Alterations in male reproductive hormones in relation to environmental DDT exposure

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Highlights

Chronic DDT exposure may contribute to altered male reproductive hormone homeostasis.

Men living in IRS villages incurred highest degree of homeostatic derangement.

Men with high DDE, DDT concentrations had high T, E_2, but low FSH, LH concentrations.

Abbreviations

AR	Androgen receptor
BDL	Below detection limit
BFRs	Brominated flame retardants
BMI	Body mass index
b-T	Bioavailable testosterone
CYP19	Aromatase
DDE	1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene
DDT	1, 1, 1-trichloro-2,2-bis (p-chlorophenyl) ethane
DHT	Dihydrotestosterone
E_2	Estradiol
ECL	ElectroChemi-Luminescence
ER	Estrogen receptors
FSH	Follicle stimulating hormone
f-T	Free testosterone
HBCD	Hexabromocyclododecane
HPT	Hypothalamic-pituitary-testicular
IRS	Indoor residual spraying
LH	Luteinizing hormone
LOD	Limit of detection
PBDEs	Polybrominated diphenyl ethers
PCBs	Polychlorinated biphenyls
POPs	Persistent organic pollutants
SHBG	Sex hormone-binding globulin
SHSPH	School of Health Systems and Public Health
t-T	Total Testosterone
T	Testosterone
ΣDDT	Sum of DDT isomers

ABSTRACT

DDT [1, 1, 1-trichloro-2,2-bis (p-chlorophenyl)-ethane] compounds are used for indoor residual spraying (IRS) to control malaria mosquitoes. DDT is an endocrine disruptor chemical in experimental conditions, but little is known of adverse effects related to living conditions with continual uptake across a time span by all possible means of exposure. Based on estrogenic and/or anti-androgenic effects found in animal studies, we hypothesized that that chronic DDT/DDE exposures in men may be associated with changes in male reproductive hormones. We tested this hypothesis by compared the magnitude and direction of associations between DDT and DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene) concentrations and male reproductive hormones in samples collected from IRS and non-IRS areas.

We sampled a cross-section of 535 men (aged 18-40 years). Men living in IRS villages had significantly higher DDT and DDE concentrations compared with men from non-IRS villages. Men with DDT or DDE uptake (as reflected in detectable plasma concentrations) had significantly higher total-, free and bio-available testosterone (T), and lower follicle stimulating hormone (FSH) concentrations; lower luteinizing hormone (LH) concentrations were only evident with DDT uptake. To establish a dose-dependent effect, four sub-categories were defined. Men with the highest DDT (74-519 µg/g) and DDE (173-997 µg/g) concentrations had significantly higher total-, free and bio-available T, and lower FSH concentrations compared with subjects with non-detectable isomer concentrations. Estradiol concentrations were significantly higher in men with DDT and DDE concentrations in both the third (DDE: 27-172 µg/g; DDT: 5-73 µg/g) and fourth (DDE: 173-997 µg/g; DDT: 74-519 µg/g) categories. Men from IRS villages were significantly more likely to have higher total and bioavailable T as well as higher estradiol concentrations OR=2.5 (95% CI 1.2, 3.2); OR 2.5 (95% CI 1.6, 4.0) and OR=2.3 (95% CI 1.3, 4.1) compared to men from non-IRS villages, after controlling for age, BMI, personal use of pesticides, and smoking.

Men living in IRS villages with life-long exposure (17.6 (±6) years) at the current residence with multiple exposure modalities incurred the highest degree of homeostatic derangement over and above circulating isomer concentrations. Further studies are needed to elucidate the health implications of these findings.

KEYWORDS: DDT, malaria, testosterone, estradiol, male reproductive hormones

1. Introduction

DDT is an insecticide sprayed onto the inside walls of homes for indoor residual spraying (IRS) to control malaria mosquitoes in several endemic countries and areas (WHO 2011). The DDT used for spraying (termed 'technical DDT') contains 65%–80% of the active insecticidal ingredient, 1, 1, 1-trichloro-2, 2-bis (4-chlorophenyl) ethane (*p,p′*-DDT) and 15%-21% of the less insecticidal 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl) ethane (*o,p'*-DDT) (Bouwman 2004). DDT bio-accumulates in fatty tissue as the metabolite 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (*p,p′*-DDE) (referred to as DDE) (ATSDR 2002; Smith 1991). Both DDT and DDE are persistent organic pollutants (POPs) and are hormonally active substances (ATSDR 2002).

In vitro, DDT and DDE impact on various biochemical and physiological processes, including estrogen receptors (ER) and androgen receptors (AR), which ultimately influence complex hormonal regulatory systems. Technical DDT (mixture of *p,p′*- and *o,p′*-isomers) is estrogenic, but has less agonist activity than estradiol as monitored by ER-positive cell lines (Chen et al. 1997; Dees et al. 1997) and also stimulates ERα- and ERβ-mediated transcription (Lemaire et al. 2006). DDE competitively binds with the androgen receptor and blocks androgen-induced transcription (Kelce et al. 1995; Kelce and Wilson 1997). DDE also stimulates aromatase (CYP19) activity in cultures of human ovarian (Younglai et al. 2004) and endometrial cells (Holloway et al. 2005), thereby significantly increasing local estradiol (E_2) concentrations through elaboration from testosterone.

In vivo DDT exposure to adult male (Krause 1977) and juvenile rats (Rhouma et al. 2001) resulted in lower testosterone concentrations and inconsistent follicle-stimulating hormone (FSH) and luteinizing hormone (LH) concentrations, but others reported reduced dihydrotestosterone (DHT), increased estradiol (E_2) and no significant changes in testosterone, LH and FSH concentrations (O'Connor et al. 2002). *In vivo* DDE exposure resulted in significantly increased estradiol and T, but decreased FSH concentrations (O'Connor et al. 2002). Multigenerational *in vivo* reproductive toxicity studies using DDT in mice (Tarjan and Kemeny 1969; Turusov et al. 1973), rats (Ottoboni 1969; 1972) and dogs (Ottoboni et al. 1977) were not contributing to a clearer understanding of DDT effects. Collectively, the *in vitro* and *in vivo* evidence supports the estrogen-like properties of DDT (Metcalf and Nicola 1995) and the AR antagonist properties of DDE (ATSDR 2002; Danzo 1997).

The epidemiologic associations of DDT and DDE exposure with male sex hormones in humans are inconsistent and the comparison of findings is confounded by differences in exposure concentrations, duration, pathways/routes and study populations (Ayotte et al. 2001; Blanco-Muñoz et al. 2012; Bonde et al. 2008; Cocco et al. 2004; Dalvie et al. 2004a; Hagmar et al. 2001; Haugen et al. 2011; Martin Jr et al. 2002; Rignell-Hydbom et al. 2004). Most reported studies arise from the United States and Europe, where the use of DDT has been banned since the late 1970s, such that exposures were low and indirect. In men from IRS-related exposure, Mexican men with non-occupational DDT exposure had significant correlation between DDE concentrations and the ratio of bioavailable to total testosterone (Ayotte et al. 2001). Workers spraying DDT in the Limpopo province of South Africa had positive correlations between both E_2 and t-T concentrations and ∑DDT isomers (Dalvie et al. 2004b). Young men living in an IRS area had impaired semen quality associated with environmental DDT exposure (Aneck-Hahn et al. 2007) and weak associations with a high incidence of sperm with DNA breaks (De Jager et al. 2009).

