Veterinary growth promoters in cattle feedlot runoff: estrogenic activity and potential effects on the rat male reproductive system

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ABSTRACT

The contribution of veterinary growth promoters (VGP) to the environmental burden of endocrine disrupting chemicals (EDCs) is largely unknown. At cattle feedlots, the excrement of cattle may contain VGPs, which can contaminate aquatic systems and pose reproductive health risks. The study identifies VGPs used at cattle feedlots in South Africa and confirms associated estrogenic activity in feedlot runoff water. Using a rat model, we investigate the potential reproductive health effects and thyroid function of an environmentally relevant mixture of VGPs. Collected water samples had low levels of selected VGPs and estrogenic activity was detected in the T47D-KBluc bioassay. Rats exposed to VGP had significant adverse effects on male reproductive health, including shortened anogenital distance, lowered sperm counts, disorganized seminiferous tubules and thyroid parameters. In conclusion, VGP can contribute to complex environmental EDC mixtures and may adversely affect the reproductive and thyroid health of both humans and wildlife. The varied topography of individual cattle feedlots will govern the rate and extent of effluent runoff, thus continuous monitoring of VGPs in aquatic systems surrounding cattle feedlots is necessary.

Keywords:

Androgenic, bioassays, cattle feedlots, endocrine disrupting chemicals, estrogenic, South Africa, veterinary growth promoters

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are natural or synthetic compounds that disrupt hormone dependent processes and elicit adverse health effects in both animals and humans (Sifakis et al. 2017). While the list of chemicals with suspected EDC activity is growing, the contribution of veterinary growth promoters (VGPs) to the environmental burden of EDCs remains largely unknown. In the European Union (EU), hormones are banned as growth promoters, but can still be used as estrus inductors or suppression agents (Casewell et al. 2003). Even at small quantities, VGPs have very high biological activity prompting continuous environmental monitoring. In an effort to identify various active veterinary medicines that pose environmental risk, Kools et al. (2008) estimated that in 25 EU countries, antibiotics (5393tons), anti-parasitics (194tons) and hormones (4.6tons) were the most widely used. The contribution of these drugs to overall environmental contamination is unknown, since relative usage rates differ by agricultural activity, country, prescribing practices and preferences (Daughton 2007).

Agriculturally, VGPs are used in animal husbandry activities and in livestock production. Primary VGPs used in feedlot operations include the estrogenic compounds zearanol (Lega et al. 2017), zilpaterol (Papich 2011) diethylstilbestrol (DES) (Raun and Preston 2002) and the androgenic compounds, trenbolone (Jones et al. 2014) and methyltestosterone (Papich 2011). South Africa is an important livestock producer with about 13.8 million cattle with the feedlot industry contributing up to 80% of the total beef production (DAFF 2015). In South Africa, VGPs are approved for use in beef products under the Register Act 36 of 1947. Approved VGPs include estradiol, progesterone and testosterone (natural), zearanol and trenbolone (synthetic) (SAFA 2016). While these VGPs are used to promote the growth of livestock, such as cattle, they are also associated with negative impacts on water sources (Vasconcelos et al. 2007) and human health (Bridges and Bridges 2001).

Humans are largely exposed to VGPs through the ingestion of meat products. Zearanol is a non-steroidal anabolic estrogenic growth promoter that increases weight gain in animals (Lange et al. 2002) and has been associated with an increased risk of developing breast cancer in women (Belhassen et al. 2015). Zilpaterol is a synthetic beta-adrenergic agonist with effects similar to norepinephrine that is administered to improve feed efficiency and promote muscle gain in cattle (Papich 2011). Zilpaterol accumulates in the liver (68 µg/kg), kidney (62 µg/kg) and muscle (73 µg/kg) tissue of feedlot cattle (Tulliez 2000), levels above the average daily intake suggested by the Food and Agriculture Organization of the United Nations (FAO/WHO 2014). Diethylstilbestrol (DES) was used as a VGP in livestock and was banned in 1971, but may still be used illegally in certain livestock producing countries (Raun and Preston 2002). Diethylstilbestrol is a potent estrogen and has been shown to increase thyroid-binding globulin (Papich 2011). In South Africa, estradiol, progesterone and testosterone (natural), zearanol, zilpaterol and trenbolone acetate are approved VGPs (SAFA 2016), while DES, tylosin, spiramycin, bacitracin and virginiamycin were banned because of their structural relatedness to therapeutic antimicrobials used in humans (Eagar et al. 2012). Trenbolone is a synthetic androgen steroid used to enhance growth and feed efficiency in beef cattle (Lange et al. 2002). In feedlots, trenbolone is administered as trenbolone acetate, which is then hydrolyzed to 17β-trenbolone (Kolodziej et al. 2013). Trenbolone metabolites are ultimately mobilized in feedlot runoff (Jones et al. 2014), resulting in ongoing scrutiny to reveal the consequences of its presence in the environment (Bertram et al. 2015). Methyltestosterone is an anabolic androgen agonist administered to promote growth and stimulate erythropoiesis (Papich 2011) and is illegally used to promote cattle growth (Miller 2002). The hormone 17β-estradiol (E_2) can be used alone or in combination with other synthetic hormones, such as testosterone, progesterone or trenbolone acetate, to improve the rate of weight gain and feed efficiency in cattle (Passantino 2012). The use of VGPs will depend on the size of the feedlot and number of cattle kept in the feedlot, and subsequently the impact of feedlot on the surrounding environment will depend on the topography of the surrounding area and proximity to water sources.

