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REPRODUCTIVE HEALTH EFFECTS DUE TO PESTICIDE EXPOSURE AMONGST BOYS IN THE RURAL WESTERN CAPE, SOUTH AFRICA.

Dr René Glynnis English
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REPRODUCTIVE HEALTH EFFECTS DUE TO PESTICIDE EXPOSURE AMONGST BOYS IN THE RURAL WESTERN CAPE, SOUTH AFRICA.

Dr René Glynnis English

Thesis presented for the Degree of

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April, 2011

Supervisor:

Mohamed Aqiel Dalvie, PhD
Associate Professor
Programme Leader (Chemical exposures and toxicity)
Centre for Occupational & Environmental Health Research (COEHR)
School of Public Health & Family Medicine
Health Sciences Faculty, University of Cape Town
Executive Summary

Introduction

Contemporary agricultural pesticides have been shown to possess hormonally-active properties, and have been associated with declining male reproductive health. These chemicals act by disrupting the normal functioning of the male endocrine system, through their actions on the hypothalamic-pituitary-gonadal axis. Reported male reproductive abnormalities are reproductive organ defects, cancers and declining fertility. Cryptorchidism, hypospadias, testicular cancer and poor sperm quality (testicular dysgenesis syndrome) have also been reported to be associated with these endocrine-disrupting chemicals.

The pesticides most frequently used in South Africa, for which there is some evidence of male reproductive effects are: chlorpyrifos (organophosphate), endosulfan (organochlorine), azinphos-methyl (organophosphate), atrazine (triazine), simazine (triazine), deltamethrin (pyrethroid), and penconazole (azole). Use of hormonally-active contemporary pesticides is high in agriculturally-intense areas of the Western Cape Province, South Africa. Evidence also exists to suggest that environmental and occupational exposures, particularly of farm workers, are high in these areas. Pesticides have been detected in sediments, local water sources, wheat and fish. Farm workers report occupational and environmental exposures. Knowledge, attitudes and practices regarding pesticide use, handling and exposure reduction have also been shown to be poor amongst farm workers. Higher blood chlorpyrifos and endosulfan levels have also been detected amongst exposed farm workers.
Studies have also shown that farm dwellers, particularly children, are exposed to pesticides, either environmentally or through coming into contact with contaminated equipment, soil, water, food, air and clothes.

Males are said to be more susceptible to the effects of endocrine disruptors particularly during critical periods of development such as in utero, neonatally and during puberty. Laboratory studies have provided evidence that pesticides administered during embryonic and neonatal developmental periods, and during lactation resulted primarily in reduced testicular weights, histological changes, biochemical changes and decreased serum and testicular testosterone. Studies where the pesticides were administered during puberty showed a reduction in the serum and testosterone levels, and minimal or no effects on the number of Leydig cells or on the weight of the testis. Very few epidemiological studies have been conducted which investigate the reproductive health effects of exposure to contemporary pesticides on the reproductive health of exposed males. One study, however, showed that boys exposed to endosulfan had reduced sexual maturity rating scores and serum testosterone levels compared to a control group in a neighbouring town.

Given the high rates of contemporary endocrine use, and evidence of environmental exposure to pesticides in the Western Cape, there is need for a study to investigate the potential reproductive health effects of exposed boys.
This study therefore aims to investigate the adverse reproductive health effects of environmental pesticide exposure in male children and adolescents, based on their lifetime residential history (namely, having lived on a farm or not).

**Methods**

An analytical cross-sectional study was conducted. Male children and adolescents (boys), nineteen years and younger, who resided and attended primary or secondary schools (n=8) in three agriculturally-intense areas in the Western Cape were included in the study. The study was conducted between April 2007 and March 2008. Exposed boys were selected from amongst boys who lived or had lived on farms. Unexposed boys were selected from amongst boys who lived or had lived in towns. Boys were recruited from local schools in the areas. After consent was signed, the boys’ mothers or legal guardians were interviewed using a standardized questionnaire which included demographics; birth, general, medical and genital health histories; environmental exposure to pesticides; mothers’ personal habits during pregnancy; and the boys’ reproductive history, alcohol consumption, and smoking history. The cumulative lifetime and intensity of pesticide exposure were also determined. Thereafter, bloods were drawn to measure serum oestradiol, luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone and sex hormone-binding globulin (shbg). The boy was then examined using a structured questionnaire and genital abnormalities were excluded. Testicular volume and consistency were recorded. Height and weight were measured. Data were collected onto an electronic questionnaire loaded onto a cellphone. The data were automatically uploaded to a central website and then downloaded into a
spreadsheet and statistical package for further analysis. Lifetime history of living on a farm was determined using information on place of residence at the time of the interview (current farm or town residence), as well as prior residential history (previous farm or town residence). Boys were included in the exposure group if they had lived on a farm all their lives, or if they had lived both on a farm and in a town and were born on a farm and/or spent the first three years of their life on a farm and/or spent more than 3 years of their first 12 years on a farm. Boys who did not meet the latter criteria or who only lived their entire lives in a town, were included in the unexposed group. The primary exposure variable was therefore dichotomous (exposed; unexposed). Exploratory data analysis, univariate, bivariate and regression analyses were conducted. The primary outcome variables were the baseline reproductive hormonal levels (FSH, LH, E2, testosterone), sexual maturity development (Tanner stage scores, sum of right and left testicular volumes) and anthropometric measurements (height, weight, body mass index (BMI)).

**Results**

Two-hundred and sixty-nine participants were included in the study. The median ages of the two groups were 11.3 years for the farm group compared to 12.0 years for the non-farm group (p=0.037). Non-farm boys had higher household incomes (R2500) versus R1800 for the farm group (p=0.001). There were no statistically significant differences for the medical and genital histories between the two groups. Overall, the rates of disease were low for both groups. Pesticide use was more prevalent (61%) in the homes of the farm group compared to the non-farm group (47.8%). More boys (65.5%) in the farm group reported eating fruit
from their home gardens compared to the non-farm boys (6.5%) (p<0.001). Boys living on farms were exposed to 60 days of spraying (range: 0 to 90 days). The median distance of the spraying area from their homes was 20 meters (range: 7 to 50 meters). High rates of pesticide drift (44.4%), outside contact during spraying (40.0%) and the drinking of water from unprotected sources (43.3%) were reported in the exposed group. Thirty-nine percent of mothers reported having worked in the fields when spraying occurred during their pregnancies.

The median LH levels for the age category > 14 years was lower in the farm group (1.9; IQR: 0.05-4.80) compared to the non-farm group (3.85; IQR: 0.30-7.20) (p=0.015). Oestradiol levels were statistically significantly elevated in farm boys for both the 5-9 years age category ((48.1; IQR: 9.2 - 70.9) versus (25.5; IQR: 9.2 - 41.2) (p = 0.023)) and for the 9.1 to 11 years age category ((38.4; IQR: 9.2 - 75.6) versus (28.3; IQR: 9.2 - 45.6) (p = 0.015)), when compared to non-farm boys. In general farm boys were also shorter and lighter than non-farms boys. For the age category 5 to 9 years, the farm boys were statistically significantly shorter (123.05cm versus 131.00cm) (p=0.04), and lighter (23.00kg versus 25.00kg) than the non-farm boys (p=0.03). The median ages at Tanner stages 1 to 3 were lower than for the non-farm group (not statistically significant). For Tanner stages 4 and 5, however, the ages were higher. After controlling for confounders, farm boys had lower levels of testosterone (beta (β) = -0.346 nmol/L; CI: -1.123 - 0.436 nmol/L), LH (β = -0.284 IU/L; CI:-0.483 - -0.084 IU/L), smaller testes (β = -1.745 mL; CI:-4.248 - 0.758 IU/L), were shorter (β = -3.417 cm; CI: -6.382 - -0.453 cm) and lighter (β = - 2.258 kg; CI: -4.441 - -0.753 kg). They also had higher levels of oestradiol (β = 8.074 pmol/L; CI: 2.339 - 13.808 pmol/L).
pmol/L) and FSH (β = 0.634 IU/L; CI:0.186 - 1.081 U/L). There was no statistically significant difference or effect on Tanner stage.

**Discussion**

In this study, farm residence was used as a proxy for exposure. Associations were observed between serum reproductive hormone levels and the exposure. Boys who lived on farms were shown to have lower serum testosterone and LH levels, and higher FSH and oestradiol levels, as compared to non-farm boys. Furthermore, exposed boys were also shown to be smaller and lighter than non-farm boys. No statistically significant difference for Tanner stage was shown. Their testicular volumes were also lower. Overall, these results are consistent with the hypothesis that exposure to endocrine-disrupting contemporary pesticides negatively impact on male reproductive health systems. These findings are also consistent with laboratory and epidemiological studies. Furthermore, the results also indicate that farm boys have higher exposures to household and environmentally-occurring pesticides. This supports the findings of other studies conducted in the same areas. The limitations to the study, however, are that reliance on parental reports could have introduced recall bias. Exposure misclassification could have occurred due to non-farm boys being inadvertently exposed to pesticides. Furthermore, selection bias could have been introduced due to the limiting of the selection of participants to school-going boys, thus potentially missing a group of boys who do not attend school. Measurement bias could have been introduced during the interview, physical examination, timing of blood collection, or laboratory testing of the samples. Another major limitation is the absence of biomarker data.
Known and unknown confounders were collected and controlled for during the analysis. For all the limitations, however, relevant procedures were put in place to minimize the introduction of biases.

Conclusion

This study provides evidence that environmental exposure to hormonally active contemporary agricultural pesticides may adversely affect the reproductive health of pubertal boys. Initiatives to change knowledge, attitudes and practices through the education of farmers, farm workers and other rural residents about the harmful effects of pesticides and the need to reduce exposure are required.
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PART A: PROTOCOL

1. Introduction

1.1 Problem Statement

Environmental chemicals, acting as hormonally-active substances, have been hypothesized to be associated with declining male reproductive health. More recently, further evidence has emerged to suggest that many contemporary agricultural pesticides have hormonally active properties and have the potential to cause male reproductive health effects in exposed persons. This has major public health implications for South Africa, the highest pesticide user in Southern Africa, and may have far reaching consequences on the reproductive health and capability of humans.

1.2 Literature Review

1.2.1 Background

Environmental chemicals which possess hormonally-active properties have been associated with the abnormal release of male reproductive hormones, increased reproductive organ defects, and declining sperm quality and fertility in both wildlife,
laboratory animals and humans, as will be presented below. These endocrine-disrupting (ED) chemicals exert their effects by disrupting the normal functioning of the male endocrine system, namely the hypothalamic-pituitary-gonadal (HPG) axis. ED substances target oestrogen, androgen, aryl-hydrocarbon and thyroid receptors and hormone synthesis pathways. Pesticides are also said to cause reproductive health effects through non-endocrine mechanisms.

Pesticides are widely used in agricultural, horticultural and vector control programmes, and the positive impacts of pesticide use on agricultural productivity and disease control are well-known. However, these agents are toxic. Emerging evidence to suggest a link between contemporary pesticide use and declining male reproductive health is of concern, especially in countries like South Africa where pesticide use is substantial, yet poorly regulated. Furthermore, pesticide use in the Western Cape, a province in South Africa is also high. Many of the most frequently used pesticides in the aforementioned region have been shown to be toxic to the male reproductive health systems of laboratory animals and wildlife, and have been associated with adverse male reproductive health outcomes in humans. The section below will discuss these studies in more detail.
1.2.2 Laboratory studies

1.2.2.1 Pesticides with known endocrine disrupting activity

This section will present the male reproductive health effects of hormones which have had their specific endocrine-disrupting hormonal activity identified by means of in vitro studies. Both laboratory and epidemiological studies will be presented.

The organochlorine, endosulfan, has been shown to have both anti-androgenic and oestrogenic in vitro activity, and has been associated with reduced secretory capacity of adrenal cells in frogs. In male rats administered endosulfan doses of 5, 10 and 15 mg/kg for 30 days, decreased serum testosterone, testes and accessory gland weights, sperm quality and reduced fertility were demonstrated. A study conducted on Indian boys exposed to endosulfan through aerial spraying showed that the exposed boys had reduced sexual maturity ratings and serum testosterone when compared to unexposed boys from a neighbouring town. Fenarimol, a pyrimidine pesticide, has been shown to have anti-androgenic, oestrogenic and aromatase activity. Sexual differentiation and reduced breeding performance in male rats were demonstrated in two studies which investigated the effects of this pesticide on the male reproductive system. Chlorpyrifos, an organophosphate pesticide, was shown to be weakly oestrogenic and was associated with reduced serum luteinizing hormone (LH) levels in ewes. Studies conducted on American
men demonstrated a statistically significant negative association between the urinary metabolite of chlorpyrifos, TCPy, and reduced sperm quality, serum testosterone and androgen index. Deltamethrin, a pyrethroid pesticide, has been shown to have weak oestrogenic activity. Administration of oral doses of 4 mg/kg from the first day of pregnancy to the twenty-first day of lactation resulted in a change in reproductive behavior and physiology of male rat offspring. Dichlorvos, an organophosphate pesticide, which is an androgen-receptor antagonist, was associated with reduced sperm quality, serum testosterone and testicular damage in rats who received dosages of 1, 2 and 4 mg/kg for 6 weeks. Iprodione, a chloranaline pesticide, has been shown to have anti-androgenic effects and aromatase inhibition properties. The offspring of male rats who were treated with 31, 63, 125 and 250 mg/kg per day dosages of iprodione were shown to be poorly sexually differentiated.

1.2.2.2 Pesticides with unknown endocrine disrupting activity

The endocrine disrupting activities of the pesticides presented below have not been assessed via in vitro studies. However, laboratory and epidemiological studies have demonstrated that these pesticides are associated with adverse male reproductive health effects.

Cypermethrin, a pyrethroid pesticide, has been shown to increase testicular weight, decrease serum testosterone, LH, follicle stimulating hormone (FSH) and sperm quality; and
reduce fertility in rats fed regular doses.\textsuperscript{30} Rabbits administered 24mg/kg every second day for 12 weeks were shown to have reduced plasma testosterone and sperm quality.\textsuperscript{31} \textbf{Fenvalerate}, a pyrethroid pesticide, was associated with elevated levels of serum LH and FSH, and with reduced serum levels of testosterone and poor sperm quality in rats administered increasing doses for 15 and 30 days.\textsuperscript{32} These findings were supported by two other studies.\textsuperscript{33} \textsuperscript{34} Studies conducted on humans showed that low level exposed factory workers had reduced sperm quality.\textsuperscript{35} \textbf{Glyphosate}, an organophosphate pesticide, was associated with \textit{in vitro} reduction of human and rabbit sperm quality.\textsuperscript{36} \textbf{Parathion}, a glycine pesticide, was associated with statistically significant reduction in sperm quality and non-significant reduction in fertility amongst exposed rats.\textsuperscript{37} In another study, exposed Chinese factory workers also had statistically significantly reduced sperm quality and raised aneuploidy.\textsuperscript{38}

\textbf{1.2.3 Exposure of Western Cape residents to pesticides}

Previous studies have shown that rural residents living in agriculturally-intense areas are environmentally exposed to contemporary pesticides as will be presented below. A survey conducted by Dalvie et al\textsuperscript{39} demonstrated that rural residents are at increased risk through their exposure to contaminated food, soil, water and spray drift. Water contamination was identified as a common source of exposure as a series of studies have demonstrated the presence of pesticides such as endosulfan, chlorpyrifos, fenarimol, deltamethrin and iprodione in rural surface water, groundwater and drinking water.\textsuperscript{40} \textsuperscript{41} \textsuperscript{42} In one study,
endosulfan was detected in ground, surface and drinking water.\textsuperscript{42} Although low levels of endosulfan contamination were reported in that study, the European Drinking Water Standard of 0.1µg/L was exceeded in some sites. The most common areas of higher levels of endosulfan were Grabouw (69%), Hex River (64%) and Piketberg (39%). In another study, nine different pesticides were detected in a number of rural dams, and endosulfan was present in 26 of 27 dams in one town.\textsuperscript{43} The highest detected concentration was 626 µg/L. Increased endosulfan levels were also recorded in a large river in this rural region after the first rains.\textsuperscript{44}

A survey of the knowledge and attitudes towards pesticides in water sources was undertaken in the areas of the Western Cape, where many of the above studies were conducted, and confirmed that rural residents are exposed to potentially contaminated environmental sources through a number of environmental routes.\textsuperscript{39} Many residents reported that they used groundwater (from springs and boreholes) and mountain dams as their primary water sources both for drinking and for domestic use. About a third lived within 10 meters of the nearest spraying site, and almost half used pesticides within their homes (41%) and in their gardens (33%). Farm workers were also at risk of increased exposure due to unsafe application methods and poor working conditions. This is of concern as other studies have shown that the families of farm workers are at increased risk of pesticide exposure due to indirect exposure through coming into contact with the clothes of those who are occupationally exposed.\textsuperscript{45} 46 47
It is interesting to note, however, that despite increasing evidence of male reproductive health effects due to contemporary pesticides exposure, few studies evaluating the reproductive health effects of these pesticides amongst boys living in high exposure areas have been conducted. Puberty is considered to be a critical time period in the sexual and reproductive life of a male. And change to the hormonal environment at or during this and other critical developmental periods (e.g. pre-natally and intra-uterine) may result in adverse male reproductive health effects. As presented above, Saiyed conducted a study in India to determine the impact of endosulfan exposure primarily through aerial spraying on childhood male reproductive development. The primary outcomes were to estimate the sexual maturity rating (SMR) scale using Tanner staging, serum hormones and endosulfan residues. Anthropometric measurements were also evaluated. Finally, the study aimed to determine whether endosulfan exposure predicted various serum hormone levels and delayed sexual maturity. The exposed group was school boys who permanently resided in a village below a cashew nut plantation where endosulfan was aerially sprayed. The control group was selected from an unexposed population who lived approximately 20km away. The mean ± standard error serum endosulfan levels were shown to be statistically significantly higher (p<0.001) in the exposed (7.47 ± 1.19ppb) compared to the controls (1.37 ± 0.40ppb). Aerial exposure to endosulfan was negatively associated with the SMR scoring for pubic hair, testicular, penile and serum testosterone levels on multiple regression testing. Serum levels of luteinizing hormone were statistically significantly and positively associated with aerial spraying when controlling for age. However, response rates in the study were low and this may have biased the findings. Other epidemiological studies investigating male reproductive health effects of endocrine-disrupting pesticides amongst
infants born to exposed women also demonstrated adverse effects associated with exposure.\textsuperscript{49, 50} A study conducted in South Africa demonstrated birth abnormalities in the offspring of women who transported water intended for domestic use in pesticide-contaminated containers.\textsuperscript{51}

In conclusion, boys who live in agriculturally-intense areas where exposure to contemporary pesticides with endocrine-disrupting properties is high may be at increased risk of developing adverse male reproductive health at critical stages in their development. This could be an important public health problem.