To the best of our knowledge, the impact of concurrent exposure to both DDT and DDE on male reproductive hormones (hypothalamic-pituitary-testicular (HPT) hormones); has not been reported in animal models or of men living in a currently IRS area. Changes in hormones of men from an IRS area seem particularly important as in theory, simultaneous exposure to DDT and DDE may cause estrogenic, anti-androgenic effects or a combination. We tested the hypothesis that exposure to DDT has anti-androgenic and/or estrogenic effects and changes in reproductive hormone concentrations. We compared male reproductive hormones and DDT and DDE concentrations in samples collected from men living in IRS and non-IRS areas to determine the difference in DDT and DDE concentrations and whether these DDT and DDE concentrations were associated with changes in male reproductive hormones.

2. Materials and Methods

2.1. Study Design and Population

This cross-sectional, observational study was part of a larger ongoing study that commenced in 2003 to evaluate the effects of indoor residual spraying (IRS) on the reproductive health of young men from Limpopo, South Africa.

2.2. Study Area

The Limpopo Province is situated in the north-eastern corner of South Africa and is divided into five districts, including the Vhembe District. The Vhembe district is a malaria-endemic area where housing comprises traditional mud dwellings with thatch (straw grass) roofs or brick and cement houses. The inside of unpainted brick, cement, and daub houses are sprayed annually with DDT to control malaria mosquitos. DDT is usually not sprayed on painted surfaces.

The participants were from rural IRS villages or from nearby non-IRS villages in the Thulamela Local Municipality. The decision to spray villages depends on the Department of Health, based on the number of malaria cases. Volunteers comprised men, between 18 and 40 years old, living in these communities. Full detail on the recruitment and questionnaire was reported elsewhere (Aneck-Hahn et al. 2007; De Jager et al. 2009). Participants from sprayed and non-sprayed villages volunteered, but we excluded men who had lived in study villages for less than a year, those younger than 18 or older than 40 years, those with neuropsychiatric disorders or who appeared intoxicated.

All participants provided informed consent and were interviewed using a structured questionnaire, which detailed their general history, personal use of insecticides, diet, smoking and drinking habits, illegal substance use, exposure to other insecticides and fertility history. Physical measurements included participants' weight and height and the body mass index (BMI) was calculated. Blood samples were collected at the Tshilidzini Hospital and Thohoyandou Health Care Centre. The Limpopo Provincial Government's Department of Health and Social Development (July 11, 2002) and the Ethics Committee of the Faculty of Health Sciences, University of Pretoria (UP) (Reference 43/2003) approved the study.

2.3. Biochemical analyses

Data were collected during each of six five-day visits to the study area (between November 2003 and October 2007). We collected venous blood samples from participants between 08:00 – 10:00. The samples were centrifuged at 670×g for 10 min at room temperature and stored in 500 µL aliquots at -20°C on site and during transport. At the UP laboratory, samples were stored at -80°C until analyzed. Hormones (abbreviation and kit catalog number) measured, in serum with the Cobas® 6000 analyser (Roche Products (Pty) Ltd Diagnostics Division using ECL (ElectroChemi-Luminescence) immunometric detection,

were: Luteinizing hormone (LH; 11732234122); Follicle-stimulating hormone (FSH; 11775863122), estradiol (E_2; 03000079190); total testosterone (t-T; 05200067160), and human sex hormone-binding globulin (SHBG; 03052001160). We measured serum albumin on the general automated platform to calculate bioavailable testosterone (b-T) and free testosterone (f-T) using the calculator available at http://www.issam.ch/freetesto.html, following Vermeulen's formula.

2.4. Body burden

We used a Shimadzu GCMS-QP2010 gas chromatograph/mass spectrometer to measure 1,1,1-trichloro-2,2′bis(p-chlorophenyl) ethane (DDT) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (DDE) as reported (Aneck-Hahn et al. 2007). We could not detect *o,p'*-DDD, *p,p'*-DDD, *o,p'*-DDE in 66 to 75% of the men and this report focuses therefore on DDT and DDE concentrations. Total cholesterol and triglycerides were determined by enzymatic methods (Aneck-Hahn et al. 2007) and the total plasma lipid level was calculated according to (Rylander et al. 2006). The lower limit of detection (LOD) for DDT and DDE were both 0.02 μg/g lipid.

2.5. Data analysis

Data were analyzed in Stata 11 (StataCorp. 2009. Stata Statistical Software: Release 11. College Station, TX: StataCorp LP). Descriptive analyses included the calculations of means, standard deviations, medians and ranges for continuous variables and frequencies for categorical variables. We used the Mann Whitney U tests and T-test for continuous variables and Chi square test for dichotomous variables for comparisons between IRS and non-IRS villages. Non-parametrically distributed hormone concentrations were $\log_{10}$-transformed before inclusion in linear regression models.

2.5.1. IRS vs. non-IRS villages

Simple linear regression models explored associations between response variables (hormones concentrations) and exposure of interest (DDT and DDE concentrations). Potential confounding and interaction was assessed by analyzing the association of exposure and effect with predictor variables: IRS, age, age of puberty onset, weight, height, body mass index (BMI), smoking and drinking habits, drug use, exposure to other insecticides, and history of any testicular problem (trauma, torsion, infections, malformations). To further explore the impact of timing and duration of exposure to DDE and DDT we explored the associations of

DDE and DDT levels with participants' mothers place of birth, the time each participant had lived in the village and the percentage of his life that this represented, Dichotomous predictor variables with fewer than five observations in a category were excluded from the analysis. Statistically significant associations of the aforementioned predictor variables were taken into account to generate multivariate models. Final multivariate regression models were created for each hormone and each isomer controlling for age, BMI, personal use of insecticides other than DDT (yes or no), and smoking (yes or no).

2.5.2. Effect of DDT and DDE on hormone concentrations

DDT and DDE concentrations were dichotomized as uptake or no uptake. Uptake of DDT and DDE were reflected as detectable plasma concentrations (=/>LOD in plasma). Lipid–adjusted DDT and DDE concentrations were further divided into four categories, resulting in equivalent percentages of observations in each category. Category 1 of each isomer consisted of observations with below the level of detection. Since Category 1 represented 28% and 32% of the DDT and DDE values respectively, Categories 2, 3 and 4 were created by assigning equivalent percentages of observations with detectable values (22-24%) in ascending order. DDE Category 1 was BDL; Category 2 was 0.5-26 µg/g lipid; Category 3 was 27-172 µg/g lipid, Category 4 was 173-997 µg/g lipid, respectively. DDT Category 1 was BDL, Category 2 was 0.03 – 4 µg/g lipid, Category 3 was 5 – 73 µg/g lipid and Category 4 was 74-519 µg/g lipid. Logistic regressions tested for relationships between hormone concentrations (response variables) and DDT and DDE concentration (predictor variables) expressed as a dichotomous variable (uptake vs. no uptake) and as a categorical variable as described above. Multivariate logistic models were adjusted for age, BMI, personal use of other insecticides, and smoking.