Veterinary growth promoters used to promote livestock growth have been associated with negative impacts on water sources (Vasconcelos et al. 2007). In the United States, effluent from beef cattle

concentrated animal feedlot operations displays androgenic activity *in vitro*, due, in part, to the presence of a steroid used to promote growth in beef cattle (Gray et al. 2006). The effluent from cattle feedlots may concentrate in local aquatic systems and pose a potential health risk to the surrounding biota (Bartelt-Hunt et al. 2012, Adeel et al. 2017). At feedlot sites, resident fish species have increased somatic growth and lower reproductive rates (Leet et al. 2012). According to Johnson et al. (2005), the estrogenic activity in surface waters close to livestock farms may be high enough to cause endocrine disruption in some aquatic organisms. Similarly, intensive livestock farming is associated with biologically active concentrations of natural estrogens in surface water in North America, Israel, Ireland and Denmark (Matthiessen et al. 2006). While the presence of these compounds in the environment is undisputed, additional research is needed to determine the toxicological or environmental implications of groundwater contaminated with multiple steroids and pharmaceuticals (Bartelt-Hunt et al. 2011).

In South Africa (SA), no research has investigated EDCs associated with feedlot activities. With the growing concern of human exposure to complex EDC mixtures, research is needed to assess environmental mixtures of concern. In SA, people living in rural areas often depend on environmental water for drinking and household purposes, increasing their exposure to potentially harmful EDCs. This study aimed to identify compounds used at cattle feedlots in SA, to confirm estrogenic activity in runoff water and to investigate the potential reproductive health effects and thyroid function of environmentally relevant mixtures of VGPs in a rat model.

MATERIALS AND METHODS

Feedlot sites

After surveying a number of feedlots throughout SA (DAFF 2015), we realized that each feedlot has a unique topography. To determine the possible implications of feedlot activity on associated aquatic systems, we purposively selected two feedlots close to perennial water systems. We identified 11 sampling sites, three at Feedlot A and eight at Feedlot B. We collected water samples from boreholes within the feedlots, settling ponds, surface water and water from the closest river.

Chemical analysis

Sample collection and preparation

All samples were collected in 11 glass bottles, pre-rinsed with methanol and deionized water. Clear bottles were wrapped in foil to protect the content from light. The samples were stored in a refrigerator at 4°C until analysis. Samples with a high concentration of suspended matter were centrifuged at 4000 rpm for 10 minutes to avoid clogging the filter paper. The samples were filtered through glass micro fiber filters prior to analyses, to avoid clogging the solid phase extraction (SPE) cartridges.

The samples were analyzed with the LC-MS/MS system. In this study, analytes included both natural and synthetic hormones, Androstanolone, Androstenedione, Androsterone, Diethylstilbestrol, β-Estradiol, Estriol, Estrone, Ethisterone, α-Ethynyl estradiol, Medroxyprogesterone, Methyltestosterone, Nandrolone, Norgestrel, Progesterone, Testosterone, Trenbolone, α-Zearalanol, β-Zearalenol, Zilpaterol. We expected these compounds to occur in and around feedlots in South Africa. We analyzed a limited number of metabolites and excluded conjugated compounds from the chemical analysis. For most of the compounds, we set the quantification limits at 10 ng/l, except for Zilpaterol which had a quantification limit of 50 ng/l.

Sample extraction

To quantify estrogenic hormones, we extracted hormones by concentrating and separating the hormones from interfering compounds. The method comprised a number of steps, 1) pre-preparation,

2) enzymatic hydrolysis of the conjugates and 3) SPE. In step two, we used *Helix Pomatia* juice (β-glucuronidase-aryl sulfatase) to enhance the deconjugation of the hormones from the matrix (Le Bizec et al. 1993). Briefly, samples were hydrolysed overnight (15 h) at 52°C with *H Pomatia* to produce the free steroid following the modified method by Le Bizec et al. 1993, since it was expected that many water samples would contain urine, and thus deemed it necessary to deconjugate the steroids. For the SPE, 1l of water was passed through an Oasis Hydrophilic-Lipophilic Balance (HLB) cartridge. The cartridges were subsequently eluted with acetonitrile for LC-MS/MS analysis.

LC-MS/MS analysis

We identified the hormones in the water samples using an Applied Biosystems API 4000 QTRAP LC-MS/MS system equipped with a Shimatzu Prominence autosampler and binary LC pump. All compounds were monitored with two Multiple Reaction Monitoring (MRM) transitions per compound.