1.3 Justification and Purpose

The above literature review provides evidence to suggest that there is an association between contemporary agricultural pesticide use and abnormal male reproductive health effects. Very few studies, however, have been conducted to investigate the reproductive health effects of pesticides on boys living in exposed areas, such as in rural areas of the Western Cape, where agricultural pesticide use is high and pesticide levels have been detected in the environment.\textsuperscript{5, 14, 15, 16, 17}

The purpose of the proposed study is therefore to investigate the effects of contemporary endocrine-disrupting agricultural pesticides, which includes many pesticides commonly used
in the South African agricultural industry, on the male reproductive system of school-going boys. This study is important for developing appropriate pesticide monitoring legislation and programmes in the Western Cape, as well as for the rest of South Africa. This research will also be able to contribute to improving the quality of rural life whilst at the same time promoting environmental justice and empowerment of rural communities and those who are most at risk.

1.4 Aim

This study aims to investigate the adverse reproductive health effects of environmental pesticide exposure in male children and adolescents, based on their lifetime residential history.

The study forms part of a larger one that aims to investigate the adverse reproductive health effects of environmental pesticide exposure in male children and adolescents living in three rural agricultural and neighbouring non-agricultural areas in the Western Cape Province, South Africa. Data for the current study has already been collected and this protocol serves to present the methods for data analysis and reporting, as presented in the objectives listed below.*

* Dr English acquired the data after collection by the study team and was primarily responsible for data analysis.
1.5 Objectives

1. To characterise the demographic, general and reproductive health information of the participants.
2. To characterize the environmental exposures to pesticides of all participants, based on their lifetime living history (namely, living on a farm or not on a farm).
3. To measure the height, weight and body mass index (BMI) of the boys.
4. To assess the physical sexual maturity of the boys.
5. To characterize baseline pituitary and gonadal endocrine levels of the boys.
6. To measure confounders of the various reproductive health outcomes such as lifetime phyto-estrogen intake, including dietary content of soya beans and other vegetables, low birth weight and mother’s habits during pregnancy.
7. To investigate the effect of lifetime residence on a farm on the reproductive outcomes of the boys whilst controlling for relevant confounders.

2. METHODS

2.1 Study Design

An analytical cross-sectional study of male children and adolescents (hereafter referred to as boys), nineteen years and younger, residing in and attending primary or secondary schools in three rural areas in the Western Cape Province of South Africa was conducted.
The study took place between April 2007 and March 2008. Exposed participants were selected from amongst boys who lived or had lived on farms. Unexposed boys were selected from amongst boys who lived or had lived in neighbouring towns (non-farm areas).

2.2 Population

The sampling frame was all boys who attended primary and secondary schools (n=8) in three agriculturally intense and neighbouring non-agricultural rural areas in the Western Cape Province. Pesticides had previously been detected in water supplies of the agriculturally-intense areas. These included the Hex River Valley, where grape farming is practised; Grabouw, where pome fruit farming is predominantly practised; and Piketberg, where wheat and fruit farming is practised. A map depicting these areas is presented in Appendix 1.

2.3 Sampling

2.3.1 Sample size calculation

The sample size calculations were based on the mean (3.57 ± 3.15 miu/ml) blood FSH levels as the latter was found to be the most variable hormone in a previous study investigating dichlorodiphenyltrichloroethane (DDT) and reproductive health effects. A two-sample test of equality of means [Stata Corporation, 2003] indicated a sample size of 174,
split as 116 exposed and 58 unexposed assuming an 80% power, a 40% difference in blood FSH and a confidence level of 95%. The age-groups were as follows: 5 to 9 years (representing the period before the start of pubertal development); 9.1 to 11 years (representing the period at the start of pubertal development); 11.1 to 14 years (representing the period of advanced and end of pubertal development); and > 14 years (representing the time period after pubertal development has been attained).

2.3.2 Sampling

A list of schools in the selected areas was provided by the Western Cape Department of Education. Boys were recruited from the most accessible primary and secondary schools (n=8) that had learners from both farms and neighbouring towns. Prior to the start of the 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 consent 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 on a farm or not at the time of the study. 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.56
Selected boys and their parents or guardians were invited to participate in the study and were asked to be present at the school on specified dates.

2.4 Recruitment procedures

The field team consisted of a field coordinator, 3 interviewers, a male nurse (who drew the bloods), a bio-technician (who processed the biological samples before these were sent to the laboratory), and a male nurse who examined the reproductive system of the boys.

The mothers were encouraged to be present for the interviews as it was assumed that they would be able to provide more reliable information regarding the boys’ developmental and personal patterns, and their own personal and occupational habits during their pregnancies. On arrival, the parent or legal guardian signed the informed consent form in the language of their choice. The interviewer administered the questionnaire to the parent or legal guardian. Thereafter, the boys were examined by a male study nurse. After the physical examination, the boy was seen by the second study nurse who drew the bloods. A technician then processed the blood samples and prepared these for transportation on ice to the laboratory for storage. These processes and procedures are described in more detail in the sections below.
2.5 Measurements and Instruments

2.5.1 Questionnaire

Trained interviewers conducted face-to-face interviews with the parent or legal guardian and boy in their language of preference. Questions on sexual development, however, were also asked directly of the boys in the presence of the interviewee in the language of preference. The responses were recorded directly onto an electronic questionnaire which was pre-loaded onto a cell phone using mobile technology (Mobile Researcher, Clyral). At the end of each interview the completed questionnaires were downloaded onto a central website and were accessed by the Principal Investigator. This information was then exported into Microsoft Excel® and Stata 10 [Stata Corporation, Texas, USA] for further data management and analysis.

The questionnaire was developed by the study team, led by the Principal Investigator, and was based on previous local studies in similar populations.\textsuperscript{57, 58, 59, 60} 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. A copy of the questionnaire can be viewed in Appendix 2. 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. 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.

2.5.2 Physical examination structured record form

After completion of the questionnaire a trained male medical study nurse physically examined the boy and recorded the following information onto a structured record form (Appendix 3). Height, weight, secondary sexual characteristics and sexual maturity rating (SMR) according to the Tanner scoring system were recorded. Genital anatomical abnormalities including the presence of congenital hydroceles, undescended testes, congenital inguinal hernias and hypospadias, the presence of infection, previous injury or tumours were also assessed for. Testicular consistency and size were also recorded. The SMR was derived by assessing pubic hair (adrenal maturation) and penis development (testicular maturation) and then determining a combined score using Tanner stages, but with
stages five and six merged. Testicular volume was assessed using a standardized set of wooden testicular beads [Orchidometer, Kabi, Japan]. Height and weight were recorded according to standardized methods, and using calibrated instruments. Photographs showing the various Tanner Stages were used as a reference (Appendix 4). The nurse was trained by a local reproductive health specialist who demonstrated how to perform the anthropometric measurements, 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.

2.5.3 Blood endocrine measurements

Bloods were drawn from each boy by a study nurse, as early as logistically possible and preferably before 13h00. Two six millilitre (mL) venous whole blood samples were collected from each boy by the study nurse after the physical examination was completed. The samples were allowed to clot, and were then centrifuged at 5000 revolutions per minute in the field by a qualified laboratory technician using a portable centrifuge. The supernatant of the blood samples were stored 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 analysed for baseline FSH, luteinizing hormone (LH), testosterone, oestradiol (E2), and sex hormone-binding globulin (SHBG). LH was measured with the MAIAclone IRMA kit (Bologna, Italy) [Biochem Immuno Systems62 63], FSH and total testosterone with ACS-180 competitive chemiluminescent automated systems (New York, USA) [Bayer Corporation, 200064],
oestradiol with an in-house radioimmunoassay (Nivellus, Belgium) [Biosource-Europe SA, 1994\textsuperscript{65}], and SHBG with the IRMA kit from Orion Diagnostica (Finland) [Orion Diagnostica, 1990\textsuperscript{66}]. Baseline hormonal levels were compared to age-related laboratory normal ranges.

2.6 Pilot study

A pilot study was conducted on three boys from two of the study areas to field-test the questionnaire, the procedures for testing the participants and the logistics for the main study.

2.7 Training

Prior to commencing the study, the fieldworkers underwent extensive training on the study methods, administration of the questionnaires, methods for and assessment of the reproductive system, drawing of bloods, and on the processing and transporting of the blood samples.
2.8 Logistics

Arrangements were made with the schools, community and farmers participating in the study for a date and suitable venue to conduct interviews, tests and training; and for setting up a fridge and centrifuge for temporary storage of biological samples and spinning of blood samples. The construction of the questionnaire and training of interviewers were conducted during January 2008 to March 2008. The pilot study was conducted in April 2008. The main study was performed during May 2008 to July 2008. Laboratory analysis of biological samples, entering of data and data analysis took place during May 2008 to August 2010.

2.9 Consideration of potential biases, and validity and reliability of instruments

The introduction of selection, measurement and confounding biases was considered prior to and during the conduct of the study. To minimize selection bias, boys were randomly selected by exposure status. To reduce recall bias and reporting bias, questions were kept simple and unambiguous, and every effort was made to interview the mother. To reduce measurement bias, field staff were trained to conduct measurements consistently and similarly for all boys. Interviews and examinations were conducted in rooms where privacy was adhered to in order 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 associations between exposure and the various outcome measurements.

2.10 Exposure measures

Lifetime history of living on a farm was determined using information on place of residence at the time of the interview (current farm or town residence), as well as prior residential history (previous farm or town residence). Boys were included in the exposure group if they had lived on a farm all their lives, or if they had lived both on a farm and in a town and were born on a farm and/or spent the first three years of their life on a farm and/or spent more than 3 years of their first 12 years on a farm. Boys who did not meet the latter criteria or who only lived their entire lives in a town, were included in the unexposed group. The primary exposure variable was therefore dichotomous (exposed; unexposed).

2.11 Outcome measures

The primary outcome variables were the baseline reproductive hormonal levels (FSH, LH, E2, testosterone), sexual maturity development (Tanner stage scores, sum of right and left testicular volumes) and anthropometric measurements (height, weight, body mass index (BMI)). These were also dichotomised using quartiles (25th, 50th, 75th percentiles) as cut-offs.

* Dr English was responsible for data analysis. She joined the study team after the data were collected.
except Tanner stage. Other dichotomised outcome variables included anthropometric measurements below the 25th or 50th percentile for age (according to CDC growth charts, which are standard throughout the world).67 The categorization of outcomes was based on that used in previous studies.57 58 59 60

2.12 STATISTICAL ANALYSIS

2.12.1 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 in order 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 characterisation.
2.12.2 Data analysis*

All analyses will be performed using Stata 10 statistical software (StataCorp, Texas, USA).

First, exploratory data analysis will be conducted. Data for each variable will be examined for missing data, for any obvious abnormalities, and for the type of coding used. Continuous variables will be assessed for normality using histograms and Shapiro-Wilk tests. The exposure variable will be created based on a history of the boy having lived on a farm for all or most of their lives. Categorical data with dichotomous responses will be recoded into numeric responses, and those with more than one response will be recorded into numeric or dummy variables, where indicated. Numeric outcome variables will be categorised as described below. Bivariate analysis will be conducted. Box and whisker plots will be used to depict the association between continuous and the exposure variables. Scatter plots will be used to depict the association between the categorical continuous variables. Frequency distributions will be explored to determine measures of central tendency or dispersion, and for categorical data frequency tables will be generated to derive proportions.

To test for the 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

* Dr English joined the study team after the data were collected. She is primarily responsible for data analysis. This section of the protocol is therefore written in future tense.
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. 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. These confounders will be selected on an *a priori* basis and will be based on biological plausibility and/or on bivariate testing (where associations with a significance of p<0.1 are found). Analysis will also be conducted per age-category using the four age categories: 5 to 9 years; 9.1 to 11 years; 11.1 to 14 years and > 14 years. The outcome measure will be dichotomised using quartile values (derived as part of the analysis) as cut-offs. Other dichotomised outcome variables will include anthropometric measurements below the 25th or 50th percentile for age (according to CDC growth charts) – also calculated as part of data analysis. Sensitivity analysis will be conducted excluding participants who lived both on farms and towns during their lifetime.

For model building, a logical variable selection model will be used to select the variables. First, potential confounders will be fitted to the model. Then, the other variables will be added one at a time. The variable that statistically significantly reduces the deviance when compared to the null model will be chosen as the baseline model. Secondly, the variables will be added to the baseline model. The combination of variables that results in the biggest change to the variance will be the chosen model. As a final step, the variables will once again be added to the model to assess whether in the presence of other variables, they make a reduction in the deviance. Confounders will be selected in an *a priori* basis, based
on literature, biological plausibility, and using bivariate testing, if p<0.1. Age and household income (a marker of socio-economic status) were selected \textit{a priori} for all outcomes. For all the hormonal outcomes SHBG and the other reproductive hormones measured were also included \textit{a priori}. The confounders were included in the regression model during the model building process. A logical variable selection model was used to select the best model. Regression diagnostics was applied to determine the goodness of fit of the model and to assess for outliers or influential observations. Then variables that are exposure/risk factors are added one at a time, and their significance will be assessed for by looking at the reduction in the deviance.

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 standardised 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. Ethics

During the entire study, ethical research principles of respect for persons, beneficience and justice were adhered to.

3.1 Ethical approval

The study was conducted in accordance with the Declaration of Helsinki of the 25th World Medical Assembly. Ethical approval for the study was granted by the University of Cape Town’s Research Ethics Committee (Appendix 5).

3.2 Informed consent

Consent was obtained from the Department of Education. The Principals of the schools in the study areas contacted the male pupils and their parents. Inclusion of the boys in the study occurred if the parents or legal guardians, or the eligible boys (eighteen years and older) gave informed consent to participate. The consent forms were available in English, Afrikaans, and Xhosa and were read by the parent or legal guardian at first contact (Appendix 6). If the parent or legal guardian was illiterate, the form was read and explained to them in the relevant language. After answering any potential questions and ensuring that the parent or legal guardian fully understood the procedures and what was expected of them, their signatures on the document (or fingerprint if illiterate) were requested. Participants were permitted to withdraw from the study at any time, and this was made clear
in the consent form and verbally. It was also explained that refusal to participate, or withdrawal from the study would in no way affect the treatment of the parent or legal guardian or boy by the investigators or school staff. No study procedures were performed without the consent form being signed.

3.3 Respect for participants

All information relating to the study participants is to be confidentially maintained through storage at and in a secure site. Confidentiality will be preserved in that only the research team will access the data and only group results will be reported on. During the study a medical referral process was established in the event that any medical concerns were noted during the conduct of the study. The investigators provided the participants with information about where to seek appropriate medical advice.

A number of other ethical considerations were taken into account during the design of this study. These included:

- Fair selection of study participants: clear inclusion/exclusion criteria.
- Favourable harm/benefit ratio: the study was conducted in a sensitive manner; a system for reporting health problems identified during the study was established.
- Collaborative partnership: the study will be reviewed by the scientific committee of the University of Cape Town.
Social value: a communication plan was put into place.

4. Communication

The potential beneficiaries of this research are the communities living in the Western Cape Province who are exposed to contemporary pesticides. The results of the study will be disseminated in the following ways:

The outputs of the study will be presented through or at:

- Peer review articles on male reproductive health effects of pesticides in the Western Cape.
- Conference and seminar presentations on the male reproductive health effects of pesticides in the Western Cape
- A report of the study to various stakeholders including the South African Medical Research Council, Western Cape and National Departments of Health and Environmental Health and Tourism.
- A workshop to local stakeholders on the results of the study.
The outcomes will provide:

- Information on the male reproductive health effects of pesticides in the Western Cape. This study could prompt further study on the health effects of endocrine disrupting agricultural pesticides in South Africa and other countries, where these chemicals are extensively used.
- Recommendations for policies regarding health effects of pesticides. The study could have a significant impact on pesticide legislation locally and internationally thus involving government, industry, farmers and the rest of the rural community.
- Ideas and content for training of rural farming communities in pesticide safety and risk perception.
- Enhanced community capacity to seek redress of environmental threats to their health.

5. Capacity building initiatives in the communities

This project is particularly geared towards rural communities, generally the most marginalised people in South Africa, who are at risk of pesticide exposure. The particular communities participating in this study will benefit from provision of information, education and training on pesticide health effects, and if evidence of adverse health effects is demonstrated. Communities will be assisted to lobby for control measures to reduce or remove the risks.
6. References

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London L, Dalvie MA, Cairncross E, Solomon A. The quality of surface and groundwater in the rural Western Cape with regard to pesticides. 2000. WRC Report No: 795/1/00. WRC, Pretoria.

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PART B: STRUCTURED LITERATURE REVIEW

1. Introduction

1.1 Background

Pesticides are widely used globally. Agricultural, horticultural and vector control programmes are the greatest consumers of pesticides, and evidence shows that use of these chemicals impact positively on agricultural productivity, forestry activities, and disease burdens caused by insects and rodents. The positive impacts of pesticide use are therefore indisputable, but many of these agents are also known to affect the health of humans and animals. This fact is of public health concern, particularly in developing countries where pesticide use is on the increase, but poorly regulated.

Over the past few decades, the decline in male reproductive health has been associated with the ubiquitous use of hormonally-active chemicals, including older pesticides such as DDT (dichlorodiphenyltrichloroethane) and chlordanes. It is hypothesized that low-dose exposure to these hormonally-active pesticides can have dramatic health consequences at a population level, particularly when exposure occurs at critical time periods in the development of males (namely, prenatally, neonatally or during puberty). Contemporary agricultural pesticides have also been shown to be hormonally active. However, only a few studies have investigated the reproductive health effects of these pesticides, particularly in developing countries, such as South Africa, where pesticide use is widespread. There is...
therefore undoubtedly a need to explore the link between contemporary endocrine-disrupting pesticide exposure during critical developmental time periods and adverse male reproductive health effects in those who are at risk of exposure.