2.5.3. Sensitivity analysis

In order to estimate the potential influence of prenatal exposure to DDT and DDE in addition to the postnatal exposure, statistical models were ran including only the men who were born at their village of recruitments and had lived there all their lives; these models were compared to those including all men. After calculating dfbetas for the models, observations outside the limits ($2/\sqrt{N}$) were excluded and graphical representations of the models with and without these observations were compared.

3. Results

Participants included 535 men, aged between 18 and 40 years old, of whom 56% lived in IRS villages and 44% lived in non-IRS villages. Of the 535 men, 188 (35%) reported smoking between 1 and 20 cigarettes per day; on average four cigarettes per day over 6 years (Table 1). The mean (±SD) age of the participants was 22 (±4) years and the mean time lived at their village was 18 (±6) years. On average, the participants had lived 82% of their lives in their village of birth and 98% reported being breastfed. Concentrations of crude DDE and DDT ranged from non-detectable levels to 6621 and 2644 µg/dL, respectively. Lipid-adjusted concentrations of DDE and DDT ranged from non-detectable levels to 997 and 519 µg/g, respectively. Since fewer than five participants reported being on medication, drinking more than three alcoholic drinks per week or using recreational drugs, these characteristics were excluded. Maternal birth place was closely associated to the participants' birthplace, and percentage of life lived at the village of origin was most of their lives in most cases (r=0.86), so these variables were not included in the evaluation of associations between exposures and outcomes of interest.

3.1. IRS vs non-IRS villages

Men from IRS villages were significantly older, had a higher mean BMI, and more frequently reported cigarette smoking and the use of other insecticides, compared to men from non-sprayed villages ($p<0.05$). Men from IRS villages spent on average 77 (±28) % of their lives in the village of birth compared to 89 (±24) % from non-IRS villages.. Men from IRS villages had significantly higher median concentrations of DDT (43 µg/g lipid) and DDE (134 µg/g lipid) compared to men from non-IRS villages (both BDL)The main characteristics of all the men and their differences by village of origin are shown in Table 1.

Table 1.

Main characteristics, lipid-adjusted DDT and DDE concentrations of men from Limpopo, South Africa. Men were from indoor residual sprayed (IRS) villages or non-IRS villages.

Characteristic	All men (N=535)	Non-IRS villages (N=234)	IRS villages (N=301)
Age (years): median (range)	21 (18, 44)	20 (18-36)	22 (18, 44)*
Years at current residence: median (range)	19 (1, 37)	19 (1, 36)	19 (1, 37)
Percentage of life at current residence: median (range)	99 (4, 100)	99 (4, 100)	92 (5, 100)*
Self-reported age at puberty onset (years): median (range)	15 (10, 22)	15 (10, 21)	15 (10, 22)
BMI (kg/m^2): median (range)	20 (14, 34)	19 (14, 27)	20 (15, 34)*
Smoking: (%)	35	28	40*
Was breastfed: (%)	98	97	98
Personal use of other than DDT insecticides: (%)	46	36	56*
DDE (µg/g lipid): median (range)	27 (BDL, 997)	BDL (BDL, 25)	134 (BDL, 997)*
DDT (µg/g lipid): median (range)	4 (BDL, 519)	BDL (BDL, 86)	43 (BDL, 519)*

*Statistically significant difference (P value <0.05) between IRS and non-IRS villages based on Mann-Whitney U test for continuous variables and Chi squared test for dichotomous variables.

BDL: below detection limit

3.2. Relationship between DDT and DDE and hormone concentrations

Mean concentrations of total testosterone, free testosterone, bioavailable testosterone and estradiol were consistently higher in groups with DDT or DDE uptake compared to no uptake (data not shown). Men with DDE uptake had significantly higher total testosterone (23±8 vs 21±7 nmol/L, p=0.01), free testosterone (0.46±0.2 vs 0.41±0.1 pmol/L, p<=0.001), bioavailable testosterone (11.3±4 vs 9.9±3 nmol/L, p<=0.001) but lower FSH (3.9±2 IU/L, p=0.02) and not significantly different LH. Estradiol level was significantly higher (131±38

vs 120±47 pmol/L, p=0.008). Men with DDT uptake had significantly higher free testosterone (0.45±0.2 vs 0.41±0.1pmol/L, p=0.01, bioavailable testosterone (11.1±4 vs 10.2±3 nmol/L, p=0.03), lower FSH (3.8±2 vs 4.9±5 IU/L, p=0.001) and lower LH (4.5±2 vs 5.1±3IU/L, p=0.02). Estradiol was significantly higher (130±37 vs 121±49 pmol/L, p=0.03).

In Figure 1 the violin plots show the full distribution of the total testosterone concentrations for each DDE category for IRS villages.

