THE RELATIONSHIP BETWEEN URINARY ORGANOPHOSPHATE PESTICIDE RESIDUES AND REPRODUCTIVE DEVELOPMENT AMONG BOYS LIVING IN THE RURAL WESTERN CAPE

PEARL NALEDI MONATE

MNTPEA001

MASTER OF PUBLIC HEALTH (EPIDEMIOLOGY)

SCHOOL OF PUBLIC HEALTH AND FAMILY MEDICINE

Supervisor:

Mohamed Aqiel Dalvie,PhD

Professor
Director of Centre for Environment and Occupational Health Research
School of Public Health and Family Medicine
UCT Faculty of Health Sciences

Co-Supervisor:

Wisdom Basera, MPH

School of Public Health and Family Medicine

UCT Faculty of Health sciences

PART 0: PREAMBLE

Declaration

I, **Pearl Naledi Monate,** hereby declare that the work on which this dissertation is based is my original work (except where acknowledgement indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Dedication

To God almighty, thank you for the power and strength you gave me to complete this work and to my parents Michael and Sarah Schwenzfeier I am beyond grateful, thank you for your unconditional love, support and encouragement throughout this academic journey.

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Dissertation Abstract

Background

Many contemporary agricultural pesticides are hormonally active, but few previous studies have investigated their effect on the reproductive health and growth of pubertal boys. A previous analysis found significant differences in serum reproductive hormone levels and lower anthropometric measurements as well as non-significant lower sexual maturity ratings and testicular sizes in farm boys compared to non-farm boys from the rural Western Cape in South Africa.

Methodology

This analysis included 183 out of 269 school boys residing on farms and neighbouring non-farming areas who provided urine samples in a cross-sectional study. Measurements included a questionnaire, clinical assessment of sexual maturity development (SMD), anthropometric measurements (height, weight and body mass index (BMI)), serum reproductive hormones (including luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone and oestradiol (E2)) and urinary levels of 3 dialkyl phosphates (organophosphate pesticide metabolites) including di-ethyl, di-methyl and di-methyl triphosphate (DEP, DMP and DMTP).

Results

The median (interquartile range) of age and sum dialkyl phosphates of the school boys was 12 years (9-13 years) and 68.3 ng/mL (27.9-129.5 ng/mL) respectively. There were consistent, mostly non-significant associations with some dose response relationships between urinary levels of dialkyl phosphates and adverse effects on outcomes including SMD, serum reproductive hormones and anthropometric development. The strongest results included a

strong positive association and dose response found between serum oestradiol > the 50th percentile and quartiles DMTP (odd ratio and confidence interval for highest and lowest quartile: 7.4; 1.7-32.4) and between BMI <50th percentile and quartiles of DMTP (odd ratio and confidence interval for highest and lowest quartile: 3.2; 1.2-9.0).

Conclusion

The results provide some preliminary evidence that organophosphate pesticides exposure could alter the reproductive hormone levels and adversely affect the body size of school boys. There was also lack of evidence of other adverse effects on reproductive development. These findings require further investigation in a larger longitudinal study with seasonal bio-monitoring for pesticides.

Table of Contents

TABLE OF TABLES

PART A: PROTOCOL

PART B: STRUCTURED LITERATURE REVIEW

PART C: MANUSCRIPT

PART A: PROTOCOL

1.0 Introduction

1.1 Literature Review

1.1.1 Background

Commercial farming in South Africa is substantial and this has made the country the top producer of agricultural products in Sub-Saharan Africa (Quinn et al. 2011a; Dabrowski et al. 2014). The growing population and continued immigration from neighbouring countries, has led to an increase in population and subsequent food demand that resulted in increased use of pesticides in South Africa making it the largest user of pesticides in Southern Africa (Dalvie et al. 2009b; Quinn et al. 2011a; Dabrowski et al. 2014). Pesticides used are applied using different methods in order to reach the biological target, however, they tend to reach non-targeted areas (Eskenazi et al. 1999; Quinn et al. 2011a), and they have been detected in different environmental media (Dalvie et al. 2003; Dabrowski et al. 2014; Mwanga et al. 2016). Hence, there is need to make the pesticides application process as efficient as possible to minimise their release into the environment and human exposure (Eskenazi et al. 1999), including communities around farming areas, farm workers and consumable farm products. The detection of agricultural pesticides in non-targeted areas (e.g. at people's household) has now become a public concern (English et al. 2012; Kjeldsen et al. 2013). Pesticides exposure is associated with several adverse health effects including negative impacts on male reproductive health and development as many are hormonally active substances (Toppari et al. 1996; English et al. 2012; Kjeldsen et al. 2013) with the ability to disrupt physiological processes.

Several studies conducted in the rural Western Cape, one of the agriculturally intense provinces in the country, reported the presence of pesticides in environmental media including underground and surface water used for human consumption (Rother and London 1998; Dalvie et al. 2003; Rother et al. 2008; Stehle et al. 2016). This movement of pesticides beyond targeted areas constitute to health risk of rural residents who use untreated water (Stehle et al. 2016). Continuous pesticides exposure in rural communities in the Western Cape may have consequences in the development and health of children since they are more vulnerable (English et al. 2012).

1.1.2 Exposure of children to pesticides

Children can be exposed to pesticides through air, food, water and soil (Mnif et al. 2011). These exposures can occur in homes, schools and the outside environment such as recreational parks and playgrounds. Those who live in farms and rural farming communities have additional exposure to pesticides drift from sprayed fields, contaminated equipment and their parents' protective clothing and shoes (Mnif et al. 2011). Children are exposed to pesticides from water when drinking and when swimming in water bodies that are contaminated. A survey conducted in the Western Cape to assess knowledge and attitudes in the rural Western Cape regarding pesticides in water sources, documented that 48% of participants reported swimming in farm dams (Dalvie et al. 2004). The presence of pesticides in water has been identified as a common source of exposure following reports from several studies that have been conducted in the Western Cape where pesticides such as endosulfan, ipradione, deltamethrin, chlorpyrifos and fenarimol have been detected in ground water, drinking water and rural surface water (London et al. 2000; Dalvie et al. 2003; Dalvie et al. 2004; Bollmohr et al. 2007; Dabrowski and Balderacchi 2013; Dabrowski et al. 2014; Stehle et al. 2016).

1.1.3 Exposure to pesticides and childhood development

Many contemporary pesticides are endocrine disruptors and may result in adverse male reproductive health effects (Mnif et al. 2011; English et al. 2012). Childhood and puberty are important developmental stages and critical periods in sexual and reproductive life of males (English et al. 2012). During these stages, children experience physical development and hormonal changes. When endocrine disrupting pesticides enter the body, they can interfere with physiological processes by imitating the actions of natural hormones (Mnif et al. 2011). Endocrine disruptors can also obstruct the metabolism, transportation and elimination of natural hormones (Mnif et al. 2011). By interfering with hormonal signalling systems, this may result in adverse reproductive health effects such as altered sex-hormone levels and low testicular volume in boys during puberty (Landrigan et al. 2003; Mnif et al. 2011; English et al. 2012).

1.1.4 Organophosphate (OP) Pesticides

"Pesticides are chemical substances intended to kill and destroy unwanted and harmful weeds and pests" (Touart 1995). There are several groups of pesticides such as herbicides, fungicides, rodenticides, bactericides, larvicides and insecticides. Organophosphate pesticides are mostly used as insecticides that include malathion, dichlorvos, parathion, glyphosate and chlorpyrifos among others. OP pesticides are used mainly for domestic and agricultural purposes to kill insects that affect people's health such as mosquitoes and termites and also to kill insects in crops and stored products (Touart 1995). Studies have associated OP pesticides to have negative effects on a few body organs, causing liver damage and toxic hepatitis.

OP pesticides have also been suggested to have effect on the nervous system damaging the brain and nerves, disturbing other physical processes in human development, some OP pesticides have been associated with adverse male reproductive development which will be discussed in a later section (Eskenazi et al. 1999). In the last few decades OP pesticides were the most commonly used pesticides worldwide (Dalvie et al. 2009a; Quinn et al. 2011b; Dabrowski 2015).

1.1.5 OP pesticide use in the Western Cape (WC) Province

There has been an increase in the use of pesticides in South Africa over the past decades with an estimate of over 3000 pesticides registered for use (Quinn et al. 2011a; Dabrowski et al. 2014; Dabrowski 2015; Stehle et al. 2016). In a study by (Dalvie et al. 2009a), it was found that between 1994 and 1999, the amount of pesticides sold to the five major agricultural sectors of the Western Cape increased from 5400 tons to >6800 tons. In the year 1999, the overall kilograms of pesticides sold for a single hectare increased by 47%. Dithiocarbamates and organophosphates were the top chemical groups, apart from inorganic pesticides, sold in high quantities in both years (Dalvie et al. 2009a). A subsequent study by (Dabrowski 2015), documented an increase in some of the pesticides quantities.

Western Cape is one of the provinces that has agricultural-intense areas. The province also experiences increase in pesticides use. Pesticides such as chlorpyrifos, endosulfan, malathion, mancozeb, dichlorvos and fenthion have been used commonly in the WC (Stehle et al. 2016).

Studies conducted in WC have shown that residents are occupationally and environmentally exposed to pesticides (Dalvie et al. 2003; English et al. 2012; Motsoeneng and Dalvie 2015; Mao 2016). A survey that was conducted by (Dalvie et al. 2004) assessing the knowledge and attitudes in the rural Western Cape towards pesticides in water sources demonstrated that residents living in and around agricultural intense areas are at increased risk of pesticide exposure through spray drift, water, soil and contaminated food (Dalvie et al. 2004). In this survey, many residents reported that they used groundwater (springs and boreholes) and water from mountain dams for drinking and domestic use. A third of residents lived within 10m of spraying sites and many of them used pesticides for household purposes (41%), and in gardens (33%) (Dalvie et al. 2004). A field survey by (Stehle et al. 2016), on fruit orchards in the Louren River catchment, reported a total of 15 different pesticides in the in-stream surface water. Among them were OP pesticides azinphos-methyl and malathion. The results showed that, malathion had the highest erosion rill (92.9 µg/l) and in-stream (19.5 µg/l) concentrations. However, it was stated that it could have been due to direct application on buffer strip vegetation.

A previous study conducted by (Dalvie et al. 2003), investigating the contamination of ground and surface water in three intensive agricultural areas in the Western Cape reported pesticides detected in water sources. Several pesticides were detected but endosulfan was found to be prevalent, both in surface water, ground water and drinking water. The overall results of these studies show that residents are exposed to OP pesticides from pesticide contaminated food and drinking water.

1.1.6 Male reproductive health effects due to pesticides currently used in South African agriculture

In total, it is estimated that there are approximately 100 contemporary agricultural pesticides globally that are endocrine disruptor chemicals, of which 21% are herbicides, 31% fungicides and 46% insecticides (Mnif et al. 2011).

In laboratory studies, pesticides associated with adverse male reproductive effects include chlorpyrifos, cypermethrin, fenvalerate, glyphosate, deltamethrin, endosulfan and dichlorvos. Among these pesticides are OP pesticides; chlorpyrifos, which was associated with decreased spermatid number, sperm count, daily sperm production and sperm motility. And it has also been found to decrease levels of blood testosterone, decreased body weight, and relative weight of reproductive organs and increased luteinizing hormone (LH) and follicle stimulating hormone (FSH) in male rats (Mosbah et al. 2016). In another study that was conducted on albino rats, chlorpyrifos was associated with reduced testosterone and epidydimal sperm viability, motility and count (Ventura et al. 2016). A study conducted in ewes associated chlorpyrifos with reduced serum LH levels (Rawlings et al. 1998). Dichlorvos, has also been found to be associated with reduced serum testosterone, sperm quality and testicular damage in rats given doses of 1, 2 and 4 mg/kg for 6 weeks (Okamura et al. 2005).

Few previous epidemiological studies (Meeker et al. 2004; Lifeng et al. 2006; Meeker et al. 2006a; Meeker et al. 2006b; Meeker et al. 2008; English et al. 2012) have investigated the association between contemporary endocrine disrupting pesticides and male reproductive development and health and one of the investigations was on the OP pesticide chlorpyrifos.

There was a significant negative relationship between the urinary metabolite of chlorpyrifos, TCPY, and serum testosterone, reduced sperm quality and androgen index in a study that was conducted in American men (Meeker et al. 2004; Meeker et al. 2006b). This study is the first study in South Africa investigating the association between pesticides exposure and reproductive health of boys in the rural Western Cape (English et al. 2012). The first analysis of the data found that farm boys were significantly shorter with less weight compared to non-farm boys. The farm boys also had lower serum luteinizing hormone and increased serum estradiol and follicle stimulating hormone. A second analysis by (Ochieng et al. 2013), developed an environmental pesticide exposure index and found increased pesticide exposure associated with lower height and weight among farm boys. Another analysis, indicated some delays in pubertal development of boys in the rural Western Cape when compared to boys from other settings possibly due to nutritional, socio-economic and environmental exposures (Mao 2016).

1.2 Research Question

Boys living in rural communities in the Western Cape are environmentally exposed to pesticides which include organophosphate pesticides (OP) which may have effects on male reproductive development. Are urinary levels of dialkyl phosphate metabolites among these boys negatively associated with their reproductive development?

1.2.1 Aim

The current study aims to investigate the association between male reproductive development and exposure to organophosphate pesticides (OP) based on the urinary levels of OP metabolites among school going boys living in rural areas in the Western Cape Province in South Africa.

1.2.2 Objectives

-To determine the exposure of boy participants to OP pesticides through bio-monitoring of urinary OP metabolites.

-To determine reproductive health and developmental outcomes including sexual development (tanner staging), testicular volume, reproductive hormone levels and anthropometric measurements in boys.

-To measure the characteristics that may confound the association between pesticides exposure and reproductive/pubertal development in boys.

-To investigate the association between OP pesticide exposure and the male reproductive/developmental health outcomes while controlling for confounders amongst boy participants.

2.0 Methodology

2.1 Study Design

This is a sub-study of an analytical cross-sectional study that was conducted to investigate the adverse reproductive health and developmental effects of pesticides exposure on boys living in the rural agricultural and neighbouring non-agricultural areas in the Western Cape Province, South Africa. Previous sub-studies (English et al. 2012; Ochieng et al. 2013), used farm residence and environmental indices to quantify the pesticide exposure of the boy participants. This sub-study will use urinary levels of OP metabolites as the exposure index to investigate the relationship between OP pesticide exposure of the boys and reproductive development. The main study was conducted from April 2007 to March 2008.

2.1.1 Study population and sampling

The study population was boys residing in the rural Western Cape of South Africa. The sampling frame was all boys who attended primary and secondary schools in three rural agriculturally intense areas; Hex River Valley, Piketberg and Grabouw located in the Western Cape Province, where pesticides have been previously detected in environmental media. The sampling frame included both boys living on farms and those living in neighbouring rural communities.

The Western Cape department of Education provided a list of primary and secondary schools in the selected areas. Boys were recruited from the most accessible primary and secondary schools that had learners from both farms and neighbouring towns. Prior to the start of study, the principal investigator engaged with the school principals and held meetings with the staff to inform them about the proposed study. Parents were asked in advance, by means of letters distributed to schools to provide provisional written permission for their children to be recruited. Once the school and parents agreed and the boys were recruited into the study, parents were informed of the details about when the study was to start and what was to be expected of them. Boys whose parents consented (n=492) were stratified according to age, and according to whether they had lived in a farm or not at the time of the study (Appendix A). At each school, where the number of consenting boys living on farms exceeded the number of boys to be selected, random systematic sampling was used to select the boys. Selected boys and their parents or guardians were invited to participate in the study and were asked to present at the school on specified dates.

2.1.2 Inclusion and Exclusion Criteria

The study included boys who were between the ages of 5 and 19 years, either attending primary or secondary school in the three rural agricultural communities. This included those who lived on farms and those living in neighbouring communities. The boys who did not meet these inclusion criteria were excluded.

2.1.3 Sample Size

This sub-study will use all the 269 samples that were collected from the main study. In the initial study, the calculation for sample size were based on blood FSH which was found to be the most varying hormone in previous studies investigating dichloro-diphenyl-trichloroethane (DDT) reproductive health effects. Where, using the mean (3.57±3.15 miu/ml), a two-sample test of equality of means using Stata 13 [Stata Corporation, 2013] indicated a sample size of 174 assuming an 80% power, 40% difference in blood FSH and 95% confidence interval. The boys' age was divided into four groups; 5 to 9 years, 9.1 to 11 years, 11.1 to 14 years and >14 years.

2.2 Measurements and Instruments

2.2.1 Physical Examination

A trained male study nurse performed a physical examination on boys recording height, weight, secondary sexual characteristics and sexual maturity rating (SMR) on a structured record form (Appendix B). SMR was assessed using Tanner Stages. (Marshall and Tanner 1969) The secondary sexual characteristics assessed included the presence of genital anatomical abnormalities such as congenital hydrocoeles, undescended testes, congenital inguinal hernias and hypospadias.

Additionally, the presence of infection, previous injury or tumors was also recorded. Testicular consistency and size were also recorded. Testicular volume was assessed using a standardized set of wooden testicular beads [Orchidometer, Kabi, Japan]. Height and weight were recorded per standardized methods using calibrated instruments. Photographs showing the various Tanner Stages were used as a reference (Appendix C). Nurses were trained by a local reproductive health specialist who demonstrated how to perform the anthropometric measurements, how to use the orchidometer and assess the sexual maturation score using visual material and through demonstration on a subject. Training was conducted over a period of 2 days at the UCT teaching hospital, Groote Schuur adolescent Health Unit.

2.2.2 Blood Hormones

The male study nurses also collected blood samples from each boy, in the morning preferably before 1300 hours. Two six milliliters (ml) venous whole blood samples were collected from each boy by the study nurse. These were put in an in-field refrigerator and then transported on ice to the National Health Laboratory Sciences (NHLS) facility at Groote Schuur Hospital in Cape Town for reproductive hormone analysis within 24 hours. The samples were analyzed for baseline FSH, luteinizing hormone (LH), testosterone, oestradiol (E2), and serum hormone binding globulin (SHBG). LH was measured with the MAIAclone IRMA kit (Bologna, Italy) [Biochem Immuno Systems] (Systems 1985; Systems 1995), FSH and total testosterone with ACS-180 competitive chemiluminescent automated systems (New York, USA) [Bayer Corporation, 2000] (Corporation 2000), oestradiol with an in-house radioimmunoassay (Nivellus, Belgium) [Biosource-Europe SA, 1994] (Biosource-Europe 1994), and SHBG with the IRMA kit from Orion Diagnostica (Finland) [Orion Diagnostica, 1990] (Diagnostica 1990). Baseline hormonal levels were compared to age-related laboratory normal ranges.