1.2 Objectives of the literature review

The overall objective of this literature review is to examine and present evidence that justifies the need for a study which explores the association between environmental exposure to pesticides during critical developmental time periods and adverse reproductive health in boys living in an agriculturally-intense area in the Western Cape Province in South Africa.

1.3 Overview of the literature review

The first section of this literature review will provide a brief overview of pesticides, with a focus on their routes of exposures, toxic effects and endocrine-disrupting properties. To better understand the potential mechanisms of action of endocrine disruptors (ED) on male reproductive development, a section describing the normal development of the male reproductive system will follow. Thereafter, a section reviewing the reproductive health effects of commonly used Western Cape Province pesticides will be presented. A brief overview of pesticide exposure in the Province will follow.
Literature search strategy; potential inclusion and exclusion criteria, quality criteria to include or exclude studies.

2. Pesticides

2.1 Definition of pesticides

The Food and Agricultural Organization of the United Nations defines pesticides as being\textsuperscript{11}:

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 animal feedstuffs, or which may be administered to animals for the control of insects, arachnids, or other pests in or on their bodies. The term includes substances intended for use as a plant-growth regulator, defoliant, dessicant, fruit-thinning agent, or an agent for preventing the premature fall of fruit, and substances applied to crops either before or after harvest to prevent deterioration during storage or transport.

Pesticides are natural or synthetic substances used, amongst others, as insecticides, herbicides and fungicides. They contain the active ingredient together with other chemicals
(e.g. solvents) and impurities (e.g. chloroform, carbon tetrachloride and dioxin), which may be more toxic than the compound itself. Active ingredients include chemical groups such as organochlorines, organophosphates and pyrethroids which have different toxic effects.

2.2. Routes of exposure

According to Davies et al,\textsuperscript{12, 13} human exposure to pesticides can be intentional (suicides or homicides) or unintentional (occupational or non-occupational), may occur over short or long periods of time, and can occur at high or low levels. Typical exposure routes are through ingestion (food and drinking water), inhalation (air and dust) and skin absorption (clothing or direct contact). Thus, individuals who work on or live close to farming areas may be exposed to pesticides through sprays in the air, contaminated soil and produce, contact with contaminated water, through direct chemical contact, or indirectly, through coming into contact with contaminated objects or clothes.\textsuperscript{14} Furthermore, infants and children may have higher exposures than would normally be considered, as they often engage in activities such as crawling and playing outside which increases their contact with contaminated dusts, soil and water.\textsuperscript{15, 16, 17, 18} They are also more susceptible to adverse effects of the pesticides due to their physiology, lower immune status, lower body weights and higher exertion levels.\textsuperscript{19, 20} Furthermore, children who live on farms or who have parents who work on farms may have higher exposures than those who do not, due to aerosol drift or through contact with contaminated clothes, shoes or skin of exposed parents.\textsuperscript{22, 23, 24, 25, 26, 27, 28} Prenatal exposure occurs via transplacental transfer of pesticides from the exposed mother to the child.\textsuperscript{7}
2.3 Toxic effects

Pesticides can have acute or chronic toxic effects, the latter occurring primarily after long-term exposure to low doses.\textsuperscript{\ref{1}} Major factors affecting pesticide toxicity are the dose; route of exposure; effect of the pesticide and its metabolites; accumulation and persistence in the body; and the health status of the individual. Malnutrition and dehydration can also increase sensitivity to pesticides.\textsuperscript{\ref{1}} Chronic exposure can result in bone marrow defects, cancer, cytogenetic changes, neurotoxicity, immune system enzyme induction, and skin or reproductive effects.\textsuperscript{\ref{1}} The mechanisms of action vary according to the class and chemico-physical composition of the pesticide.\textsuperscript{\ref{14}}

2.4 Endocrine-disrupting properties of pesticides

In wildlife, animals and humans, endocrine-disrupting pesticides have been shown to exert their toxic effects through interfering with the normal functioning of the endocrine system. These substances can mimic, block and/or alter the chemical activity of endogenous hormones,\textsuperscript{\ref{29}} and can disrupt or change the activity of hormone signaling systems.\textsuperscript{\ref{30}} These chemicals are said to “interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for maintenance of homeostasis (normal cell metabolism), reproduction, development and/or behavior”.\textsuperscript{\ref{31}} Given the widespread environmental occurrence of ED, these chemicals have been reported to be
associated with observed changes in secular trends of hormone-sensitive conditions, as first documented by Rachel Carsons in her book *The Silent Spring*. Specific associations have been declining male reproductive health in wildlife, animals and humans, and reduced hatching success and feminization of different species in birds, fish, reptiles and mammals.

The mechanisms through which ED act on the male endocrine system are postulated to be through their actions on the oestrogen, androgen or aryl-hydrocarbon receptors or on hormone synthesis pathways, thereby interfering with the male hypothalamic-pituitary-gonadal (HPG) axis. Other mechanisms may be through the chemical’s effect on the thyroid or non-endocrine systems, or through interference with hormone signalling systems.

In general, reported male reproductive health abnormalities are reproductive organ defects, cancers, decreased sperm quality and declining fertility. More specifically, testicular dysgenesis syndrome, which is characterised by cryptorchidism, hypospadias, testicular cancer and poor sperm quality has been hypothesised to result from hormonal imbalance caused by ED during reproductive organ development. Increased rates of these disorders and breast cancer have also been reported. To better understand the potential mechanisms of action on the male endocrine system, the section below will provide an overview of the normal development of this system.
3. Development of the male reproductive health system

In males, the major endocrine system involved in reproduction is the HPG axis, and the production of male sex hormones is largely uniform except during puberty. Testosterone produced during the foetal or neonatal periods are said to programme the development and functioning of the male hypothalamus and brain during that period and throughout the life of the individual, thus rendering the foetus and neonate vulnerable to external influences that may have permanent adverse consequences.\textsuperscript{41, 42}

The reproductive system starts to develop about four weeks after conception and continues through to puberty.\textsuperscript{43, 44, 45, 46} Mammalian embryos can develop into phenotypic females or males. \textit{In utero} the testicular Leydig cells of genetically-determined males produce androgens which stimulate the development of the internal and external genitalia and results in masculinisation of the brain.\textsuperscript{47} Failure to produce testosterone during this critical period will result in a phenotypic female with testes indicating that interference with the hormonal milieu during this developmental phase will result in permanent developmental changes. Reproductive system development is arrested soon after birth until puberty.

Puberty is defined as the period when a sexually immature child becomes a sexually fertile adult and is characterized by physiological, morphological and behavioural changes that
accompany maturation of the gonads. The typical age of onset of puberty in males varies but is said to occur between the ages of 9 and 14 years. Environmental and genetic determinants influence pubertal onset but the exact factors and biological processes that trigger puberty remain unclear. What is known is that about two to four years prior to development of external signs of sexual maturation, a range of internal changes start to occur. The earliest changes are characterized by increased plasma concentrations of adrenal androgens which stimulate pubic and axillary hair growth (adrenarche). These changes typically occur around the age of eight years.

At the start of puberty, gonadotrophin-releasing hormone (GnRH) is secreted by the hypothalamic GnRH neuronal terminals and stimulates the pituitary to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH) gonadotropes, which in turn stimulate gonadal sex steroid secretion. GnRH stimulates acute and pulsatile secretion of LH, whereas FSH release is much slower and longer. LH stimulates the Leydig cells to release testosterone, which in turn stimulates the Sertoli cells to produce sperm. Testosterone, however, is primarily responsible for the development of secondary sexual characteristics in males. Testosterone is also converted to the hormone dihydrotestosterone (DHT) through the action of the enzyme 5-alpha reductase and inhibits the release of LH and GnRH. Under the action of the enzyme aromatase, testosterone is converted into oestradiol (aromatization), and this activity occurs at multiple sites in the male (namely, in the bone, muscles, the cardiovascular system, adipose tissue, the pituitary gland, the brain and reproductive systems). Half of the oestradiol is derived from extraglandular aromatization of testosterone and about one-quarter from testicular secretion. Oestradiol, however, is a
more potent inhibitor of both LH and FSH secretion than testosterone. Very low levels of oestrogen are also said to be present in boys before puberty. FSH stimulates the Sertoli cells to produce sperm and to secrete a protein hormone, Inhibin B. Inhibin B selectively inhibits FSH secretion. The schemata in Appendix 7 graphically depict the hormonal regulation of the male endocrine system. Proteins that bind sex steroids also influence the availability of these hormones. Sex hormone-binding globulin (SHBG) binds approximately 97% to 98% of testosterone, therefore only 2% to 3% of the latter is free and biologically-active.

The features of puberty are characterised by a pubertal growth spurt, the development of secondary sexual characteristics, the attainment of fertility and psychological and social development. The earliest pubertal event is an increase in the growth velocity which is influenced by the secretion of growth hormone and the sex steroids. Testicular androgens control genitalia development, laryngeal enlargement and therefore deepening of the voice. Under the influence of FSH, the testicular volume increases from about 2 mL to 4 mL around the age of 12 years due to seminiferous tubule proliferation. LH causes testosterone secretion by the interstitial Leydig cells. The scrotum also enlarges and thickens and then pubic hair growth follows. The penis enlarges after testicular enlargement and doubles in size during puberty. Although the timing of puberty may differ between boys, the sequence of pubertal changes remains uniform. The Tanner stages of development are listed in Appendix 8, and illustrate the stages of sexual maturation during puberty. The endpoints of interest in males therefore include pubertal development, reproductive organ abnormalities,
reproductive hormones, semen quality and reduced fertility. The exposure time window is in-utero, child and adult.

4. Reproductive health effects of commonly used contemporary pesticides in the Western Cape

South Africa is a developing country and is the highest pesticide user in Southern Africa, and possibly in Africa. Reports suggest that widespread local pesticide use is on the increase. A large number of agricultural chemicals are used in South Africa and more than 52% of pesticides sold to the largest eight crop sectors in South Africa have endocrine-disrupting properties. The pesticides most frequently used in the country, for which there is some evidence of male reproductive effects resulting from exposure are: chlorpyrifos (organophosphate), endosulfan (organochlorine), azinphos-methyl (organophosphate), atrazine (triazine), simazine (triazine), deltamethrin (pyrethroid), and penconazole (azole).

In the Western Cape Province agricultural sector pesticide use is also substantial. Below are summaries of the results of laboratory and epidemiological studies which examine the reproductive health effects of endocrine-disrupting pesticides of more commonly used pesticides in the Province (Table 1).
Table 1: Reproductive health effects of contemporary pesticides commonly used in the Western Cape Province, South Africa.

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>In vitro/In vivo endocrine activity</th>
<th>Author</th>
<th>Study subjects</th>
<th>Reproductive health effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endosulfan (organochlorine) insecticide</td>
<td>Anti-androgenic &amp; estrogenic</td>
<td>Goulet and Hontela, 2003(67)</td>
<td>Frogs</td>
<td>Reduced secretory capacity of adrenal cells of frogs.</td>
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<td></td>
<td>[Andersen et al, 2002]</td>
<td></td>
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<tr>
<td>Choudhary and Joshi, 2003(68)</td>
<td></td>
<td>Male rats</td>
<td></td>
<td>Reduced serum testosterone, testes &amp; accessory glands weight, sperm quality and fertility in male rats administered orally at 5, 10 and 15mg/kg for 30 days.</td>
</tr>
<tr>
<td>Saiyed et. al., 2003(95)</td>
<td></td>
<td>Indian boys</td>
<td></td>
<td>Reduced sexual maturity rating and serum testosterone in environmentally exposed Indian boys.</td>
</tr>
<tr>
<td>Procloraz (fungicide)</td>
<td>Anti-androgenic, estrogenic and inhibit aromatase activity [Anderson et al, 2002]</td>
<td>Noriega et. al., 2005(69)</td>
<td>Pregnant rats</td>
<td>Sexual differentiation in male offspring of pregnant rats treated with 31, 63, 125 and 250 mg/kg per day.</td>
</tr>
<tr>
<td>Vinggaard &amp; Noriega state that disruption of reproductive development and functions have several modes of action.</td>
<td>AR antagonist (endocrine activity) Gray 2006</td>
<td>Wilson et al 2004(63)</td>
<td>Rats</td>
<td>Reduced fetal testis testosterone and increased progesterone production 10-fold in rats.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blystone et al, 2007(71)</td>
<td>Rats</td>
<td>Statistically significantly decreased fetal testosterone levels at high doses for rats whose pregnant mothers received 1, 7.8, 15.6, 31.3, 62.5, 125mg/kg/day.</td>
</tr>
<tr>
<td>Pesticide</td>
<td>In vitro/In vivo endocrine activity</td>
<td>Author</td>
<td>Study subjects</td>
<td>Reproductive health effects</td>
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<tr>
<td></td>
<td></td>
<td>Blystone et al, 2007(72)</td>
<td>Rats</td>
<td>Statistically significant delay in preputial separation, decreased serum and testicular testosterone, delayed rat pubertal development in rats dosed with 0, 31.3, 62.5, 125mg/kg/day.</td>
</tr>
<tr>
<td>Fenarimol</td>
<td>Anti-androgenic <a href="64">Vinggaart et al, 2005</a></td>
<td>Gray et al, 1989(73)</td>
<td>Rats</td>
<td>Sexual differentiation and reduced breeding performance in male rats.</td>
</tr>
<tr>
<td></td>
<td>Anti-androgenic, estrogenic and inhibit aromatase activity <a href="65">Anderson et al, 2002</a></td>
<td>Vinggaart et al, 2005(64)</td>
<td>Rats</td>
<td>Sexual differentiation and reduced breeding performance in male rats.</td>
</tr>
<tr>
<td>Chlorpyrifos (organophosphate) insecticide</td>
<td>Weak estrogenic activity <a href="65">Andersen et al., 2002</a></td>
<td>Rawlings 1998(75)</td>
<td>Ewes</td>
<td>Decrease in serum LH of ewes.</td>
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<td></td>
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<td>Meeker et. al., 2004(97)</td>
<td>American men</td>
<td>Statistically significant negative association of urinary metabolite, TCPY (median = 3.2 ug/L) with sperm quality, serum testosterone and androgen index in American men.</td>
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<tr>
<td></td>
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<td>Akhtar et al, 2009(66)</td>
<td>Male rats</td>
<td>Reduced testis weight, morphological changes in reproductive organs, reduced testicular enzymes and decreased sperm counts at 9mg/kg/day in male rats fed 3,6,9 mg/kg/day for 90 days.</td>
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<tr>
<td>Pesticide</td>
<td>In vitro/In vivo endocrine activity</td>
<td>Author</td>
<td>Study subjects</td>
<td>Reproductive health effects</td>
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<tr>
<td>Deltamethrin</td>
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<td>Male rats</td>
<td>In utero and lactational exposure results in changes in reproductive behaviour and physiology of male rats administered orally at 4 mg/kg from day 1 of pregnancy to day 21 of lactation.</td>
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<td></td>
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<td>Issam et al, 2009(77)</td>
<td>Male rats</td>
<td>Hypospermatogenesis in testis, statistically significant decrease in FSH, LH, testosterone at highest doses in male rats given deltamethrin subcutaneously at 2 ppm for 30 days, 20 ppm for 45 days, and 200 ppm for 60 days.</td>
</tr>
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<td></td>
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<td>Zeng et al, 2009(79)</td>
<td>Rats</td>
<td>Dose-dependent increasing apoptosis of Leydig cells in male offspring of pregnant rats fed 1, 4, 8, 16, 20, 24 mg/kg/day from day 12-17 post-conception.</td>
</tr>
<tr>
<td>Cypermethrin</td>
<td>(pyrethroid)</td>
<td>Eibertieha, 2001(80)</td>
<td>Rats</td>
<td>Increase in testis weight, reduced fertility, decrease in serum Testosterone, LH, FSH and lower sperm quality in rats fed 13.15, 18.93 and 39.66 mg per day.</td>
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<tr>
<td></td>
<td></td>
<td>Yousef et. al, 2003(81)</td>
<td>Rabbits</td>
<td>Reduced plasma testosterone &amp; sperm quality in rabbits administered 24 mg/kg every 2nd day for 12 weeks.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wang et al, 2010a(82)</td>
<td>Mice</td>
<td>Reduced weight of testis, histological changes in reproductive organs, decreased testosterone synthetic enzyme, decreased serum and testicular testosterone and decreased number of spermatzoa in adulthood of mice whose mothers were exposed to cypermethrin 25mg/kg daily from postnatal day 0-21.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wang et al, 2010b(83)</td>
<td>Pubertal mice</td>
<td>Decreased serum and testicular testosterone, mRNA levels of steroidogenic acute regulatory protein, number of sperm. Number of Leydig cells and testis weight unaffected in mice whose pregnant mothers were exposed to cypermethrin</td>
</tr>
<tr>
<td>Pesticide</td>
<td>In vitro/In vivo endocrine activity</td>
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<tr>
<td>Glyphosate (chlorophenoxy herbicide)</td>
<td></td>
<td>Yousef et. al, 1996(89)</td>
<td>Humans and rabbit sperm</td>
<td>In vitro reduction in human and rabbit sperm quality.</td>
</tr>
<tr>
<td>Fenvalerate (Pyrethroid)</td>
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<td>Tan et al, 2004(98)</td>
<td>Factory workers</td>
<td>Reduced sperm quality amongst low level exposed factory workers.</td>
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<tr>
<td></td>
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<td>Xu et al, 2002(90)</td>
<td>Male rats</td>
<td>Elevation of serum LH and FSH, decrease in serum testosterone and sperm quality in male rats dozed 2, 4, 12 &amp; 60 mg/kg for 15 and 30 days.</td>
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<tr>
<td></td>
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<td>Mani et al., 2002(91)</td>
<td>Rats</td>
<td>Decrease in sperm production, testosterone marker enzymes and serum testosterone in rats for 3 months.</td>
</tr>
<tr>
<td>Pesticide</td>
<td></td>
<td>Ahmed et al, 2009(84)</td>
<td>Dwarf goats</td>
<td>Statistically significant decreased ejaculatory volume, motility percentage, mass activity and concentration of spermatozoa. More alkaline semen. Grossly decreased weight of testis in goats dipped with 1, 1.0, 1.4, 1.8, 1.6% cypermethrin on days 1 and 15. Semen collected at Day 2, then fortnightly till day 75.</td>
</tr>
<tr>
<td>DNOC (4,6-dinitro-orthocresol) insecticide</td>
<td></td>
<td>Takahashi et. al., 2004(85)</td>
<td>Rats</td>
<td>Reduction in sperm quality of rats treated 4, 7.5 &amp; 15 mg/kg for 5 days.</td>
</tr>
<tr>
<td>Parathion (Organophosphate)</td>
<td></td>
<td>Padungtrod et.al, 1999(86)</td>
<td>Chinese factory workers</td>
<td>Statistically significant increase in semen aneuploidy and decrease in sperm quality amongst Chinese factory workers.</td>
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<tr>
<td>Padungtrod et.al, 2000(87)</td>
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<td>Rats</td>
<td>Statistically significant reduction in sperm quality and non-significant reduction in fertility amongst exposed rats.</td>
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<tr>
<td>Piña-Guzmán et al, 2009(88)</td>
<td></td>
<td>Mice</td>
<td>DNA alterations in spermatozoa, decreased sperm quality and decreased fertilizing capacity in mice who receive methyl-parathion at 20mg/kg. Spermatozoa is collected from the vas deferens at 7 and 28th day post-administration.</td>
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<td>Pesticide</td>
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<td></td>
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<td>Xu et al, 2004(92)</td>
<td>Rats</td>
<td>Testicular lesions, reduced sperm motility in rats dosed 3.3 mg/kg.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xia et al, 2004(99)</td>
<td>Chinese factory workers</td>
<td>Statistically significantly reduced sperm quality and raised aneuploidy amongst Chinese factory workers.</td>
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<tr>
<td></td>
<td></td>
<td>Zhang et al, 2010(93)</td>
<td>Mice</td>
<td>Decreased sperm count, histology, serum and testicular testosterone in adult mice given fenvalerate 60mg/kg/day from postnatal day 35-63.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zhang et al, 2009(94)</td>
<td>Mice</td>
<td>Decreased testis weight, increased histological changes, decreased serum and testicular testosterone in mice whose pregnant mothers received fenvalerate 60mg/kg/day postnatal day 0-21.</td>
</tr>
</tbody>
</table>

4.1 Laboratory studies

Endosulfan\textsuperscript{67, 68} (organochlorine), procloraz\textsuperscript{69, 70, 71, 72} and fenarimol\textsuperscript{73} (pyrimidine) which are anti-androgenic and estrogenic in vitro, have been found to reduce sperm quality, reproductive hormone levels and fertility in adult male rats as well as reduce sexual differentiation in the male offspring of female rats.