Bioassays for estrogenic activity

Water sample collection

Surface samples were collected as grab samples from various water sources in the vicinity of Feedlots A and B. Water samples were collected in 1l - glass Schott bottles, pre-washed with HPLC grade ethanol (Sigma-Aldrich, St. Louis, MO, USA). The samples were kept at 4°C and analysed within 10 days after collection.

Extraction procedure

To extract potential estrogen-like compounds, we performed a SPE according to the protocol described in Bornman et al. (2007). The extracted samples were stored at –20°C prior to analysis.

Recombinant yeast screen assay

We performed the YES assay according to the assay procedure (Routledge and Sumpter 1996) and analysis described in Bornman et al. (2007) and Aneck-Hahn et al. (2008). Serial dilutions were made from the water samples and controls in 96-well microtiter plates. Each plate contained at least one row of blanks (assay medium and solvent ethanol). A standard curve for E_2 (Sigma-Aldrich, St. Louis, MO, USA) was drawn ranging from 1 x 10^{-8} M to 4.8 x 10^{-12} M (2.724 µg/l to 1.3 ng/l) and extended to a concentration of 1.19 x 10^{-15} M (3.24 x 10^{-13} g/l). After a 3-day incubation period, we checked color development of the medium from day 3 to 5 at an absorbance (abs) of 540 nm for color change and 620 nm for turbidity of the yeast culture. Absorbance was measured on a Titertek Multiskan MCC/340 plate reader to obtain data with the best contrast. After incubation, the control wells appeared light orange in color, due to background expression of β-galactosidase and turbid due to the growth of the yeast. Positive wells were indicated by a deep red color accompanied by yeast growth. Clear wells, containing no growth indicated lysis of the cells and color varied from yellow to light orange. All experiments were performed in quadruplicate. The following equation was applied to correct for turbidity:

Corrected value = test abs (540 nm) - [test abs (620 nm) - median blank abs (620 nm)].

The E_2 standard curve was fitted (sigmoidal function, variable slope) using Graphpad Prism (version 4), which calculated the minimum, maximum, slope, EC_{50} value and 95% confidence limits. The detection limit of the yeast assay was calculated as absorbance elicited by the solvent control (blank) plus three times the standard deviation. The estradiol equivalents (EEq) of the water samples were

interpolated from the E_2 standard curve and corrected with the appropriate dilution factor for each sample on day 4.

T47D-KBluc assay

We processed the extracts as described in Wilson et al. (2004). Ninety-six well luminometer plates were seeded at 5 x 10^4 cells per well and allowed to attach overnight. We prepared dosing dilutions in growth media containing 5% dextran-charcoal treated FBS (Hyclone, Laboratories, Logan, UT, USA). Vehicle (ethanol) did not exceed 0.2%. Each plate contained agonist positive control (E_2), negative control (vehicle only), antagonist control (E_2 plus ICI) and background control (vehicle plus ICI). Each sample was tested alone, and in the presence of 0.1 nM E_2 or ICI. Cells were incubated for 24h with 100 µl/well dosing solution at 37°C, 5% CO_2. After the incubation period, cells were washed with phosphate buffered saline (PBS) at room temperature and lysed with 25 µl lysis buffer (Promega, Madison, WI, USA). Luciferase activity was determined using a LUMIstar OPTIMA luminometer and quantified as relative light units. Each well received 25 µl reaction buffer (25 mM glycylglycine, 15 mM $MgCl_2$, 5 mM ATP, 0.1 mg/ml BSA, pH 7.8), followed by 25 µl, 1 mM D-luciferin (Promega, Madison, WI, USA) 5s later. Relative light units were converted to a fold induction above the vehicle control value. The E_2 standard curve was fitted (sigmoidal function, variable slope) using Graphpad Prism (version 4), which calculated the minimum, maximum, slope, EC_{50} value and 95% confidence limits. The EEq of extracts with greater than a twofold induction above the vehicle control were interpolated from the E_2 standard curve and corrected with the appropriate dilution factor for each sample.

Reproductive and thyroid parameters using the rat model

Test system and experimental design

This study was approved by the University of Pretoria Animal Ethics Committee (Project no: H031-07). The Organization for Economic Cooperation and Development (OECD) 415 protocol for Reproductive Toxicity Studies (one generation) was modified to accommodate one control and three experimental groups (OECD 1983). Using this design, we studied the endocrine disrupting effects of maternal (P1) exposure to known VGPs on the fertility and reproductive parameters in lifetime exposed F1 males. The study was conducted at the University of Pretoria Biomedical Research Centre (UPBRC). Seven-day pregnant, female (P1) Sprague-Dawley rats (n=20) were randomly divided (n=5 per group) into a control group (Group 1) and three experimental groups (Groups 2, 3, and 4) (Fig. 1). All the animals were housed in standard polycarbonated cages in temperature controlled (22 ± 2ºC) rooms with a constant humidity (55 ± 10%) and 12-hour day/night cycles. The animals were fed a stock pellet diet (Epol ®: Epol mice cubes, lot nr. 30101, Pta, SA) and had free access to food and tap water.