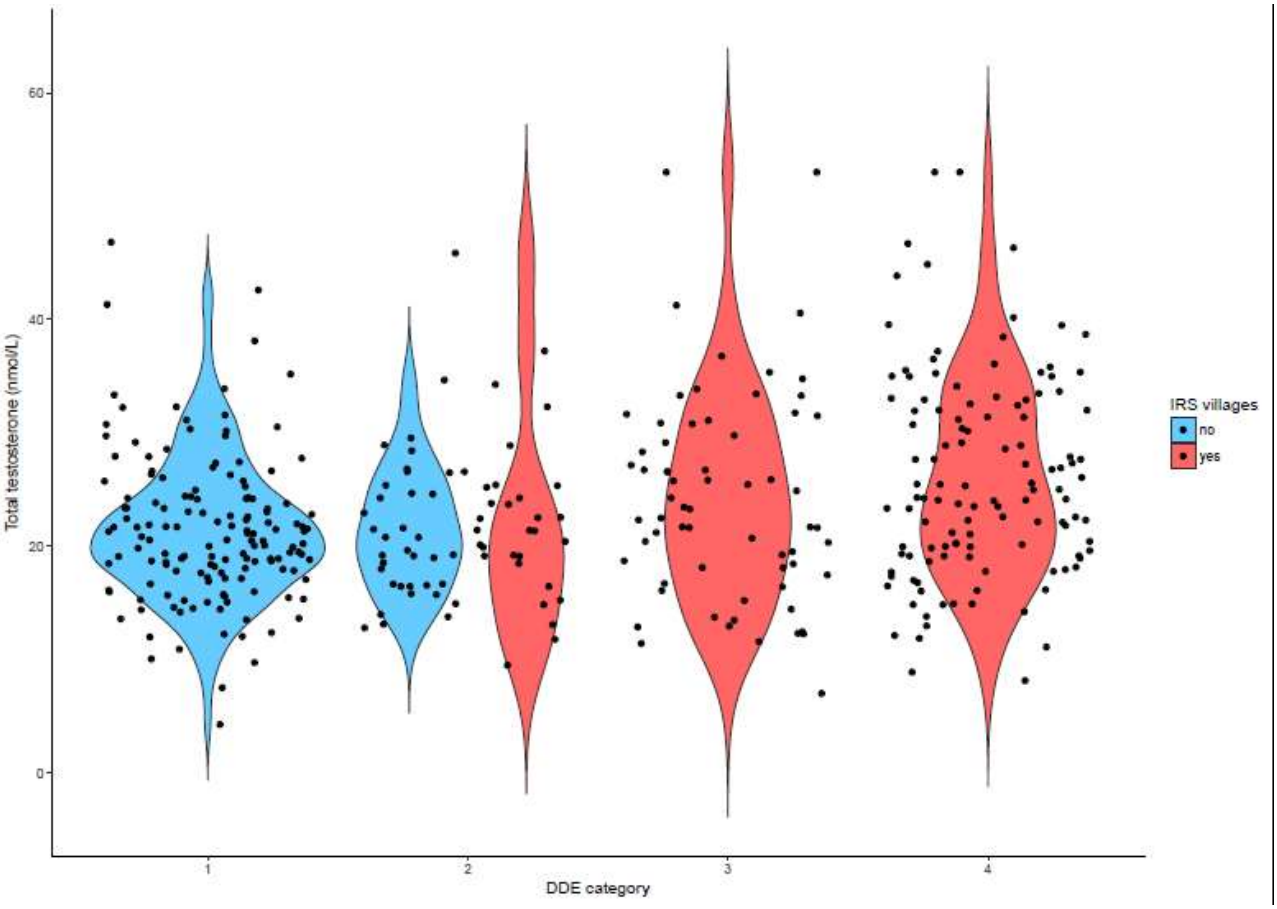


Fig. 1. Violin plots show the distribution of total testosterone concentrations per DDE category. The blue color represents sample points from villages where no residual indoor spraying (IRS) occurs and the red color represent sample points from IRS villages. The hormone values were adjusted for BMI, smoking, and time at day of blood sampling. DDE Category 1 was BDL; Category 2 was 0.5-26 µg/g lipid; Category 3 was 27-172 µg/g lipid, Category 4 was 173-997 µg/g lipid.

Crude linear regression results comparing the highest category of exposure compared to the lowest three showed that men whose DDE concentrations were in the highest category (173-997 µg/g lipid) had mean total testosterone concentrations that were 4.8 (CI 95% 3.3, 6.3) nmol/L higher than men in the three lower categories (BDL -172 µg/g lipid) g. Likewise,

men whose DDT concentrations were in the highest category (77-519 µg/g lipid) had mean total testosterone concentrations 5.9 (CI 95% 4.4, 7.4) nmol/L higher than men in the three lower categories (BDL - 4 µg/g lipid). Similarly, men with DDE concentrations in the highest category had significantly higher log transformed estradiol concentrations, and lower log transformed FSH concentrations (data not shown). There were no associations between LH, SHBG and DDE as well as DDT.

Results of multivariate regressions between hormones and each isomer category controlling for age, BMI, personal use of pesticides, and smoking are summarized in Table 2. The first category for both isomers included concentrations below the detection limit (BDL).

Table 2. Multivariate linear regression associations between hormone concentrations and categorical lipid-adjusted DDE and DDT concentrations in men from Limpopo, South Africa (controlled by age, BMI, smoking and personal use of other than DDT insecticides).

Hormone	**DDE (µg/g)**		**DDT (µg/g)**	
	Category 1 compared to	Coefficient (95% Cl)	Category 1 compared to	Coefficient (95% CI)
Total testosterone (nmol/L)	Category 2	-1.06 (-2.99, 0.87)	Category 2	**-2.05 (-3.74, -0.36)**
	Category 3	0.19 (-1.58, 1.95)	Category 3	-1.59 (-3.24, 0.06)
	Category 4	**3.92 (2.08, 5.77)**	Category 4	**4.18 (2.38, 5.98)**
Free testosterone (pmol/L)*	Category 2	0.029 (-0.07, 0.13)	Category 2	-0.01 (-0.10, 0.08)
	Category 3	0.004 (-0.08, 0.09)	Category 3	-0.07 (-0.16, 0.01)
	Category 4	**0.17 (0.08, 0.26)**	Category 4	**0.16 (0.07, 0.25)**
Bioavailable testosterone (nmol/L)*	Category 2	0.03 (-0.06, 0.12)	Category 2	-0.26 (-0.11, 0.06)
	Category 3	0.04 (-0.05, 0.11)	Category 3	-0.04 (-0.13, 0.04)
	Category 4	**0.21 (0.12, 0.29)**	Category 4	**0.18 (0.09, 0.27)**
E_2 (pmol/L)*	Category 2	0.01 (-0.08, 0.09)	Category 2	-0.02 (-0.10, 0.06)
	Category 3	**0.14 (0.06, 0.23)**	Category 3	**0.12 (0.04, 0.20)**
	Category 4	**0.09 (0.02, 0.17)**	Category 4	0.06 (-0.01, 0.13)
SHBG (nmol/L)*	Category 2	**-0.21 (-0.34, -0.08)**	Category 2	**-0.20 (-0.32, -0.08)**
	Category 3	-0.11 (-0.23, 0.01)	Category 3	**-0.12 (-0.23, -0.003)**
	Category 4	-0.03 (-0.15, 0.10)	Category 4	.005 (-0.12, 0.13)
FSH (IU/L)*	Category 2	-0.09 (-0.25, 0.08) -0.11 (-	Category 2	-0.06 (-0.21, 0.09) -0.07 (-
	Category 3	0.27, 0.05) **-0.16 (-0.30, -**	Category 3	0.22, 0.09) **-0.16 (-0.30, -**
	Category 4	**0.1**)	Category 4	**0.02)**
LH (IU/L)*	Category 2	0.02 (-0.11, 0.15) -0.04 (-	Category 2	**-0.14 (-0.25, -0.02)** -0.05 (-
	Category 3	0.17, 0.09) **-0.14 (-0.25,-0.02)**	Category 3	0.18, 0.07) **-0.20 (-0.31, -**
	Category 4		Category 4	**0.09)**

DDE Category 1 was BDL (N=170); Category 2 was 0.5-26 µg/g lipid (N=97); Category 3 was 27-172 µg/g lipid (N=132), and Category 4 was173-997 µg/g lipid (N=133).
DDT Category 1 was BDL (N=152), Category 2 was 0.03 – 4 µg/g lipid (N=126), Category 3 was 5 – 73 µg/g lipid (N=126), and Category 4 was 74-519 µg/g lipid (N=130).
* Log transformed

The multivariate linear regression between hormones and isomers, as continuous variables, and controlling for age, BMI and smoking, resulted in similar associations and patterns as the bivariate analysis (Table 3). DDT and DDE exposure were significantly positively associated with total-, free and bio-available testosterone, non-significantly associated with lower FSH and LH, whereas little effect was seen on estradiol (Table 3).