2.2.3 Questionnaire

Trained interviewers conducted face-to-face interviews with the parent or legal guardian in their language of preference. The boys were asked questions on sexual development in the presence of the interviewee in their preferred language. The responses were recorded directly on to an electronic questionnaire which was preloaded on to the cellphone using mobile technology (Mobile Researcher, Clyral). At the end of each interview the completed questionnaires were downloaded on to a central website and were accessed by the Principal Investigator. This information was then exported in to Microsoft Excel® and Stata 13 [Stata Corporation, Texas, USA] for further data management and analysis.

The questionnaire (Appendix D) was developed by the study team, led by the Principal Investigator, and was based on previous local studies in similar populations. English, Afrikaans and Xhosa versions of the questionnaire were developed. The latter questionnaires were back-translated to English during their development to ensure validity and reliability of the questions. The questionnaires were administered by fieldworkers, who were extensively trained in conducting interviews, on the content, and on the various terminologies in the questionnaire. The questionnaire included questions on: demographics, birth weight, general medical history, genital health history and lifetime environmental exposure to pesticides including a summary of lifetime diet and dietary content of soya beans and other vegetables. Items on the mothers' personal habits during pregnancy included questions on alcohol consumption, smoking, diet and the use of soya milk after conception or birth. Questions on the boys' reproductive history, alcohol consumption, and smoking history were also included and the cumulative lifetime and intensity of pesticide exposure were also determined. Environmental exposure to pesticides was assessed from factors such as place of residency, years of residence in agriculturally-intense areas and proximity of the pesticide spraying.

Domestic use of pesticides, domestic water sources, use of empty pesticide containers and pesticide exposure through diet were also measured. The interviewers administered the questionnaires to the parent regardless of the child's age.

2.2.4 Urine sample collection

The study nurses also collected a spot urine samples from participants into a colourless clear plastic urine container (50 mL) sealed with a plastic cap. The samples were kept on dry ice before they could be transported and stored in a refrigerator at -20 °c at the School of Public Health and Family Medicine before being analysed at the Clinical Pharmacology laboratory at UCT. The samples were analysed for the three di-alkyl phosphates metabolites (DEP: Diethyl phosphate, DMTP: dimethyl thiophosphate, DMP: dimethyl phosphate), which were by far the most commonly detected.

Isoniazid and Acetyl Isoniazid were analyzed for with a validated liquid chromatography tandem mass spectrometry assay developed at the Clinical Pharmacology Laboratory at UCT. Samples were processed with a protein precipitation extraction method using Isoniazid-d4 and Acetyl Isoniazid-d4 as internal standards, followed by high performance liquid chromatography with MS/MS detection using an AB SCIEX API 3000 instrument. The analyte, metabolite and internal standards were monitored at mass transitions of the protonated precursor ions m/z 138.11, m/z 180.16, m/z 142.21 and m/z 184.21 to the product ions m/z 79.10, m/z 121.10, m/z 83.10 and m/z 83.20 for Isoniazid, Acetyl Isoniazid, Isoniazid-d4 and Acetyl Isoniazid-d4, respectively. The calibration curves fitted quadratic (weighted by 1/concentration) regressions over the ranges 0.102 to 26.0 µg/ml for Isoniazid and 0.0501 to 25.6 µg/ml for Acetyl Isoniazid.

The combined accuracy (%Nom) and precision (%CV) statistics of the limit of quantification, low, medium, and high-quality controls (3 validation batches, N=18) of the analyte and metabolite were between 92.2% and 107%, and 2.9% and 10.9%, respectively.

2.2.5 Pilot Study

Three boys from two study areas who met the eligibility criteria were used to pilot the study before the main study was conducted, to field test the procedures.

2.2.6 Training

All the field workers went through a two days intensive training on the study methods, physical assessment, sample collection, and on processing and transporting samples.

2.3 Consideration of potential biases, validity and reliability

The introduction of selection, measurement and confounding biases was considered prior to and during the conduct of the study. To reduce recall bias and reporting bias, questions were kept simple and unambiguous, and questions from previous surveys were largely used. To reduce measurement bias, field staff with similar education and age were trained to conduct measurements in a standardized manner for all boys. Interviews and examinations were conducted in rooms where privacy was adhered to ensure that the interviewees felt comfortable to share difficult information. Field staff were also trained to conduct the interview in a non-threatening manner.

To minimize confounding, all questionnaires were designed in such a way as to include potential confounders, and during the analysis stage univariate and multivariate analysis will explore the relationships between exposure and the various outcome measurements.

2.3.1 Exposure measures

The study measures the exposure of boy participants to OP pesticides based on examination of OP metabolites in urine. All boys were included in the study if they lived in rural agricultural areas or neighbouring communities.

2.3.2 Outcome measures

The primary outcome is male reproductive health, and other developmental outcomes such as sexual development, testicular volume and levels of reproductive hormones (FSH, LH, E2 and testosterone) and anthropometric growth (height, weight and body mass index) of boy participants.

2.4 Statistical Analysis

2.4.1 Data Management and quality assurance

In the field, all completed questionnaires and data sheets were checked for completeness, consistency and logical reasoning by a single field supervisor with a tertiary qualification in environmental health. This was done before the subject left the examination site. A checklist was placed on the outside of each envelope, which contained each participant's data. This was done to facilitate completeness of the data collection process. Where indicated, missing or relevant data were obtained before the interviewee and participant left the venue.

Field workers were available in the study region to follow up on subjects who had missing or inconsistent questionnaire data, particularly for exposure characterization.

2.4.2 Data Analysis

The exposure variable comprises of the individual levels of OP metabolites, including dialkyl phosphates (DAP) metabolites (dimethylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethylthiophosphate (DETP) and diethyldithiophosphate (DEDTP)) which are detectable in urine. The individual metabolites as well as the sum of the metabolites will be analysed as both continuous and categorical variables using medians, tertiles and quartiles as cut-offs.

The primary outcome variables are sexual maturity development (Tanner stage scores, sum of right and left testicular volumes), baseline reproductive hormone levels (FSH, LH, E2 and testosterone) and the anthropometric measurements (height, weight and BMI). These variables are continuous variables and they will be dichotomised using quartiles (25^{th}, 50^{th} and 75^{th}) as cut-offs, except for Tanner stage. The anthropometric measurements will also be dichotomised below the 25^{th} or 50^{th} percentile for age based on the Centre for Disease Control and Prevention (CDC) growth charts. The categorization of outcomes is based on that used in previous studies. The following table presents variables that will be used in statistical analysis.

Table 1: List of exposure and outcome variables

Variable Name	**Original Measurement Scale**	**Units/categorisation**
Age	Numerical- continuous	Years
Height	Numerical- continuous	Centimetres
Weight	Numerical- continuous	Kilograms
BMI	Continuous	Kg/m^2
Tanner Stages	Continuous	1-5
Testicular Volume	Continuous	mL
Luteinising Hormone (LH)	Continuous	IU/L
Follicle Stimulating Hormone (FSH)	Continuous	IU/L
Testosterone	Continuous	nmol/L
Estradiol (E2)	Continuous	pmol/L
SHBG	Continuous	nmol/L
Birth weight	Numerical- continuous	kilograms
Household income	continuous	Rands
Medical history	Categorical	Diabetes/TB/Fits/Asthma/HIV/Mumps
Water sources	Categorical	Municipal/Mountain/borehole/River/Farm dam/Rain/Other
Household pesticides exposure	Categorical	Yes/No
Smoking	Categorical	Yes/No
Alcohol intake	Categorical	Yes/No
Maternal alcohol intake during pregnancy	Categorical	Yes/No
Maternal smoking during pregnancy	Categorical	Yes/No
Maternal education	Categorical	Yes/No
Maternal employment status	Categorical	Working/Not working/Other
Self-reported pesticides exposure	Categorical	Yes/No
Phyto-oestrogen intake	Categorical	Yes/No
Exposure variable: OP metabolites		
DMP	Continuous	ng/mL
DMTP	Continuous	ng/mL
DMDTP	Continuous	ng/mL
DEP	Continuous	ng/mL
DETP	Continuous	ng/mL
DEDTP	Continuous	ng/mL

Stata 14 statistical software will be used for data analysis. Data will be explored for any abnormalities, missing data and assessment of distribution by histogram and box-and-whisker plots. Frequency tables and scatter plots will also be displayed to help detect patterns and relationships between variables. The individual OP metabolites concentrations will be used to generate exposure variables.

To test for the bivariate association between the outcome and exposure variables a t-test for independent samples will be used if data are normally distributed, or a Wilcoxon sum rank test if the data are skewed. Chi-square testing will be conducted for categorical variables. A Fisher's exact test will be used when frequencies are less than 5 in bivariate analysis. Confounders for the association between the various exposures and the individual outcomes will be tested for using regression analysis techniques. Significance will be measured at the 10% level. The confounders will therefore be included in the regression model during the model building process. Confounders are first identified a priori based on biological plausibility. Then additional confounders with association $p<0.1$ in bivariate analysis are added, then stepwise regression is performed.

For model building, a logical variable selection model will be used to select the variables. First, potential confounders will be added one by one to see which one reduces the deviance the most. The outcome with the confounder that reduces the deviance the most is then used as the baseline model. Other confounders are then added one at a time, forcing a priori confounders back in to the model. The combination of variables that results in the biggest change to the variance will be the chosen model.

Regression diagnostics will be applied to determine the goodness of fit of the model. Collinearity will be assessed by calculating the simple correlation coefficients and variable inflation factors. Outliers and influential points will be assessed using regression diagnostic techniques. Studentised residual values of >2 will determine the presence of outliers. Influential points will be assessed by examining Cook's distance (0.33), dfit ($>2\sqrt{k/n}$) and dfbeta's ($>2/\sqrt{n}$).

The association between the exposure and the categorical form of the outcome variable will be explored using logistic regression modelling while controlling for confounding. Regression diagnostics to assess the form of the linear predictor and to assess for outliers or influential observations will be done. Outliers will be identified if the standardized residuals are >2 or <-2. Influential observations will be identified by determining the effect that the covariate pattern has on the estimated model (>2(p/n)).

3.0 Ethics

The ethical research principles such as respect for persons, justice and beneficence were adhered to.

3.1 Ethical Approval

The study was granted approval by the University of Cape Town Research Ethics Committee (Rec Ref: 279/2005) (Appendix E) and department of Education. The study was conducted in accordance with the Declaration of Helsinki. Informed consent was also obtained (Appendix F).

3.2 Risk and Benefits

Participants were exposed to minimal risk during the study and where medical problems were identified in participants, a system of referral for further medical attention was established with the help of the study team. The following were also considered as potential risk; phlebotomy, as taking blood can cause bruising, pain, bleeding, and rarely muscle damage or infection where the needle was inserted.

3.2.1 Potential Benefits to Community and Health Services

The potential beneficiaries of this research are participants and residents of rural communities in Western Cape who are environmentally exposed to different types of pesticides. The community will continue to receive education and information on pesticides found in environmental media particularly OP pesticides. This sub-study will also form as a baseline for further research investigation on the association of OP pesticides and boys' reproductive development in Western Cape Province. In addition to other studies that have been conducted on pesticides in South Africa, this study could influence the pesticides management policy of South Africa and contribute towards the strategies and possible formulation of health policies to ensure future protection of boys from the adverse effects of exposure to hormonally active agricultural pesticides.

3.3 Informed Consent

Permission to conduct the study was also obtained from the Department of Education, and from primary and secondary school principals. The principals contacted the male students and their parents in each school. Before participants were enrolled, parents/legal guardians and boys who were 18 years and above signed written informed consent form. Informed consent forms were available in three languages; English, Afrikaans and Xhosa. Participants and their parents were given informed consent forms in their preferred language. A witness was used where parents/guardians were assessed to be illiterate (could not read and/or write) to read and explain the informed consent form to them. Parents/guardians who were illiterate used thumb print as a sign of their signature in the informed consent form. The participants, parents/guardian were also given time to think and make decisions without any pressure after reading the consent form.

Before signing the informed consent form, parents/guardians were given the opportunity to ask questions and seek clarity concerning the study. It was emphasized to them that participation in the study is voluntary and they are allowed to withdraw from the study at any time they wish to, and that their decision to withdraw will not affect the relationship between them, their children, school staff and research team in any way.

3.3.1 Privacy and Confidentiality

Participants' information and their confidentiality will be preserved throughout the study and at all times. Confidentiality will be maintained through proper handling of soft and paper records. Participants' information will be stored in secured database, password protected and access to data will be limited to the study team involved in data analysis. Data will be de-identified before analysis. The results will be grouped before reporting and no individual participant's result will be reported.

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PART B: STRUCTURED LITERATURE REVIEW

1.0 Introduction

1.1 Background

Organophosphate (OP), were first manufactured in the 1900's in Germany and were introduced worldwide as pesticides during Second World War in 1941 (WHO 1990; Goodman 1996; Ngoula et al. 2007). OP pesticides became more popular in the 1980s after some organochloride pesticides that were regarded as environmentally persistent were banned (Wessels et al. 2003; Sharpe and Irvine 2004). OP pesticides are still currently widely used worldwide.

There are various sectors that use OP pesticides in South Africa including agriculture, government, commercial and veterinary. Currently, agricultural sector is the highest user of OP pesticides as well as other types of pesticides (Quinn et al. 2011). Because of the positive benefits of pesticides in crop productivity and reducing disease burdens caused by pests, OP pesticides are primarily used for agricultural purposes to control pests and for public health protection against vector-borne diseases like malaria (WHO 1990; IA 1994; Wessels et al. 2003).

There is evidence suggesting that some OP pesticides are toxic to humans. However, evidence from the existing literature shows that there is increase in use of OP pesticides in developing countries regardless of their toxic effects to humans and animals (Dalvie et al. 2009a; Quinn et al. 2011; Dabrowski 2015). In addition, it has been discovered that these pesticides are inadequately managed and not strictly regulated in developing countries.

The increased use, toxicity and mismanagement of pesticides has become a public health concern (IA 1994; Harrison et al. 1997; Curl et al. 2002; Damstra et al. 2002; Sharpe and Irvine 2004; Bradman et al. 2007).

Several OP pesticides have been identified as endocrine disrupting chemicals which are chemicals that mimic, enhance, or inhibit the action of hormones. Endocrine disruptors have been linked to the declining male reproductive health particularly male fertility, decreased sperm quality and reproductive organ defects in humans and animals (Landrigan et al. 2003; Mnif et al. 2011a; WHO 2012). These health effects have been hypothesized to result from exposure during early development, including the pubertal period in boys. OP pesticides use in South Africa is widespread, with Western Cape (WC) being one of the major provinces that use OP pesticides because of its extensive agricultural sector (Quinn et al. 2011; Dabrowski 2015).

1.2 Objective of the literature review

This literature review will firstly give an overview of human exposures to OP pesticides; use of OP pesticides in WC and South African agriculture; presence of OP pesticides in environmental media and rural residents in WC; endocrine disruption and male reproductive health effects due to OP pesticides. Secondly, a systematic review of male reproductive health effects due to pesticides currently used in agriculture will be conducted.

1.3 Search strategy

This literature review was conducted from electronic and paper sources. Google scholar was used as the main search engine. Additional databases like PubMed, Scopus and Medline were used. The following search terms were used to search for literature in general review: organophosphates, pesticides, endocrine disrupting chemical, hormonally active agent, reproductive development, sexual development, and reproductive hormone. Search terms for the literature review includes pesticides and male reproduction, pesticides and reproductive health, endocrine disruption and reproductive health, pesticides and anthropometric measurements, pesticides and reproductive hormones. The reviews only included publications in English and focused on both laboratory and epidemiological studies. The reference list in articles that were found during search were also used to search for additional articles. Non-electronic sources were also used such as dissertations to obtain additional information.

2.0 Literature review

2.1 Environmental exposure to organophosphate pesticides

According to WHO, a pesticide is “any substance or mixture of substances intended for preventing, destroying, or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during, or otherwise interfering with, the production, processing, storage, transport, or marketing of food, agricultural commodities, wood and wood products, or animals feedstuffs, or which may be administered to animals for the control of insects, arachnids, or other pests in or on their bodies” (WHO 1990).

OP pesticides are a group of chemical pesticides that contains a phosphate ester. OP pesticides inhibit the neurotransmitter enzyme in the nervous system and brain, acetylcholinesterase which breaks down the neurotransmitter, acetylcholine into acetyl and choline and therefore leading to build-up of acetylcholine in nerve endings. The latter process is toxic for insects and could also be toxic in humans (Colborn 2006; Gilden et al. 2010).

As with other pesticides, people can be exposed to OP pesticides in different ways in an occupational or non-occupational setting. In most cases, the exposure to OP pesticides is unintentional but there are instances where the exposure is intentional (Davies et al. 1980; Davies et al. 1982; WHO 1990). Exposure to OP pesticides varies according to the length of time and the level of exposure to these pesticides (IA 1994).

The common routes of OP pesticide exposure for humans are through ingestion, inhalation and skin contact (clothes and shoes) (Gilden et al. 2010; Giudice 2016). Skin contact can also occur during cleaning of empty pesticide containers for re-use. Ingestion of fresh vegetables and fruits from farm or eating contaminated food also pose exposure to pesticides residues found in food. This can also occur due to the use of pesticide contaminated water to wash fruits or crops before consumption. Water sources contaminated with pesticides such as dams and rivers, are a source of human exposure through dermal contact e.g. swimming or ingestion when drinking. In addition, exposure to humans may occur through inhalation of air and dust contaminated by pesticides from spray drift and while playing in recreational parks (Simcox et al. 1995; Gilden et al. 2010).