Chemicals and oral dosing procedure

The VGPs were selected according to the expected range of VGPs found in the feedlot water samples. Dosing, by oral gavage was set at the detection limit of the specific compound with an additional 20% added, to make the dosage environmentally relevant.

Four experimental groups were used in this study:

Group 1 - Control group – Cottonseed oil [Sigma-Aldrich, Steinheim, Germany; as vehicle;

Group 2 – Estrogenic group – 0.12 µg/kg zilpaterol (supplied by a feedlot owner), 0.24 µg/kg diethylstilbestrol (DES) (Sigma, 99%) and 2.4 µg/kg α-Zearalanol (Sigma, 97%);

Group 3 - Androgenic group – 12 µg/kg β-trenbolone (Dr Ehrenstorfer GmbH, 95%) and 6 µg/kg methyltestosterone (Dr Ehrenstorfer GmbH);

Group 4 - mixture-exposed group – 0.12 µg/kg zilpaterol (supplied by a feedlot owner), 0.24 µg/kg DES (Sigma, 99%), 2.4 µg/kg α-Zearalanol (Sigma, 97%), 12 µg/kg β-trenbolone (Dr Ehrenstorfer GmbH, 95%) and 6 µg/kg methyltestosterone (Dr Ehrenstorfer GmbH).

All chemical mixtures were administered by oral gavage. The dose rate for all groups, including the control group, was 1 ml/kg adjusted to the body mass of each animal. The rats were weighed and dosed at the same time each day to exclude any external variables. F1 males were exposed *in utero* for 14 days, during lactation for 20 days (Postnatal day (PND) 1 – PND 20) and directly for 70 days (PND 21- PND 90). After dosing at PND 90, the adult F1 males were euthanized with an overdose of isoflurane by insufflation (Isofor®, Safeline Pharmaceutical [Pty] Ltd., South Africa) under controlled conditions.

Tissue collection and histology preparation

After termination, we measured the anogenital distance (AGD), and weighed the testes, epididymides, seminal vesicles and prostate glands. The left testis was fixed for histological evaluation. For thyroid hormone analysis, blood was collected via cardiac puncture and allowed to clot. To determine T_3 and T_4, blood samples were centrifuged at 3000rpm for 15min to obtain serum. The testes, epididymis, seminal vesicles and liver were fixed in Bouin's Fluid [15 parts picric acid (BDH laboratory Supplies, Poole BH15 1TD, England); 5 parts 40% formalin (Merck, Darmstadt, Germany); 1 part glacial acetic acid (Merck, Darmstadt, Germany)], following standard protocols. The tissues were embedded in paraffin blocks and sections of 3µm thick were made and collected on SuperFrost slides (Menzel-Glaser, Germany; catalogue number: J1800AMNZ). Slides were stained with a modified periodic acid-Schiff's reaction (PAS) and counterstained with hematoxylin. A computer software program on spermatogenesis, STAGES 2.1 (Vanguard Media Inc., Illinois, UDA), was used for the staging process together with a histological atlas by Russell et al. (1990). Staging of spermatogenesis was done using an Olympus BX 41 microscope and Altra 20 Olympus camera with 10x, 20x and 100x objectives. For each of the 47 F1 male rats, thirty randomly selected seminiferous tubules were assessed to identify and classify the 14 stages of spermatogenesis. For these thirty tubules, we horizontally and vertically measured the tubular diameter, seminiferous epithelium and lumen diameter. The mean values of the horizontal and vertical measurements for each parameter were statistically analysed (Patrick et al. 2016).

Cauda epididymal sperm count

The cauda epididymis was separated from the caput-corpus and placed in a petri dish containing 2ml phosphate buffered saline (PBS). The cauda epididymis was macerated to expel the sperm into the medium, which was then transferred to a Falcon tube. Sperm were counted using the Neubauer method, and the sperm count was expressed as million/ml (World Health Organization 1999).

T_3 and T_4 determinations

A commercial kit Coat-A-Count Total T_3 (PITKT3-5, 2006-12-29; Cat no TKT31) is a solid-phase radioimmunoassay designed to identify the concentration of total circulating triiodothyronine (T3) in serum or plasma. Coat-A-Count Canine T_4 is a solid-phase radioimmunoassay to identify the concentration of total thyroxine (T4) in canine serum.

Statistical Analysis

We used the Wilcoxon Rank Sum test to compare the control group and the treatment groups, with a Bonferroni adjusted level of significance (0.05/4 = 0.012). For male F1 data, we also used the Kruskal-Wallis All-Pair-wise Comparisons Test to compare groups. Only significant findings > 0.012 and < 0.05 are reported.

RESULTS

Chemical analyses

We detected low levels, below the quantification limit of 10 ng/l, of estrone, ethinylestradiol, DES and testosterone in a few water samples. In Feedlots A and B, zilpaterol was detected at concentrations ranging from not detected (nd) to 3373 ng/l.