The anti-androgenic pesticide, iprodione\textsuperscript{74} (chloranaline) has been found to cause male reproductive abnormalities in rats. Chlorpyrifos\textsuperscript{75} (organophosphate) and deltamethrin\textsuperscript{76, 77} (pyrethroid), both estrogenic in vitro, have been found to decrease serum LH in ewes and cause changes in reproductive physiology and behaviour in the male off-spring of pregnant rats. The organophosphate, dichlorvos\textsuperscript{78, 79}, which has been shown to be an androgen
receptor antagonist, has been found to damage the testis and negatively impact sperm quality and serum testosterone in rats.

Quite a few pesticides, not found or tested for in vitro for endocrine disrupting activity - including the pyrethroid, cypermethrin,\textsuperscript{80, 81, 82, 83} the dinitrophenol, 4,6-dinitro-o-cresol (DNOC),\textsuperscript{85} the organophosphate, parathion\textsuperscript{86, 87, 88}, the glycine, glyphosate,\textsuperscript{89} and the pyrethroid, fenvalerate,\textsuperscript{90, 91, 92, 93, 94} - have been associated with male reproductive health effects in laboratory animals and humans. Other associated effects include an increase in semen aneuploidy, reduced serum levels of reproductive hormones, reduced sperm quality, testicular lesions and reduced fertility (Table 1).

Deeper analysis of the aforementioned studies suggest that there is a difference in outcomes between laboratory studies where the endocrine-disrupting pesticides were administered at different developmental time periods (Table 1) Pesticides administered during embryonic, neonatal or lactation developmental periods resulted primarily in reduced testicular weights, histological changes, biochemical changes and decreased serum and testicular testosterone levels.\textsuperscript{77, 79, 82, 93} The decreased hormone levels, however, tended to correct post-weaning or during adulthood. Testicular developmental changes, however, were permanent. Studies where the pesticides were administered during puberty, suggested that there was little or no effect on the number of Leydig cells or on the weight of the testis, but the serum and testicular testosterone levels were decreased.\textsuperscript{72, 83, 88, 94}
4.2 Epidemiologic studies

A cross-sectional analytical study conducted on rural Indian male schoolchildren aged 10 to 19 years of age, who were environmentally exposed to aerial spraying of endosulfan found reduced sexual maturity rating scores and serum testosterone levels amongst 117 exposed boys compared to 90 boys in the same age groups living in a control area (Table 1).\textsuperscript{95} A statistically significant negative association between male reproductive outcomes (sperm quality, serum testosterone and androgen indices) and urinary chlorpyrifos levels in urban American men has also been shown.\textsuperscript{96, 97} Negative associations between sperm quality and exposure to fenvalerate\textsuperscript{98, 99} and parathion\textsuperscript{96} were also found in cross-sectional studies conducted on male factory workers.

5. Western Cape rural residents are exposed to contemporary pesticides

Despite a paucity of data on the extent and nature of local occupational and environmental exposure to agricultural pesticides, research has shown that Western Cape rural residents are environmentally and occupationally exposed.\textsuperscript{60, 61} A number of studies have reported on the presence of pesticides in sediments and local water sources, such as boreholes, rivers and dams.\textsuperscript{100-105} Pesticides have also been detected in locally grown and imported raw wheat intended for commercial use in South Africa.\textsuperscript{106} Other studies have reported increased exposure of farm dwellers due to spraying activities on the farm, or a lack of knowledge of pesticide exposures and health risks.\textsuperscript{107}
Groundwater is an important source of drinking, or other domestically-consumed water in the Western Cape. The following chemicals have been found in groundwater, surface water, and drinking water in rural areas in the Province: endosulfan; chlorpyrifos; fenarimol; deltamethrin; and iprodione. Endosulfan and chlorpyrifos were studied in more detail and were found to exceed the World Health Organisation’s (WHO) drinking water standard of 0.1 µg/L in about a third of the samples.

Other pesticides such as atrazine were also detected in water entering irrigation systems in the Western Cape from runoff due to maize farming. Constant pesticide pollution of ground and surface water in three rural Western Cape agricultural districts, namely dams and boreholes in rural communities and two major dams contributing to municipal water supplies was shown. Endosulfan were detected in a major river, from orchard run-off. The maximum whole fish organochlorine load reached high levels if fish were eaten daily. Another study showed consistently low-levels of endosulfan in rural water sources in the Western Cape, with the pesticide being present in 26 of 27 dams. Nineteen of the identified contaminated sites were sources for drinking or domestically-used water. Chlorpyrifos levels amongst rural residents in the Western Cape were found to be higher than those found among American men from the general population and similar to endosulfan, fenvalerate and parathion levels measured in environmentally and occupationally exposed populations in India and China, where statistically significant associations between exposures to adverse male reproductive effects were found. A study
investigating pesticide exposure among 25 farm workers on three farms in the Hex River Valley, Western Cape, one of the study areas in the proposed study, found high blood serum endosulfan levels in both applicators and non-applicators during the spraying season as well as in the pre-season (mean = 530 ± 0.05 µg/L).¹¹²

Furthermore, a survey in a rural area in the Western Cape showed that about 50% of respondents used borehole water and 20% used surface water on a daily basis.¹¹³ Another survey of farm workers in the Western Cape reported that 18% of farm workers reported that spray drifted into their homes. Thirty to 60% reported living less than 10 meters away from orchards, vineyards or fields subject to pesticide application (drift exposure).¹¹⁴ About 50% reported using pesticides obtained from the farm for home purposes. Levels of awareness of the health effects of pesticides were also shown to be low which contributed to increased exposure. Use of untapped water for domestic purposes and farm dam swimming were also commonly reported.¹¹³

6. Conclusion

In many agricultural settings endocrine-disrupting pesticides are commonly used, released into the environment and absorbed by humans. Evidence exists to suggest that temporal changes in male reproductive health are as a result of exposure to hormonally active chemicals. Both laboratory and epidemiological studies provide evidence to suggest that contemporary agricultural pesticides reduce male reproductive health especially sperm
quality, testosterone levels and sexual development, and negatively impacts on growth. Rural residents in the Western Cape are environmentally exposed to pesticides and agricultural pesticides have been detected in the rural environment and in humans in the Western Cape, South Africa. Furthermore, evidence exists to suggest that knowledge, attitudes and practices regarding pesticides, pesticide use and exposures are low amongst those most at risk of exposure. Clearly, ongoing and increased use of these toxins in agricultural practices place those who manufacture and work with these substances, their families, and others who are environmentally exposed at higher risks of adverse health effects. Only one epidemiological study could be found that has investigated early male reproductive health effects in humans consequent to environmental exposure to contemporary agricultural pesticides, and none investigating these effects in communities exposed to many of these pesticides. The proposed study thus presents the opportunity to fill this important gap in the literature.
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Davies H. An assessment of the suitability of a series of Western Cape Farm Dams as water bird habitats. MSc (ConservationBiology) thesis. Zoology Department, University of Cape Town 1997.

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PART C: JOURNAL READY MANUSCRIPT

1. Preparation for submission

1.1 Journal

Prepared for the purposes of submission to the *Environmental Health Perspectives* Journal. The *Instructions to Authors* can be found in Appendix 9.* The author has adhered to all the Instructions set out by the Journal, except that the Tables and Figures are included in the text.

1.2 Short running head

Reproductive health effects in boys and pesticides.

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* For the purpose of the thesis, variations from the journal article requirements are allowed. The variations are that the figures and tables are inserted in the text, and the article refers to supplementary material in the Appendices section of the thesis. The latter would otherwise be referred to as ‘not shown’ or ‘loaded information’. The font has been changed to Arial. Pages and lines are not numbered. Author names, affiliations and contact details have not been included. These changes are in accordance with the thesis outline.
1.3 Key words

Endocrine disruptors

Contemporary agricultural pesticides

Environment

Male reproductive health effects

Male puberty

Sex hormones

Height

Weight

Sexual maturity rating

1.4 Acknowledgments/grant support

National Research Foundation (SA), Medical Research Council (SA), University of Cape Town Research Committee, Associate Prof Melissa Perry (Harvard School of Public Health), Professor Mary Lee (University of Massachusetts), Algernon Africa (University of Cape Town)
1.5 Disclaimers/Competing interests Declaration

Nil.

1.6 Abbreviations and definitions used in manuscript

SMR – sexual maturity rating

PI – primary investigator

NHLS – National Health Laboratory Service

LH – luteinizing hormone

E2 – oestradiol

FSH – follicle stimulating hormone

SHBG – sex hormone-binding globulin

BMI – body mass index

IU/L – International Units per Litre

mL – millitre

µg/L – microgram per litre
1.7 Outline of manuscript headers

Abstract

Introduction

Methods and Materials

  Participants

  Questionnaire

  Physical examination

  Reproductive hormone measurements

  Sample size calculation

  Statistical analysis

Results

  Participation

  Exposure assessment

  Demographic, socioeconomic and medical histories

  Pesticide exposure, phyto-oestrogen intake and exposures during pregnancy

  Hormones

  Sexual and anthropometric reproductive health
Associations between exposure and reproductive health outcomes

Discussion

Conclusions

References
2. Abstract

Background

Few studies have investigated reproductive health effects due to contemporary agricultural pesticide exposure amongst boys.

Objectives

To determine the association between lifetime pesticide exposure and the reproductive health of boys.

Methods

We conducted a cross-sectional study in rural areas. Boys living on farms and those not living on farms were recruited from local schools. The study included a structured questionnaire (items on demographics, birth weight, general and reproductive health, phyto-oestrogen intake, residential history, pesticides exposures, mother’s exposures during pregnancy); sexual maturity development ratings recorded according to Tanner Stage;
testicular volume; anthropometric measurements (height, weight and BMI); and measurement of sex hormone levels (LH, FSH, testosterone, oestradiol) in blood.

**Results**

The participants included 269 boys of which 177 (65.8%) were classified as farm and the rest as non-farm residents (median ages: 11.3 vs 12.0 years). After controlling for confounders, farm boys had lower levels of testosterone (beta (β) = -0.346 nmol/L; CI: -1.123 - 0.436 nmol/L), LH (β = -0.284 IU/L; CI: -0.483 - -0.084 IU/L), and smaller testes (β = -1.745 mL; CI: -4.248 - 0.758 IU/L), and were shorter (β = -3.417 cm; CI: -6.382 - -0.453 cm) and lighter (β = -2.258 kg; CI: -4.441 - -0.753 kg). They also had higher levels of oestradiol (β = 8.074 pmol/L; CI: 2.339 - 13.808 pmol/L) and FSH (β = 0.634 IU/L; CI: 0.186 - 1.081 U/L). There was no statistically significant effect on Tanner stage.

**Conclusions**

These results provide evidence that environmental exposure to contemporary pesticides may be associated with adverse reproductive and developmental effects in boys.
3. Paper

3.1 Introduction:

Environmental chemicals acting as hormonally active substances\(^1\) have been hypothesized to be the underlying cause of declining male reproductive health.\(^2\)\(^3\) These chemicals have been shown to cause male reproductive effects such as abnormal secretion of reproductive hormones, reproductive organ defects and a decrease in sperm quality and fertility in laboratory animals as well as in wildlife.\(^4\) Many contemporary agricultural pesticides are hormonally active with the potential to cause male reproductive health effects in exposed persons.\(^5\)

South Africa is the highest pesticide user in Southern Africa and our prior work has shown that pesticide use in the Western Cape Province agricultural sector is substantial.\(^6\)\(^7\)\(^8\) Some of the most commonly used pesticides in the Western Cape, which we have identified in previous surveys,\(^6\)\(^7\)\(^8\)\(^9\) are toxic to the male reproductive system in laboratory animals and/or wildlife or have been related to adverse male reproductive outcomes in humans. The organochlorines, endosulfan\(^10\)\(^11\) and procloraz,\(^12\)\(^13\)\(^14\)\(^15\) and the pyrimidine, fenarimol,\(^16\) are anti-androgenic and estrogenic \textit{in vitro},\(^17\) and have been found to give rise to abnormal sexual differentiation including feminized \textit{in utero} exposed males, reduction in sperm quality, decreased testosterone concentrations and reduced fertility in adult male rats. The anti-androgenic chloranaline pesticide, iprodione,\(^18\) has been found to cause male reproductive developmental abnormalities including delayed puberty in rats. The organophosphate,
chlorpyrifos, and the pyrethroid, deltamethrin, are oestrogenic in vitro, and were shown to decrease serum luteinising hormone (LH) in ewes and alter reproductive physiology and behavior in male offspring exposed during gestation. The organophosphate, dichlorvos, known to be an androgen-receptor antagonist, was associated with testis damage, inferior sperm quality and reduced serum testosterone in rats. Several other contemporary used pesticides including the pyrethroid, cypermethrin, the dinitrophenol, 4,6-dinitro-o-cresol (DNOC), the organophosphate, parathion, the glycine, glyphosate, and the pyrethroid, fenvalerate were associated with male reproductive effects in laboratory animals and humans including increases in semen aneuploidy, reduced serum levels of reproductive hormones, reduced sperm quality, testicular lesions, involving vacuolation of endoplasmic reticulum and necrosis of Sertoli cells, and reduced fertility.

Very few epidemiological studies have been conducted which investigates the association between contemporary agricultural pesticide exposure and male reproductive health especially amongst boys. Endosulfan has, however, been associated with lower sexual maturity ratings and serum testosterone in environmentally exposed Indian boys. Urinary 3,5,6-trichloro-2-pyridinol (TCPy), the metabolite of chlorpyrifos, was associated with reduced sperm quality and serum testosterone in American men. Negative associations between sperm quality and exposure to fenvalerate and parathion were also found in cross-sectional studies conducted on male factory workers. No epidemiological study investigating the effect of contemporary pesticides on pubertal growth of boys could be found in the literature.
Our previous work has shown that rural residents are exposed to pesticides through exposure to contaminated food, soil, water and spray drift, in addition to occupational exposure.\textsuperscript{45} Endosulfan, chlorpyrifos, fenarimol, deltamethrin and iprodione have been detected in Western Cape rural surface and ground waters, including drinking water\textsuperscript{9 45 46}, with endosulfan and chlorpyrifos exceeding the World Health Organisation’s drinking water standard of 0.1 \textmu g/L in about a third of samples.\textsuperscript{45} Recently, chlorpyrifos levels amongst farm workers in the Western Cape\textsuperscript{47} were also found to be higher than those found among Northern American men from the general population where statistically significant associations between exposures and adverse male reproductive effects were found.\textsuperscript{40 41} In addition, Western Cape rural residents were shown to be exposed to a number of different pesticides that can affect male reproduction rather than to just a single pesticide as was the case in all the epidemiological studies reviewed here.\textsuperscript{9 45 46} No studies on the reproductive health effects of agricultural pesticide exposure on boys had previously been conducted in South Africa.

This study therefore aims to investigate whether exposure of young boys to pesticides in agriculturally-intense areas of the Western Cape Province has discernable effects on reproductive development and growth. The paper presents the results of a study which uses the lifetime living history of the boy as an exposure index thus comparing boys who live on farms to those who do not.
3.2 Methods and Materials

Institutional Review Board (IRB) approval for the study was granted by the ethics review board of the University of Cape Town (REC REF: 279/2005).