Pesticide exposure in children is higher than adults because of their behaviour, activities and diet (Eskenazi et al. 1999; Curl et al. 2002; Lambert et al. 2005; Gilden et al. 2010). This is also because their bodies are still going through physiological development, with their elimination and detoxification capacities still immature which put them at higher risk to adverse health effects of pesticides than adults (Eskenazi et al. 1999; Lambert et al. 2005). Children also engage in recreational activities that expose them to contaminated soil, dust and water such as swimming in rivers and playing in recreational parks. Children who live near farming areas may have higher exposure to OP pesticides than those who do not live in farming areas because of the proximity to fields where these OP pesticides are used (Bradman et al. 2007; Ochieng et al. 2013).

Extensive use of OPs such as malathion in homes for protection against pests also put children at low level chronic exposure. Furthermore, children can experience "carry-home" exposure from their parents' contaminated clothes, shoes and vehicles particularly those whose parents are occupationally exposed to pesticides (Eskenazi et al. 1999; Curl et al. 2002; Lambert et al. 2005; Arcury et al. 2006). In a study in agricultural communities of farm worker (n=218) households that was conducted in Washington State, take-home OP pesticides exposure among agricultural workers and their children was evaluated, and it was found that take-home exposure pathway leads to household contaminations. Azinphos-methyl, which is an OP pesticide was detected in homes in higher concentrations than other pesticides (Curl et al. 2002).

2.2 Factors influencing pesticide toxicity to humans

There are several factors that influence pesticides toxicity in humans (WHO 1990). The adverse health outcomes from exposure to pesticides are largely influenced by quantity of pesticides that one is exposed to. The route of exposure also has effect on the severity of adverse health outcomes from exposure to pesticides (WHO 1990; Giudice 2016). Toxicity also depends on how easily the pesticide is absorbed, its accumulation and persistence in human body. Some pesticides are metabolized, some are excreted unchanged while others are stored in the fat (WHO 1990; IA 1994; Gilden et al. 2010).

An individual's health status is also important in responding to the toxic effects of pesticides, particular people who have malnutrition and those who are dehydrated as these states of health are likely to increase sensitivity to pesticides (WHO 1990). Water deprivation may make people more susceptible to effects of anticholinesterase pesticides.

3.0 Pesticides used in Western Cape and its presence in the environment

3.1 Usage of pesticides in Western Cape Province

Developing countries have experienced a noticeable increase in use of pesticides in the last 20 years, particularly in Africa. OP pesticides have been the most used insecticides in developing countries since some organochloride pesticides that were regarded as environmentally persistent were banned (Pope 1999).

South Africa is leading in use of pesticides in Sub-Saharan Africa (Osibanjo et al. 2002). More than 52% of pesticides sold to the largest crop sectors in South Africa possibly have endocrine disrupting properties (English et al. 2012; Mao 2016). As a developing country, it is estimated that there are over 3000 registered pesticides in South Africa (Quinn et al. 2011; Dabrowski et al. 2014; Dabrowski 2015; Stehle et al. 2016).

A study conducted in South Africa assessing the change in quantity and acute toxicity of pesticides sold to SA crop sectors between 1994 and 1999 found that the amount of pesticides sold to the five major agricultural sectors increased by 5400 tons to >6800 tons (Dalvie et al. 2009a). In a subsequent study that evaluated the development of pesticides use maps for South Africa (Dabrowski 2015), it was established that WC province is the leading province using OP pesticides. This study documented increase in pesticides quantities such as chlorpyrifos, parathion and dichlorvos and others in year 2009. The WC province was found to be the highest user of OP pesticides including chlorpyrifos, azinphos-methyl and fenthion and the second highest user of dichlorvos. The amount of use in WC province alone is estimated to be 16027kg of chlorpyrifos, 205kg of fenthion, 42105kg of azinphos-methyl and 911kg of dichlorvos in 2009 (Dabrowski 2015).

3.2 The presence of pesticides in environmental media and residents of rural Western Cape

Previous studies in rural WC found the presence of pesticides in surface and groundwater sources including drinking water (Dalvie et al. 2003; Dalvie et al. 2004). In one of the studies, the endocrine disrupting pesticide endosulfan, was detected in the sampling sites in all 3 study areas including Grabouw (69%), Hex River (46%) and Piketberg (39%) and in both surface

water (47%) and groundwater (32%). About 30% of sampling sites exceeded the European drinking water standard of 0.1 µg/L. Other endocrine disrupting pesticides not monitored regularly but detected in study included chlorpyrifos, azinphos-methyl, fenarimol, iprodione, deltamethrin, penconazole and prothiofs (Dalvie et al. 2003).

Sebastian also conducted a water survey of the fruits orchards of Lourens River catchment in WC. The survey found that a total of 15 different pesticides, inclusive of 8 insecticides and 7 fungicides were detected in the in-stream surface water. The most often detected pesticides included malathion, azinphos-methyl and pyrimethanil. Malathion had the highest erosion rill (92.9 µg/L) and in-stream (19.5µg/L) concentrations (Stehle et al. 2016).

In a study investigating knowledge, attitudes and practices of residents regarding water in rural WC, it was found that most residents identified groundwater from springs and boreholes (30-60%), mountain dams (40-65%) and water from farm dams (>40%) as their main water sources. Forty-eight percent of participants also said they swim in farm dams. About a third of residents revealed living not beyond 10m to site of spraying (Dalvie et al. 2004). Another study investigated the presence of pesticide residues in wheat produced and imported in South Africa and assessed their health risks, pesticides were detected in all local and imported samples. Eight different pesticides were detected in total and multiple pesticides were detected in about 39% imported and 30% local samples. Permethrin (19%) and chlorpyrifos (17%) were among the most detected pesticides. Nine (11%) samples exceeded the EU wheat MRL for permethrin (0.05mg/kg) which included 7 (10%) local samples and 2 (15%) imported samples (Dalvie and London 2009). Moreover, endosulfan and chlorpyrifos was also detected in blood and urine respectively among farm workers in studies conducted in WC, where serum endosulfan and

urinary levels of dialkyl phosphates (non-specific OP metabolite) were found to be high when compared to populations in other countries (Dalvie et al. 2009b). Additionally, urinary levels of DAPs were detected in farm women and in non-farm residents of nearby towns (Dalvie et al. 2011).

4.0 Endocrine disruption and the male reproductive system

4.1 Endocrine disrupting chemicals

WHO defines an endocrine disruptor (ED) as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or (sub) population" (WHO 1990). Endocrine disrupting properties have been identified in different chemicals that are both synthetic and naturally-occurring (Sharpe and Irvine 2004; Pronczuk de Garbino and Organization 2005; Özen and Darcan 2011). Synthetic pesticides are reported to be of potential hazard to public health and they are widely dispersed in the environment (WHO 1990).

The endocrine system comprises of a network of glands, hormones and receptors (Crisp et al. 1998; Gore et al. 2014). It controls the bodily functions such as reproduction, metabolism, behaviour and immunity. It is also influential during adulthood in regulating bodily functions and the reproduction process. This system is also important during childhood, as it controls growth and development. This makes children to be particularly susceptible to endocrine disrupting chemicals (EDCs) during development. During their critical developmental stages when programming of the endocrine system is occurring, exposure to these chemicals may result in changes in growth, development, reproduction and behaviour (Pronczuk de Garbino

and Organization 2005; Gilden et al. 2010; Özen and Darcan 2011; Giudice 2016). However, exposure to EDCs at other developmental time periods might not have any negative health effects. The developmental stage during which an individual is exposed has influence in the nature and severity of health outcomes (Pronczuk de Garbino and Organization 2005).

EDCs interfere in some way with the normal functioning of the endocrine system and can cause adverse health effects in both animal and human. The mechanism through which EDCs act varies (Den Hond and Schoeters 2006; Özen and Darcan 2011). Some bind to hormone receptors and act as agonists while others block receptors acting as inhibitors. This can disrupt the activities of hormone signalling system (Pronczuk de Garbino and Organization 2005; Gilden et al. 2010). Some EDCs may cause obstruction of natural hormone synthesis, metabolism, transportation and elimination of natural hormones that are responsible for development and reproduction (Solomon and Schettler 2000; Pronczuk de Garbino and Organization 2005; Den Hond and Schoeters 2006). Disturbance between various components of the endocrine system may also have different consequences at various life stages and may negatively impact other body systems. Some EDCs such as chlorpyrifos and endosulfan are moderately persistent and can be transported over long distances (Pronczuk de Garbino and Organization 2005).

4.2 Effect of EDCs on the male reproductive system

There are several hormones involved during development of the male reproductive system. At the beginning of pubertal changes, the hypothalamus produces gonadotrophin-releasing hormone (GnRH) that triggers secretion of gonadotrophins such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH). FSH regulates gonads on the production of sex

steroids like testosterone and estrogen. Testosterone stimulates growth of reproductive tissues and estrogen induces sperm maturity (Bousfield et al. 1994).

There are some agents that have been pinpointed to have consequences on pubertal development in humans (Özen and Darcan 2011). Though GnRH hormone is vital for male reproductive system, the neurons of this hormone are sensitive to urban environmental toxicants (Gore 2001). EDCs may affect puberty onset by increasing GnRH production (Özen and Darcan 2011). During puberty, all reproductive hormones increase to high levels during adulthood. Lower secretions of FSH and LH can result in failure of gonadal function, which leads to failure in production of expected number of sperms (Perry 2008). Clinical disorders of hormone synthesis result in abnormal growth and also have irreversible negative impact on reproductive health (Perry 2008).

Puberty is characterized by development of secondary sexual characteristics, increase in secretion of growth hormone and sex steroids and attainment of fertility (Den Hond and Schoeters 2006). Secondary sexual characteristics include laryngeal enlargement and deepening voice, increase in testes volume from 2-4 ml around age 12, scrotum enlargement, growth of pubic hair and enlargement of the penis to double its size (Den Hond and Schoeters 2006). (Marshall and Tanner 1969), explained variation in tanner stages that illustrates sexual maturation during puberty and further states that pubertal development follows a certain pattern. EDCs may exhibits hormonal or anti-hormonal activity such as anti-androgenic (e.g. endosulfan may result in lower testosterone and sexual maturity rating) and oestrogenic activities (e.g. glyphosate may result in poor sperm quality) which can affect sexual development and functioning (Damstra et al. 2002; Sharpe and Irvine 2004).

5.0 Adverse health effects due to agricultural pesticides currently in use in South Africa

Adverse health effects due to pesticides include both acute or chronic health effects but this review will focus on the latter for which research is lacking.

Chronic health effects associated with pesticides include cancers, respiratory effects such as asthma, neurotoxicity and reproductive health effects by endocrine disruptors (Gore 2001; Gore et al. 2014; Gore et al. 2015). Many pesticides are hormonally active (EDC pesticides, that are identified through laboratory testing), and can cause adverse male reproductive health effects through oestrogenic, anti-androgenic and other mechanisms as discussed in section 4.2 (English et al. 2012; Mao 2016).

5.1 Endocrine disrupting pesticides used in the Western Cape crop sector

Table 2 summarizes the laboratory and epidemiological evidence on adverse chronic male reproductive health effects due to agricultural pesticides currently used and/or used in the last 15 years in WC crop sector. There are 13 pesticides listed in Table 2 including chlorpyrifos, parathion, azinphos-methyl, cypermethrin, endosulfan, prochloraz, fenvalerate, deltamethrin, iprodione, glyphosate and dichlorvos of which some can induce in vitro endocrine activity in animals and humans. Among these pesticides are seven OP pesticides.

5.1.1 Laboratory evidence of male reproductive health effects due to agricultural pesticides from laboratory studies

This section summarizes laboratory studies. These include:

(a) Chlorpyrifos has been in use since 1965. Chlorpyrifos has been banned for domestic purposes in developed countries like USA in 2001 (Ruiz 2017), but its use in developing countries has continued until 2010 in SA when it was banned for use in homes and gardens. Nonetheless, it still remains one of the most widely used OP pesticide in the agricultural sector (Dabrowski 2015) in SA. Evidence from laboratory studies suggests that chlorpyrifos may be linked to male reproductive effects following exposure. It was found to cause reduction in testicular weight, sperm count, motility and viability, serum FSH, LH and testosterone levels in adults male albino rats (Alaa-Eldin et al. 2017). In vitro studies indicates that chlorpyrifos is weakly oestrogenic (Gore 2001) and therefore is suspected to be an endocrine disruptor (Mnif et al. 2011a).

(b) Pirimiphos-methyl was found to significantly increase testis and epididymis weights in rats receiving doses of 62.5 or 125mg/kg and a significant decrease in serum total protein, sperm density and motility, and fertility (Ngoula et al. 2007). It is used widely particularly in Africa because of its affordability. It is rapidly absorbed, metabolized and excreted in animals such as rats and dogs (Ngoula et al. 2007).

(c) Parathion and Methyl parathion are highly acutely toxic OP ester insecticides, were introduced in 1949. They are mainly used as insecticides and acaricides to protect agricultural products such as vegetables, soybeans and citrus fruits. Methyl parathion is still in use in SA. Methyl parathion has been associated with a decrease in sperm quality and fertility in laboratory animals (Joshi et al. 2003; Pina-Guzman et al. 2009). Parathion and methyl parathion are also suspected to be endocrine disruptors (Mnif et al. 2011b).

(d) Dichlorvos is still in use in SA. A laboratory study where 34 wistar rats were divided into 4 groups and were injected with dichlorvos at 0, 1, 2 or 4mg/kg for 6 days in a week for 9 weeks. It was found that exposure to dichlorvos slightly decreased sperm

motility in 1 and 4mg/kg groups (Okamura et al. 2005), it is also suspected to be an endocrine disruptor (Mnif et al. 2011a).

(e) Azinphos-methyl (AZP) is one of the most commonly used pesticides worldwide. It is mainly applied on fruits plants such as apples and pears in the WC province. In WC province alone, it is estimated that 52000 kg of active ingredients is applied in fruit orchards per year (Dabrowski and Schulz 2003). Similar to chlorpyrifos, azinphos-methyl has been detected following runoff and spray drift activity in the WC province in Lourens River and it has been found to be persistent in pond water (Dabrowski and Schulz 2003). One laboratory study found male reproductive health effects with exposure to AZP. It is also suspected to be an endocrine disruptor (Orton et al. 2011).

(f) Malathion was introduced in the 1950s, is widely used in home, garden, field, animals and public health programmes and it has a relatively low acute toxicity when compared to other OP pesticides. It is suspected to be an endocrine disruptor (Mnif et al. 2011a) and has the ability to bind to thyroid hormone receptors. In one study it decreased testes weight and significantly reduced FSH, LH and testosterone levels in male wistar rats compared to the control group (Flehi-Slim et al. 2016).

(g) Glyphosate, a systemic herbicide that has both agricultural and non-agricultural uses. It kills different types of plants such as weeds, grass and shrubs but when applied at lower rates it acts as plant-growth regulator. It was found to reduce spermatids number, seminiferous tubules and increase abnormal sperm morphology in male rats that were exposed to soy milk supplemented with glyphosate (Nardi et al. 2017). In experimental study that assessed endocrine disruption and cytotoxicity in human JAr cells in vitro due to glyphosate, it was found that glyphosate caused cytotoxicity and reduced progesterone synthesis (Young et al. 2015).

5.1.2 Epidemiological evidence of male reproductive health effects from pesticides currently or recently used in the Western Cape

A summary of epidemiological evidence in Table 2. There is limited epidemiological data globally with only 7 studies found that have investigated effects on male reproductive health outcomes due to exposure to these pesticides. All the studies were cross-sectional and there were thus no longitudinal studies.

Three small studies found some evidence that exposure to OP pesticides parathion, malathion and pyrethroid fenvalerate may increase semen aneuploidy and reduce sperm quality among Chinese and Malaysian workers (Padungtod et al. 1999; Padungtod et al. 2000; Lifeng et al. 2006; Hossain et al. 2010). A study of American men from the general population, urinary TCPY, chlorpyrifos metabolite, was negatively associated with semen quality, serum testosterone and androgen index (Meeker et al. 2004; Meeker et al. 2006). Another 2 studies among American and Spanish men found dialkyl phosphates negatively associated with semen quality and altered reproductive hormone levels (Melgarejo et al. 2015; Omoike et al. 2015). Only 1 study was conducted among boys (Padungtod et al. 1999; Padungtod et al. 2000). A study of 207 Indian boys found exposure to aerial application of endosulfan to decrease sexual maturity rating and serum testosterone (Saiyed et al. 2003).

In a previous analysis of the current study in the rural WC of 269 boys aged 5 to 19 years in which pesticide bio-monitoring data were not available, found significantly different levels of the reproductive hormones LH, FSH and oestradiol and non-significantly lower levels of testosterone, sexual maturity rating and testicular volumes compared to non-farm boys (Ochieng et al. 2013).

Boys who lived on farms were also significantly shorter with less weight than boys who lived in neighbouring communities. Additionally, anthropometric measurements were lower in farm boys compared to non-farm boys and negatively associated with their pesticide exposure index based on their reported intensity of pesticide spraying on farms they resided and the proximity of their homes to spraying areas (English et al. 2012).

These studies provide preliminary evidence of possible adverse male effects due to exposure of currently used agricultural pesticides including effects on reproductive development on exposed boys. This evidence needs to be explored further in larger longitudinal studies. Additionally, incorporation of pesticide bio-monitoring would strengthen exposure assessment.