Bioassays

YES assay

Only water samples from the feedlot dam at Feedlot A had an EEq value (Site 4: 0.38 ± 0.15 ng/l) (Table 1). In Feedlot B, the water from the feeding cradle (Site 8), borehole water used in the feedlot (Site 14), water downstream from the feedlot (Site 20) and the borehole downstream of the feedlot (Site 41) had points above the detection limit of the assay, but not enough points to calculate an EEq value.

T47D-KBluc assay

Nine of the 11 feedlot water samples tested positive for estrogenic activity with EEq values ranging from 0.02 ng/l to 2.57 ng/l (Table 1). Only the settling dam (Site 3) from Feedlot A and a site (Site 20) downstream from Feedlot B, exceeded the 0.7 ng/l trigger value for estrogenic activity in drinking water, which is associated with cytotoxicity. Water from the influent from the feedlot dam (Site 4, Feedlot A) and water downstream from the feedlot (Site 20, Feedlot B) tested positive for estrogenic activity and showed cytotoxicity at higher concentrations. Cytotoxicity was observed in borehole water (Site 1) 150 m upstream of Feedlot A (Table 1).

Reproductive and thyroid parameters using the rat model

F1 males

Histological examination of the testes showed that rats in the control group (Group 1) had smaller lumen diameters than rats in the androgen group (Group 3: $p = 0.0455$) and rats in the mixture group (Group 4: $p = 0.0289$). Rats in the control group (Group 1) and experimental groups had similar seminiferous tubule diameter and epithelium thickness. Further investigation of the testes indicated that all 14 stages of spermatogenesis were present. A histological examination of the testes of rats in the exposure groups showed selected seminiferous tubules containing dilated tubular lumens, marked detachment of the seminiferous tubule, apical sloughing of immature germ cells, reduced seminiferous tubule diameter with no lumen, absent seminiferous tubules and decreased cellularity of the seminiferous epithelium (groups 2–4) (Fig. 2).

Rats in the control group (Group 1) and treatment groups (Groups 2 – 4) had similar mean body mass, total testicular mass and mean epididymis mass. Rats in the control groups had shorter AGD (Group 1: 40.90 mm) compared to rats in the androgen exposed group (Group 3: 38.17 mm; $p = 0.01$) and the mixture group (Group 4: 39.42 mm; $p = 0.01$) (Table 2). The weight of prostate glands of rats from the mixture group (Group 4: 0.78 g; $p = 0.02$). was lower in comparison to animals from the control group (Group 1: 0.93 g) Similarly, rats in the androgen group (Group 3: 41.08×10^6) had a lower total sperm count ($p < 0.03$) in comparison to animals from the control group (Group 1: 57.04×10^6),.

T_3 and T_4

Rats in the control and experimental groups had similar T_3 levels. Rats in the estrogen (Group 2: 74.19 nmol/l; $p = 0.01$) and androgen groups (Group 3: 74.46 nmol/l; $p = 0.02$) had higher T_4 levels in comparison to animals in the control group (Group 1: 64.40 nmol/l) (Table 2).

DISCUSSION

This study identified the presence of VGPs in runoff from feedlot sites in South Africa. Using environmentally relevant concentrations of these VGPS, we identified endocrine disruptive effects, specifically the reproductive and thyroid parameters of the male rats in the F1 generation, following exposure *in utero,* during lactation and lifetime. Much debate surrounds the potential impact of veterinary medicines on surrounding populations and ecosystems, with the cattle feedlot industry emphatically touting the relative safety of VGPs (Sundlof and Cooper 1996). South Africa is also a relatively arid country with few perennial water sources. The levels of VGPs sampled in the areas surrounding cattle feed lots will probably depend on various climate, topographic and agricultural factors. Our findings support the continuous monitoring of VGPs associated with cattle feedlots, to ensure that the levels of these compounds do not exceed cytotoxic levels.

In this study, water samples from various points in cattle feedlots had confirmed estrogenic activity. We detected the EDC, the estrogenic activity in environmental samples. In this study, we experimentally investigated the effects of the EDCs commonly used in cattle feedlots on male reproductive health of rats. We observed that rats exposed to a control were slightly heavier than rats exposed to androgens (Group 3) and a combination of androgens and estrogens (Group 4). This slight effect is presumably due to the relatively low exposures in our study. **AGD** is an established reproductive endpoint for rodent studies examining the effects of exposure to EDCs (Thankamony et al. 2016). Reduced male AGD indicates feminization and has been observed after treatment with estrogenic compounds (Kelce et al. 1994, Gray et al. 2006, Kilian et al. 2007, Patrick et al. 2016). The shorter AGD of rats exposed to androgens observed in this study could be attributed to the conversion of methyltestosterone (a synthetic androgen) through aromatization to 17α-methylestradiol which is a potent estrogen (Hornung et al. 2004). Mammals seem to possess a single androgen receptor (AR) that mediates the effects of endogenous androgens including, testosterone, androstenedione and DHT. As a result, chemicals with either androgen or antiandrogen activity may interact with the AR, preventing endogenous hormonal action (Phillips and Foster 2008).