3.2.1 Participants

An analytical cross-sectional study of 269 boys aged 5 to 19 years, who resided in rural areas of the Western Cape Province, South Africa, was conducted between April 2007 and March 2008. Boys were recruited from the most accessible primary and secondary schools (n=8) attended by pupils living on both farms and in towns, in three agriculturally-intense areas where pesticides were detected in the environment and in farmworkers. These areas were the Hex River Valley, where grape farming was practised; Grabouw, where apple farming was predominantly practised; and Piketberg, where wheat and fruit farming were the primary agricultural activities. Boys living on farms as well as those living in neighbouring non-agricultural areas were recruited to ensure comparability between the two exposure groups. Other than age, there were no further exclusion criteria. Parents were asked in advance, by means of detailed letters distributed to parents at the schools, to provide provisional written consent for their child to be recruited to the study. Boys whose parents consented (n = 492) were stratified according to whether they lived on a farm or not at the time of the study. They were also stratified by area. All 94 boys not living on a farm were selected for the study, and 180 boys (60 in each area) out of 398, stratified by age-group, were selected from those living on farms. The age-groups were as follows: 5 to 9
years (representing the period before the start of pubertal development), 9.1 to 11 years (representing the period at the start of pubertal development), 11.1 to 14 years (representing the period of advanced and end of pubertal development) and > 14 years (representing the time period after puberty has been reached). At schools, where the number of consenting boys living on farms exceeded the number of boys to be selected, random systematic sampling was used to select those who were to be included in the study.\textsuperscript{49} Selected boys and their parents (preferably the mother) or guardians were invited to participate in the study and were asked to present at the school on specified dates. Prior to the initiation of the study, the protocol and questionnaire were piloted on 3 subjects.

3.2.2 Questionnaire

On presentation at the school, written informed consent was obtained. Thereafter, trained interviewers administered questionnaires to the participants’ parents or guardians in Afrikaans, which was the local language of preference. The questionnaire included sections on demography, general medical history, genital health history, pubertal development, the mothers’ personal habits during pregnancy, lifetime environmental exposure to agricultural pesticides, domestic pesticide use, phyto-oestrogen intake and lifestyle factors. Questions in the demographic, general medical history, genital history and environmental exposure sections were based on previous local studies in similar populations.\textsuperscript{50, 51, 52, 53}
The section on lifetime environmental exposure to agricultural pesticides was based on the participant’s lifetime residential history and elicited information on all the places the participant had resided since birth. For each location, it was established whether the home was located on a farm or not. If located on a farm, further information was collected on the frequency of pesticide spraying, the application methods used, and the proximity of the home to the location of the pesticide application. The latter also included information on the boys’ participation in spraying activities, contact with the pesticide itself, contact with contaminated surfaces while playing outside, water sources, ingestion of contaminated crops from farms and tasks performed on the farm. The questionnaire responses were entered directly onto an electronic questionnaire loaded onto cellular phones. After completion of the questionnaire, the data were transferred to a central website via the internet. The data were then manually downloaded from the website into a Microsoft Excel® spreadsheet and then transferred to the statistical package, Stata 10® by a member of the study team.

3.2.3 Physical examination

A trained male nurse practitioner conducted an examination to determine the sexual maturity rating (SMR) score of the boy. The nurse was trained by a local reproductive health specialist who demonstrated the anthropometric measurement techniques, use of the orchidometer, and assessment of the SMR score on examination and through use of visual material. Training was conducted over a period of 2 days.
The SMR was derived by assessing pubic hair (adrenal maturation) and penis development (testicular maturation) and then by determining a combined score using Tanner scores, but with stages five and six merged. Further examinations were conducted to assess for the presence of genital scars, penis and testicular abnormalities, and to assess the testicular consistency and volume. Testicular volume was assessed using a standardized set of wooden testicular beads [Orchidometer, Kabi, Japan]. The nurse also performed anthropometric measurements and recorded the boys’ heights and weights (using a calibrated scale) according to standardized methods.

3.2.4 Reproductive hormone measurements

Two six millilitre (mL) venous whole blood samples were collected from each boy by one of two qualified nurses after physical examination was completed. Blood samples were collected in the morning and as early as logistically possible (preferably before 13H00). The samples were allowed to clot, and were then centrifuged at 5000 revolutions per minute in the field by a qualified laboratory technician using a portable centrifuge. The supernatants were stored 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 NHLS laboratory determined baseline follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, oestradiol (E2) and SHBG (sex hormone-binding globulin) levels using electrochemiluminescence immunoassays (ECLIA) on a Roche Cobas Modular E170 analyser. The intra- and inter-
assay variabilities and sensitivities for FSH, LH, testosterone, E2 and SHBG as provided by the laboratory are listed in Table 2.

Table 2: Intra-and inter-assay variabilities and sensitivities for FSH, LH, testosterone, E2 and SHBG.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Intra-assay variability</th>
<th>Inter-assay variability</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td></td>
</tr>
<tr>
<td>LH (IU/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human serum 1</td>
<td>6.15 (0.08)</td>
<td>5.81 (0.12)</td>
<td>0.10</td>
</tr>
<tr>
<td>Human serum 3</td>
<td>164 (1.41)</td>
<td>159 (3.47)</td>
<td></td>
</tr>
<tr>
<td>FSH (IU/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human serum 1</td>
<td>5.97 (0.15)</td>
<td>5.33 (0.19)</td>
<td>0.10</td>
</tr>
<tr>
<td>Human serum 3</td>
<td>178 (4.54)</td>
<td>229 (10.3)</td>
<td></td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human serum 1</td>
<td>1.91 (0.05)</td>
<td>1.67 (0.09)</td>
<td>0.069</td>
</tr>
<tr>
<td>Human serum 3</td>
<td>30.6 (0.54)</td>
<td>28.2 (0.79)</td>
<td></td>
</tr>
<tr>
<td>Estradiol (pmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human serum 1</td>
<td>130 (4.28)</td>
<td>120 (5.70)</td>
<td>18.4</td>
</tr>
<tr>
<td>Human serum 3</td>
<td>467 (7.9)</td>
<td>472 (11.8)</td>
<td></td>
</tr>
<tr>
<td>SHBG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human serum 1</td>
<td>14.9 (0.17)</td>
<td>13.7 (0.24)</td>
<td>0.35 nmol/l</td>
</tr>
<tr>
<td>Human serum 3</td>
<td>219 (3.76)</td>
<td>189 (7.58)</td>
<td></td>
</tr>
</tbody>
</table>

* Data provided by National Health Laboratory Services, Groote Schuur, South Africa
3.2.5 Statistical analysis

A history of living on a farm was treated as a proxy for the boys' pesticide exposure history. A lifetime history of living on a farm was determined using information on the place of residence at the time of interview (current farm or town residence), as well as prior residential histories (previous farm or town residence). Boys were included in the exposure group if they had lived on a farm all their lives, or if they had lived both on a farm and in a town and were born on a farm and/or spent the first three years of their life on a farm and/or spent more than 3 years of their first 12 years on a farm. Boys who did not meet the latter criteria or who only lived their entire lives in town, were included in the unexposed group. The primary exposure variable was therefore dichotomous (exposed; unexposed).

The primary outcome variables were baseline reproductive hormonal levels (FSH, LH, E2 and testosterone), sexual maturity development (Tanner stage scores, sum of right and left testicular volumes) and anthropometric measurements (height, weight, body mass index (BMI)). These were all dichotomised using quartiles (25th, 50th, 75th percentiles) as cut-offs. Other dichotomised outcome variables included anthropometric measurements below the 25th or 50th percentile for age (according to CDC growth charts). The Tanner stages were dichotomized using Stage 4 and 5 as a cut-off. The categorization of outcomes was based on that used in a previous study.

55 56 57 58
Univariate and bivariate exploration of the data was performed. Bivariate analysis included regression analysis, t-test or Wilcoxon sum rank test (for linear data), and Chi-square testing (for categorical data). Multiple linear and logistic regression analyses were used to test for associations between the individual outcomes and exposure while controlling for confounding. Confounders was selected on an *a priori* basis, according to biological plausibility, or using bivariate testing, if p<0.1. Age and household income (marker of socio-economic status) were selected *a priori* for all outcomes. For all the hormonal outcomes SHBG and the other reproductive hormones measured were also included *a priori*. In regard to the latter, other hormones were included to control for their effect on a particular hormonal outcome but analysis was also conducted which excluded the other hormones from the model. Potential confounders included in the bivariate testing included all variables measuring demography, medical history, socio-economic status (other than household income), household pesticide exposure, phyto-oestrogen intake mother’s exposure during pregnancy and SHBG. The confounders were included in the regression model during the model building process. A logical variable selection model was used to select the variables. First, potential confounders were fitted to the model. Then, the other variables were added one at a time. The variable that statistically significantly reduced the deviance when compared to the null model was chosen as the baseline model. Secondly, the variables were added to the baseline model. The combination of variables that resulted in the biggest change to the variance was the chosen model. As a final step, the variables were once again added to the model to assess whether in the presence of other variables, they made a statistically significant reduction in the deviance. Regression diagnostics was applied to
determine the goodness of fit of the model and to assess for outliers or influential observations.

Analysis was also conducted per age-category using the four age categories: 5 to 9 years; 9.1 to 11 years; 11.1 to 14 years and > 14 years. Sensitivity analysis was conducted excluding participants who lived both on farms and towns during their lifetime. All analyses were performed using Stata 10 statistical software (StataCorp, Texas, USA).

3.3 Results

3.3.1 Participation

Two-hundred and sixty-nine participants were recruited from schools located in the three rural areas in the Western Cape in South Africa. The overall response rate of selected boys was 98.2 %. Thirty-seven percent (100) resided in Grabouw, 29% (78) in Hex River, and 34% (91) in Piketberg.

3.3.2 Exposure assessment

One hundred and seventy-five boys currently lived on a farm, and 94 in a town. Of these, 82.9% (223) had lived in one location all their lives, including 147 on farms and 76 in towns and were included into a farm group and a non-farm group, respectively. The remaining
17.1% (46) had lived in 2 to 4 different locations (farms and/or a town) of which (36.9%) 17 boys lived on a farm and (26.1%) 12 in a town throughout their lives. These boys were also included into the farm and non-farm groups, respectively. Seven percent of the boys (19) lived on both a farm and in a town. Sixty-eight percent (13) of this group who were born on a farm and/or spent the first 3 years of their lives on a farm, and/or spent more than 3 years of their first 12 years on a farm were included in the farm group. The other 31.6% (6) were included in the non-farm group. There were thus 65.8% (177) boys included in the farm group and the rest ((34.2%) 92) in the non-farm group.

3.3.3 Demographic, socioeconomic and medical histories

The median ages of the two groups were in the same age range (11.3 versus 12.0 years, respectively), although the non-farm group was statistically significantly older (p=0.037) (Table 3). There was also good representation of boys from the various age-groups during which pubertal development occurs in both groups. The median household income in the farm group (R1800) was statistically significantly less than in the non-farm group (R2500) although both were below R3000 (p=0.001). The median birth weight of the farm group (2.86kg) was similar to that of the non-farm group (2.90kg), however, birth weights were only available for three-quarters of the study sample.
Table 3: Demographics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Farm, (n = 177) median (IQR)†</th>
<th>Non-farm, (n = 92) median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) (n = 269)*</td>
<td>11.1 (9.25;13.1)</td>
<td>12.4 (9.46;13.29)</td>
</tr>
<tr>
<td>Age range of boys (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-9 (n = 41)</td>
<td>8.67 (8.33;8.83)</td>
<td>8.8 (8.50;8.92)</td>
</tr>
<tr>
<td>9.1-11 (n = 66) **</td>
<td>10.1 (9.50;10.67)</td>
<td>9.42 (9.33;9.92)</td>
</tr>
<tr>
<td>&gt;14 (n = 32)</td>
<td>14.88 (14.42;15.58)</td>
<td>14.83 (14.33;16.10)</td>
</tr>
<tr>
<td>Household income (Rands) (n = 262)***</td>
<td>1800 (1200;2400)</td>
<td>2500 (1500;15000)</td>
</tr>
<tr>
<td>Birth weight (Kilogram), (n = 193)</td>
<td>2.85 (2.40;3.20)</td>
<td>2.82 (2.54;3.30)</td>
</tr>
</tbody>
</table>

* p=0.037; ** p=0.004; *** p<0.001; † IQR = interquartile range

Chronic medical problems were below 3% in both groups, apart from TB (7.34%) amongst the farm boys and asthma, which ranged between 8 and 10% in both groups (Table 4). There were only two participants with foetal alcohol syndrome and both lived on farms. Two farm boys had previous unintentional pesticide poisoning. More boys were reported to have been born with abnormal testes in the farm group compared to the non-farm group, although the overall numbers were very small (10 (5.68%) vs 2 (2.20%), respectively). This difference was not statistically statistically significant at the 5% level. Three farm boys (1.7%) underwent a testicular operation within 3 months of birth of which 2 (1.1%) were for hypospadias while one non-farm boy (1.1%) underwent a hypospadia operation. Additionally, 1 farm boy (0.6%) was reported to have a hypospadia and 1 (1.1%) non-farm
boys had 1 testicle. The prevalence of mumps and other testicular problems were similar in the two groups
Table 4: Medical History

<table>
<thead>
<tr>
<th>Variable</th>
<th>Farm (n = 177), n (%)</th>
<th>Non-farm (n = 92), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General Health, n (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good to Excellent</td>
<td>168 (94.92)</td>
<td>91 (98.91)</td>
</tr>
<tr>
<td>Poor</td>
<td>9 (5.08)</td>
<td>1 (1.09)</td>
</tr>
<tr>
<td><strong>Medical History (Lifetime)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>2 (1.13)</td>
<td>0</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>13 (7.34)</td>
<td>2 (2.17)</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>2 (1.13)</td>
<td>1 (1.09)</td>
</tr>
<tr>
<td>Asthma</td>
<td>17 (9.60)</td>
<td>8 (8.80)</td>
</tr>
<tr>
<td>Heart Problem</td>
<td>2 (1.13)</td>
<td>0</td>
</tr>
<tr>
<td>HIV</td>
<td>0</td>
<td>1 (1.09)</td>
</tr>
<tr>
<td>Foetal Alcohol Syndrome</td>
<td>2 (1.13)</td>
<td>0</td>
</tr>
<tr>
<td>Mumps</td>
<td>49 (27.68)</td>
<td>30 (32.97)</td>
</tr>
<tr>
<td>Pesticide poisoning</td>
<td>2 (1.14%)</td>
<td>0</td>
</tr>
<tr>
<td>Born with abnormal testes</td>
<td>10 (5.68)</td>
<td>2 (2.20)</td>
</tr>
<tr>
<td>Previous testicular injury</td>
<td>6 (3.39)</td>
<td>2 (2.17)</td>
</tr>
<tr>
<td><strong>Previous testicular operation</strong></td>
<td>4 (2.26)</td>
<td>1 (1.09)</td>
</tr>
<tr>
<td>Reported testicular disease</td>
<td>5 (2.82)</td>
<td>2 (2.17)</td>
</tr>
</tbody>
</table>

3.3.4 Pesticide exposure, phyto-oestrogen intake and exposures during pregnancy

The use of household pesticides was prevalent in the current home of more than half of the farm (61.0%) and nearly half of the non-farm boys (47.8%) (Table 5). Only households in the
farm group used empty pesticide containers for domestic purposes, but the overall proportion was small (4.0%). Substantially more boys in the farm group (65.5%) compared to the non-farm group (6.5%) ate fruit from home gardens (p<0.001). The farm boys had exposures to pesticide drift in the home (44.4%), contact outside during spraying (40.0%) and drinking water from unprotected sources (43.3%).

The proportion of boys who ingested phyto-estogens in the form of soya, nuts and vegetables throughout their lives were similar for both groups (Table 5). Only six (3.3%) of the mothers in the farm group reported having sprayed or mixed pesticides when they were pregnant, but 39.0% (69) of them worked in the field while spraying occurred (Table 5). Alcohol consumption and smoking during pregnancy was more prevalent amongst mothers in the farm group.

Table 5 shows that the median number of days of spraying on the current farm where farm boys lived was 60 (days) and ranged from 0 to 90 days; the median distance of the farm homes from the spraying area was 20 meters (range: 7 to 50 meters).
Table 5: Exposure histories reported by parents/guardian amongst boys living on and off farms

<table>
<thead>
<tr>
<th>Pesticide exposure and phyto-oestrogen intake</th>
<th>Farm, (n = 177), n (%)</th>
<th>Non-farm, (n = 92), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pesticide use in current home*</td>
<td>108 (61.0)</td>
<td>44 (47.8)</td>
</tr>
<tr>
<td>Empty pesticide containers used in house</td>
<td>7 (4.0)</td>
<td>0</td>
</tr>
<tr>
<td>Fruit eaten from home garden**</td>
<td>116 (65.5)</td>
<td>6 (6.5)</td>
</tr>
<tr>
<td>Pesticides sprayed on farm during current year</td>
<td>171 (97.0)</td>
<td>NA</td>
</tr>
<tr>
<td>Farm spraying drifts into house</td>
<td>76 (44.4)</td>
<td>NA</td>
</tr>
<tr>
<td>Pesticide contact outside house on farm during spraying</td>
<td>70 (40.0)</td>
<td>NA</td>
</tr>
<tr>
<td>Unprotected drinking water (borehole, river, dam, rainwater)</td>
<td>72 (43.3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Phyto-oestrogen intake throughout lifetime</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soya</td>
<td>150 (85.0)</td>
<td>84 (91.3)</td>
</tr>
<tr>
<td>Nuts</td>
<td>136 (76.8)</td>
<td>86 (93.5)</td>
</tr>
<tr>
<td>Vegetables</td>
<td>172 (97.7)</td>
<td>88 (96.7)</td>
</tr>
<tr>
<td>Maternal exposure during pregnancy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sprayed pesticides</td>
<td>6 (3.3)</td>
<td>0</td>
</tr>
<tr>
<td>Work in vineyard/orchard while spraying occurred</td>
<td>69 (39.0)</td>
<td>10 (10.9)</td>
</tr>
<tr>
<td>Smoked</td>
<td>80 (45.2)</td>
<td>34 (37)</td>
</tr>
<tr>
<td>Consumed alcohol</td>
<td>36 (20.0)</td>
<td>9 (9.8)</td>
</tr>
<tr>
<td>Exposure to pesticide spraying</td>
<td>Median (IQR)†</td>
<td></td>
</tr>
<tr>
<td>Number of days per year sprayed on current farm</td>
<td>60 (0;90)</td>
<td>NA</td>
</tr>
<tr>
<td>Distance of current home from spraying on farm (m)</td>
<td>20 (7;50)</td>
<td>NA</td>
</tr>
</tbody>
</table>