Table 2: Evidence from laboratory and epidemiological studies that shows contemporary agricultural pesticides have adverse effects on male reproduction

Pesticides	In vitro/in vivo endocrine activity	Animal studies	Epidemiological studies
Methyl-Parathion (OP) Parathion (OP)		Six wistar rats weighing 150-180g were given methyl-parathion orally at doses of 30 mg/kg b.wt./day every day for 30 days. Significantly decreased weight of testis, epididymis and decreased testicular sperm counts were found in exposed males (Joshi et al. 2003). Male mice (10-12 weeks) were divided into 2 groups of six animals. One group was exposed to methyl-parathion injected as single dose of 20mg/kg (bw,i.p) and a control group received the vehicle only. Spermatozoa was collected at 7 or 28 days post-treatment to assess the effects on maturing spermatozoa and spermatocytes. Decreased sperm quality and fertility capacity were found in mice who received methyl-parathion at 20mg/kg. DNA alteration in spermatozoa (Pina-Guzman et al. 2009).	A study in China enrolled 32 men from a large pesticide-manufacturing plant exposed to OP pesticides and 43 controls from nearby factory unexposed. Statistically significant slightly reduced median sperm concentration and median percentage of normal motility in exposed than unexposed men after controlling for age were found (Padungtod et al. 1999; Padungtod et al. 2000).
Azinphos-methyl (AZP)	Anti-androgenic activity (Orton et al. 2011).	Biomphalaria glabrata specimen were exposed to different concentrations of azinphos-methyl (0.021, 0.5, 2.5 and 5mg/L) for either 2 or 14 days. Depending on concentration and exposure time, azinphos-methyl decreased number of egg masses and lower or total absence of hatchings (Kristoff et al. 2011).	
Chlorpyrifos (CPF) (OP)	Weak estrogenic Activity (Akhtar et al. 2009).	Forty adults' albino rats (10-12 weeks) weighing 190-210g were randomized in to 4 experimental groups. Control group were given chlorpyrifos 6.75mg/kg b.w/orally daily and treatment was given by oral gavage for 12 weeks. CPF reduced testicular weight, decreased sperm count, motility and viability. Significantly increased percent of morphologically abnormal spermatozoa, increments in sperm DNA fragmentation index	Urinary levels of the chlorpyrifos metabolite, TCPY, were associated with poorer sperm quality, decreased testosterone and lower androgen index in American men, a cross sectional study that included 272 male subjects (Meeker et al. 2004; Meeker et al. 2006).

		when compared to control group. Serum FSH, LH, testosterone levels were decreased significantly compared to control group (Alaa-Eldin et al. 2017).	
		Experimental study was performed on 32 adult male wistar rats, weighing 200-220g. Rats were allocated in to 4 groups of 8 rats each. The control group received 1ml/kg/day of distilled water and group II received 20 mg/kg/day, group III received 1ml/kg/day of NSO orally every day for 4 weeks. Group IV received dose level of 1ml/kg/day of NSO and then after 30 minutes received 20mg/kg/day of CPF. CPF decreased spermatid number, sperm count, daily sperm production and sperm motility. It increased dead sperm and abnormal sperm when compared to control group. Level of testosterone, body weight and relative weight of reproductive organs were decreased, LH and FSH were significantly increased in comparison with controls (Mosbah et al. 2016).	
Pirimiphos-methyl (OP)	Anti-androgenic (Orton et al. 2011).	Twenty-four adult wistar rats were divided in to 4 groups of 6 rats each and orally treated with 0, 41.67, 62.5 or 125mg/kg of pirimiphos-methyl for 90 days. Significantly increased ($p<0.05$) relative testis and epididymis weights in rats receiving doses of 62.5 or 125mg/kg. A significant decrease ($p<0.05$) in serum total protein, sperm density and motility, and fertility. Histological findings also indicated inhibition of spermatogenesis, rare faction of leydig cells and aodema in testes when compared to control group (Ngoula et al. 2007).	
Dichlorvos (OP)	AR antagonist (Andrade et al. 2002).	Thirty-four wistar rats (10-week-old) divided in to 4 groups and injected subcutaneously with Dichlorvos 0, 1, 2 or 4mg/kg, dissolved in saline, 6 days in a week for 9 weeks. Slightly decreased sperm motility, but significantly in the 1 and 4mg/kg groups (Okamura et al. 2005).	

Malathion (OP)		Male wistar rats divided in to 3 groups and given 1 ml corn oil containing 1.3, 13.7 and 137mg/kg wt/day, respectively. Decreased testes weight ($p< 0.05$) and significantly reduced FSH, LH and testosterone levels than the control group ($p< 0.05$) (Flehi-Slim et al. 2016). Wistar rats were administered malathion orally at dose of 50, 150 and 250 mg/kg/body wt/day for 60 days. Exposed group had a reduction in the weight of testes, epididymis, seminal vesicle and ventral prostate and there was significant suppression of testosterone (Choudhary et al. 2008).	A cross-sectional study among 152 male farmers in different communities in Malaysia where farmers were divided in to a group exposed to malathion (n=15), one exposed to paraquat (n=39), one exposed to both paraquat and malathion (n=8) and a control group (n=90). The exposed group had significantly greater risk of having abnormal semen quality in volume ($p=0.000$), sperm concentration ($p=0.000$), motility of sperms ($p=0.000$) (Hossain et al. 2010).
Fenvalerate (pyrethroid)		Male mice administered fenvalerate (60mg/kg) by gavage daily from postnatal day 35 to 63. Significantly decreased sperm count in mice treated with fenvalerate, decreased spermatogenic cells and increased the number of apoptosis cells in testes (Zhang et al. 2010).	Hundred male Chinese participants were involved in the study of which 32 workers were exposed to fenvalerate, 46 were internal and 22 external control groups. Subjects were monitored for 3 consecutive days. Sperm progression and count were significantly lower in the exposed group than other groups. A significant decrease in sperm motility was also observed among Chinese factory workers exposed to fenvalerate (Lifeng et al. 2006).
Endosulfan (organochlorine)	Estrogenic Anti-androgenic (Sinha et al. 2001; Akhtar et al. 2009).	Hybrid male mice (10-12 weeks old) sacrificed and epididymis removed and transferred to a dish containing in vitro fertilization medium. Exposure of sperm to the highest concentration of endosulfan significantly increased sperm in vitro chromatin decondensation ($p<0.005$). Endosulfan impaired sperm viability, motility and DNA fragmentation (Sanchez et al. 2017).	A cross-sectional study that enrolled 117 male school children and adolescents aged 10 to 19 years in India, environmentally exposed to aerial spraying of endosulfan and the control group, which were 90 males not exposed to endosulfan; endosulfan level in the study group was significantly higher ($p<0.001$), the exposed group had a lower sexual maturity rating score and serum testosterone levels compared to the control group (Saiyed et al. 2003).
Iprodione (fungicide)	Anti-androgenic (Wolf et al. 1999).	Sprague-Dawley weanling rats dosed with 0, 50,100 or 200 mg/kg/day of iprodione from post-natal day 23 to 51/52. Progression of preputial separation was delayed by iprodione at 100 and 200mg and decreased androgen sensitive seminal vesicle	

		and epididymides weights at 200mg. Decreased Serum testosterone levels. Iprodione also reduced ex vivo testis production of testosterone and progesterone (Blystone et al. 2007b).	
Prochloraz (PCZ) (fungicide)	Estrogenic Anti-androgenic (Andersen et al. 2002; Vinggaard et al. 2005; Earl Gray et al. 2006).	Sprague dawley rats dosed with 0, 31.3, 62.5 or 125 mg/kg/day of PCZ from postnatal day 23 to 42 or 51. Significant delay in preputial separation at 125mg/kg/day PCZ and several of the androgen-dependent organ weights were decreased significantly. At both ages, serum testosterone levels and ex vivo testosterone release from the testis were significantly decreased (Blystone et al. 2007a).	
Deltamethrin (pyrethroid)	Weakly estrogenic (Andersen et al. 2002; Andrade et al. 2002).	60 Female wistar rats (100 days old) divided in to four groups. One was a control group, 3 groups were given 3 different doses of deltamethrin (1.0, 2.0 and 4.0mg/kg body weight). Significantly reduced testis and epididymis weights in exposed animals given highest dose of deltamethrin (4.0mg/kg/day) (Andrade et al. 2002).	
Glyphosate (OP)	In experimental study that looked at glyphosate endocrine disruption and cytotoxicity in human JAr cells in vitro. It was found that glyphosate caused cytotoxicity ($p<0.001$), significantly decreased progesterone synthesis ($p<0.01$) and caused cell death at lower concentrations (Young et al. 2015).	23 days old male wistar rats, where treatment was administered daily through gavage for 35 days. The study evaluated the pubertal toxicity of a soy milk rich feeding (soy supplemented with glyphosate) at doses of 50 and 100mg/kg during pre-pubertal period in male rats. The exposed group showed decrease in spermatids number and increase of epidydimal tail mass and decrease in diameter of seminiferous tubules compared to controls. Animals that received soy and 100mg/kg glyphosate also showed increase in abnormal sperm morphology (Nardi et al. 2017).	

OP insecticides			A cross-sectional study that enrolled 356 men aged 20-55 years from the U.S National Health and Nutrition Examination Survey who were exposed to organophosphate insecticides found a statistically significant inverse relationship between diethyl phosphate and testosterone (β: -2.4%, 95% CI: -3.7;-1.2%) (Omoike et al. 2015).
OP pesticides			In an observational cross-sectional study, 116 men aged 25-38 years who attended infertility services of university hospital in the Murcia Region in Southern Spain were enrolled. This study found that there was a significant positive association between DEDTP concentrations and LH (β=11.4, 95% CI 0.81-22.1) as well as FSH levels (β=3.2, 95% CI 0.08-6.2) (Melgarejo et al. 2015)

6.0 Conclusion

Humans including children are exposed to many agricultural pesticides which could cause long-term health effects. These pesticides reach environmental areas that were not targeted and are later detected in consumables. Residents of rural WC, who are exposed to agricultural pesticides, have been shown to have limited knowledge about the risks of the pesticides that they are exposed to. Persistent use of these pesticides in agriculturally intense areas may therefore result in health problems. The declines in male reproductive health and development may be due to exposure to EDCs which include pesticides. There is laboratory evidence that contemporary agricultural pesticides cause adverse effects on male reproductive health and growth but very limited epidemiological data especially among boys.

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PART C: JOURNAL READY MANUSCRIPT

Preparation for submission

1.1 Journal

Prepared to be submitted for publication in the Environment International Journal. The journal's instructions for authors (Appendix G) have been adhered to except insertion of tables in the text.

1.1.1 Short running head

Urinary Organophosphate pesticides metabolites and male reproductive development.

1.1.2 Competing financial interest declaration

Nil

1.1.3 Abbreviations

OP- Organophosphate

DEP- Diethyl phosphate

DMP- Dimethyl phosphate

DMTP- Dimethylthio phosphate

DAP- Dialkyl phosphate

BMI- Body mass index

FSH- Follicle stimulating hormone

LH- Luteinizing hormone

E2- Oestradiol

SHBG- Serum hormone binding globulin

SMR- Sexual maturity rating

WC- Western Cape

µg/L- Microgram per litre

IU/L- International units per litre

1.1.4 Key words

Organophosphorus pesticide

Urinary dialkyl phosphate

School boy

Reproductive health effects

Anthropometric measurements

Agriculture

2.1 Abstract

Background

Many contemporary agricultural pesticides are hormonally active, but few previous studies have investigated their effect on the reproductive health and growth of pubertal boys. A previous analysis found significant differences in serum reproductive hormone levels and lower anthropometric measurements as well as non-significant lower sexual maturity ratings and testicular sizes in farm boys compared to non-farm boys from the rural Western Cape in South Africa.

Objectives

This analysis investigated the association between urinary levels of dialkyl phosphate (DAP) metabolites resulting from exposure to organophosphate pesticides and the reproductive health and growth of the boys.

Methods

This analysis included 183 out of 269 school boys residing on farms and neighbouring non-farming areas who provided urine samples in a cross-sectional study. Measurements included a questionnaire, clinical assessment of puberty development, anthropometric measurements (height, weight and body mass index (BMI)), serum reproductive hormones (including luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone and oestradiol (E2)) and urinary levels of 3 dialkyl phosphates (organophosphate pesticide metabolites) including di-ethyl, di-methyl and di-methyl triphosphate (DEP, DMP and DMTP).

Results

The median (interquartile range) of age and sum dialkyl phosphates of the school boys was 12 years (9-13 years) and 68.3 ng/mL (27.9-129.5 ng/mL) respectively. There were consistent, mostly non-significant associations with some dose response relationships between urinary levels of dialkyl phosphates and adverse effects on outcomes including puberty development, serum reproductive hormones and anthropometric development. The strongest results were a strong positive association and dose response found between serum oestradiol > the 50^{th} percentile and quartiles DMTP (odds ratio and confidence interval for the highest quartile relative to the lowest: 7.4; 1.7-32.4) and between BMI $<50^{th}$ percentile and quartiles of DMTP (odds ratio and confidence interval for the highest quartile relative to the lowest: 3.2; 1.2-9.0).

Conclusion

The results provide some preliminary evidence that organophosphate pesticides exposure could alter the reproductive hormone levels and adversely affect the body size of school boys. These findings require further investigation in a larger longitudinal study with seasonal bio-monitoring for pesticides.

3.1 Introduction

A number of agricultural pesticides in current use could possibly have reproductive adverse effects in exposed people as they are hormonally active (Andersen et al. 2002) particularly during early developmental stages (WHO 2012). These includes several OP pesticides such as chlorpyrifos, pirimiphos-methyl, parathion, dichlorvos, azinphos-methyl and malathion which are suspected endocrine disruptors and have been found to cause adverse male reproductive effects such as reproductive developmental abnormalities including delayed puberty and subfertility, reduced levels of reproductive hormones and reduced sperm quality in laboratory animals (Joshi et al. 2003; Choudhary et al. 2008; Flehi-Slim et al. 2016; Mosbah et al. 2016; Alaa-Eldin et al. 2017). These OP pesticides are commonly used in South African (SA) agriculture, top user of pesticides in sub-Saharan Africa. In Western Cape (WC) province, one of the most important agricultural areas in SA, chlorpyrifos and other non-OP pesticides, have been detected in the rural environment including drinking water and among farm workers (Dalvie et al. 2003; Dalvie et al. 2009; Dalvie and English 2013).

There are few epidemiological studies that have investigated the association between exposure to currently used agricultural pesticides and male reproductive health particularly among boys (Perry 2008). According to (Saiyed et al. 2003) endosulfan was associated with lower sexual maturity ratings (SMR) and serum testosterone in environmentally exposed Indian boys. Three small studies found some evidence that exposure to OP pesticides parathion and malathion and the pyrethroid fenvalerate may increase semen aneuploidy and reduce sperm quality among Chinese and Malaysian workers (Joshi et al. 2003; Orton et al. 2011; Flehi-Slim et al. 2016; Alaa-Eldin et al. 2017).

In a study of American men from the general population, urinary TCPY, the chlorpyrifos metabolite, was negatively associated with semen quality, serum testosterone and androgen index (Meeker et al. 2004; Meeker et al. 2006).

In addition to the studies described above, a previous analysis of the current study in the rural Western Cape of 269 boys aged 5 to 19 years in which pesticide bio-monitoring data were not available, found significantly different levels of the reproductive hormones LH, FSH and oestradiol and non-significantly lower levels of testosterone, SMR and testicular volumes compared to non-farm boys (English et al. 2012). Boys who lived on farms were also significantly shorter with less weight than boys who lived in neighbouring communities. Additionally, anthropometric measurements were lower among farm boys compared to non-farm boys and negatively associated with their pesticide exposure index based on their reported intensity of pesticide spraying on farms they resided and the proximity of their homes to the spraying areas (Ochieng et al. 2013). These studies provide preliminary evidence of possible adverse male effects due to exposure to currently used agricultural pesticides including effects on reproductive development on exposed boys. Additionally, incorporation of pesticide bio-monitoring would strengthen exposure assessment.

This sub-study aims to investigate the reproductive development and health effects due to exposure of rural boys in the WC province to organophosphate pesticides based on urinary levels of OP metabolites.

4.1 Methods and Materials

4.1.1 Study design, population and sampling

This is a sub-study of a cross-sectional investigation of the adverse reproductive health and developmental effects of agricultural pesticides exposure on boys living in the rural Western Cape in South Africa. Previous sub-studies have used farm residence and environmental indices to quantify the pesticide exposure of the boy participants. In this analysis, urinary levels of DAP metabolites are used as the exposure index to investigate the relationship between OP pesticide exposure of the boys and reproductive development. The study enrolled 269 boys from primary and secondary schools in three agricultural intense areas (Hex River Valley, Piketberg and Grabouw) attended by pupils from farms and neighbouring non-farm areas. The study was conducted from April 2007 to March 2008. The sampling has been described in detail elsewhere (English et al. 2012).

Briefly, the 8 most accessible but representative schools (primary and secondary schools) from the three study areas were chosen for the study. To cover the full age range for pubertal development, boys aged from 5 to 19 years were chosen. Boys' parents (n = 489) who consented to participate in the study were stratified according to whether they had lived on a farm or not at the time of the study and by area. Then 274 boys were selected including all boys (n = 180) living on a farm and 94 not living on farms. The former group was chosen by random systematic sampling, stratified equally by age groups (5-9; 9.1-11; 11.1-14; >14 years). A further 5 boys all who lived on a farm did not participate in the study leaving only 269 participants.

The study sample for this analysis include all 183 boys from whom urine sample were collected and analysed for DAP metabolites.

4.1.2 Questionnaire

Trained interviewers performed interviews on the parent or legal guardian of the boy in the language of their preference (after back translation into English, Afrikaans or Xhosa). The questionnaire was based on previous studies among similar populations in the same setting (Dalvie et al. 1999; Dalvie et al. 2004a; Dalvie et al. 2004b). The questionnaire included questions on demography, boy's medical history, environmental exposure to pesticides, mother's personal habits during pregnancy and questions on the boy's lifetime diet and other lifestyle factors. The responses were recorded directly on to an electronic questionnaire using mobile technology. At the end of each interview the completed questionnaires were transferred to a central website where they were accessed by the principal investigator. The data were later downloaded from the website in to Microsoft excel® spreadsheet and then imported in to the statistical package (Stata 14, StatCorp, Texas USA).

4.1.3 Physical examination

A trained male study nurse, performed a blinded physical assessment of the secondary sexual characteristics, SMR and anthropometric measurements on the boys. SMR was assessed according to Tanner stage for development of the genitalia, not of pubic hair development which reflects adrenarchal rather than testicular maturity (Marshall and Tanner 1969). Height and weight were recorded according to standardized methods and using calibrated instruments.

Testicular volume was measured using a standardized set of wooden testicular beads [Orchidometer, Kabi, Japan]. The secondary sexual characteristics assessed, included the presence of genital anatomical abnormalities such as congenital hydrocoeles, undescended testes, congenital inguinal hernias and hypospadias. In addition, the presence of infection, previous injury or tumours were also recorded. The study nurse was trained over a period of 2 days by a local reproductive health specialist who demonstrated how to perform measurements and how to assess sexual maturation and score using visual material.