Elevated levels of endogenous estrogens or estrogenic EDCs may also lead to permanent disturbances in prostate growth (Prins et al. 2006). In this study, rats exposed to a mixture of androgens and estrogens (Group 4) had significantly lighter mean seminal vesicle and mean prostate mass than rats in the control group. The effect could be attributed to synergistic estrogenic activity caused by the conversion of methyltestosterone to estradiol (Hornung et al. 2004) in these rats. Studies with rodents have indicated that estrogens play a physiological role in prostate development. Rats exposed postnatally to PCBs had a lower prostate and seminal vesicle mass compared to control (Faroon and Ruiz 2015), and rats exposed to DES (estrogenic) also had lighter seminal vesicles (Prins et al. 2001). In our study, rats exposed to androgens (Group 3: $p = 0.0455$) and the mixture of estrogens and androgens (Group 4: $p = 0.0289$) had a significantly wider **lumen diameter** compared to rats in the control group. In mammals, tubular lumen dilatation or contraction is influenced by the rate of seminiferous tubule fluid secretion, the rate of transport from the tubule, rate of reabsorption in the rete and epididymis, all functions that are androgen dependent (Creasy 2001). Although all spermatogenic stages were present in the rats in our study, the wider lumen diameter may be due to altered fluid retention indicating disruption of the seminiferous tubules, ultimately negatively influencing spermatogenesis.

In this study, rats exposed to androgens (Group 3) had a significantly lower total sperm count compared to rats in other groups. Low **sperm counts** have been associated with *in utero* and early life exposure to estrogenic chemicals (Sharpe and Skakkebaek 1993, de Jager et al. 1999, Patrick et al. 2016). Xenobiotic androgen receptor agonist and estrogenic compounds can cause reduced testosterone production in the testis, together with a reduced release of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. Estradiol also exerts a negative feedback on FSH secretion, resulting in depressed spermatogenesis (Carreau et al. 2011). In vertebrates both the differentiation and development of the male reproductive system are under the influence of the androgen, testosterone (Bergman et al. 2013). Rodents have a "male programming

window", when the fetal testes synthesize testosterone and the entire program of development of the male reproductive tract occurs (Welsh et al. 2008). It is likely that the male rats in our study exposed *in utero* to the various compounds suffered from some interference with their "male programming window".

In vertebrates, **thyroid hormones** regulate several major functions including metabolism, growth and development (Bergman et al. 2013). In this study, rats exposed to estrogen (Group 2) and androgen (Group 3) compounds had significantly higher T_4 levels compared to the control group. This increase in T_4 may be associated with exposure to estrogens, which stimulate production of thyroid-hormone-binding globulin (TBG) (Santin and Furlanetto 2011) , leading to a subsequent increase in T_4 levels (Bisschop et al. 2006). Yamamoto et al. (2003) investigated the effects of maternal exposure to DES on male and female offspring, and found that pups exposed to 1.5 µg/kg/day DES had significantly increased T_4 levels. Our findings were similar even though rats in the estrogen group (Group 2) were only exposed to 0.24 µg/kg/day DES, but which together with the other estrogenic compounds in the group may have had an additive effect. We did not observe any differences in T_3 levels between rats in the control group and rats in the experimental groups.

Conclusion

In this study, we screened water sources around cattle feedlots, and found VGPs, which displayed estrogenic activity. We experimentally showed that these VGPs have adverse effects on the reproductive parameters of male rats. While iodine and selenium deficiency may also affect male reproductive parameters, our experiments showed clear differences between the control and experimental groups. Most EDCs have the potential to accumulate in agricultural and aquatic food chains (Bergman et al. 2013). Humans and wildlife that are exposed to EDCs during vulnerable periods of development may undergo congenital changes, which is of great concern.

Conflict of interest declaration

The authors declare that there is no conflict of interest.

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Table 1: Estrogenic activity of water samples collected from cattle feedlots in South Africa using the YES and T47D-KBluc bioassays

Feedlot	**Site number**	**Site description**	**YES: EEq (ng/l)**	**T47D-KBluc: EEq (ng/l)**	**Chemical analysis* Zilpaterol (ng/l)**
A	1	Borehole 150m upstream	**	**	69
A	3	Settling dam	**	2.57 ± 0.39	3373
A	4	Influent feedlot dam	0.38 ± 0.15**	0.32 ± 0.04**	nd
B	8	Feeding cradle	n/q**	0.02 ± 0.004	252
B	13	Borehole 1a in feedlot	**	0.13 ± 0.03	nd
B	14	Borehole 2 in feedlot	n/q**	0.14 ± 0.02	nd
B	20	Downstream from feedlot	n/q**	0.94 ± 0.67**	220
B	39	Borehole 1b in feedlot	<dl	<dl	nd
B	41	Borehole downstream	n/q	0.47 ± 0.01	86
B	45	Reservoir water	<dl	0.25 ± 0.14	nd
B	46	4km downstream	<dl	0.04 ± 0.007	nd

<dl - Below detection limit of the assay plate
n/q - EEq not quantifiable, for less than 3 point above the dl were obtained
* - Only Zilpaterol was quantifiable
** - Cytotoxicity present