Table 3. Associations between continuous hormone concentrations and lipid adjusted continuous DDE and DDT concentrations by multiple linear regression*****

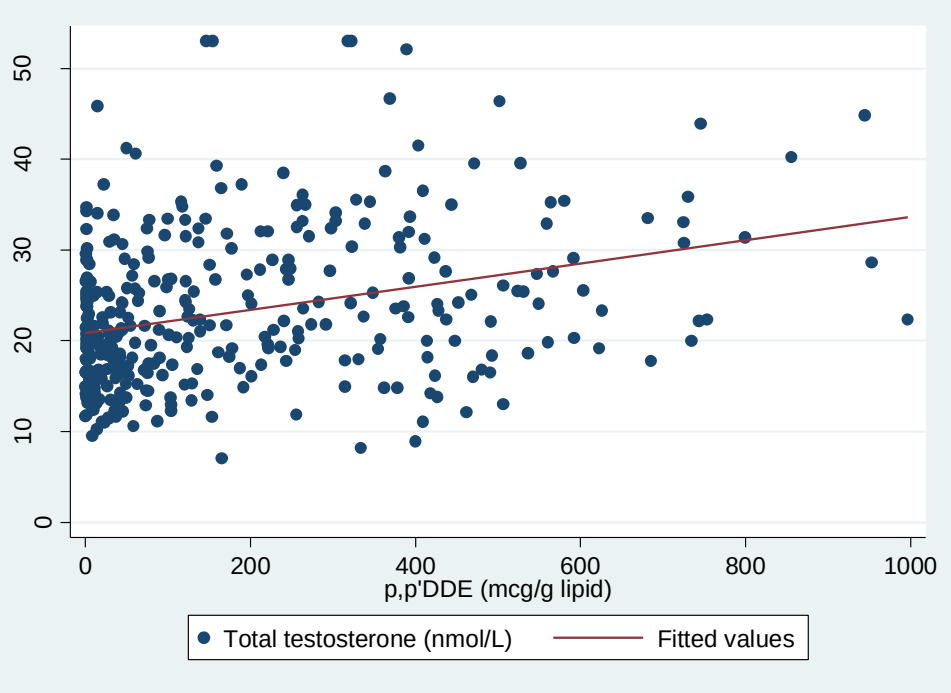
Hormone	DDE (µg/g)				DDT (µg/g)			
	N	Coefficient	95% Confidence interval		N	Coefficient	95% Confidence interval	
Total testosterone (nmol/L)	521	**0.0112**	**0.0077**	**0.0147**	523	**0.0252**	**0.0178**	**0.0326**
Log transformed Free testosterone (pmol/L)	519	**0.0004**	**0.0002**	**0.0006**	521	**0.0009**	**0.0005**	**0.0013**
Log transformed Bioavailable testosterone (nmol/L)	519	**0.0004**	**0.0003**	**0.0006**	521	**0.0009**	**0.0006**	**0.0013**
FSH (IU/L)	403	-0.0002	-0.0005	0.0001	405	-0.0003	-0.001	0.0003
LH (IU/L)	401	-0.0002	-0.0004	5.03e-06	403	-0.0004	-0.0009	0.0001
E_2 (pmol/L)	401	0.0001	-0.00003	0.0003	403	0.0002	-0.0001	0.0006

* Adjusted linear regression β coefficient and 95% confidence interval (CI) for the association between DDT and DDE serum concentrations and reproductive hormone concentrations**.** Adjusted by age, BMI, personal use of pesticides, and smoking (yes vs. no)

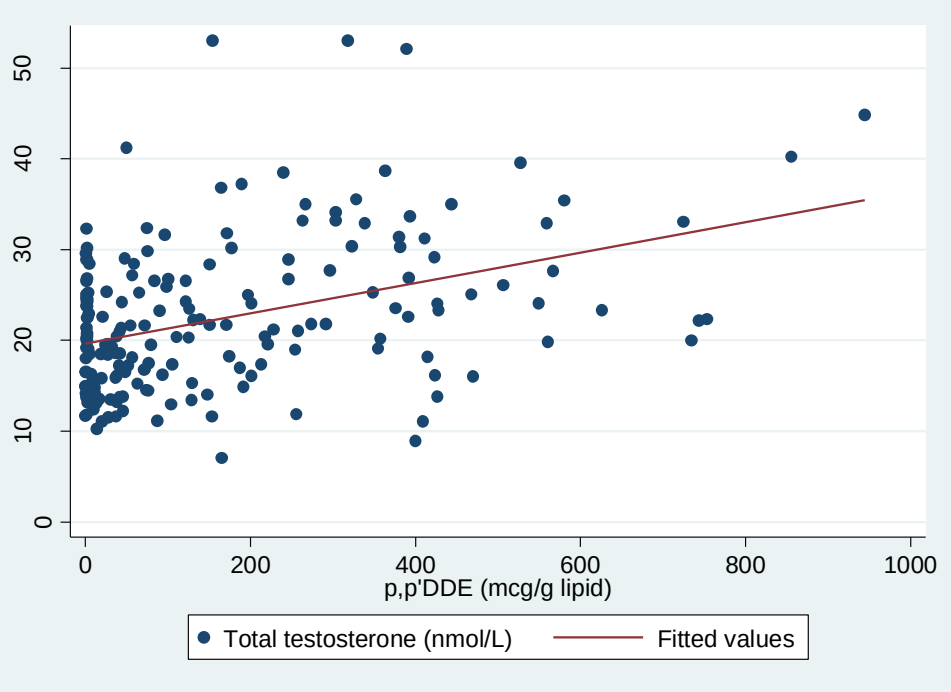
Positive associations, indicating increase in hormone concentrations per increase in isomer are shaded in blue. Negative associations are shaded in pink.

Multivariate analyses showed that for every 1µg/g lipid increase in DDE, total testosterone increased by 0.01 nmol/L ($p<0.001$), therefore an increase of 100µg/g of DDE would result in an increase of total testosterone of 1.1 nmol/L. For DDT, an increase of 100 µg/g lipid would result in a total testosterone increase of 2.5 nmol/L ($p<0.001$).

The sensitivity analysis to determine the influence of prenatal in addition to postnatal exposure to DDE and DDT showed that men who were born and had lived all their lives at their village of origin were no different to all the men in terms of the association between the exposures of interest and total testosterone levels analyzed in a dichotomous and a continuous way (Figure 2). Furthermore, excluding from the analysis the eight observations with dfbetas <0.11 and the seven observations with dfbetas >0.11, did not alter the results in terms of coefficients or P values (0.02 and <0.001, respectively, in both cases).