4.1.4 Reproductive hormone measurements

A study nurse collected 2 X 6ml venous whole blood samples from each boy participant. Blood samples were collected before 1300 hours. Samples were allowed to clot, then centrifuged at 5000 revolutions per minute by a qualified laboratory technician. The supernatants were then put in an in-field refrigerator and transported on ice to the National Health Laboratory Sciences (NHLS) laboratory at Groote Schuur Hospital in Cape Town for analysis of reproductive hormones within 24 hours. Serum reproductive hormones including, follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, oestradiol (E2) and serum hormone binding globulin (SHBG) were measured using electrochemiluminescence immunoassays (ECLIA) on a Roche Cobas Modular E170 analyser.

4.1.5 Pesticides bio-monitoring

Spot urine samples were collected by a study nurse in a colourless clear plastic urine container (50ml) sealed with a plastic cap. The samples were kept on dry ice in the field and during transportation and then stored in a refrigerator at -20°C at the School of Public Health and

Family Medicine before being analysed at the Clinical Pharmacology laboratory in University of Cape town (UCT). The samples were later analysed for the three DAP metabolites (DEP, DMTP and DMP) which were the most commonly detected. Analysis were done with a validated liquid chromatography tandem mass spectrometry assay developed at the Clinical Pharmacology Laboratory at UCT.

4.1.6 Statistical Analysis

The exposure variables of interest were urinary levels of the individual DAP metabolites (DMP, DEP and DMTP) and the sum of these metabolites. DAPs were analysed as both continuous and categorical variables using medians, tertiles and quartiles as cut-offs.

The primary outcome variables were anthropometric measurements (height, weight, BMI), baseline serum reproductive hormonal levels (LH, testosterone, E2, FSH) and sexual maturity rating (Tanner stage scores and testicular volume) which were analysed as both continuous variables and dichotomous variables. Baseline serum reproductive hormones were dichotomized using 25^{th} and 50^{th} percentiles as cut-offs and tanner stage using stage 4 and 5 as cut-off. Anthropometric measurements were also dichotomized using the 25^{th} and 50^{th} percentiles for age according to the CDC growth chart (CDC 2009).

Data was explored using both the univariate and bivariate analysis. Simple regression analysis, t-test or Wilcoxon sum rank test (for continuous data) and chi-square test or Fisher's exact test (where expected frequency was <5) were used during exploratory bivariate analysis.

Both logistic and linear regression were used to test for association between individual outcomes and exposure variables. Confounders were first selected a priori based on biological plausibility. Additional confounders were then identified based on bivariate testing (where associations with a significance of $p<0.1$ are found) and then through model building. Household income, age, SHBG and other reproductive hormones were selected a priori for all hormonal outcomes. Other hormones were included to control for their effect on individual hormonal outcomes. For all other outcomes, only age and household income were selected a priori. Mother's exposure during pregnancy, household pesticide exposure, phyto-oestrogen intake, medical history, socio-economic status, birthweight and demographic variables were included in bivariate analysis as potential confounders.

A logical variable selection model was used to select confounders to the model in addition to the a priori variables. Variables were added to an empty model one at a time and the variable that significantly reduced the deviance the most when compared to null model, was chosen as the baseline model. Then the other variables were added to the baseline model and the combination of variables that resulted in the biggest change to the variance was chosen as the model with the a priori variables forced into the model. Regression diagnostics were applied to determine the goodness of fit of the model. Stata 14 statistical software (StataCorp, Texas, USA) was used for data analysis.

The University of Cape Town's Faculty of Health Sciences. Research Ethics Committee granted the main study approval (REC REF: 279/2005) as well as this sub-study (HREC Ref: 480/2017). Approval was also obtained from the Department of Education in South Africa to

conduct the study at schools. Consent was obtained from the parents/legal guardians of participating boys as well accent form the boys before conducting the tests on them.

5.1 Results

Participation

The 183 boys from whom urine samples were collected in the main study, formed the study sample for this sub-study of which 128 (70%) were “farm boys” (defined as boys who only lived on a farm their whole lives or who were born and lived on a farm for the first 3 years of their lives or spent 3 of their first 3 years on a farm) and 55 (30%) were “non-farm boys”.

5.1.1 Socio-demographic characteristics, Phyto-oestrogen intake, reported pesticide exposures and exposures during pregnancy

The socio-demographic and exposure history of boys are presented in Table 1. Non-farm boys were significantly older than farm boys (Table 1). The median birthweight of farm boys was not significantly different to that of non-farm boys. Although the percentage of parents employed was higher for farm boys, the median parental income was significantly higher for non-farm boys. The proportion of parents with a secondary and tertiary education was also higher for non-farm boys.

Reported phyto-oestrogen intake (regular intake of soya, nuts and vegetables throughout their lives) were similar for both farm and non-farm boys. Reported pesticide exposures from household use, use of empty containers, swimming in nearby dam, pesticide drift, drinking from unprotected sources and eating from a home garden were much higher in farm boys than

in non-farm boys. The main sources of exposure among the farm boys were household pesticide use (98.4%), fruits eaten from home garden (65.6%), drinking water from unprotected sources (50.4%) and swimming in dams and rivers (38.3%).

The reported general health status for farm boys were not significantly different to that of non-fam boys. Exposures during pregnancy from maternal alcohol consumption, smoking, pesticide spraying or working in the fields during spraying, was higher among farm boys as expected but also prevalent among non-farm boys.

Table 1: Socio-demographic characteristics, Phyto-oestrogen intake, reported pesticide and pregnancy exposures

Variable	n	Farm boys (n=128)	Non-farm boys (n=55)
		Median (IQR)	
Socio-demographic characteristics			
Age(years)*	180	11 (9;13)	12.5 (12;13)
Age groups (years)			
5-9	40	9 (8;9)	9 (9;9)
>9-11	42	10 (10;11)	10 (10;11)
>11-14	84	13 (12;13.5)	13 (12;13)
>14	14	15 (15;16)	16.5 (15;18)
Birthweight (grams)	134	2800 (2500;3220)	3000 (2600;3500)
Income (USD) **	178	131.4 (87.6;175.2)	182.5 (102.2;343.1)
		N (%)	
Parental employment: Working	123	94 (73.4)	29 (52.7)
Not working	51	30 (23.4)	21 (38.2)
Parental education: Primary	107	83 (65.4)	24 (44.4)
Secondary	74	44 (34.7)	30 (55.6)
Tertiary	5	1 (0.78)	4 (7.3)
Phyto-oestrogen intake (lifetime):			
Soya	161	111 (86.7)	50 (90.9)
Nuts	150	100 (78.1)	50 (90.9)
Vegetables	181	127 (99.2)	54 (98.2)
Pesticides exposure history:			
Pesticides use in current home	125	123 (98.4)	2 (3.6)
Empty pesticides containers used in house	4	4 (3.2)	0
Swimming in nearby dam or river	56	49 (38.3)	7 (12.7)
Farm spraying drifts in to house	54	53(43.1)	1(1.8)
Drinking water from unprotected water source (mountain, dam, rainwater, borehole, river)	62	61(50.4)	1(1.9)
Fruits eaten from home garden	89	84 (65.6)	5 (9.1)
Medical History:			
Diabetes	2	2 (1.6)	0
Asthma	17	11 (8.6)	6 (10.9)
Tuberculosis	12	10 (7.8)	2 (3.6)
Mumps	54	37 (28.9)	17 (31.5)
General health:			
Excellent to good	176	122 (95.3)	54 (98.2)
Poor	7	6 (4.7)	1 (1.8)
Maternal exposure during pregnancy			
Smoked	74	58 (45.3)	16 (29.1)
Alcohol consumption	27	24 (18.8)	3 (5.5)
Sprayed pesticides	6	6 (4.7)	0
Work in vineyard/orchard during spraying	51	46 (35.9)	5 (9.09)

*p<0.001 ; **p=0.0046; IQR= Interquartile range

5.1.2 Urinary levels of dialkyl phosphate metabolites

The three DAP metabolites measured were detected in the urine samples of virtually all farm and non-farm boys but was significantly higher among farm boys (Table 2). Nineteen participants had urinary levels of DEP below the limit of detection, one participant for DMTP and two for DMP. Samples below limit of detection were assigned a value calculated by dividing the value below limit of quantification (BLQ) 0.78 ng/ml by square root of 2 (Hardt and Angerer 2000) (Table 2).

Table 2: Urinary levels of DAP (ng/mL) among farm and non-farm boys

Organophosphate metabolites (ng/ml)	n	Farm Residents	Non-Farm Residents	Overall
		Median (IQR)		
ΣDAP	183	78.1 (34.0;161.6)*	51.4 (15.7;86.4)	68.3 (27.9;129.5)
DEP	183	6.6 (2.9;15.0)**	2.3 (0.9;9.3)	5.5 (2.2;13.6)
DMP	183	37.8 (15.4;74.7)***	26.1 (7.2;55.6)	32.6 (12.6;62.8)
DMTP	183	23.8 (9.6;52.4)****	12.5 (5.9;27.7)	16.7 (7.6;43.4)

Samples below limit of detection were assigned a value calculated by dividing the BLQ value (0.78 ng/ml) by square root of 2 (Hardt and Angerer 2000).
IQR= Interquartile range
ΣDAP: sum of 3 dialkyl phosphates metabolites; DEP: diethyl phosphate; DMP: dimethyl phosphate; DMTP: dimethyl thiophosphate
*p=0.001 ; **p<0.001 ; ***p=0.012 ; ****p=0.0027

5.1.3 Descriptive result of health outcomes

Levels of reproductive hormones outside the laboratory ranges for age was found in both the farm and non-farm boys, but consistently higher in the former group when considering the different age groups (Table 3). There was no significant difference in the median tanner stage among farm and non-farm boys for all age groups (Table 3). The median sum testicular volume of non-farm boys was non- statistically significantly higher than those of farm boys for all age groups.

Table 3: Reproductive hormones and sexual reproductive health

Outcomes (by age group)	n	Farm Boys (n=128)	Non-Farm Boys (n=55)
		Median (IQR)	
FSH (IU/L)	177	**1.9 (0.9;3.9)**	**2.0 (1.3; 3.7)**
5-9		0.65 (0.4;1.0)	0.8 (0.9; 1.5)
>9-11		1.7 (0.9; 2.8)	1.2 (0.6; 1.3)
>11-14		3.3 (2.3; 5.4)	2.1 (1.6; 4.0)
>14		6.3 (3.9; 8.8)	4.6 (3.7;5.2)
LH (IU/L)	177	**0.55 (0.1;1.6)**	**1.0 (0.3; 2.4)**
5-9		0.05 (0.05; 0.1)	0.1 (0.05; 0.3)
>9-11		0.2 (0.1; 0.7)	0.1 (0.05; 0.1)
>11-14		1.5 (1.1; 2.5)	1.3 (0.8; 2.7)
>14		2.2 (1.8; 3.9)	4.2 (2.3; 5.0)
Oestradiol (pmol/L)	179	**47.9 (31.6; 63.8)**	**44.5 (31.3; 59.1)**
5-9		38.5 (25.0; 57.4)	31.3 (22.1; 38.3)
>9-11		39.3 (29.0; 47.8)	24.6 (23.6; 30.0)
>11-14		56.5 (42.0; 75.8)	45.8 (41.2; 59.1)
>14		80.7 (57.8; 96.6)	88.3 (71.1; 100.9)
Testosterone (nmol/L)	180	**0.4 (0.05; 2.8)**	**1.3 (0.05; 5.9)**
5-9		0.05 (0.05; 0.1)	0.05 (0.05; 0.05)
>9-11		0.05 (0.05; 0.4)	0.05 (0.05; 0.05)
>11-14		2.7 (0.95; 8.6)	2.05 (0.3; 5.0)
>14		8.8 (4.2; 14.4)	14.3 (9.6; 19.1)
SHBG (nmol/L)	178	**86.5 (61.6; 128.2)**	**75.0 (48.7;126.6)**
5-9		116.05 (98.9; 147.3)	114.5 (62.4; 158.9)
>9-11		104.8 (78.4; 136.5)	131.2 (128.5; 147.5)
>11-14		62.7 (47.3; 85.2)	68.5 (47.7; 102.4)
>14		38.3 (36.1; 74.0)	45.0 (36.6; 63.9)
Testicular Volume (ml)*	174	**10.5 (7; 30)**	**27 (7; 35)**
5-9		5 (5;7)	7 (4;9)
>9-11		9 (7;11)	7 (6;8)
>11-14		27 (18;40)	35 (11;35)
>14		36.5 (35;42.5)	45 (45;45)
Tanner stage	182	**2 (1 ;3)**	**2 (1;4)**
5-9		1 (1;1)	1 (1;2)
>9-11		2 (1;2)	1 (1;1)
>11-14		3 (2;3.5)	3 (2;4)
>14		4 (3.5;4)	4 (4;4)
Hormones outside laboratory normal ranges		**N (%)**	
Low FSH	14	13 (10.5)	1 (1.9)
High FSH	18	13 (10.5)	5 (9.4)
Low LH	39	33 (26.6)	6 (11.3)

High LH	1	1 (0.81)	0
Low Testosterone	65	46 (36.2)	19 (35.9)
High Testosterone	15	10 (7.9)	5 (9.4)
Low Oestradiol	22	17 (13.5)	5 (9.4)
High Oestradiol	4	4 (3.2)	0
Low SHBG	1	1 (0.78)	0
High SHBG	144	105 (82.0)	39 (70.9)
* Sum of right and left testicular volumes			

More than 25% and 50% of boys respectively fell below the CDC 25th and 50th percentile for age for both height and weight but not for BMI.

Table 4: Anthropometric measurements

Outcomes (by age groups in years)	**Farm Residents (n=127)**	**Non-Farm residents (n=55)**
	Median (IQR)	
BMI (kg/m^2)	**23.7 (20.9;27.8)**	**27.6 (22.9;31.3)**
5-9	20.2 (18.4; 22.5)	23.8 (22.4; 27.8)
>9-11	22.8 (20.9; 24.9)	21.7 (21.3; 22.9)
>11-14	26.3 (24.7; 29.8)	28.1 (25.7; 31.7)
>14	31.4 (28.9; 36.3)	31.1 (27.6; 35.2)
Height (cm)	**136.3 (127.5; 146.7)**	**145.2 (136.3; 162)**
5-9	125 (119.3; 128.8)	126.3 (118.5; 140.1)
>9-11	133 (129.1; 138.0)	134.3 (126.7; 136.5)
>11-14	146.7 (139.2; 154.2)	148.9 (138.5; 162)
>14	159.8 (146.9; 169.6)	160.1 (155.6; 165.5)
Weight (kg)	**32 (27; 40)**	**43 (31; 48)**
5-9	25.5 (22.5; 28.0)	30.0 (28.0; 39.0)
>9-11	30.0 (27.0; 34.0)	29.0 (28.0; 30.0)
>11-14	39.5 (34.5; 46.5)	43.0 (36.5; 51.0)
>14	49.0 (42.5; 63.0)	50.0 (45.0; 55.0)
BMI (kg/m^2)	**N (%)**	
≤ CDC 50th percentile for age	49 (38.6)	19 (34.6)
≤ CDC 25th percentile for age	25 (19.7)	7 (12.7)
Height (cm)		
≤50th CDC percentile for age	100 (78.7)	36 (65.5)
≤25th CDC percentile for age	79 (62.2)	28 (50.9)
Weight (kg)		
≤50th CDC percentile for age	92 (72.4)	29 (52.7)
≤25th CDC percentile for age	58 (45.7)	18 (32.7)
CDC: USA Centre for Disease Control and Prevention		

5.1.4 Relationship between DAP metabolites and health outcomes

Table 5 gives a summary of multiple logistic regression results for the association between DAP metabolites and health outcomes including reproductive hormones, anthropometric measures, SMR and testicular volume while controlling for confounding. The models presented in Table 5 include dichotomous outcomes with cut-off at the mean and an ordinal exposure variable with cut-offs at the quartiles of the dialkyl phosphate metabolites which produced the strongest exposure-response relationships. Analyses using models with outcomes with different cut-offs or continuous exposures and those using multiple linear regression analyses was also conducted (Appendix H).

ΣDAP levels were positively associated with lower FSH and LH levels with the odds of having FSH and LH levels below the 50% percentile higher among those in the 2^{nd}-4^{th} quartile DAP levels compared to the reference category (the first quartile). There was also an incomplete dose response in the associations for FSH and LH with the association for the 3^{rd} and 4^{th} quartile **Σ**DAP levels higher than that for the 2^{nd} quartile but the association for the 3^{rd} quartile higher than that for the 4^{th} quartile. The association between FSH and 3^{rd} quartile **Σ**DAP levels was statistically significant with the odds of having FSH levels < 50^{th} percentile 4.22 (95% CI: 1.06;16.7) times that of the first quartile (p=0.04). The DAP metabolites which had the strongest associations with LH and FSH were DMP and DMTP for which similar results to that of **Σ**DAP levels were found for FSH but much stronger associations for LH.

ΣDAP levels were positively associated with higher oestradiol levels with the odds of having oestradiol levels above the 50% percentile higher among those in the 2^{nd}-4^{th} quartile DAP levels compared to the reference category (the first quartile). There was also a dose response in the

associations with oestradiol from the 2nd – 4th quartile **Σ**DAP levels. None of these associations were statistically significant (p-value>0.5). The DAP metabolite which had the strongest association with oestradiol was DMTP followed by DMP. The association between oestradiol and DMTP (3rd and 4th quartile) levels were statistically significant with the odds of having oestradiol levels above the 50th percentile as 5.32 (95% CI 1.22; 23.29) and 7.42 (95% CI :1.69; 32.39) times that of the first quartile, respectively.

There were no strong associations found between testosterone levels and DAP levels. The results of both logistic and linear regression analysis where other hormones were excluded as confounders in the model were consistent with these results (data not shown).

ΣDAP levels were positively associated with lower height, weight and BMI with the odds of having height and BMI measurement below the 50% percentile and weight below the 25% percentile, higher among those in the 2nd-4th quartile DAP levels compared to the reference category (the first quartile). There was also a dose response relationship in the associations with weight and BMI from the 2nd-4th quartile **Σ**DAP levels. The strongest associations were that for the 4th quartile for both weight and BMI with the odds of having weight lower than the 25th percentile and BMI below the 50th percentile 2.59 (95% CI: 0.94;7.11) and 2.56 (95% CI: 0.95; 6.83) times that of the first quartile, respectively. DMTP was the metabolite with the strongest association with anthropometric measures. The associations between BMI and the 4th DMTP quartile was statically significant with the odds ratio = 3.239 (95% CI: 1.17; 8.97).

