seminiferous tubule degeneration (36). Similarly, rats exposed to a mixture of EDCs showed abnormal testicular histology, with signs of seminiferous tubule degeneration (37).

The degeneration of the seminiferous tubules might be attributed to the loss of germ cells through apoptosis (304). During spermatogenesis, up to 75% of potential spermatozoa has been estimated to undergo **apoptosis** (305, 306). In so doing, apoptosis serves as a mechanism to remove excess germ cells or germ cells not able to pass the control points of the cell cycle (307). The process of apoptosis (216), commonly mediated by the caspase family (47), especially the main executioner caspase 3, plays an integral role in spermatogenesis.

In this study, compared to the control group (group 1; 29.13%) there were no significant differences in caspase 3 positive seminiferous tubules in the exposed groups. These findings are inconsistent with findings that adult male rats exposed for 10 days to 20, 60 and 100mg/kg *p,p'*-DDE showed an increase in testicular apoptosis, with a significant increase in messenger RNA (mRNA) expression of caspase 3 in at the highest concentration (308). Following caspase activity the cell undergoes DNA fragmentation and the detection of the fragmented DNA gives an indication of cells in the final stages of cellular death (238). DNA fragmentation is ideally detected using the TUNEL assay (249). In the current study, compared to the control group (group 1; 3.68%), there were no significant differences in the TUNEL-positive cells in the exposed groups compared to the control group. These findings are inconsistent with similar studies where male rats exposed to 5mg/kg ZEN (46), 8.75mg/kg DM for 15 days (309), *p*-NP (247, 310) induced apoptosis and fragmented DNA was detected using the TUNEL assay.

Whilst there were no significant differences in the caspase 3 or TUNEL positive cells, the clustered distribution of positive cells in this study was in accordance with the observation in literature that apoptosis can primarily affect a single member of a spermatogonial cell cohort. Thus, the affected non-viable spermatogonia cells will remain joined to viable spermatogonial cells, accounting for clusters of positive caspase 3 or TUNEL positive cells (311, 312). It has been reported that intercellular bridges allow the sharing of molecular signals, which may also act in a similar way in spermatocytes and spermatogonia (313). It is, therefore, plausible that exposure to the selected EDCs used in this study may act in a similar way, resulting in incomplete spermatogenesis. In the DDT, DDE and mixture groups of this study; incomplete

spermatogenesis was observed in the testicular histology and confirmed by the Johnsen score.

This study addressed a gap in testing strategies by modifying a standard OECD 415 protocol reproductive toxicology protocol to include a longer prenatal period of exposure and adding non-standard endocrine sensitive endpoints, such as AGD, testosterone, testicular STAGES, caspase 3 immunohistochemistry, TUNEL *in situ* hybridization and the Johnsen score. The modified protocol used in this study allowed for a more detailed assessment of histological changes. In this study, to the best of our knowledge, the Johnsen score for assessing spermatogenesis has been used for the first time to assess the effects of EDCs on spermatogenesis in a rat model.

The data from this study indicated that *in utero*-, lactational- and direct exposure to a DDT, DDE, DM, p-NP and phytoestrogens had negative effects on male reproductive organs and associated endocrine-sensitive endpoints. Since these EDCs, in particular DDT and DDE have long half-lives, the long-term and potential multiple-generational effects need to be addressed in future studies. From the results of this study, the testes were the apparent target of the selected EDCs. The abnormal testicular histology, apical sloughing, seminiferous tubule disorganization, interstitial necrosis and reduced seminiferous tubule diameters, warrants investigation into the possible molecular and biochemical mechanisms and changes that *in utero*-, lactational- and direct exposure to these EDCs had on testicular function. In particular, the results from this study suggested further investigation into the effects on Sertoli cells and function. Since this reproductive toxicology study constitutes *in utero*-, lactational and direct exposure to environmentally relevant concentrations of EDCs present in a currently malaria-vector control area, these results are relevant to potential effects in humans following similar exposures. For most of the outcomes of this study, all the experimental groups showed similar adverse effects and there was no clear indication if DDT or DDE was more disruptive. The findings of this study add to the growing, scientific-based appeals for the development of alternatives to DDT as a matter of global importance.

# Chapter 7: Conclusions

1. Exposure both to single chemicals (DDT or DDE) and a mixture of chemicals caused significant changes in some endocrine sensitive endpoints.

2. The AGD was significantly shorter in the mixture group of estrogenic chemicals, emphasizing that the synergistic effect of the chemicals may play a key role in the androgen:estrogen ratio during masculinization of the male foetus and may have possible developmental consequences due to this imbalance.

3. Total testosterone concentrations were significantly higher in the DDE and mixture group. In the DDE group this might have been a result from the androgen blocking properties of DDE. The mechanism of higher testosterone and estrogenic chemical exposure in the mixture group is unclear.

4. Although normal seminiferous tubules were present, the testicular histology in all exposure groups was adversely affected in a significant proportion of the tubules, with apical sloughing, seminiferous epithelium disorganization, interstitial necrosis, significantly reduced seminiferous tubule diameters, epithelial thickness and lumen diameter.

5. The Johnsen score allowed for an in depth assessment of the testicular histology, where exact criteria were used to classify seemingly “normal” seminiferous tubules into complete and incomplete spermatogenesis categories. The Johnsen score should be routinely used in any animal testicular evaluation or any reproductive toxicity protocol.

6. The mean liver mass and HSI were significantly higher in the exposed groups. Hepatocyte disorganization and steatosis were present in the liver tissue. Therefore, reproductive effects may be secondary to liver toxicity. This is a possibility that needs to be addressed in future research.

7. The findings of the study raise concern about the potential risk associated with DDT exposures to mothers living in malaria-areas and the reproductive health of their male offspring.

# Chapter 8: Recommendations

1. It is essential to include non-standard, endocrine-sensitive endpoints such as AGD, testosterone, testicular STAGES and the Johnsen score, to investigate the health effects of exposure to EDCs.

2. Future studies on exposure to EDC multi-generational studies would be needed to determine whether effects shown in this study may be transmittable to future generations, even in the absence of exposure.

3. Future studies are needed to determine early cellular and molecular events in the development of the testis and other male accessory glands that may be perturbed by EDCs, such as those evaluated in this study.

4. Alternative safer chemicals are needed to protect people against malaria and should be high on the international agenda.

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