* p=0.038; ** p<0.001; † Interquartile range
3.3.5 Hormones

For all age groups except the 5 to 9 year old category, the FSH levels were higher in the farm group compared to the non-farm group (Table 6). Oestradiol levels were higher amongst farm boys in all the age groups, except among the > 14 year old boys. The median oestradiol levels in the age groups 5 to 9 years (48.1 pmol/L vs 25.5 pmol/L; p-value=0.023) and 9.1 to 11 years (38.4 pmol/L vs 28.3 pmol/L; p-value=0.015) were statistically significantly higher in the farm versus the non-farm groups. Levels for LH were slightly higher in the non-farm group for the age group >14 years (3.85 IU/L vs 1.90 IU/L), but not statistically significant. For testosterone, the differences between the two groups were most obvious for the age group >14 years, with the non-farm boys having levels higher than the farm boys (10.7 nmol/L vs 8 nmol/L), but not statistically significant. For the age category 11.1 to 14 years, the levels of all the hormones were higher in the farm group. For the > 14 year old farm boys, the FSH levels were elevated, but the LH, oestradiol and testosterone levels were lower. The proportion of boys above and/or below the normal laboratory hormone ranges were higher in the farm group, except for testosterone, where the proportions of boys falling outside of the normal range were lower (Table 6).
Table 6: Reproductive Hormone levels among boys with and without a history of farm residence

<table>
<thead>
<tr>
<th>Reproductive hormones (units) per age category (years)</th>
<th>Farm, median (IQR)†</th>
<th>Non-farm, median (IQR)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (IU/L)</td>
<td>n = 163</td>
<td>n = 87</td>
</tr>
<tr>
<td>5-9</td>
<td>0.7 (0.3-2.9)</td>
<td>1.1 (0.6-1.5)</td>
</tr>
<tr>
<td>9.1-11</td>
<td>1.0 (0.1-5.5)</td>
<td>0.7 (0.3-2.7)</td>
</tr>
<tr>
<td>11.1-14</td>
<td>2.8 (0.4-13.0)</td>
<td>2.1 (0.4-8.9)</td>
</tr>
<tr>
<td>&gt;14</td>
<td>5.1 (1.0-11)</td>
<td>4.5 (1.3-5.3)</td>
</tr>
<tr>
<td>LH (IU/L)</td>
<td>n = 163</td>
<td>n = 88</td>
</tr>
<tr>
<td>5-9</td>
<td>0.05 (0.05-0.5)</td>
<td>0.07 (0.05-0.3)</td>
</tr>
<tr>
<td>9.1-11</td>
<td>0.1 (0.6-1.7)</td>
<td>0.1 (0.05-0.9)</td>
</tr>
<tr>
<td>11.1-14</td>
<td>1.3 (0.05-4.7)</td>
<td>1.1 (0.05-5.3)</td>
</tr>
<tr>
<td>&gt;14 *</td>
<td>1.9 (0.05-4.8)</td>
<td>3.85 (0.3-7.2)</td>
</tr>
<tr>
<td>Oestradiol (pmol/L)</td>
<td>n = 166</td>
<td>n = 87</td>
</tr>
<tr>
<td>5-9 **</td>
<td>48.1 (9.2-70.9)</td>
<td>25.5 (9.2-41.2)</td>
</tr>
<tr>
<td>9.1-11 ***</td>
<td>38.4 (9.2-75.6)</td>
<td>28.3 (9.2-45.6)</td>
</tr>
<tr>
<td>11.1-14</td>
<td>49.6 (9.2-122.9)</td>
<td>44.9 (9.2-106.4)</td>
</tr>
<tr>
<td>&gt;14</td>
<td>73.4 (9.2-143.7)</td>
<td>86.1 (44.1-112.5)</td>
</tr>
<tr>
<td>Testosterone (nmol/L)</td>
<td>n = 166</td>
<td>n = 88</td>
</tr>
<tr>
<td>5-9</td>
<td>0.05 (0.05-0.9)</td>
<td>0.05 (0.05-0.2)</td>
</tr>
<tr>
<td>9.1-11</td>
<td>0.07 (0.05-18.2)</td>
<td>0.05 (0.05-0.6)</td>
</tr>
<tr>
<td>11.1-14</td>
<td>1.8 (0.05-19.3)</td>
<td>1.35 (0.05-17.1)</td>
</tr>
<tr>
<td>&gt;14</td>
<td>8.0 (0.05-20.5)</td>
<td>10.7 (0.3-19.2)</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>n = 165</td>
<td>n = 86</td>
</tr>
</tbody>
</table>
### Reproductive hormones (units) per age category (years)

<table>
<thead>
<tr>
<th>Age Category (years)</th>
<th>Farm, median (IQR)†</th>
<th>Non-farm, median (IQR)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-9</td>
<td>128.5 (11.3-179.3)</td>
<td>124.6 (87.2-198.3)</td>
</tr>
<tr>
<td>9.1-11</td>
<td>105.1 (37.3-185.5)</td>
<td>120.2 (46.7-180.4)</td>
</tr>
<tr>
<td>11.1-14</td>
<td>80.5 (18.3-1131.6)</td>
<td>79.6 (28.5-197.1)</td>
</tr>
<tr>
<td>&gt;14</td>
<td>56.3 (36.1-157.1)</td>
<td>46.3 (21.4-164.3)</td>
</tr>
</tbody>
</table>

### Hormone levels outside of the normal range

<table>
<thead>
<tr>
<th>Hormone Levels</th>
<th>Farm n (%)</th>
<th>Non-farm n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High LH</td>
<td>0 (0.87)</td>
<td>0</td>
</tr>
<tr>
<td>Low LH</td>
<td>38 (25.00)</td>
<td>18 (20.93)</td>
</tr>
<tr>
<td>High FSH</td>
<td>15 (10.34)</td>
<td>5 (6.02)</td>
</tr>
<tr>
<td>Low FSH*</td>
<td>16 (10.96)</td>
<td>3 (3.70)</td>
</tr>
<tr>
<td>High testosterone</td>
<td>12 (11.76)</td>
<td>9 (18.75)</td>
</tr>
<tr>
<td>Low testosterone</td>
<td>53 (37.06)</td>
<td>37 (48.68)</td>
</tr>
<tr>
<td>High Oestradiol</td>
<td>1 (2.56)</td>
<td>0</td>
</tr>
<tr>
<td>Low Oestradiol</td>
<td>6 (13.64)</td>
<td>2 (10.00)</td>
</tr>
</tbody>
</table>

*p=0.015; **p=0.023; ***p=0.015; †IQR = interquartile range

### 3.3.6 Sexual health and anthropometric results

Farm boys > 14 years had lower BMIs (18.21) compared to non-farm boys (20.08). This difference was not statistically significant at the 5% level (Table 7). Farm boys were shorter than non-farm boys across all the age categories. The difference was statistically significant for the age category 5 to 9 years (123.05cm vs 131.00cm; p=0.04). For all the age categories, except the 9.1 to 11 years category, the farm boys were weighed less than the non-farm boys. The difference was statistically significant for the age category 5 to 9 years (23.00kg vs 25.00kg; p=0.03).
There was no statistically significant difference in the median Tanner Stage of the different age categories between the two groups (Table 7). The median age of boys > 14 years who had reached puberty (Tanner Stage = 5) was 14.8 years in the farm group, and 14.2 years in the non-farm group. The median age of boys at the other Tanner Stages were also not statistically significantly different. The median ages of the farm boys for the Tanner stages 1 to 3 were lower compared to the non-farm boys (Table 7). However, for stages 4 and 5, the median ages were higher in the farm compared to the non-farm boys.

On comparison of the median combined volume of the testes, there was no difference between the two groups for the age categories < 11 years. However, farm boys aged 11.1 to 14 years (18mL versus 27mL) and > 14 years had smaller median testicular volumes (35mL versus 43mL) when compared to the non-farm boys. These differences were not statistically significant at the 5% level.

Larger proportions of boys fell below the 25th and 50th percentile for age for BMI, weight and height (Table 7).
Table 7: Anthropometric and sexual reproductive health

<table>
<thead>
<tr>
<th>Variable</th>
<th>Farm, (n = 177), Median (IQR) †</th>
<th>Non-farm, (n = 92), Median (IQR) †</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age Group: 5-9 years</td>
<td>15.57 (14.19;16.81)</td>
<td>16 (13.99;17.09)</td>
</tr>
<tr>
<td>Age Group: 9.1-11 years</td>
<td>17.03 (15.98;18.11)</td>
<td>17 (15.23;19.87)</td>
</tr>
<tr>
<td>Age Group: 11.1-14 years</td>
<td>17.89 (17.23;19.12)</td>
<td>18.43 (17.12;20.45)</td>
</tr>
<tr>
<td>Age Group: &gt;14 years</td>
<td>18.21 (17.79;21.26)</td>
<td>20.08 (18.16;20.64)</td>
</tr>
<tr>
<td><strong>Height (cm)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age Group: 5-9 years*</td>
<td>123.05 (116.10;127.10)</td>
<td>131.00 (120.20;135.00)</td>
</tr>
<tr>
<td>Age Group: 9.1-11 years</td>
<td>132.05 (127.30;138.40)</td>
<td>134.30 (126.70;138.70)</td>
</tr>
<tr>
<td>Age Group: 11.1-14 years</td>
<td>145.50 (136.70;153.80)</td>
<td>147.30 (138.00;162.00)</td>
</tr>
<tr>
<td>Age Group: &gt;14 years</td>
<td>153.75 (145.85;163.10)</td>
<td>157.35 (152.40;165.00)</td>
</tr>
<tr>
<td><strong>Weight (kg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age Group: 5-9 years**</td>
<td>23.00 (20.00;26.00)</td>
<td>25.00 (24.00;28.00)</td>
</tr>
<tr>
<td>Age Group: 9.1-11 years</td>
<td>29.50 (27.00;34.00)</td>
<td>29.00 (28.00;35.00)</td>
</tr>
<tr>
<td>Age Group: 11.1-14 years</td>
<td>38.00 (34.00;45.00)</td>
<td>43.00 (33.00;52.00)</td>
</tr>
<tr>
<td>Age Group: &gt;14 years</td>
<td>45.00 (39.00;49.00)</td>
<td>50.00 (44.00;57.00)</td>
</tr>
<tr>
<td><strong>Tanner Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age Group: 5-9 years</td>
<td>1 (1;1)</td>
<td>1 (1;1)</td>
</tr>
<tr>
<td>Age Group: 9.1-11 years</td>
<td>1.5 (1;2)</td>
<td>1 (1;2)</td>
</tr>
<tr>
<td>Age Group: 11.1-14 years</td>
<td>2 (2;3)</td>
<td>3 (2;4)</td>
</tr>
<tr>
<td>Age Group: &gt; 14 years</td>
<td>3.5 (2.5;4)</td>
<td>4 (3.5;4)</td>
</tr>
<tr>
<td><strong>Age at Tanner Stage (years)</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Variable                  | Farm,  
(n = 177), Median (IQR)† | Non-farm,  
(n = 92), Median (IQR)† |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1 (n=107)</td>
<td>9.25 (8.75;10.50)</td>
<td>9.33 (9.00;11.33)</td>
</tr>
<tr>
<td>Stage 2 (n=78)</td>
<td>11.38 (10.42;12.67)</td>
<td>12.29 (9.92;12.92)</td>
</tr>
<tr>
<td>Stage 3 (n=39)</td>
<td>12.75 (12.42;13.83)</td>
<td>13.33 (13.00;13.92)</td>
</tr>
<tr>
<td>Stage 4 (n=36)</td>
<td>14.00 (13.50;14.92)</td>
<td>13.33 (12.83;15.00)</td>
</tr>
<tr>
<td>Stage 5 (n=8)</td>
<td>14.84 (13.21;15.58)</td>
<td>14.21 (12.88;15.38)</td>
</tr>
<tr>
<td>Sum of testes (mL),</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age Group: 5-9 years</td>
<td>5 (5;7)</td>
<td>5 (5;7)</td>
</tr>
<tr>
<td>Age Group: 9.1-11 years</td>
<td>7 (5;9)</td>
<td>7 (5;9)</td>
</tr>
<tr>
<td>Age Group: 11.1-14 years</td>
<td>18 (11;35)</td>
<td>27 (9;35)</td>
</tr>
<tr>
<td>Age Group: &gt;14 years</td>
<td>35 (19;43)</td>
<td>43 (35;45)</td>
</tr>
<tr>
<td>Height, weight and BMI &lt; 25th &amp; 50th percentile</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Body Mass Index,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50th percentile for age</td>
<td>68 (41.30)</td>
<td>38 (38.64)</td>
</tr>
<tr>
<td>≤25th percentile for age</td>
<td>38 (41.30)</td>
<td>68 (39.64)</td>
</tr>
<tr>
<td>Height,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50th percentile for age</td>
<td>97 (54.80)</td>
<td>37 (40.22)</td>
</tr>
<tr>
<td>≤25th percentile for age</td>
<td>53 (29.94)</td>
<td>15 (16.30)</td>
</tr>
<tr>
<td>Weight,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50th percentile for age</td>
<td>95 (53.67)</td>
<td>41 (44.57)</td>
</tr>
<tr>
<td>≤25th percentile for age</td>
<td>55 (31.07)</td>
<td>18 (19.57)</td>
</tr>
</tbody>
</table>

* p=0.04; ** p=0.03; † Interquartile range
3.3.7 Associations between exposure and reproductive health outcomes

Table 8 presents a summary of the results of the linear regression analysis which explores the association between lifetime residence on a farm and the various reproductive, sexual and anthropometric outcomes, whilst controlling for confounding.

FSH and oestradiol levels were 0.634 IU/L (95% CI: 0.186;1.081) and 8.074 pmol/L (95% CI: 2.339;13.808) higher and testosterone and LH levels were 0.346 nmol/L (95%CI: -1.123;0.436) and 0.284 IU/L (95%CI: -0.483;-0.084) lower, respectively, amongst the farm boys compared to the non-farm boys when adjusting for confounding. All these associations were statistically significant (p < 0.05) apart from the association between testosterone and farm residence. The results in Table 8 were supported by linear regression analysis excluding other hormones as confounders and also by logistic regression analysis whereby hormonal outcomes were dichotomized at the 25th, 50th and 75th percentile (Appendix 10).

The boys in the farm group were 3.417 cm shorter and weighed 2.258 kg less than boys in the non-farm group when adjusted for confounding. The association between exposure and height was statistically significant (95%CI:-6.382;-0.453) as was the association between the exposure and weight (95%CI:-4.441;-0.753). The association between exposure and BMI was not statistically significant. The results in Table 8 were supported by logistic regression
analysis whereby the anthropometric outcomes were dichotomized at the 25th, 50th and 75th percentile (Appendix 10).

The farm boys had 1.745 ml smaller testes and lower SMR (β = - 0.066) than non-farm boys when adjusted for confounding (Table 8). These effects were not statistically significant. The results reported in this paragraph were supported by logistic regression analysis whereby the outcomes were dichotomized at the 25th, 50th and 75th percentile (Appendix 10). The association between the exposure and the various outcomes could not be explored per age-group at multivariate level due to low sample sizes within the strata. Excluding the 19 boys who had lived on both farms and towns from the analysis slightly reduced the precision due to a decrease in sample size but not the direction of the associations.
Table 8: Association between pesticide exposure (lifetime residence on a farm) and reproductive health outcomes using linear regression modelling

<table>
<thead>
<tr>
<th>Outcomes</th>
<th>n</th>
<th>B-coefficient (SE)</th>
<th>p-value (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH*</td>
<td>242</td>
<td>0.634 (0.230)</td>
<td>0.006 (0.186;1.081)</td>
</tr>
<tr>
<td>Testosterone*</td>
<td>242</td>
<td>-0.346 (0.397)</td>
<td>0.385 (-1.123;0.436)</td>
</tr>
<tr>
<td>Oestradiol*</td>
<td>242</td>
<td>8.074 (2.911)</td>
<td>0.006 (2.339;13.808)</td>
</tr>
<tr>
<td>LH*</td>
<td>242</td>
<td>-0.284 (0.101)</td>
<td>0.006 (-0.483;-0.084)</td>
</tr>
<tr>
<td>Testicular size **(ml)</td>
<td>234</td>
<td>-1.745 (1.270)</td>
<td>0.171 (-4.248;0.758)</td>
</tr>
<tr>
<td>Height **(in centimeters)</td>
<td>187</td>
<td>-3.417 (1.505)</td>
<td>0.024 (-6.382;-0.453)</td>
</tr>
<tr>
<td>Weight **(in kilograms)</td>
<td>187</td>
<td>-2.258 (1.108)</td>
<td>0.043 (-4.441;-0.753)</td>
</tr>
<tr>
<td>BMI**</td>
<td>187</td>
<td>-0.241 (0.379)</td>
<td>0.525 (-0.988;0.505)</td>
</tr>
<tr>
<td>Tanner Stage **(1-5)</td>
<td>242</td>
<td>-0.066 (0.112)</td>
<td>0.560 (-0.267;1.556)</td>
</tr>
</tbody>
</table>

shbg = sex hormone binding globulin; * Adjusted for shbg, age, income and each of the other reproductive hormones;

** Adjusted for shbg, age, income
3.4 Discussion

In this study, we used a history of farm residence as a proxy for pesticide exposure. We observed associations between serum reproductive hormone levels and exposure. The difference in hormonal levels measured amongst farm boys compared to non-farm boys (Table 7) is consistent with the hypothesis that environmental exposure to hormonally active contemporary agricultural pesticides disrupts the male hypothalamus-pituitary-gonadal (HPG) axis of pubertal boys. Laboratory studies suggest that pesticides administered during embryonic, neonatal or lactation developmental periods result in permanent testicular developmental changes, whereas pesticides administered during puberty may not result in testicular development changes but may affect male reproductive hormone levels during puberty. The effects could be due to faster conversion of testosterone to oestradiol leading to inhibition of LH, or it could be due to reduced spermatogenesis due to less testosterone, which results in less inhibin B and therefore to weaker inhibition of FSH.