ΣDAP levels were positively associated with lower Tanner stage and testicular volume with the odds of having Tanner stage < 4 and testicular volume (sum of left and right testicles) below

the 50% percentile, higher among those in the 2nd-4th quartile DAP levels compared to the reference category (the first quartile) apart from the association for the 3rd quartile DAP for Tanner stage. There was no evidence of a dose response in these associations. The DAP metabolite which had the strongest associations with Tanner stage and testicular volume were DMTP followed by DMP. The association between testicular volume and 3rd quartile DMTP was statistically significant with the odds of having lower testicular volume 3.7 (95% CI: 1.0; 13.2) times than that of the first quartile.

Table 5: Relationship between health outcomes and DAP metabolites using multiple logistic regression

Outcomes	DAP Metabolites			
	Odds Ratio (95% Confidence Intervals); Reference: DAP first quartile			
	N	Second Quartile	Third Quartile	Fourth Quartiles
Hormonal Outcomes (Adjusted for age, income & SHBG and other 3 hormones)				
FSH < 50th percentile #				
DEP	168	1.636 (0.433; 6.184)	5.009 (1.166; 21.525)*	1.455 (0.379; 5.591)
DMP	168	1.107 (0.309; 3.959)	2.495 (0.629; 9.896)	4.024 (0.943; 17.172)
DMTP	168	0.939 (0.251; 3.520)	3.994 (0.891; 17.902)	3.315 (0.826; 13.311)
ΣDAP	168	2.098 (0.525; 8.383)	4.222 (1.067; 16.704)*	3.373 (0.859; 13.232)
LH < 50th percentile #				
DEP	162	0.242 (0.016; 3.682)	52.713 (1.105; 2514.47)	5.167 (0.180; 148.142)
DMP	162	6.336 (0.345; 116.301)	31634.46(0.611; 1.64e+09)	19.529 (0.338; 1128.77)
DMTP	162	103.19(1.775;6000.023)*	33.89 (0.550;2088.806)	5.456 (0.268; 111.100)
ΣDAP	162	3.002 (0.296; 30.470)	7.672 (0.371;158.831)	6.367 (0.358; 113.287)
Testosterone < 50th percentile #				
DEP	168	0.597 (0.067; 5.326)	0.279 (0.033; 2.354)	0.046 (0.004; 0.480)
DMP	168	0.214 (0.027; 1.673)	0.032 (0.003; 0.368)	0.130 (0.015; 1.110)
DMTP	168	1.992 (0.238; 16.677)	0.892 (0.117; 6.821)	0.164 (0.018; 1.522)
ΣDAP	168	1.665 (0.193; 14.387)	0.141 (0.014; 1.376)	0.103 (0.012; 0.905)
Oestradiol > 50th percentile $				
DEP	165	0.865 (0.233; 3.209)	1.524 (0.429; 5.402)	2.464 (0.677; 8.975)
DMP	165	1.181 (0.344; 4.057)	1.209 (0.338; 4.331)	1.705 (0.467; 6.216)
DMTP	165	1.847 (0.428; 7.977)	5.321 (1.215; 23.295)*	7.419 (1.699; 32.399)*
ΣDAP	165	1.248 (0.329; 4.721)	1.784 (0.479; 6.640)	3.371 (0.976; 11.651)
Anthropometric Outcomes (Adjusted for age, income)				
Height (m) <50th percentile #				
DEP	175	0.542 (0.191; 1.534)	0.903 (0.305; 2.669)	0.462 (0.163; 1.313)
DMP	175	2.614 (0.934; 7.312)	1.766 (0.649; 4.809)	1.537 (0.568; 4.159)
DMTP	175	1.126 (0.404; 3.141)	0.829 (0.308; 2.232)	0.978 (0.336; 2.852)
ΣDAP	175	1.821 (0.665; 4.987)	1.049 (0.389; 2.826)	1.291 (0.463; 3.599)
Weight (kg) <25th percentile $				
DEP	175	0.599 (0.226; 1.588)	1.496 (0.599; 3.736)	1.341 (0.527; 3.415)
DMP	175	2.362 (0.914; 6.104)*	1.666 (0.644; 4.308)	1.985 (0.747; 5.273)
DMTP	175	1.537 (0.597; 3.958)	1.008 (0.388; 2.617)	2.599 (0.941; 7.184)
ΣDAP	175	1.237 (0.486; 3.148)	1.394 (0.544; 3.569)	2.598 (0.949; 7.114)
BMI <50th percentile #				
DEP	175	0.852 (0.314; 2.309)	1.539 (0.597; 3.968)	2.311 (0.899; 5.941)
DMP	175	1.764 (0.651; 4.779)	2.066 (0.770; 5.540)	3.443 (1.270; 9.329)
DMTP	175	1.668 (0.619; 4.488)	1.843 (0.684; 4.968)	3.239 (1.169; 8.974)*
ΣDAP	175	1.051 (0.399; 2.771)	1.223 (0.469; 3.184)	2.559 (0.959; 6.830)
Sexual Maturity Outcomes (Adjusted for age, income, SHBG)				
Tanner Stage < stage 4 $				
DEP	170	0.294 (0.041; 2.107)	1.123 (0.185; 6.833)	0.555 (0.076; 4.043)
DMP	170	4.507 (0.658; 30.892)	0.208 (0.026; 1.648)	1.693 (0.276; 10.389)
DMTP	170	5.313 (0.626; 45.075)	0.918 (0.155; 5.424)	5.507 (0.623; 48.661)
ΣDAP	170	2.144 (0.340;13.499)	0.430 (0.070;2.634)	3.845 (0.527; 28.036)
Testicular Volume (ml) <50th percentiles #				
DEP	170	0.350 (0.099; 1.241)	0.821 (0.239; 2.809)	0.463 (0.128; 1.667)
DMP	170	0.832 (0.245; 2.829)	1.159 (0.349; 3.860)	1.449 (0.399; 5.257)
DMTP	170	1.918 (0.532; 6.922)	3.685 (1.031; 13.175)*	1.187 (0.325; 4.337)
ΣDAP	170	1.729 (0.501; 5.973)	1.603 (0.467; 5.502)	1.459 (0.417; 5.105)

*Statistically significant results, p<0.05.

6.1 Discussion

The number of statistically significant associations between adverse health outcomes (reproductive hormones, anthropometric outcomes and reproductive development) and urinary levels of dialkyl phosphates of the participating boys found in this study was low but there were consistent non-significant positive associations with evidence of dose response effects for some associations. The lack of significant associations could be due to the reduced sample size due to pesticide bio-monitoring measurements not being available for all the boys who participated in the main study.

With regard to reproductive serum hormone levels, the lower LH levels and higher oestradiol levels associated with increased urinary dialkyl phosphate levels (increased odds of LH levels below the 50^{th} percentile and oestradiol levels higher than the 50^{th} percentile in boys with dialkyl phosphate levels > the 3^{rd} quartile compared to those in the 1^{st} quartile) is consistent with the significantly lower LH levels associated with farm boys when compared with non-farm boys found in the previous analysis as dialkyl phosphates levels were higher in farm boys (English et al. 2012). The results in this study indicates that boys with ΣDAP levels > 68 µg/mL have > 3 times the odds of LH levels below the median and > 1.3 times the odds of oestradiol less than the median. The lower testosterone levels in the 1^{st} quartile DAP levels compared to those in the higher quartiles DAPs are also consistent with the lower testosterone levels among farm boys compared to non-farm boys found in the previous analysis but the associations in both studies were not significant. The weaker associations with serum testosterone relative to other hormones could be due to its higher variability as indicated in Table 3 and therefore requiring a higher sample size.

The lower FSH levels associated with higher levels of DAPs is not consistent with the significantly higher levels of FSH among farm boys as found in the previous analysis. A reason for the difference in the findings could be that the previous analysis did not focus only on OP pesticides and that other non-OP pesticides could be responsible for increased FSH levels in farm boys. The effect of OP pesticides on reproductive hormone levels is consistent with the theory that pesticides that are hormonally active can cause interruption to the male hypothalamic pituitary–gonadal (HPG) axis of pubertal boys and laboratory evidence of effects on reproductive hormone levels after administration of current-use OP pesticides (Saiyed et al. 2003; Flehi-Slim et al. 2016; Mosbah et al. 2016; Alaa-Eldin et al. 2017). An explanation for higher oestradiol and lower FSH, LH and testosterone levels due to OP pesticides could be aromatization of testosterone to oestradiol by these pesticides leading both to inhibition of LH and FSH. However, the boys are likely exposed to different OP pesticides which may result in a mixture of estrogenic, anti-androgenic and other endocrine disrupting effects on the HPG axis (Daston et al. 2003; Kortenkamp 2007).

Chlorpyrifos and malathion decreased serum FSH, LH and testosterone levels (Choudhary et al. 2008; Flehi-Slim et al. 2016; Mosbah et al. 2016; Alaa-Eldin et al. 2017), and parathion decreased serum testosterone levels (Flehi-Slim et al. 2016) in laboratory animals. The only epidemiological studies that investigated the effects of current use pesticides on male reproductive hormones include a cross-sectional study of Indian boys that found exposure to aerial application of endosulfan, a non OP pesticide to be significantly associated with lower serum testosterone and a study among US men (Meeker et al. 2004; Meeker et al. 2006; Blystone et al. 2007; Choudhary et al. 2008; Flehi-Slim et al. 2016; Mosbah et al. 2016; Alaa-

Eldin et al. 2017) has found urinary TCPY, the chlorpyrifos metabolite, to decrease serum testosterone levels.

The reason for the stronger exposure-outcome associations found in these studies could be due to a bigger sample size in both studies, the study of a pesticide that is a stronger reproductive toxin as in the case of endosulfan and a more highly exposed study sample as was the case in the endosulfan study.

An alteration of hormone levels by pesticides could lead to adverse effects on reproductive development and both effects can result in reduced semen quality and fertility in men (WHO 2012). The lower tanner stage scores and testicular volumes associated with urinary DAPs (ΣDAP levels > 68 µg/L have > 2 times the odds of tanner stage score below 3 and > 1.4 times the odds of testicular volumes below the median after controlling for confounding) is consistent with the lower tanner stage scores and testicular volumes found in farm boys compared to non-farm boys although the results in both analyses were not significant (English et al. 2012). In the Indian study, a significant delay in sexual maturity was found due to endosulfan exposure (Saiyed et al. 2003).

The negative association between anthropometric measurements (height, weight and BMI) found in this study is also consistent to those found in the previous analyses that compared boys living on farms to those not living on farms and using exposure indices based on the reported intensity of spraying on farms and proximity of the participant to the nearest spraying area (Ochieng et al. 2013). The results in this analysis indicates that boys exposed > 68 µg/L have 1.2 times the odds of weight measurements below the 25^{th} percentile of CDC reference values and > 1 times the odds of BMI below the 50^{th} percentile of CDC growth reference

values. There is laboratory evidence that administration of chlorpyrifos to male rats reduces body weight (Mosbah et al. 2016).

Although nutritional status (measured by dietary intake data), an important confounder for anthropometric measurements was not measured in the study, this does not affect the current study as participating boys were recruited from neighbouring areas ensuring that their dietary intake was not substantially different. Household income was low for the whole study sample and it was also included as a confounder in the analysis (socioeconomic and nutritional status indicator) (Marmot 2004).

The biological mechanism for the effect of hormonally active pesticides on anthropometric measurements is through alteration of growth hormone release by the pituitary gland. No previous epidemiological studies on the effect of current use agricultural pesticides on anthropometric measurements were found in the literature. There is evidence from laboratory studies on the effect of DDT on pubertal growth and from epidemiological studies among DDT-exposed boys of reduced height measurements, although results are contradictory, as discussed earlier (Gladen et al. 2000; Karmaus et al. 2002; Gladen et al. 2004; Ribas-Fitó et al. 2006; Burns et al. 2012).

Urinary DMP and DMTP were the metabolites that had the strongest associations with health outcomes in this study. With DAP metabolites being non-specific markers of OP pesticide exposure, the specific pesticides associated with the health outcomes in this study cannot be identified.

The levels of urinary DAP's measured among both farm and non-farm boys in this study were substantially higher than those measured in US general populations indicating a relatively high exposed study sample (Dana B. Barr 2004). The relatively high levels of DAPs found in non-farm boys in addition to farm boys is not corroborated by the low prevalence of reported domestic pesticide use and other exposure activities (Table 1). A possible explanation for the low prevalence of reported pesticide exposures could be due to a reporting bias in that parents/guardian did not want to reveal household chemical exposures. The use of illegal street pesticides has been reported in low-income urban settings and might be prevalent in these settings as well (Rother 2010). An important reason for the high levels of urinary DAPs among farm and non-farm boys is their exposure to contaminated water and food and pesticide drift.

An important limitation in this study is the cross-sectional design as associations are determined at one point in time and therefore precluding the establishment of the temporality of the association. Additionally, with the individual variability in the reproductive outcomes measured cross-sectionally being high, the power of the study to detect associations is lowered. Another important limitation in this study is the single urine sample which represents short-term exposure as OP pesticides are eliminated from the body within a few days. Additionally, the urine samples were not collected during the peak spraying season (October -February). However, OP pesticides and environmental DAPs are known to be present in the environment for a longer period than in the human body and repeated exposures are likely (Lucyna Kapka-Skrzypczak 2011). A longitudinal study design in which OPs measurements are repeated a few times per year and the outcomes followed up would significantly strengthen a future study.

Another limitation is the selection of only school boys as boys who do not attend schools may have higher exposure to pesticides than those who attend schools, and this would underestimate the strength of associations. Recall bias related to questions on the boys' childhood and mothers' pregnancy in the parent/guardian questionnaire is possible and the provision of incorrect information in the absence of the biological mother, is possible, but this would likely be prevalent equally in boys with low and high exposures.

7.1 Conclusion

This study found high levels of urinary DAPs and consistent non-significant associations and some dose response relationships between DAP metabolites and reproductive and growth outcomes of boys. This provide some evidence that currently used agricultural OP pesticides alter reproductive hormone levels and decrease sexual maturity ratings and anthropometric measurements of these boys residing in the rural Western Cape. A larger cohort study with repeated measurements of urinary OP pesticides is recommended. Policy and interventions to reduce pesticide exposure among these boys is warranted.

8.1 Acknowledgements

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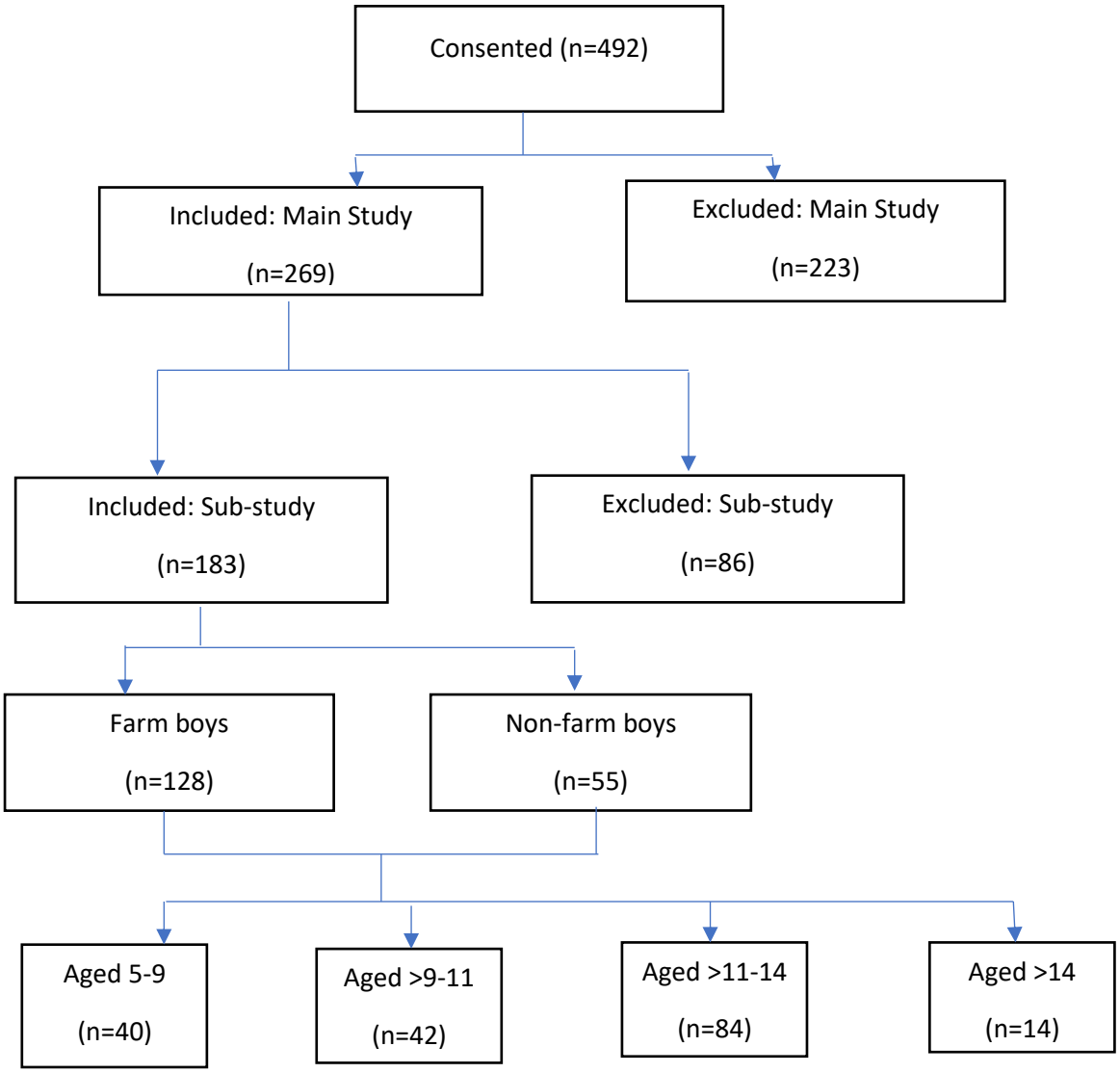
PART D: APPENDICES

Appendix A: Study flow-diagram



Appendix B: Structured record form

PHYSICAL EXAMINATION

Study Number: _____

DATE: _____

PHYSICIAN: _____

Measures/evaluations of height, weight, testes disposition, varicocele and hydrocele have been performed with the man in standing position.