Including all men in the study from IRS and non-IRS villages (N= 535)



Including only the men who were born in their village of origin (IRS or non-IRS) (N=325)

Fig. 2. Sensitivity analysis for total testosterone

Binomial logistic regressions investigating the relationship between isomers (uptake versus no uptake, predictor variable) and hormone variables (response variables) produced larger and more significant odds ratios compared to models with isomer as a continuous predictor variable. Men with DDE uptake were nearly twice as likely to have both total testosterone and bioavailable testosterone concentrations above reference values for the laboratory compared to men with no uptake OR=1.9 (95% CI 1.1, 3.2) and OR 1.8 (95% CI 1.1, 3.0). Furthermore, using the type of village (IRS vs. no IRS) as a dichotomous predictor variable produced the largest and most significant odds ratios (Table 4). Men from IRS villages were significantly more likely to have higher total and bioavailable testosterone as well as estradiol concentrations OR=2.5 (95% CI 1.2, 3.2); OR 2.5 (95% CI 1.6, 4.0) and OR=2.34 (95% CI 1.3, 4.1) compared to men from non-IRS villages, after controlling for age, BMI and smoking.

Table 4. Associations by logistic regression between hormones at reference cut-off points and DDE or living in DDT sprayed villages*

Hormone	**N**	**Uptake vs. no uptake of lipid adjusted DDE**			**IRS village vs. non-IRS village origin**		
		Odds Ratio	**P value**	**95% Confidence interval**	**Odds Ratio**	**P value**	**95% Confidence interval**
Total testosterone (nmol/L) Reference: < 27.8	521	**1.86**	**0.03**	**1.07, 3.21**	**2.47**	**0.001**	**1.47, 4.13**
Bioavailable testosterone (nmol/L) Reference: <13.5	519	**1.83**	**0.02**	**1.11, 3.01**	**2.48**	**<0.001**	**1.55, 3.97**
Estradiol (pmol/L) Reference: < 156	401	1.65	0.09	0.93, 2.94	**2.34**	**0.003**	**1.33, 4.11**
FSH (IU/L) Reference: <12.4	401	1.72	0.27	0.65, 4.52	1.10	0.831	0.45, 2.68
LH (IU/L) Reference: <8.6	401	0.94	0.89	0.36, 2.42	0.83	0.851	0.33, 2.11

*Models adjusted by age, BMI, personal use of pesticides and smoking (yes vs. no)

4. Discussion

This study reports the concentrations of male reproductive hormones from men living in an area where IRS is used for malaria vector control and one where IRS is not used. We determined if DDT and DDE concentrations differed between IRS and non-IRS villages and whether those DDT and DDE concentrations were correlated with hormone concentrations. Men living in IRS villages had significantly higher DDT and DDE concentrations compared

to men from non-IRS villages. Men with DDT or DDE uptake, especially those who had the highest DDT (category 4: 74 -519 µg/g lipid) and DDE (category 4: 173-997 µg/g lipid) concentrations had significantly higher total-, free and bio-available testosterone. Living in IRS villages instead of non-IRS villages thus increased the risk of having higher testosterone and estradiol concentrations. Other studies on human field exposure to DDE and DDT have yielded contrasting results. Exposure to DDE and DDT is likely influenced by the time from last exposure, frequency of spraying and the point of exposure. These factors need to be considered when evaluating the hormonal response of humans to DDE and DDT exposure.

Indoor residual spraying with DDT is recommended practice for the control of mosquitoes in malaria endemic areas (WHO 2016). Indoor residual spraying is recommended where the vectors mainly sleep and feed indoors and where people sleep indoors at night (WHO 2016). In our study area, in the Vhembe district, IRS rounds are performed annually just before the peak season for vectors, as part of a long-term malaria control programme. Various insecticides have been used since the early 1930's, with DDT being introduced in 1946 (Mabaso et al. 2004). In this study, the greatest uptake of DDE (Category 4: 173 to 997 µg/g lipid) was well above the DDE concentrations measured in most studies. The ongoing use of DDT for IRS can result in exposure to DDT for as much as 8 hours per day during activities such as eating, sleeping, bathing and more. Indoor air samples analysed two months after IRS had median 2700 ng/ m3 ΣDDT concentrations two months after IRS (Van Dyk et al. 2010); considerably higher than the EPA inhalation unit risk of 0.097 ng/m3 (ATSDR 2002), suggesting continuous contamination from sprayed surfaces into the environment. Indoor residual spraying may thus also cause water and food contamination and eventually cause human uptake. Females living in IRS houses had DDT and DDE serum concentrations that were significantly positively associated with DDT and DDE concentrations measured in undisturbed house dust (Spearman's rho=0.68 and 0.54, respectively) (Gaspar et al. 2015), suggesting that contamination of the home environment may lead to uptake . In our study, men from IRS villages had DDE concentrations up to 200x higher than those from non-IRS villages, indicating significant uptake of DDE from their environment. The only other study that reports comparable, but lower concentrations of DDT and DDE uptake are those for malaria spray workers working in the Limpopo province of South Africa (Dalvie et al. 2004a; Dalvie et al. 2004b). Malaria spray workers would not necessarily have come from villages where IRS rounds were conducted. Many studies report on the effects of historical and indirect DDT exposure, which may lead to the contrasting hormonal responses.

Positive hormonal responses have been reported in few cases. Direct, occupational exposure to *p-p'*-DDT caused increased testosterone concentrations (β±SE 0.17±0.07, P=0.014) in Limpopo spray-workers (Dalvie et al. 2004a). Men from Greenland and Kharkiv, with greater DDE uptake had higher free testosterone and FSH concentrations (Giwercman et al. 2006). Our findings do not concur with several epidemiological studies reporting that DDE exposure was associated with lower testosterone concentrations, but associations were not significant in these studies in contrast to ours. These included Mexican flower workers (Blanco-Muñoz et al. 2012), Norwegian men from southern Norway (Haugen et al. 2011) and African-American farmers from North Carolina (Martin Jr et al. 2002). Other studies reported no changes in testosterone, FSH or LH concentrations in Swedish and Latvian men (Hagmar et al. 2001), in previous DDT spray-workers (Cocco et al. 2004) and Swedish fishermen (Rignell-Hydbom et al. 2004). Testosterone was lower or remained steady in response to much lower DDT exposure concentrations than those seen in our total study population (median DDE 26.13 µg/g lipid). Median DDE concentrations were 0.677 µg/g lipid in the Mexican flower workers study (Blanco-Muñoz et al. 2012), 0.83 µg/g in the Swedish/Latvian study (Hagmar et al. 2001) and 1.2 µg/g in the North Carolina study (Martin Jr et al. 2002), 0.396 µg/g in the spray-worker study (Cocco et al. 2004) and 0.24 µg/g lipid in Swedish fishermen (Rignell-Hydbom et al. 2004). These DDE concentrations are similar to the DDE concentrations we measured in non-IRS villages (DDE Category 1 and 2) of the Limpopo province, South Africa (Table 2, Figure 1 this study) and are likely to account for the absence of any hormone changes in those studies. In the comparative exposure group, DDE Category 2 (0.5-26 µg/g lipid), T-concentrations were slightly higher than T-concentrations associated with DDE Category 1 (Fig 2). Two papers related to IRS activity, reported either no association (Dalvie et al. 2004a) or a negative correlation between DDE and testosterone concentrations (Ayotte et al. 2001). DDE exposure in the last study ranged between 17.0 and 177.2 µg/g lipid and was similar to our Category 3 (Ayotte et al. 2001), but our results showed positive associations.