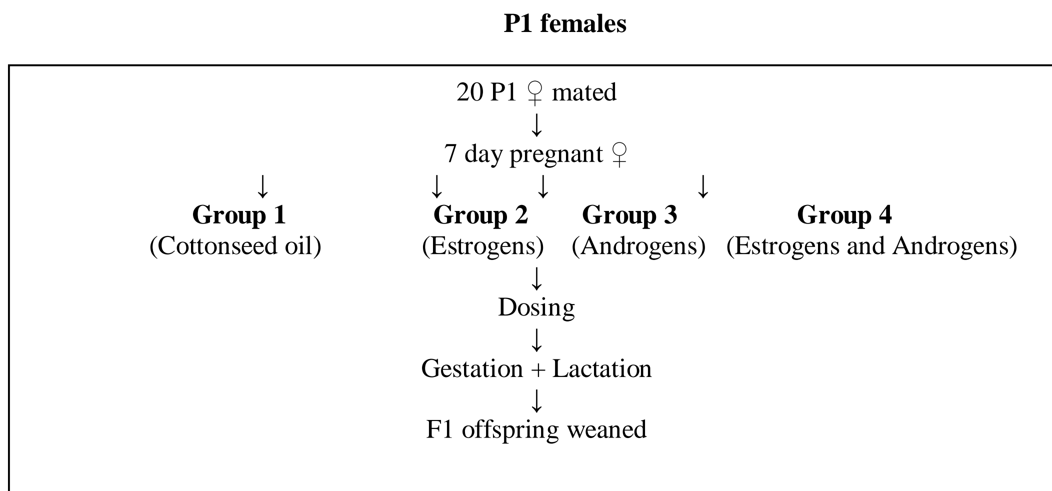
Table 2: The reproductive parameters of the F1 males of the different treatment groups, using the Wilcoxon rank sum test

Variables	Group 1	Group 2	1:2	Group 3	1:3	Group 4	1:4
	Mean	**Mean**	***p*-value**	**Mean**	***p*-value**	**Mean**	***p*-value**
Body mass (g)	374.76	374.00	ns	360.64	ns	358.04	ns
Anogenital distance (mm)	40.90	38.83	0.067	38.17	**0.01**	39.42	0.09
Seminal vesicle mass (g)	0.75	0.79	ns	0.68	ns	0.62	**0.01**
Epididymal mass (g)	0.56	0.57	ns	0.58	ns	0.59	ns
Testicular mass (g)	1.78	1.83	ns	1.79	ns	1.86	ns
Seminiferous tubule diameter (µm)	306.83	312.57	ns	316.83	ns	314.78	ns
Seminiferous epithelium thickness (µm)	100.19	101.70	ns	103.68	ns	102.56	ns
Lumen diameter (µm)	114.04	120.60	ns	121.69	**0.05**	121.56	**0.03**
Total sperm count ($x10^6$)	57.04	46.30	ns	41.08	**0.03**	47.033	n/s
Prostate mass (g)	0.93	0.88	ns	0.86	ns	0.78	**0.02**
T_3 (nmol/l)	1.21	1.27	ns	1.24	ns	1.30	ns
T_4 (nmol/l)	64.40	74.20	**0.01**	74.46	**0.02**	64.80	ns

Group 1: Control
Group 2: Zilpaterol (0.12 µg/kg), DES (0.24 µg/kg), α-Zearalanol (2.4 µg/kg)
Group 3: β-Trenbolone (12 µg/kg), Methyltestosterone (6 µg/kg)
Group 4: Zilpaterol, DES, α-Zearalanol, β-Trenbolone, Methyltestosterone
Statistically significant: $p < 0.005$

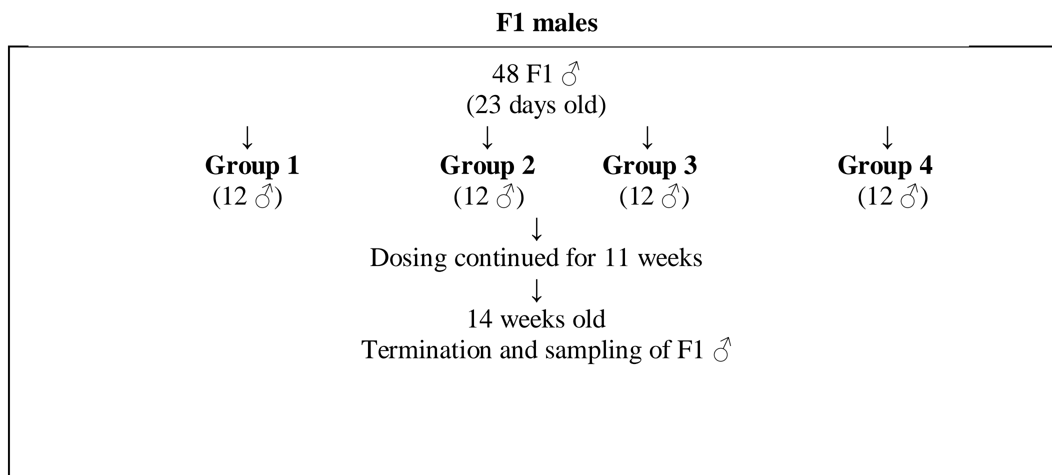
1. Gestational + lactational exposure

P1 females



2. Direct exposure

F1 males



Direct exposure of F1 generation *In utero* exposure of F1 generation
= Lifetime exposure of F1 generation