Evaluation of pubic hair should be according to the stages of Tanner, for which illustrations have been provided.

For evaluation of testes size, the orchidometer provided has to be used.

HEIGHT: _____ cm WEIGHT: _____ kg

Birth weight _____(kg)

GENITAL REGION:

Scars due to surgery: No: _____ Yes: _____

(describe as "other remarks")

Pubic Hair and Penis: Tanner stage: _____

(1-5)

Penis: Normal: _____ Abnormal: _____

(describe as "other remarks")

Testes size: Left: _____ ml Right: _____ ml

Testes consistency: Left: _____ Right: _____

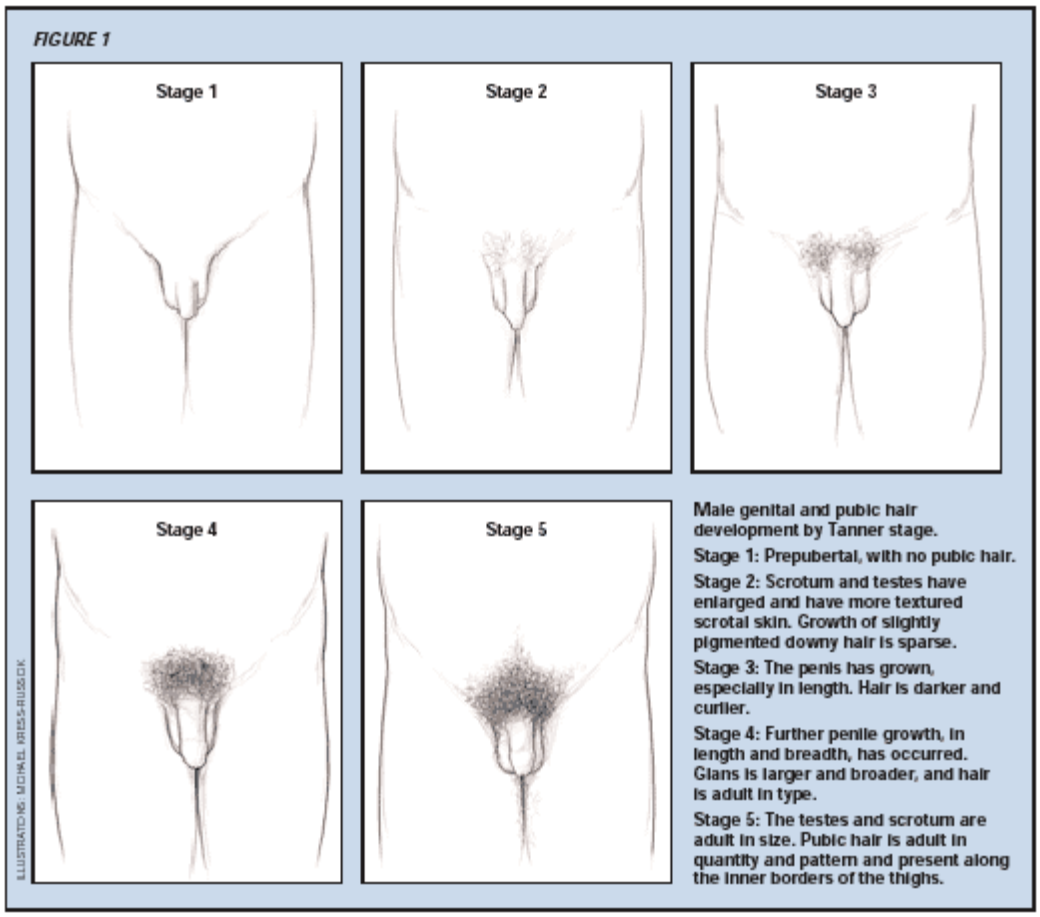
(N = normal, S = soft, H = hard)

Testes abnormailty: Left: _____ Right: _____

(N = no, Y = yes)

OTHER REMARKS

Appendix C: Tanner Stage diagrams



Source: http://www.childclinic.net/pain/tanner_puberty_boys_img.gif

Appendix D: Questionnaire

CHILD QUESTIONNAIRE

(Male reproductive health effects due to pesticides amongst farm residents in the Western Cape)

Date _____ Room Temperature _____

Survey Number _____

Name of the Interviewer _____

Study Area _____

School _____

Source of drinking water _____

Specify the source of drinking water _____

Details of parent:

Relationship to participants: mother, father, other (circle which one is applicable)

If other, specify _____

Highest Standard/Grade passed at school: _____

Diplomas/Tertiary Education: _____ (Y/N)

Employment status _____ (yes, no, student, retired, other)

If employed, Job Title: _____

If farm worker, Exposure group: _____

(Supervisor, Sprayer/Mixer, Non- Sprayer Farmworker, Non Farmworker)

Marital Status _____

(Married, living with someone as married, widowed, divorced, separated, single with girl friend, single with no girlfriend)

What is your monthly household income (in Rands)? _____

How often do your family go hungry or have no food to eat:

Never _____

Seldom____

Sometimes_____

Often_____

Details of son:

Date of birth _____ Age (_____)

Gender: ____ (Male/Female)

Birth weight:_____(kg)

Current standard/grade at school: _____

Address _____

A. GENERAL MEDICAL HISTORY

A1. How do you judge your son's health in general? _____

(Excellent, Very good, Good, Bad)

A2**.** Did he have/does he have:

Disease	**Yes, No, Don't Know**	**Year Diagnosed**
Diabetes		
TB		
Fits		
High Blood Pressure		
Asthma		
Heart Problems		
Back Problems		
HIV		
Foetal Alcohol Syndrome		
Other Specify:		

A3.a) Did he have /does he have any other chronic illnesses (longer than three months) apart from those listed above? __ (1 = Yes, 2 = No)

b) If yes, specify _____

A4. Has he taken any daily medication during the last 3 months? ___ (Yes, No)

A5. Has he ever been poisoned by pesticides? _____ (Yes, No, Don't know)

If yes, give details (date, name of doctor, name of hospital)

_____

B. GENITAL HEALTH HISTORY AND PUBERTY

B1. Did your son ever had mumps? ___ (Yes, No, DN)

B2. If yes, how old was he when he had mumps? _____years old

B3. Do you think your child has already entered puberty? _____ (Yes No)

If : Yes

a. At what age do you think your child entered puberty?

_____ years, _____ months

b. What was the first sign of puberty you saw in your child?

_____

If : NO (not yet entered puberty)

c. At what age do you expect your child to enter puberty?

_____ years, _____ months

d. What is the first sign of puberty you expect to see?

_____

B4. Would you say that your son's growth spurt (in height) has started yet? (A growth spurt is defined as growth in height that is faster than usual.)

_____

(No, Yes, barely, Yes, definitely, Development completed, Don't know)

If yes, at what age _____(years)

B5. Would you say that growth of his underarm and pubic hair has started yet?

_____

(No, Yes, barely, Yes, definitely, Development completed, Don't know)

If Yes, at what age? _____(years)

B6. Have you noticed any changes in his skin, especially pimples?

_____

(No, Yes, barely, Yes, definitely, Development completed, Don't know)

B7. Have you noticed a deepening of his voice?

_____

(No, Yes, barely, Yes, definitely, Development completed, Don't know)

If yes, at what age _____(years)

B8. Has he started to grow hair on his face

_____

(No, Yes, barely, Yes, definitely, Development completed, Don't know)

B9. Compared with other boys his age, would you say your son's physical development is:

_____

(much earlier than the other boys, somewhat earlier than the other boys, about the same as the other boys, somewhat later than the other boys, much later than the other boys)

B10. Was your son born with abnormally developed testicles? ___ (yes, no, DN)

If Yes, did he go for an operation or received medication?

_____ . What was the date he went for an operation or received medication?

_____

B11. Has your son ever had an injury, resulting in swelling/discolouring in the testicular area? ____ (yes, no, DN)

B12. Has he ever had an operation in the testicular area?

If YES, which date?

B13. Has he been sterilized? _____(Yes, No)

B14. Has your son ever had any other diseases in the testicular area?

____ (Yes, No, Don't Know)

If "Yes", specify and give the date

_____

B15. Did your son already had his first wet dreams? _____

If yes, at what age? _____

B16. From the diagram, what stage of development do you consider your child?

Pubic hair and genital development:_____ (a, b, c, d or e)

C. LIVING HISTORY

Please answer the following questions regarding the places where your son has lived in his lifetime (C1-C16 is for current residence, Sections CA-CD is only applicable for residences before current residence starting from the most recent one)

C1 Where does he live currently? _____ (Name of town or city)

C2 For how long has he been living there? _____(years, months)

C3 Is his home located on a farm, town or city? _____

C3 If the place was on a farm, what kind of farm

C4 If his home is located on a farm, how far from the house is the nearest Vineyard/field? _____ (meters)

C5 Are pesticides sprayed on the vineyard/field during the year? ____ (yes, no, DN)

IF No (go to C7)

IF YES, complete the following:

How many times a year are pesticides applied by means of

a) a tractor with a boom sprayer _____ (number of times a year)

b) a tractor with persons using hand or backpacks? _____ (number of times a year)

c) aeroplane _____ (number of times a year)

C6 Does the pesticides spraying come into the house? ____ (yes, no, DN)

C7 Does your son come into contact with pesticides outside the house while spraying occurs (for e.g. playing near spraying area)? _____ (yes, no)

C8 Does your son go into in the field/vineyards soon after spraying or come into contact with sprayed surfaces? ____ (yes, no)

C9 What are the sources of drinking water at his house? _____
(municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

C10 What are the sources of water for recreational use (bathing, washing of clothes) at his house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

C11 Does your son play swim or play in dams/rivers? _____(yes, no)

If yes, where is the dam/river located

_____

(on farm, just outside farm, more than 100m away, out of town)

C12 Does your son perform help on the farm? _____ (yes, no)

If Yes,

What does he do _____ and

How often? _____

(every day, twice a week, once a week, once a month, school holidays)

C13 Is he involved in spraying or mixing pesticides? _____ (yes, no)

C14 Does he work in the pesticide store? _____ (yes, no)

C15 Does your son come into contact with empty pesticide containers? ___(yes, no)

If yes, how _____ (for eg play, drinking water, burning)

C16 Does your son eat from the crops in the vineyard/field soon after spraying?

_____ (yes, no)

The following questions are about the place your son lived before his current home

CA1 Where did you son live before? _____ (Name of town or city)

CA2 For how long did he live there? _____(years, months)

CA3 Was that home located on a farm, town or city**? _____**

C3 If the place was on a farm, what kind of farm

CA4 If his home was located on a farm, how far from the house was the nearest vineyard/field?

_____ (meters)

CA5 Was pesticides sprayed on the vineyard/field during the year?

____ (yes, no, DN)

IF No (go to C7)

IF YES, complete the following:

CA6 Did the pesticides spraying come into the house? ____ (yes, no)

CA7 Did your son come into contact with pesticides outside the house while spraying occurs (for e.g. playing near spraying area) ? _____ (yes, no)

CA8 Did your son go into in the field/vineyards soon after spraying or come into contact with sprayed surfaces? ____ (yes, no)

CA9 What were the sources of drinking water at his house? _____

(municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

CA10 What were the sources of water for recreational use (bathing, washing of clothes) at his house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

CA11 Does your son play swim or play in dams/rivers? _____(yes, no)

If yes, where is the dam/river located

_____

(on farm, just outside farm, more than 100m away, out of town)

C12 Did your son help on the farm? _____ (yes, no)

If Yes,

What did he do? _____ and

How often? _____

(every day, twice a week, once a week, once a month, school holidays)

CA13 Was he involved in spraying or mixing pesticides? _____ (yes, no)

CA14 Did he work in the pesticide store? _____ (yes, no)

CA15 Did your son come into contact with empty pesticide containers? ___(yes, no)

If yes, how _____ (for eg play, drinking water, burning)

CA16 Did your son eat from the crops in the vineyard/field soon after spraying?

_____ (yes, no)

The following questions are about the place your son lived before his previous home

CA1 Where did you son live before? _____ (Name of town or city)

CA2 For how long did he live there? _____(years, months)

CA3 Was that home located on a farm, town or city**? _____**

C3 If the place was on a farm, what kind of farm

CA4 If his home was located on a farm, how far from the house was the nearest vineyard/field?

_____ (meters)

CA5 Was pesticides sprayed on the vineyard/field during the year?

____ (yes, no, DN)

IF No (go to C7)

IF YES, complete the following:

CA6 Did the pesticides spraying come into the house? ____ (yes, no)

CA7 Did your son come into contact with pesticides outside the house while spraying occurs (for e.g. playing near spraying area)? _____ (yes, no)

CA8 Did your son go into in the field/vineyards soon after spraying or come into contact with sprayed surfaces? ____ (yes, no)

CA9 What were the sources of drinking water at his house? _____

(municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

CA10 What were the sources of water for recreational use (bathing, washing of

clothes) at his house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

CA11 Does your son play swim or play in dams/rivers? _____(yes, no)

If yes, where is the dam/river located

_____

(on farm, just outside farm, more than 100m away, out of town)

C12 Did your son help on the farm? _____ (yes, no)

If Yes,

What did he do? _____ and

How often? _____

(every day, twice a week, once a week, once a month, school holidays)

CA13 Was he involved in spraying or mixing pesticides? _____ (yes, no)

CA14 Did he work in the pesticide store? _____ (yes, no)

CA15 Did your son come into contact with empty pesticide containers? ___(yes, no)

If yes, how _____ (for e.g. play, drinking water, burning)

CA16 Did your son eat from the crops in the vineyard/field soon after spraying?

_____ (yes, no)

D. HOUSEHOLD PESTICIDE EXPOSURE

D1 Do you use any pesticides in your garden or in your home (e.g. doom, rat poison, fleas)? _____ (yes, no)

D2 If yes, for how long have you been using pesticides at home?

_____ (number of years)

D3 How frequently do you use pesticides at home _____

(every day, 3 times a week, once a week, once a month, less than once a month)

D4 Do you have your house fumigated?

If yes, for how long? _____(number of years)

How frequently?

_____

(every day, 3 times a week, once a week, once a month, less than once a month)

D5 Does any person in the house work with pesticides?

If yes, how many? _____

Since when has there been a person that work with pesticides? _____ (year)

Does any pesticide contaminated clothes get washed at home_____ (yes,no)

If yes, does it get washed with the rest of the washing? _____ (yes, no)

D6 Does your son eat fruit or vegetables from your garden _____ (yes, no)

D7 Do you use empty pesticide containers at home for domestic purposes

If yes, what do you use them for? _____

Since when have you been using empty containers at home _____ (year)

E. DIET

E1 Does your son eat meat/fish? _____ (Yes, No)

E2 How many times a week does he eat meat/fish _____

E3 In his lifetime, how many times a week did he eat meat/fish _____

E4 Does he eat vegetables? ___ (Yes, No)

E5 How many times a week does he eat vegetables _____

E6 How many times a week does he eat soy products ____

E7 In his lifetime, how many times a week did he eat vegetables _____

E8 In his lifetime, how many times a week did he eat soy products ____

E9 Does your son like to eat nuts? __

How many times a week does he eat nuts? ____

E10 In his lifetime, how many times a week did he eat nuts? ___

E11 Was he on soya milk after birth? ___

For how long? _____

E12 Does your son eat meals provided by the school?

If yes, what do they provide? _____

Please specify the meals

_____

F. MOTHERS HABITS DURING PREGNANCY

F1 When you were pregnant with this son, did you spray or mix pesticides ___?

If yes, for how many weeks____?

F2 During the pregnancy, did you work in the vineyard/orchard while pesticides were sprayed? ____ (Yes, No)

F3 Did you work in the vineyard/orchard while pesticides were not sprayed?___ (Yes, No)

F4 During the pregnancy, did you smoke?___ (Yes, No)

If yes, how many cigarettes per day? ____

F5 During the pregnancy, did you drink alcohol?__ (Yes, No)

If yes, how many bottles per week? ____

(if papsak, estimate number of bottles)

F6 During the pregnancy, how many times a week did you eat meat/fish _____

F7 During the pregnancy, how many times a week did you eat vegetables _____

F8 During the pregnancy, how many times did you eat soya beans or soy products ____

F9 During the pregnancy, how many times a week did you eat nuts ___

G. SMOKING AND ALCOHOL

G1 Does your son smoke currently or did he smoke before? _____ (yes, no)

If yes, for how long? _____ (number of years)

G2 Does anyone in the house smoke? _____ (yes, no)

G3 Does your son drink alcohol currently or did he drink alcohol before? _____ (yes, no)

If yes, for how long? _____ (number of years) and how many bottles per week? _____ (estimate if papsak)

G4 Does your take drugs or smoke dagga currently or before? _____ (yes, no)

If yes, for how long? _____ (number of years)

Appendix E.1: Ethics letter

UNIVERSITY OF CAPE TOWN

Research Ethics Committee
E52 Room 24,Old Main Building Groote Schuur Hospital, Observatory, 7925
Queries : Lamees Emjedi
Tel : (021) 406-6338 Fax: 406-6411
E-mail : lemjedi@curie.uct.ac.za

12 August 2005

REC REF: 279/2005

Dr MA Dalvie
Public Health & Family Medicine

Dear Dr Dalvie

ENDOCRINE DISRUPTING EFFECTS OF PESTICIDES AMONGST MALE FARM RESIDENTS IN THE WESTERN CAPE

Thank you for submitting your study to the Research Ethics Committee for review.

*It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study on the 4 August 2005.*

The REC requests the following changes be made to the documentation and that amended copies be submitted:

- *Adolescent to sign assent (include a form).*
- *Change contact person of REC as Mr Fula has left.*

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROF T. ZABOW
CHAIRPERSON

Appendix E.2: Ethics letter

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E53-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • Facsimile [021] 406 6411
e-mail: preaward@curie.uct.ac.za

07 September 2005

REC REF: 279/2005

Dr MA Dalvie
School of Public Health and Family Medicine

Dear Dr Dalvie

ENDOCRINE DISRUPTING EFFECTS OF PESTICIDES AMONGST MALE FARM RESIDENTS IN THE WESTERN CAPE

Thank you for your letter to the Research Ethics Committee dated 20 July.