In the current study, men with higher DDT and DDE concentrations (Categories 3 and 4) had higher estradiol concentrations. Malaria spray-workers from Limpopo with mean DDE = 65.0 ± 48.8 µg/g lipid concentrations also had higher estradiol concentrations (Dalvie et al. 2004b). In contrast, Taiwanese men approximately 10 years after DDT was last sprayed, had median DDE level 4.057 µg/g lipid-adjusted which were negatively correlated with estradiol concentrations (r =0.239, P=0.018) (Asawasinsopon et al. 2006). At these exposure concentrations (our Category 2), we observed a negative association of estradiol with DDT.

Most studies found no significant associations between DDE and E_2 concentrations (Blanco-Muñoz et al. 2012; Cocco et al. 2004; Giwercman et al. 2006; Haugen et al. 2011); or did not measure E_2 at all (Ayotte et al. 2001; Bonde et al. 2008; Hagmar et al. 2001; Martin Jr et al. 2002).

Men from IRS villages had DDE concentrations up to 200x higher than those from non-IRS villages, indicating significant uptake of DDE from their environment. A single previous study reported significantly higher T concentrations in response to IRS in malaria spray-workers from Limpopo Province, South Africa, with DDE concentrations of 65.0 (±48.8) µg/g) (Dalvie et al. 2004a), similar to DDE level Category 3 in this study. At DDE Category 3, we measured somewhat higher t-T concentrations compared to DDE categories 1 and 2, but significantly higher t-T concentrations were measured in DDE Category 4 compared to Category 1. The DDE concentrations measured in Category 4 ranged between 173-997 µg/g lipid and were well above the DDE concentrations measured in most studies. This may be attributed to the ongoing annual application of DDT to control malaria vectors. People living in IRS villages are likely to have indoor exposure to DDT for at least 8 hours per day for activities such as eating, sleeping and sbathing. Indoor air samples analysed two months after IRS had median 2700 ng/ m^3 ΣDDT concentrations two months after IRS (Van Dyk et al. 2010); considerably higher than the EPA inhalation unit risk of 0.097 ng/m^3 (ATSDR 2002). The authors concluded that open water and food products in the houses may become contaminated as a result of IRS. DDT and DDE concentrations in undisturbed house dust from IRS houses were significantly positively associated with serum concentrations in females living in those houses (Spearman's rho=0.68 and 0.54, respectively) (Gaspar et al. 2015), confirming that contamination of the home environment may be an important factor/source of DDT and DDE exposure.

Physiologically, high concentrations of T may result from DDE blocking the AR. Testosterone is metabolized either by 5-α-reductase to DHT or by hepatic aromatase to E_2 which may explain the high estradiol concentrations. Both T and E_2 give negative feedback to the pituitary, reducing LH and FSH secretion, which will impair testicular function. LH binds to receptors on the Leydig cell and lower LH stimulation will limit the production of T. It is biologically plausible that the "balance" of the pituitary will be re-set/adjusted in line with the higher T concentrations. Since DDT is estrogenic, it might also increase the negative feedback on the pituitary, but the possibility seems remote as E_2 has a higher potency that DDT. The mean age of all men in our study was 21.8 (±4) years and had spent 17.6 (±6) years at their current residence. *In utero* exposure, in particular during the vulnerable window

period of fetal urogenital development, might have occurred in many participants. Altogether 98% reported being breastfed and in this area may imply an extensive period up to two years (Bouwman et al. 2012) and breast milk samples from this area were reported to contain significant concentrations of DDT and DDE (Bouwman et al. 2012; Darnerud et al. 2011), might have contributed to the body burden of these chemicals. Further exposure to DDT might have resulted from IRS programmes. The precise timing and duration of exposure to DDT and DDE was impossible to determine in the current study, but it seems possible that many of these men might have been exposed in utero, during breastfeeding, as toddlers and continuously for their whole life. Our results are not only applicable to this currently DDT sprayed area in Limpopo Province, but to other areas in South Africa, Africa and elsewhere in the world where DDT IRS is being used.

A final consideration is whether the combination of high T and E_2 in concert with low LH and FSH concentrations and the associated high DDE and DDT concentrations are reasons for concern. Our previous findings regarding semen quality of men (n=311) from the same area, showed low mean ejaculate volume, lower sperm count and impaired sperm motility in men with the highest quartile DDE concentration (Aneck-Hahn et al. 2007). Low LH and FSH concentrations in the current study will result in impaired spermatogenesis (Ramaswamy and Weinbauer 2014). It seems possible that both androgen receptor antagonism by DDE and diminished LH and particularly FSH can account for the low sperm count and function (Moline et al. 2000; Mortimer et al. 2013; Robaire et al. 2007; Turek 2012). Also, sperm motility seemed to be additionally impaired by direct toxicity, possibly due to DDE exposure representing a non-genomic mechanism (Tavares et al. 2015).

This study has several strengths. Blood sampling for male reproductive hormones was done before 10:00 to avoid any interference by the normal circadian rhythm of T, peaking at 09:00 and reaching its lowest point (nadir – 20% less than peak value) in mid-afternoon (Kaufman and Vermeulen 2005). The timing of sampling was noted in the studies of Blanco-Munoz et al. (2012); (Cocco et al. 2004) and (Haugen et al. 2011) Haugen et al. (2011); presumably Martin Jr et al. (2002); or not reported (Ayotte et al. 2001; Dalvie et al. 2004b; Giwercman et al. 2006). The study population was relatively homogenous for factors such as diet, breastfeeding, and socioeconomic status, which can reduce uncontrolled confounding. This current study is the first to address the effects of DDT and DDE exposure on male hormones in men living under IRS-exposed conditions.

This study has some limitations. Although there may be selection bias as men included in this study of hormone concentrations were also those who delivered a semen sample for analysis (separate study), the existing data in the large cohort may also be strength. The participation rate was high and only some men (n=50 total) could not submit a semen sample and were excluded from this study. However, there is no reason to believe that men who did not produce a semen sample had a different exposure status than men who produced a semen sample; thus, results would not be affected. We were also not able to consider the potential confounding effects of other chemical exposures such as polychlorinated biphenyls (PCBs), other POPs, PBDEs or phthalates. Low concentrations of PCB and brominated flame retardants (BFRs) (specifically polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) (Darnerud et al. 2011), but high concentrations of DDT and DDE were reported in milk samples from this area (Bouwman et al. 2012). Finally, in the statistical analysis we have included all known potential confounders, but we cannot exclude some residual confounding.