A possible explanation for higher FSH and oestradiol levels and lower LH and testosterone levels amongst exposed boys, is that exposure to a number of different pesticides may result in a mixture of oestrogenic, androgenic and other endocrine disrupting effects on the HPG axis. For instance, chlorpyrifos, cypermethrin, endosulfan, deltamethrin, dichlorfos, fenvelerate and prochloraz, all detected in environmental media and residents of the rural Western Cape, have been shown to be hormonally active and have been shown to decrease one, two or all of LH, FSH or testosterone in laboratory animals and or humans.
Additionally, fenvalerate has been shown to increase serum LH, FSH as well as decrease serum testosterone in male rats.\textsuperscript{33} \textsuperscript{34} \textsuperscript{35} \textsuperscript{36} \textsuperscript{37} Alterations in serum LH, FSH and testosterone as well as serum oestradiol have also previously been found as a result of other endocrine disrupting substances such as DDT and PCB in laboratory studies and in humans.\textsuperscript{2} \textsuperscript{63} The non-significant effect on testosterone could be due to a lack of statistical power. Although the number of boys (n = 242) whose testosterone levels were included in multivariate analysis (Table 8) was more than that determined by sample size calculations, the testosterone levels in this study were more variable than those in a similar cross-sectional study (average values were not presented in an Indian study but the range was 0.05 to 9.00nmol/L compared to 0.05 to 20.00nmol/L for this study).\textsuperscript{39} Indian pubertal boys (mean age = 12.80 ± 2.07, n = 117) environmentally exposed to aerial spraying of endosulfan and with high serum endosulfan levels (mean = 7.47 ± 1.19 ppb) had lower SMR and lower serum testosterone levels compared to boys of a similar age in a control group (n = 90).

The lower height and weight measurements recorded amongst farm boys are also consistent with the hypothesis, as an alteration of gonadotrophin-releasing hormone (GnRH) release by the hypothalamus due to exposure to hormonally-active pesticides could have impacted on pubertal growth.\textsuperscript{64} \textsuperscript{65} \textsuperscript{66} A difference in nutritional status between farm and non-farm boys could have accounted for lower anthropometric measurements found in the former group. However, the two groups were recruited from neighbouring areas and household income which is an indicator of socio-economic status and a strong determinant of nutritional status, were low in both groups and these were controlled for in the analysis.\textsuperscript{59} No studies investigating the effect of contemporary pesticides on pubertal growth were
found in the literature, but there is laboratory and epidemiological evidence of reduced height measurements amongst DDT exposed boys.\textsuperscript{67}

The lower testicular sizes recorded amongst farm boys is also consistent with the hypothesis that a disruption of the HPG axis in these boys could have affected their reproductive organ development. The fact that this result was non-significant could be due to a lack of statistical power. No sample size calculations were calculated prior to the study for the effect of pesticides on testicular volume, but sample size calculations using the data in this study indicated that 402 boys (268 exposed, 134 unexposed) had to participate for the effect to be statistically significant. Our results do not show a statistically significant exposure effect on Tanner Stage scores between the two groups, although for the higher Tanner stages there was a slightly lower score for exposed boys. The lower testicular sizes recorded in exposed boys is therefore consistent with the literature, but not the effect on strength of the observed Tanner Stage scores especially when compared to the Indian study.\textsuperscript{39} Although we combined the scores for penis, testicular and pubic hair development, this could not have reduced the exposure effect in our study as all three scores were strongly reduced in the Indian study. A possible explanation is that the boys in our study were less exposed to anti-androgenic pesticides such as endosulfan. Mean endosulfan levels (0.53 ± 0.049 ppb) measured recently amongst adult farm workers in one of the study areas,\textsuperscript{68} during the off-season and on the first day of spraying was an order of magnitude less than that measured amongst exposed boys in the Indian study. However, chlorpyrifos levels measured amongst the farm workers recently were generally higher in comparison to those found in other settings.\textsuperscript{47}
Another possible explanation for not finding an exposure effect on SMR is that exposed boys might not have been exposed to pesticides during critical periods of development.\textsuperscript{69} Regarding the latter argument, the intensity of pesticide exposure that the farm boys experienced at critical periods of development might have affected the strength of the associations observed with SMR as well as the associations with the other outcomes. However, even if pesticides exert reproductive effects only when exposure occurs at an early stage of development, the impact at different stages of development might differ depending on the intensity, duration and timing of the exposure.\textsuperscript{69} Unfortunately this could not be explored in the study as the sample sizes for the different age-groups were not adequate. There might also be other factors in the boy’s environment such as diet and exposure other endocrine disruptors that could have diluted the effect of pesticides on SMR.

Potential limitations in the study were that parental reports of farm residence were used to determine pesticide exposure and therefore exposure misclassification could have affected the findings in the study. However, most boys (94%) had lived in one location throughout their lives or only on a farm or in a town. Sensitivity analysis excluding the boys who lived both on a farm and in a town did not change the findings. Exposure misclassification would, however, have biased the results towards the null. Exposure misclassification could also have occurred due to boys who did not reside on farms having been environmentally exposed via drinking contaminated water, food or pesticide drift. However, the level of exposure is not likely to be as high as for the farm boys. For instance, previous studies have
shown that drinking from unprotected sources is prevalent mostly on farms. An important bias is the non-selection of boys living on farms who did not attend school. These boys may have been more exposed than those who attended school. This would underestimate the strength of the association. Recall bias could also have been a factor as mother’s had to remember details of the boys’ childhoods and of their pregnancies. In the event that the mother was not present, the third party could have given incorrect information. Furthermore, measurement bias may have been introduced during the physical examination of the boy and during laboratory analysis stages. However, training and other quality control measures were introduced to reduce these biases. Another possible limitation in the study is a collection of a single hormone sample which might have been affected by the diurnal variation of sex hormone secretion. However, all samples were collected before 13h00 in order to minimize this effect. Furthermore, inclusion of all the hormones as confounders in the model could have led to over-adjustment.

The biggest limitation in this sub-study is the absence of biomarker marker data for exposure. However, the use of the dichotomous exposure index in the study is justified by the fact that most boys lived in one location throughout their lives and the fact that the findings did not change when excluding those who lived both on a farm and in a town. Farm boys were also found to be highly exposed as indicated by exposure data in Table 5 and by the results of previous exposure studies conducted in the Western Cape discussed earlier. The high prevalence of household pesticide use in the homes of the farm and non-farm groups (61.0% vs 47.8%, respectively) provides further evidence, consistent with that of previous studies in the Western Cape, that household pesticide use is an important route
of pesticide exposure that requires further investigation. Further analysis whereby the exposure data will be used to construct environmental exposure indices and pesticide analysis is currently underway. A longitudinal design would likely strengthen the measurement of the exposure-outcome relationships due to large individual variations associated with the study outcomes. A follow-up study of the boys is intended.

3.5 Conclusions

Our study provides evidence that environmental exposure to hormonally active contemporary agricultural pesticides may adversely affect the reproductive health of pubertal boys. A follow-up of the boys as well as a bigger study with more boys in the different age categories is required to support this finding. We recommend initiatives to change knowledge, attitudes and practices through the education of farmers, farm workers and other rural residents about the harmful effects of pesticides and the need to reduce exposures.
3.6 References


41 Meeker JD, Ryan L, Barr DB, Hauser R. Exposure to nonpersistent insecticides and male reproductive hormones. Epidemiology. 2006;17(1):61-68.
48 London L, Dalvie MA, Cairncross E, Solomon A. The quality of surface and groundwater in the rural Western Cape with regard to pesticides. WRC Report No: 795/1/00. 2000. WRC: Pretoria


PART D: APPENDICES
Appendix 1: Location of study areas for pesticide sampling in the Western Cape, South Africa
Appendix 2: Study questionnaire
Appendix 3: Structured record form: physical examination)
Appendix 4: Photographs showing Tanner stages
Appendix 5: Ethics letter
Appendix 6: Consent Form
Appendix 7: Schemata of reproductive health systems
Appendix 8: Tanner stages
Appendix 9: Instructions to Authors
Appendix 10: Regression analysis for main outcomes in the study
Appendix 1

Study Areas

Western Cape

Atlantic Ocean

Cape Town

Piketberg

Hex River

Grabouw

Indian Ocean

N

W

E

S

Western Cape

Study Areas

Cape Town

National Roads

Western Cape

300

0

300

600 Kilometers
Table 1: Table listing linear regression analysis for models, excluding the hormones as variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>0.412</th>
<th>0.119</th>
<th>-0.107;0.931</th>
<th>Hormones excluded from models.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>-0.742</td>
<td>0.200</td>
<td>-1.878;0.394</td>
<td></td>
</tr>
<tr>
<td>Oestradiol</td>
<td>5.769</td>
<td>0.103</td>
<td>-1.165;12.704</td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td>-0.249</td>
<td>0.090</td>
<td>-0.537;0.039</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Table listing the results of logistic regression analysis, per percentile cut-offs

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cut-off’s (percentiles) or Stages</th>
<th>OR</th>
<th>p-value</th>
<th>95%CI</th>
<th>Variables included in the model</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>25th</td>
<td>0.664</td>
<td>0.285</td>
<td>0.314;1.406</td>
<td>Age, income, shbg (latter included on bivariate testing)</td>
</tr>
<tr>
<td></td>
<td>50th</td>
<td>1.299</td>
<td>0.431</td>
<td>0.678;2.489</td>
<td></td>
</tr>
<tr>
<td></td>
<td>75th</td>
<td>1.901</td>
<td>0.072</td>
<td>0.944;3.829</td>
<td></td>
</tr>
<tr>
<td>Weight</td>
<td>25th</td>
<td>1.501</td>
<td>0.348</td>
<td>0.643;3.503</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50th</td>
<td>1.196</td>
<td>0.674</td>
<td>0.519;2.755</td>
<td></td>
</tr>
<tr>
<td></td>
<td>75th</td>
<td>3.111</td>
<td>0.066</td>
<td>0.928;10.429</td>
<td></td>
</tr>
<tr>
<td>Height</td>
<td>25th</td>
<td>1.950</td>
<td>0.129</td>
<td>0.129;4.614</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50th</td>
<td>1.528</td>
<td>0.259</td>
<td>0.732;3.949</td>
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</tr>
<tr>
<td></td>
<td>75th</td>
<td>1.475</td>
<td>0.440</td>
<td>0.655;3.949</td>
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</tr>
<tr>
<td>Tanner stage</td>
<td>25th</td>
<td>0.785</td>
<td>0.555</td>
<td>0.352;1.751</td>
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</tr>
<tr>
<td></td>
<td>50th</td>
<td>1.648</td>
<td>0.448</td>
<td>0.453;5.992</td>
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</tr>
<tr>
<td></td>
<td>75th</td>
<td>3.37</td>
<td>0.067</td>
<td>0.919;12.391</td>
<td></td>
</tr>
<tr>
<td>Stage 1</td>
<td>0.785</td>
<td>0.555</td>
<td>0.352;1.751</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td>1.474</td>
<td>0.243</td>
<td>0.769;2.824</td>
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</tr>
<tr>
<td>Stage 3</td>
<td>2.266</td>
<td>0.095</td>
<td>0.868;5.912</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 4</td>
<td>0.410</td>
<td>0.135</td>
<td>0.123;1.318</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 5</td>
<td>1.012</td>
<td>0.990</td>
<td>0.156;6.554</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td>25th</td>
<td>0.762</td>
<td>0.601</td>
<td>0.276;2.109</td>
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<td></td>
<td>50th</td>
<td>0.308</td>
<td>0.014</td>
<td>0.121;0.788</td>
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<td></td>
<td>75th</td>
<td>0.320</td>
<td>0.035</td>
<td>0.111;0.924</td>
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<tr>
<td>Testosterone</td>
<td>25th</td>
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<td>-</td>
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<td></td>
<td>50th</td>
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<td></td>
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<td>1.022</td>
<td>0.976</td>
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<td>0.375</td>
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<td></td>
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<td></td>
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<td>0.255</td>
<td>0.034</td>
<td>0.072;0.902</td>
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<tr>
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<td>-</td>
<td></td>
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<td>0.042</td>
<td>1.051;14.698</td>
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<td></td>
<td>75th</td>
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<td>0.972</td>
<td>0.216;4.912</td>
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<td>0.675</td>
<td>0.410;1.781</td>
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<td></td>
<td>50th</td>
<td>0.798</td>
<td>0.570</td>
<td>0.366;1.738</td>
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<td></td>
<td>75th</td>
<td>1.554</td>
<td>0.250</td>
<td>0.733;3.292</td>
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</tr>
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<td>0.386</td>
<td>0.284;1.626</td>
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<td>50th</td>
<td>0.417</td>
<td>0.026</td>
<td>0.193;0.902</td>
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<tr>
<td></td>
<td>75th</td>
<td>0.521</td>
<td>0.128</td>
<td>0.225;1.205</td>
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</tr>
<tr>
<td>Testosterone</td>
<td>25th</td>
<td>n too small</td>
<td>-</td>
<td>-</td>
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</table>

Hormones included in models.
<table>
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<tr>
<th>Variable</th>
<th>Cut-off’s (percentiles) or Stages</th>
<th>OR</th>
<th>p-value</th>
<th>95%CI</th>
<th>Variables included in the model</th>
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<td>0.684</td>
<td>0.340</td>
<td>0.313;1.94</td>
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<tr>
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<td>75&lt;sup&gt;th&lt;/sup&gt;</td>
<td>1.547</td>
<td>0.405</td>
<td>0.554;4.323</td>
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<td>Oestradiol</td>
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<td>0.567</td>
<td>0.108</td>
<td>0.284;1.333</td>
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<td>0.473</td>
<td>0.030</td>
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<td>0.324</td>
<td>0.303;1.484</td>
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</tr>
<tr>
<td>LH</td>
<td>25&lt;sup&gt;th&lt;/sup&gt;</td>
<td>n too small</td>
<td>-</td>
<td>-</td>
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<td></td>
<td>50&lt;sup&gt;th&lt;/sup&gt;</td>
<td>0.916</td>
<td>0.839</td>
<td>0.392;2.140</td>
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<tr>
<td></td>
<td>75&lt;sup&gt;th&lt;/sup&gt;</td>
<td>1.077</td>
<td>0.873</td>
<td>0.433;2.676</td>
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</tr>
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</table>
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 ________________________

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 girl friend)

Details of son:

Date of birth _____________   Age (____)

Gender: ____ (Male/Female)

Birth weight:__________(kg)

Current standard/grade at school: ________
Appendix 2

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:

<table>
<thead>
<tr>
<th>Disease</th>
<th>Yes, No, Don’t Know</th>
<th>Year Diagnosed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB</td>
<td></td>
<td></td>
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<tr>
<td>Fits</td>
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<tr>
<td>High Blood Pressure</td>
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<td>Asthma</td>
<td></td>
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<tr>
<td>Heart Problems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Back Problems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foetal Alcohol Syndrome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other Specify:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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
Appendix 2

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

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

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

(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
Appendix 2

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? ______________

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)
Appendix 2

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? ____________

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)
Appendix 2

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? _____________

CA4 If his home was located on a farm, how far from the house was the nearest
vineyard/field? ________ (meters)
Appendix 2

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 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)

CA12 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?
Appendix 2

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? ______________

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)

CA12 Did your son help on the farm? ______ (yes, no)

If Yes,
Appendix 2

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? ______________
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)

D. HOUSEHOLD PESTICIDE EXPOSURE

D1 Do you use any pesticides in your garden or in your home (eg 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 _____________________
Appendix 2

(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? __________

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)
Appendix 2

G2 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)

G3 Does your take drugs or smoke dagga currently or before?
   _____ (yes, no)
   If yes, for how long? _________ (number of years)
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: (N = normal, S = soft, H = hard)

Testes abnormality: (N = no, Y = yes)

OTHER REMARKS
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
12 February 2008

REC REF: 279/2005

Dr MA Dalvie
Public Health & Family Medicine

Dear Dr Dalvie

PROJECT TITLE: MALE REPRODUCTIVE HEALTH EFFECTS DUE TO PESTICIDES AMONG FARM RESIDENTS IN THE WESTERN CAPE

Thank you for your letter to the Research Ethics Committee dated 01 February 2008.

1. It is a pleasure to inform you that the Ethics Committee has approved the change in the study title. However, this will have to be reflected in a revised consent form which must carry the new title.
2. Please could you provide an annual progress report—has any enrolment taken place since the study was approved in 2005?
3. The amended protocol is approved.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS
12 February 2008

REC REF: 279/2005

Dr MA Dalvie
Public Health & Family Medicine

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Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS
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 (MBChB, Honours, MD)
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.

**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.

**Physical examination:** A doctor will assess your son’s reproductive health.

**Risks and discomforts of the research**

**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.

**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 personnel. All reports will present aggregate data in which individuals will not be identifiable.

**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
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.

**Costs to you resulting form participation in the study**

The study is offered at no cost to you.

**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.

**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.

**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.
Appendix 6

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

____________________________  _______________________
Interviewers (print) signature

Date

Witness (print) signature Date

Date:__________________________________________

Study Number _______________________________
A. Male and female reproductive systems

Source: http://www.cliffsnotes.com/study_guide/The-Male-Reproductive-System.topicArticleId-22032,articleId-22026.html
B. Hypothalamus-pituitary-gonadal axis

![Diagram of the hypothalamus-pituitary-gonadal axis](http://dels-ld.nas.edu/ilar_n/ilarjournal/45_4/graphics/45_4_471f1.jpg)

Source: http://dels-ld.nas.edu/ilar_n/ilarjournal/45_4/graphics/45_4_471f1.jpg
Figure 1: Tanner stages - male

Source: http://www.childclinic.net/pain/tanner_puberty_boys_img.gif
Environmental Health Perspectives (EHP) is a monthly open access journal of peer-reviewed research and news dedicated to the discussion of the impact of the environment on human health. All scientific articles are evaluated for scientific quality, environmental significance, appropriate degree of speculation, clarity of presentation, and conciseness. One of the overarching goals of the journal is to strive for objectivity and balance in the presentation of information.

Although EHP is sponsored by the National Institute of Environmental Health Sciences (NIEHS), its editorial policies are independent of the institute. An Advisory Board consisting of former EHP editors and non-NIEHS scientists provides guidance for journal policies and operations. Papers submitted to the journal are processed by a Board of Associate Editors. Members of the Editorial Review Board serve as a pool of potential reviewers of papers. Both the Board of Associate Editors and the Editorial Review Board are composed of leading scientists from all segments of the environmental health sciences.

EHP is the leading journal in the environmental health sciences. All papers are subjected to a preliminary screening to determine relevance, environmental significance, and creativity. In 2007, approximately 60% of papers were returned to authors without further review. The overall acceptance rate of papers submitted to the journal is approximately 20–25%.