It is a pleasure to inform you that the Ethics Committee has formally approved the changes made to the consent form:

Adding the word, "adolescent or adult" in various parts of the consent form text
Changing the name of Ethics Administrator

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROF T. ZABOW
CHAIRPERSON

Last saved by kbonani

Appendix F: Consent form

Consent to participate in a survey of investigating health effects due to occupational and environmental pesticide exposures on male farm residents in the rural Western Cape.

1. **Title of research project**

Male reproductive effects due to pesticide exposure in the Western Cape, South Africa.

2. **Names of the researchers**

Mohamed Aqiel Dalvie (BSc, Honours, MSc, PhD)
Algernon Africa (BTech)
Vicky Major
Leslie London (MBChB, Honours, MD)
Eugene Cairncross (BSc, Honours, PhD)

3. Purpose of research

The University of Cape Town is conducting this survey to investigate the reproductive health effects of pesticides on young boys and men in the Western Cape. This will be of benefit to men and boys living in farming areas and who are exposed to pesticides either at work or in
the environment.

4. Description of the research project

We will conduct tests on one day. Your son will be required to produce a urine and blood sample and undergo a physical examination and you will complete a questionnaire.

a) **Questionnaire:** A member of our study team will interview you in privacy to complete the questionnaire. You will be asked questions about general personal information about your son, his general medical health, genital health history and lifetime environmental exposure to pesticides.

b) **Urine sample:** Your son has to produce a urine sample (in privacy) in a plastic container and give it to the nurse. The sample will be analysed for pesticides.

c) **Blood sample:** A nurse will draw 10 ml blood from a vein on your son's arm. The blood will be analysed for pesticides and for the levels of hormones.

d) **Physical examination:** A doctor will assess your son's reproductive health.

5. Risks and discomforts of the research

a) From the blood tests. A single needle stick will be felt when the blood is taken. Sometimes a small bruise may occur from the needle stick, but this is minor and will heal quickly. The total amount of blood taken is quite small and the body will quickly replace it. Blood samples will be used only to measure pesticides and reproductive hormones and will be destroyed at the end of the study.

b) From the questionnaire.

There are minimal risks associated with completing the questionnaire. The only risk is loss of confidentiality about personal information but the data will be seen only by study personnel. All reports will present aggregate data in which individuals will not be identifiable.

6. Expected benefits to you and others

A doctor will examine your son's reproductive health. Refreshments will be provided as compensation for time in participating in the study. This study on the reproductive health effects of pesticides will benefit men and boys

living in farming areas and who are exposed to pesticides either at work or in the environment. Steps can be taken to reduce or prevent exposure to the pesticides or the pesticide can be banned. The blood and urine results can be used to develop ways in which the amount of pesticides in your body can be monitored.

7. Costs to you resulting from participation in the study

The study is offered at no cost to you.

8. Confidentiality of information collected

Study participants will not be personally identified in any reports on this study. The records will be kept confidential to the extent provided by law. The records, including any identification information, will be destroyed after the results have been fully analysed.

9. Documentation of the consent

One copy of this document will be kept together with our research records on this study. A second copy will be given to you to keep.

10. Contact person.

You may contact the following person for answers to further questions about the research, your rights, or any injury you may feel is related to the study.

Name of person: MA Dalvie (The principal investigator) - telephone 021 4066610
Name of person: Lamees Emjedi (Ethics administrator) - telephone 021 4066492

11. Voluntary nature of participation

Your son's participation in this project is voluntary. Subsequent to your consent, you may refuse your son to participate in or withdraw from the study at any time without penalty or loss of benefits to which you may otherwise be entitled.

12. Consent of the participant

I have read the information given above. I understand the meaning of this information. I hereby consent for my son to participate in the study.

_____

_____

Printed name of parent/ participant (adolescent or adult)

signature

_____

_____

Date

_____ _____

Interviewers (print) signature **Date**

_____ _____

Witness (print) signature **Date**

Date:_____

Study Number _____

Appendix G: Instructions to authors

ENVIRONMENT INTERNATIONAL

A Journal of Environmental Science, Risk & Health

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

ISSN: 0160-4120

DESCRIPTION

Environment International is an international, multidisciplinary Journal situated at the interface between the environment and humans. Our interest is broadly reflecting the complexity of our environment and spans sources, pathways, fate and impacts associated with air, soil, water, food, and biota and their related interactions with ecosystems and human health.

Environment International serves as a platform for academics, practitioners, regulators, and policy makers to share and discuss current developments and insights into how environmental processes and human activities shape and impact environmental and human health now and in the future. In doing so, it also brings forward opportunities to minimize public and environmental health risk and maximize resilience. The use of systematic review methods for environmental and human health risk assessment is also an area of focus and growth.

We encourage submission of all high quality environmentally and health focused papers, but would be specifically interested in the following subject areas:

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Environmental scientists, ecotoxicologists, environmental chemists, environmental health specialists, environmental regulators, ecologists, biologists, hydrologists, geologists, marine and atmospheric scientists.

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Linda Birnbaum, National Institute of Environmental Health Sciences (NIEHS), USA

Environmental Toxicology, Dioxin, Endocrine Disruptors, BPAs, Flame Retardants

Julian Blasco, Instituto de Ciencias Marinas de Andalucía (CSIC), Puerto Real (Cádiz), Spain

Metals; Pharmaceuticals; Nanoparticles; Pollution; Ecotoxicology; Risk assessment; Seawater; Sediment

Michael Bloom, University at Albany, SUNY, Rensselaer, New York, USA

Environmental Epidemiology; Reproductive Epidemiology; Endocrine Disruptors

Nicole Cardello Deziel, Yale School of Public Health, New Haven, Connecticut, USA

Exposure Science (environmental measurements, biomonitoring, geospatial models); pesticides; persistent organic pollutants; hydraulic fracturing

Corinne Charlier, CHU Sart-Tilman, Liege, Belgium

Da Chen, Jinan University, Guangzhou, China

Environmental chemistry; analytical chemistry; ecotoxicology; persistent organic pollutants; flame retardants; pesticides; mass spectrometry; gas/liquid chormatography.

Kyungho Choi, Seoul National University (SNU), Seoul, The Republic of Korea

Environmental Health, Environmental Toxicology, Exposure and Risk Assessment, Endocrine Disruption, Thyroid Hormones, Reproduction, Ecotoxicology , Persistent Organic Pollutants, Phenolics, Personal Care Products

Angel del Valls, Universidad de Cádiz, Cádiz, Spain

Julio Díaz Jiménez, Instituto de Salud Carlos III, Madrid, Spain

Time series analysis.; cold and heat waves: impact on morbi-mortality; air pollution: impact on health; prevention plans; noise and health; urban health

José Dórea, University of Brasilia, Brasilia, DF, Brazil

Raquel Duarte-Davidson, Health Protection Agency, Didcot, Oxon, England, UK
Human health risk assessment, exposure assessment, organic chemicsl (POB's, dioxins, PAHS), risk communication, contaminated land

Yanzheng Gao, Nanjing Agricultural University, Nanjing, China
Organic contaminant in soil-plant system; soil contamination and remediation; plant contamination and risk assessment; soil environmental chemistry; rhizosphere; root exudates; soil ecotoxicity; organic contaminant and DNA interaction

Stuart Harrad, University of Birmingham, Birmingham, England, UK
Gerard Hoek, Utrecht University, Utrecht, Netherlands exposure assessment,
air pollution modeling, environmental epidemiology

Barbara Hoffmann, Heinrich-Heine-Universität Düsseldorf, Dusseldorf, Germany
Milena Horvat, Jožef Stefan Institute, Ljubljana, Slovenia
Environment and health: exposure and effects of chemicals in the environment; Human exposure; Human biomonitoring; Environmental sciences (e.g. biological and geochemical cycling of chemicals); Environmental analytical chemistry; Quality systems in chemical laboratories; Metrology in environmental and health studies

Xia Huo, Jinan University, Guangzhou, China
Exposure and children's health; Early life exposure and health risk; Developmental toxicology

Kurunthachalam Kannan, University at Albany, SUNY, Albany, New York, USA
Human exposure assessment and biomonitoring

Holger Koch, German Social Accident Insurance (DGUV), Bochum, Germany
Judy LaKind, LaKind Associates, LLC, Catonsville, Maryland, USA
Biomonitoring; Risk assessment; Exposure to chemicals; Systematic reviews; Data quality

Christopher Lau, U.S. Environmental Protection Agency (EPA), Research Triangle Park, North Carolina, USA
Characterizing the chemically induced reproductive toxicity and developmental toxicity during embryonic and perinatal life stages, understanding of their modes of action, and applying such information to human health risk assessment

Dan Li, Fudan University, Shanghai, China
Gabriele Ludewig, University of Iowa, Iowa City, Iowa, USA
Toxicology, genotoxicity, telomeres, PCBs, PAHs, Benzene, Quinones, mechanisms-of-action

Lena Ma, University of Florida, Gainesville, Florida, USA
Soil pollutants and health, environmental transport and fate of pollutants, risk assessment and public health, waste treatment and disposal

Nancy Bixian Mai, Chinese Academy of Sciences (CAS), Guangzhou, China
Mandana Mazaheri, Queensland University of Technology, Brisbane, Queensland, Australia
Urban air quality; Human and environmental exposures; Green spaces and their health impacts

Luke Naeher, University of Georgia, Athens, Georgia, USA
David Oliver, University of Stirling, Stirling, Scotland, UK
Diffuse microbial pollution from agriculture; modelling & decision support in environmental systems; fate & transfer of human pathogens; recreational water quality

Nuno Ratola, Universidade do Porto, Porto, Portugal
Environmental presence and behaviour of legacy and emerging organic contaminants; Biomonitoring and chemical transport of chemicals; Advanced analytical techniques of extraction and quantification;

Field sampling campaigns and sample handling protocols; Exposure assessment and prioritisation of relevant compounds; Climate change scenarios

Benoit Roig, Université de Nîmes, Nîmes, France
Ivan Rusyn, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA Gene expression Omics
Heqing Shen, Chinese Academy of Sciences (CAS), Xiamen, China
Biological monitoring (Bio-monitoring); Endocrine disrupting chemicals; Human microbiome; Birth cohort; Male fertility; Biomarkers; Epigenetics; OMICS with emphasis toxicometabolomics

Luis Felipe Silva Oliveira, Universidad de la Costa (CUC), Barranquilla, Colombia
Nanothechnology in Real Samples (in special nanominerals and advanced electron bean); Soil and water researches; Atmosphere impacts (in special particulate matter)

Christian Sonne, Aarhus University, Roskilde, Denmark
Biological effects, environmental chemicals, infectious diseases, climate change, veterinary science, wildlife medicine, predatory mammals, raptorial birds, sea birds, fish, internal organs, reproductive organs, histopathology, morphology, skeletal system, bone density, immune system, endocrinology, PBPK modelling, blood biochemistry, implantation of PTT satellite transmitters, immobilization.

Massimo Stafoggia, Lazio Regional Health Service, Rome, Italy
Exposure assessment; statistical methods; air pollution; epidemiology; study design

Guanyong Su, Nanjing University of Science and Technology, Nanjing, China
Analytical Chemistry; Ecotoxicology; Molecular Toxicology; Environmental Monitoring; Risk Assessment; Human studies; Organic Contaminates; Flame Retardants; Urinary Biomarkers; Metabolites; Gas Chromatography-Mass Spectrometry (GC-MS); Lipid Chromatography-Mass Spectrometry (LC-MS)

Shilu Tong, Shanghai Jiao Tong University, Shanghai, China
Environmental epidemiology, climate change, planetary health, sustainable development, quantitative risk assessment, spatiotemporal modelling

Nico M. van Straalen, Vrije Universiteit Amsterdam, Amsterdam, Netherlands
Marc-Andre Verner, Université de Montréal, Montreal, Quebec, Canada
Exposure assessment, pharmacokinetic modeling, environmental epidemiology, persistent organic pollutants, risk assessment

Jun Wu, University of California at Irvine, Irvine, California, USA

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Appendix H: Supplementary Tables

Table 1: Relationship between hormonal outcomes and DAP metabolites using linear regression

	DAP Metabolites			
	B-Coefficients (95% Confidence Intervals); Reference: First quartile			
	N	**Second Quartile**	**Third Quartile**	**Fourth Quartile**
Hormonal Outcomes (Adjusted for age, income, SHBG and other 3 hormones)				
FSH:				
DEP	168	-0.0679 (-0.829; 0.693)	-0.2417 (-0.989; 0.507)	0.477 (-0.297; 1.251)
DMP	168	-0.0503 (-0.802; 0.701)	0.0155 (-0.744; 0.775)	-0.223 (-0.991; 0.544)
DMTP	168	0.380 (-0.386; 1.147)	-0.0872 (-0.845;0.670)	-0.327 (-1.118; 0.464)
ΣDAP	168	-0.0124 (-0.770; 0.746)	0.066 (-0.687; 0.818)	-0.236 (-1.006; 0.534)
LH:				
DEP	168	0.2586 (-0.051; 0.568)	0.1360 (-0.173; 0.446)	-0.0839 (-0.4049; 0.237)
DMP	168	0.1461 (-0.167; 0.459)	0.0729 (-0.248; 0.393)	0.0908 (-0.232; 0.414)
DMTP	168	-0.2536 (-0.573; 0.066)	0.0252 (-0.294; 0.345)	-0.0405 (-0.3748; 0.294)
ΣDAP	168	-0.0753 (-0.394; 0.243)	-0.0509 (-0.368; 0.267)	-0.0409 (-0.365; 0.283)
Testosterone:				
DEP	168	-0.5624 (-1.878; 0.753)	-0.666 (-1.970; 0.638)	-0.62525 (-1.962; 0.711)
DMP	168	-0.7597 (-2.037; 0.518)	-0.2965 (-1.591; 0.998)	0.3717 (-0.942; 1.685)
DMTP	168	-0.6377 (-1.979; 0.704)	-0.5795 (-1.912; 0.753)	-0.4171 (-1.819; 0.985)
ΣDAP	168	-0.0839 (-1.392; 1.224)	-0.2375 (-1.535; 1.061)	-0.4034 (-1.735; 0.928)
Oestradiol:				
DEP	168	0.0068 (-8.979; 8.992)	6.6969 (-2.301; 15.695)	5.0575 (-4.032; 14.147)
DMP	168	8.3289 (-0.479; 17.138)	5.8749 (-3.071; 14.821)	5.7713 (-3.327; 14.870)
DMTP	168	9.1195 (0.086; 18.153)*	13.1044 (4.328; 21.880)*	10.4862 (1.380; 19.592)*
ΣDAP	168	9.2867 (0.319; 18.255)*	7.1366 (-1.620; 15.893)	9.5649 (0.651; 18.478)*

*P-Value <0.05

Table 2: Relationship between sexual maturity outcomes and DAP metabolites using linear regression

	DAP Metabolites			
	B-Coefficients (95% Confidence Intervals); Reference: First quartile			
	N	**Second Quartile**	**Third Quartile**	**Fourth Quartile**
Sexual Maturity Outcomes (Adjusted for age, income, SHBG)				
Tanner stage:				
DEP	170	0.165 (-0.154; 0.484)	0.101 (-0.216; 0.418)	0.246 (-0.081; 0.574)
DMP	170	0.033 (-0.287; 0.353)	0.108 (-0.219; 0.434)	0.029 (-0.299; 0.358)
DMTP	170	-0.149 (-0.477; 0.178)	0.031 (-0.293; 0.355)	-0.173 (-0.511; 0.166)
ΣDAP	170	0.128 (-0.194; 0.449)	0.090 (-0.233; 0.413)	-0.066 (-0.392; 0.260)
Testicular volume:				
DEP	162	1.308 (-2.439; 5.056)	1.441 (-2.254; 5.136)	3.343 (-0.474; 7.159)
DMP	162	-0.512 (-4.194; 3.172)	4.076 (0.387; 7.766)*	2.781 (-0.863; 6.425)
DMTP	162	-1.366 (-5.112; 2.379)	1.188 (-2.548; 4.924)	2.429 (-1.451; 6.311)
ΣDAP	162	-0.521 (-4.225; 3.183)	2.926 (-0.791; 6.643)	2.230 (-1.469; 5.929)

*P-Value <0.05

Table 3: Relationship between anthropometric outcomes and DAP metabolites using linear regression

	DAP Metabolites			
	B-Coefficients (95% Confidence Intervals); Reference: First quartile			
	N	**Second Quartile**	**Third Quartile**	**Fourth Quartile**
Anthropometric Outcomes (Adjusted for age, income)				
Height (m):				
DEP				
DMP	175	1.731 (-2.482; 5.944)	0.855 (-3.271; 4.981)	2.562 (-1.650; 6.774)
DMTP	175	-0.802 (-4.927; 3.322)	-0.033 (-4.255; 4.188)	1.689 (-2.536; 5.913)
ΣDAP	175	0.028 (-4.165; 4.221)	3.062 (-1.066; 7.189)	0.216 (-4.101; 4.533)
	175	-0.739 (-4.811; 3.334)	2.577 (-1.649; 6.803)	-0.124 (-4.337; 4.088)
Weight (kg):				
DEP	175	2.303 (-0.882; 5.488)	-0.327 (-3.480; 2.826)	1.235 (-1.972; 4.443)
DMP	175	-0.241 (-3.399; 2.917)	-0.484 (-3.703; 2.736)	0.259 (-2.997; 3.516)
DMTP	175	-1.094 (-4.291; 2.103)	-0.034 (-3.199; 3.131)	-0.767 (-4.156; 2.622)
ΣDAP	175	-0.382 (-3.513; 2.749)	0.144 (-3.082; 3.371)	-0.738 (-4.099; 2.625)
BMI:				
DEP	175	0.585 (-0.529; 1.699)	-0.370 (-1.479; 0.739)	-0.321 (-1.436; 0.794)
DMP	175	-0.046 (-1.147; 1.054)	-0.276 (-1.400; 0.849)	-0.439 (-1.570; 0.692)
DMTP	175	-0.376 (-1.488; 0.737)	-0.707 (-1.814; 0.400)	-0.509 (-1.678; 0.659)
ΣDAP	175	-0.217 (-1.321; 0.886)	-0.843 (-1.958; 0.273)	-0.389 (-1.538; 0.761)