Fig. 1: Experimental design used to investigate the possible EDC effects of veterinary growth promoters used in cattle feedlots in South Africa on the reproductive outcome of F1 male rats

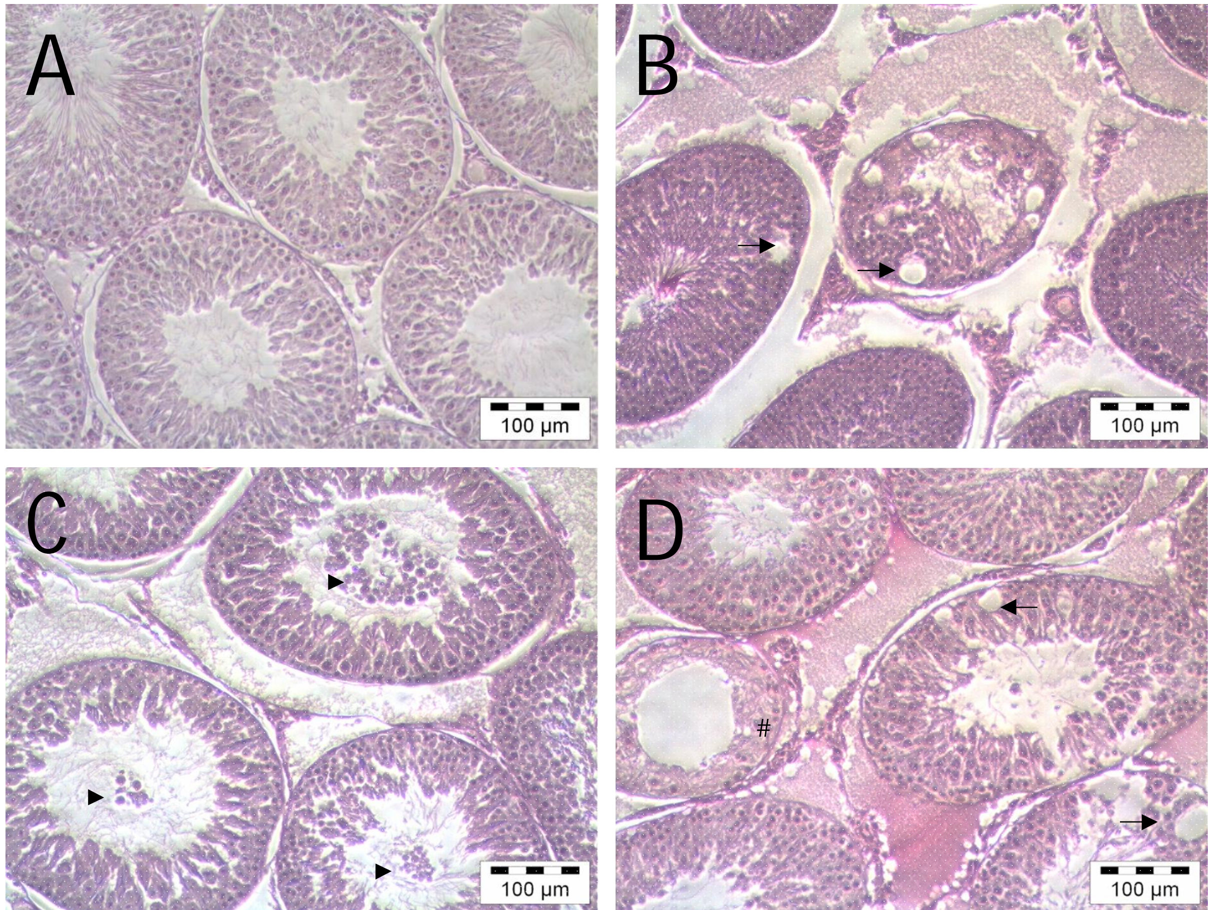


Fig. 2: Testicular histology of F1 males at PND 90 - Normal testicular histology in the Control group (A), abnormal testicular histology in Estrogenic group (B), Androgenic group (C) and in the mixture-exposed group (D); necrosis in the interstitium (*), disorganization of the seminiferous epithelium with reduced seminiferous tubule diameter and no sperm in the lumen (#), vacuoles (arrows), apical sloughing (arrowheads). A: Control – Cottonseed oil; B: Estrogenic group (Zilpaterol, DES and α-Zearalanol); C: Androgenic group: β-Trenbolone and Methyltestosterone; D: Mixture-exposed group: Zilpaterol, DES, α-Zearalanol, β-Trenbolone, Methyltestosterone.