In 2003, EHP became an open-access journal. All news and research articles published in EHP since 1972 are available free on-line (http://www.ehponline.org/). EHP is committed to promoting the discussion and exchange of information internationally, as described in detail at http://www.ehponline.org/international/.

What We Publish

The environmental health sciences include many fields of study and increasingly comprise a multidisciplinary research area. EHP publishes articles from a wide range of scientific disciplines encompassing basic research, human studies, and in vitro and in vivo animal research with a clear relationship to human health effects. Studies dealing with climate change, ecological issues, or effects on wildlife populations are welcome, but the relevance of the findings to human health should be evident. Physicians and others working in environmental medicine are encouraged to submit case reports for publication. EHP also addresses ethical, legal, social, and policy issues related to public health.

EHP provides information on emerging environmental issues through its News and Book Review sections. Although EHP welcomes ideas for News articles, Book Reviews, and Editorials, the journal typically does not accept unsolicited manuscripts of these types. Please contact the Editor-in-Chief for further information.

About Your Manuscript

Types of Manuscripts

Manuscripts in any one of the categories below are welcome. See “Manuscript Preparation” for additional details.

Correspondence (letters to the editor; ≤ 1,000 words) should address research or news articles published in the print version of the journal within the previous 6 months. Authors cited in the correspondence will be given the opportunity to respond. Correspondence may include a brief table or small figure, if it is essential to the discussion. It is permissible to include data from or re-directing of previously published materials as long as the work is cited and written permission from the authors and/or publishers has been granted for re-publication in both printed and electronic form. New data should not be included, but authors may recalculate or re-analyze data from a cited paper in support of their point(s). Correspondence is not peer-reviewed and is published at the discretion of the EHP editors. Conclusions and opinions expressed do not necessarily reflect the policies of EHP.

Research articles (≤ 7,000 words) are original manuscripts reporting scientific research and discovery in the broad field of the environmental health sciences. Original research articles may come from any field of scientific research, from the most basic molecular biology and biochemistry to atmospheric physics, ecology, and engineering, as well as related fields of social science, policy, and ethics. Manuscripts on ethical, legal, social, or policy issues may also be accepted in this category. Research articles are peer reviewed.

Commentaries (≤ 5,000 words) present information and personal insight on a particular topic. Commentaries should not be extended critiques of single articles appearing in EHP or elsewhere. Factual data should be included to substantiate arguments. Commentaries are peer reviewed.

Reviews (≤ 10,000 words) that emphasize recent developments in a particular field of research are highly desirable. Lengthy historical perspectives are not appropriate.

Meeting Reports (≤ 5,000 words) are intended to provide an overview of outcomes of conferences, symposia, or workshops. Authors should submit reports that review the state of the science for a particular area, identify research gaps and needs, and explain how the outcome of the conference addresses those gaps and needs. Meeting reports may review existing information, summarize research findings on specific topics, and recommend methods, courses of action, or other further research needs for the scientific community. De novo data, participant lists, dialogue of workgroups or committees, and discussion of the internal organization of the meeting are not allowed. Meeting Reports must be submitted to EHP no later than 9 months after the events they describe. Prospective authors should consult with the Editor-in-Chief before submitting a meeting report.

Grand Rounds articles (≤ 6,000 words) present discussions of case presentations of patients or community health issues with a clearly established link of relevance to environmental exposures and environmental health. The format requires that a case scenario be presented to illustrate the environmental issues under consideration, followed by a discussion of the clinical and public health implications of these issues. Articles should be divided into an Abstract, Case Presentation (≤ 5,000 words), Discussion, and Conclusion. Visual images (e.g., X rays, microscopic pathology) or other graphics are encouraged.

Case Reports (≤ 6,000 words) differ from Grand Rounds articles in that the diagnosis pertaining to the clinical presentation is not necessarily conclusive. Instead, evidence for an environmental etiology may be indirect—for example, a case report of hepatitis suspected to be related to a chemical that has not been previously linked with hepatitis. Similar to Grand Rounds, Case Reports should include an Abstract, Case Presentation (≤ 5,000 words), Discussion, and Conclusion. Visual images (e.g., X rays, microscopic pathology) or other graphics are encouraged.

Article Length

All word limits include tables, figures, and references. Manuscripts that do not conform to the following word limits will be returned to the author(s) for revision before the review process is initiated.

Correspondence: 1,000 words

Commentaries: 5,000 words

Reviews: 10,000 words

Research articles: 7,000 words

Meeting Reports: 5,000 words

Grand Rounds articles: 6,000 words

Case Reports: 6,000 words.

Authors should assume that each figure accounts for 250 words of the total word count. Depending on the topic and potential impact of a paper, the Editor-in-Chief reserves the right to waive word limits.

Originality of Submission

Contributions submitted to EHP must be original works of the author(s) and must not have been previously published (in print or online) or simultaneously submitted to another publication.

Scientific Integrity

EHP requires assurances that animals used in a study have been treated humanely and with regard for the alleviation of suffering. Research involving humans must have been conducted according to the Common Rule (http://ori.dhhs.gov/education/products/ucla/chapter22/page04b.htm). Research involving humans must also be approved by an appropriate institutional review board and comply with all relevant national, state, and local regulations. For research conducted outside the United States and thus exempt from U.S. federal regulations, authors must perform the research in accordance with principles of the Declaration of Helsinki (http://www.wma.net/e/policy/b3.htm).
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**PUBLIC DATABASES**

Manuscripts using microarrays must follow the Minimum Information About a Microarray Experiment (MIAME) guidelines developed by the Microarray Gene Expression Data Society (http://www.mged.org/miame). On acceptance, all integral data supporting the article’s conclusions should be submitted to the Array/Express (http://www.ebi.ac.uk/arrayexpress) or GEO (http://www.ncbi.nlm.nih.gov/geo/) databases.

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- All potential competing interests occurring during the last 3 years should be reported.
- As a condition of review and publication, authors must certify that their freedom to design, conduct, interpret, and publish research is not compromised by any controlling sponsor.
- Although not necessarily an issue for authors, editors and reviewers should disclose to the Editor-in-Chief any competing financial and nonfinancial interests that occurred within the last 3 years. Competing nonfinancial interests include former or current mentor–student relationships, faculty appointments in the same department or organization, familial relationships, service on advisory boards that oversee the research under review, collaborations, or membership in organizations that hold ideological views that are contradictory to the theme or topic under review.

For each manuscript, authors must submit a declaration of competing interests on behalf of all authors. The form is available online (http://ehp.niehs.nih.gov/cfi.pdf). A statement of disclosure must be included in the Acknowledgments section of the manuscript submitted to the journal. If a paper is accepted, a brief disclaimer describing the competing interest or a declaration of no competing interest will be published. Authors of correspondence, editors, and book reviews will also be required to submit a declaration of competing interests.

Disclosure of competing interests does not imply that the information in the article is questionable or that conclusions are biased. Decisions to publish or reject an article will not be based solely on a declaration of a competing interest.

*EHP* relies on the integrity of all authors to provide accurate disclosure statements. However, authors can expect scrutiny of their statements by the editors, reviewers, and readership. Alleged inaccuracies of declared competing interests should be addressed to the Editor-in-Chief.

- **EHP** will impose a 3-year ban on publication by any authors found to have willfully failed to disclose a competing interest. Implementation of the ban will be made in consultation with the EHP Advisory Board.
- At the discretion of the Advisory Board, a paper may also be retracted or an Expression of Concern published and appended to the online version of the article.

**MANUSCRIPT PREPARATION**

**PARTS OF A MANUSCRIPT**

Manuscripts should include the following sections in this order:

- **Author names**
- **Author affiliations**
- **Name and address of corresponding author**
- **Acknowledgments/grant support**
- **Disclaimers/Competing Interests Declaration**
- **Short running head**
- **Key words**
- **Abbreviations**
- **Outline of manuscript section headers**
- **Abstract**
- **Introduction**
- **Materials and Methods**
- **Results**
- **Discussion**
- **Conclusions**
- **References**
- **Tables**
- **Figure legends**
- **Figures**
- **Supplemental material**

All manuscripts must be submitted to *EHP* in English. Manuscript pages must be numbered consecutively, beginning with the title page, and lines should be numbered in the original submission. Standard font types (e.g., Helvetica, Times New Roman) and font size (12 point) should be used, and all parts of the manuscript, except tables, should be double-spaced. The reference list, tables, and figure legends should each begin on separate pages.

**Page 1:** The first page should include
- **a)** manuscript title,
- **b)** authors (first or second names spelled out in full),
- **c)** full address of the institution where the work was performed, and
- **d)** affiliation of each author. Titles should not exceed 20 words and should generally not contain abbreviations or numerical values. Indicate the author to whom page proofs should be sent, and include complete address for express mail service, telephone and fax numbers, and e-mail address.

**Page 2:** The second page should include
- **a)** a running title, not to exceed 50 characters and spaces;
- **b)** 5–10 key words for indexing purposes;
- **c)** acknowledgments and grant information, not to exceed 50 words;
- **d)** competing interests declaration; and
- **e)** a list of all abbreviations and definitions used in the manuscript. Nomenclature and symbols should conform to the recommendations of the American Chemical Society or the International Union of Pure and Applied Chemistry (http://www.iupac.org).

**Page 3:** Authors should provide an outline of section headers (i.e., Abstract, Introduction, Methods, etc.) to facilitate copyediting and manuscript layout.

**Page 4:** All articles must include a structured abstract, which is not to exceed 250 words and should not contain references. No information should be reported in the abstract that does not appear in the text of the manuscript. Headings to be used in the structured abstracts vary by article type.

- **Commentaries:** Background, Objectives, Discussions, and Conclusions
- **Reviews:** Objective, Data Sources, Data Extraction, Data Synthesis, and Conclusions
- **Research articles:** Background, Objectives, Methods, Results, and Conclusions (with an exception for Environmental Medicine articles: Objective, Design, Participants, Evaluations/Measurements, Results, Conclusions, and Relevance to Clinical or Professional Practice)
- **Grand Rounds Articles or Case Reports:** Context (the relevance to environmental exposures and environmental health), Case Presentation, Discussion, and Relevance to Clinical or Professional Practice

**Page 5 and subsequent pages:** Text should begin on the fifth page. Concise headings (not to exceed 8 words each) may be used to designate major sections under the headings “Materials and Methods,” “Results,” “Discussion,” and “Conclusions.” Do not include tables and figures in the text; place tables after the References section and upload figures individually.

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Journals: Writing and Editing for Biomedical Publication (http://www.icmje.org/)
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A cover letter should accompany the manuscript and include the following points:
• Assurances that the manuscript a) is an original work, b) has not been previously published whole or in part, and c) is not under consideration for publication elsewhere.
• A statement that animals used in research have been treated humanely according to institutional guidelines, with due consideration to the alleviation of distress and discomfort. The identity of the source of those guidelines must be provided.
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• Confirmation that all authors have disclosed any potential competing interests regarding the submitted article and the nature of those interests (required Competing Interest Declaration form available at http://ehp.niehs.nih.gov/cfi.pdf).
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• Information on the software programs used, file names, and the number of tables and figures for each submission (see “Files and Formats” for electronic file requirements). Inquiries may be made by e-mail (EHPmanuscripts@niehs.nih.gov), telephone (919-316-4544), or fax (919-541-0273).
• If applicable, a request to consider the submission for inclusion in the Children’s Health section of the journal.

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PREPARING TABLES AND FIGURES
Tables. Each table must begin on a separate page. Tables should be numbered with Arabic numerals, followed by a brief title (not to exceed 25 words). When setting up tables, do not use table layout format; type tables as text and use tabs to align the columns. Tables should contain no more than three layers of column headings, and the entire table should fit on one journal page or less. Tables that are more than one page may be published online as Supplemental Material. A column heading must be provided for each column. Rather than placing additional column heads in the middle of a table, a new table should be created. List abbreviations and definitions under each table. General footnotes to tables should be indicated by lowercase superscript letters beginning with “a” for each table. Footnotes indicating statistical significance should be identified in the following order: asterisks (*, **), number signs (+, ++), and daggers (′, ′′). Type footnotes directly after the abbreviations beginning on the next line.

Figures. Figure legends should be typed on one page using Microsoft Word; this page should precede the figures. Graphics must fit standard letter size paper (8.5 × 11 inches or smaller), and resolution should be at least 300 dpi. All letters, numbers, and lines should be clearly legible and easy to differentiate. Provide a key defining representational elements (e.g., dotted/dashed lines, symbols, asterisks, error bars) for each figure. All axes should be clearly labeled, giving both the measure and the unit of measurement where applicable. No lines of demarcation or measurement should appear inside the graph itself, only on the axes. Consistency among terms and styles used in figures is desirable. Photomicrographs should include a scale bar in each image, and the length should be specified in the typed figure legend (e.g., bar = 10 µm). Multiple panels within a figure should fit on one page. If you have questions or problems, contact the journal (EHPmanuscripts@niehs.nih.gov).

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The criteria for publication are weighted toward scientific quality and environmental significance. Manuscripts are also assessed according to their originality, scientific merit, appropriate degree of speculation, clarity of presentation, and conciseness.
At least two peer-reviewers will be solicited for comments on the manuscript. Authors will not know the identity of the reviewers. Peer-review is conducted electronically to accelerate the process, and each reviewer is asked to complete the review within 3 weeks.

After editorial consideration, a decision letter and reviewers’ comments will be e-mailed to authors. If a revision of the manuscript is required, the revised manuscript must be received by EHP within 6 weeks of the request. The submission will be canceled at that time unless the authors obtain prior permission from the Editor-in-Chief. Authors must submit the revised manuscript and a letter responding to reviewers’ comments.

Authors are strongly encouraged to submit the names and contact information (including e-mail addresses) of experts in their field of study to add to EHP’s scientific reviewer database.

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- Each figure as a separate file in either of the following formats: TIFF, JPEG, or EPS. Do not embed figures in a word processing file. Each figure must be labeled on the front with the figure number. For TIFF or JPEG format, the resolution should be ≥ 300 dpi for color images, 600 dpi for grayscale images, and ≥ 1200 dpi for line art (black-and-white art). JPEG files should be saved on the “highest quality” setting. Color images should be RGB and saved at a minimum of 8 bits per channel. Because figures may be reduced or enlarged to fit our layouts, sufficient resolution is essential. Vector images should be saved as editable EPS files. Any images embedded in the EPS should also be included in a separate file. Do not convert text to path outlines before submission.
- A cover letter with responses to the reviewers’ comments.

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  - (Aldridge et al. 2005; Jameson et al. 2006; Levin et al. 2007; Slotkin 2004a, 2004b; Slotkin et al. 2008)

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All manuscripts submitted but not yet accepted, unpublished data, and personal communications—any items that must be cited but are not accessible to the public—must appear in the text in parentheses but should not be listed in the references: (Ramsdell JS, Moeller PDR, personal communication); (Reeves MK, unpublished data).
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- Year of publication
- Full title of article or chapter (lower case)
- Title of journal (abbreviated according to Biois or Index Medicus) or book/proceedings in title case
- For books, city/state/country of publication and name of publisher
- Volume and inclusive page numbers
- DOI number, if available, with online publication date

If you are uncertain what to include, please include all information.

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Sample alphabetical list:


Types of references

Journal article, conventional reference


Journal article, DOI reference


Journal article, “in press”


Chapter in edited book


Agency as author


Proceedings


Additional reference examples are available on page 6.

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All nonstandard abbreviations should be defined in the text at first use: for example, organochlorine (OC) pesticides, LOD (limit of detection), polymerase chain reaction (PCR). Abbreviations for elements (e.g., Fe, Cu) and chemical compounds (e.g., polychlorinated biphenyls, PCBs; carbon dioxide, CO₂) should be spelled out on first use and abbreviated thereafter.

Units of measure should be abbreviated only when a specific amount is given (e.g., “concentration of 10 ng/mL” versus “units of nanograms per milliliter”). A list of standard abbreviations that do not need to be defined in the text is available on page 7.
TYPES OF REFERENCES

Journal article—conventional reference

Journal article—DOI reference

Journal article—conventional reference and DOI reference

Journal article, “in press”

Article in non-English language

Magazine article

Newspaper article

Chapter in edited book

Agency monograph

Book

Book, edited

Agency as author

Proceedings

Technical paper

Dissertation/thesis

Software manual

Website

Abstract

Federal regulation

Executive order; federal regulation

U.S. government document

State document

Law

Court case
### Abbreviations

All nonstandard abbreviations should be defined in the text at first use; for example, organochlorine (OC) pesticides, LOD (limit of detection), polymerase chain reaction (PCR). Abbreviations for elements (e.g., Fe, Cu) and chemical compounds (e.g., polychlorinated biphenyls, PCBs; carbon dioxide, CO₂) should be spelled out on first use and abbreviated thereafter.

The standard abbreviations listed below do not need to be defined in the text. Note that units of measure should be abbreviated only when a specific amount is given (e.g., "concentration of 10 ng/mL" versus "units of nanograms per milliliter").

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Å</td>
<td>angstrom</td>
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<tr>
<td>amu</td>
<td>atomic mass unit</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’- triphosphate</td>
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<tr>
<td>bw</td>
<td>body weight</td>
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<td>°C</td>
<td>degrees Celsius</td>
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<td>centimeter</td>
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<td>cm²</td>
<td>square centimeter</td>
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<td>cm³</td>
<td>cubic centimeter</td>
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<tr>
<td>Da</td>
<td>dalton</td>
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<tr>
<td>df</td>
<td>degrees of freedom</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine-tetraacetic acid</td>
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<td>ELISA</td>
<td>enzyme-linked immunoadsorbent assay</td>
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<td>g</td>
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<td>gal</td>
<td>gallon</td>
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<tr>
<td>ha</td>
<td>hectare</td>
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<tr>
<td>Gy</td>
<td>gray (unit of absorbed dose of ionizing radiation)</td>
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<tr>
<td>HEPES</td>
<td>2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid</td>
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<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<tr>
<td>hr</td>
<td>hour</td>
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<td>Kₘ</td>
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<td>parts per million</td>
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<tr>
<td>ppt</td>
<td>parts per trillion</td>
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<td>ribonuclease</td>
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<td>SD</td>
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<td>SDS/PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<td>SE</td>
<td>standard error, standard error of the mean</td>
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