5. Conclusions

To our knowledge, this is the first study to report that men with high DDE and DDT concentrations also had high T and E_2 concentrations, but low FSH and LH concentrations. The concurrent exposure to both DDT and DDE seemed to result from estrogenic and/or anti-androgenic effects in men living in IRS areas. The long-term implications of this hormonal imbalance for fertility and reproductive health in the IRS communities are not known, but may be evaluated with further research.

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References

Aneck-Hahn, N.H.; Schulenburg, G.W.; Bornman, M.S.; Farias, P.; Jager, C., 2007. Impaired semen quality associated with environmental DDT exposure in young men living in a malaria area in the Limpopo Province, South Africa. J Androl. 28, 423-434.

Asawasinsopon, R.; Prapamontol, T.; Prakobvitayakit, O.; Vaneesorn, Y.; Mangklabruks, A.; Hock, B., 2006. Plasma levels of DDT and their association with reproductive hormones in adult men from northern Thailand. Sci Total Environ. 355, 98-105.

ATSDR. ToxGuide for DDT/DDD/DDE. in: U.S. Department of Health and Human Services Public Health Service A.f.T.S.a.D.R., ed. Atlanta, GA, USA; 2002

Ayotte, P.; Giroux, S.; Dewailly, É.; Avila, M.H.; Farias, P.; Danis, R.; Díaz, C.V., 2001. DDT spraying for malaria control and reproductive function in Mexican men. Epidemiol. 12, 366-367.

Blanco-Munoz, J.; Lacasana, M.; Aguilar-Garduno, C.; Rodriguez-Barranco, M.; Bassol, S.; Cebrian, M.E.; Lopez-Flores, I.; Ruiz-Perez, I., 2012. Effect of exposure to p,p'-DDE on male hormone profile in Mexican flower growers. Occup Environ Med. 69, 5-11.

Blanco-Muñoz, J.; Lacasaña, M.; Aguilar-Garduño, C.; Rodríguez-Barranco, M.; Bassol, S.; Cebrián, M.E.; López-Flores, I.; Ruiz-Pérez, I., 2012. Effect of exposure to p, p'-DDE on male hormone profile in Mexican flower growers. J Occup Env Med. 69, 5-11.

Bonde, J.P.; Toft, G.; Rylander, L.; Rignell-Hydbom, A.; Giwercman, A.; Spano, M.; Manicardi, G.C.; Bizzaro, D.; Ludwicki, J.K.; Zvyezday, V., 2008. Fertility and markers of male reproductive function in Inuit and European populations spanning large contrasts in blood levels of persistent organochlorines. Environ Health Perspect. 116, 269.

Bouwman, H., 2004. South Africa and the Stockholm Convention on persistent organic pollutants: science policy. S Afr J Sci . 100, 323-328.

Bouwman, H.; Kylin, H.; Sereda, B.; Bornman, R., 2012. High levels of DDT in breast milk: Intake, risk, lactation duration, and involvement of gender. Environmental Pollution. 170, 63-70.

Chen, C.W.; Hurd, C.; Vorojeikina, D.P.; Arnold, S.F.; Notides, A.C., 1997. Transcriptional activation of the human estrogen receptor by DDT isomers and metabolites in yeast and MCF-7 cells. Biochem Pharmacol. 53, 1161-1172.

Cocco, P.; Loviselli, A.; Fadda, D.; Ibba, A.; Melis, M.; Oppo, A.; Serra, S.; Taberlet, A.; Tocco, M.G.; Flore, C., 2004. Serum sex hormones in men occupationally exposed to dichloro-diphenyl-trichloro ethane (DDT) as young adults. The Journal of endocrinology. 182, 391-397.

Dalvie, M.A.; Myers, J.E.; Lou Thompson, M.; Dyer, S.; Robins, T.G.; Omar, S.; Riebow, J.; Molekwa, J.; Kruger, P.; Millar, R., 2004a. The hormonal effects of long-term DDT exposure on malaria vector-control workers in Limpopo Province, South Africa. Environ Res. 96, 9-19.

Dalvie, M.A.; Myers, J.E.; Thompson, M.L.; Robins, T.G.; Dyer, S.; Riebow, J.; Molekwa, J.; Jeebhay, M.; Millar, R.; Kruger, P., 2004b. The long-term effects of DDT exposure on semen, fertility, and sexual function of malaria vector-control workers in Limpopo Province, South Africa. Environ Res. 96, 1-8.

Danzo, B.J., 1997. Environmental xenobiotics may disrupt normal endocrine function by interfering with the binding of physiological ligands to steroid receptors and binding proteins. Environ Health Perspect. 105, 294.

Darnerud, P.O.; Aune, M.; Larsson, L.; Lignell, S.; Mutshatshi, T.; Okonkwo, J.; Botha, B.; Agyei, N., 2011. Levels of brominated flame retardants and other pesistent organic pollutants in breast milk samples from Limpopo Province, South Africa. Sci Total Environ. 409, 4048-4053.

De Jager, C.; Aneck-Hahn, N.H.; Bornman, M.; Farias, P.; Leter, G.; Eleuteri, P.; Rescia, M.; Spanò, M., 2009. Sperm chromatin integrity in DDT-exposed young men living in a malaria area in the Limpopo Province, South Africa. Hum Reprod . 24, 2429-2438.

Dees, C.; Askari, M.; Foster, J.S.; Ahamed, S.; Wimalasena, J., 1997. DDT mimicks estradiol stimulation of breast cancer cells to enter the cell cycle. Mol Carcinog. 18, 107-114.

Gaspar, F.W.; Chevrier, J.; Bornman, R.; Crause, M.; Obida, M.; Barr, D.B.; Bradman, A.; Bouwman, H.; Eskenazi, B., 2015. Undisturbed dust as a metric of long-term indoor insecticide exposure: Residential DDT contamination from indoor residual spraying and its association with serum levels in the VHEMBE cohort. Environ Int. 85, 163-167.

Giwercman, A.H.; Rignell-Hydbom, A.; Toft, G.; Rylander, L.; Hagmar, L.; Lindh, C.; Pedersen, H.S.; Ludwicki, J.K.; Lesovoy, V.; Shvets, M.; Spano, M.; Manicardi, G.C.; Bizzaro, D.; Bonefeld-Jorgensen, E.C.; Bonde, J.P., 2006. Reproductive hormone levels in men exposed to persistent organohalogen pollutants: a study of inuit and three European cohorts. Environ Health Perspect. 114, 1348-1353.

Hagmar, L.; Bjork, J.; Sjodin, A.; Bergman, A.; Erfurth, E.M., 2001. Plasma levels of persistent organohalogens and hormone levels in adult male humans. Archives of environmental health. 56, 138-143.

Haugen, T.B.; Tefre, T.; Malm, G.; Jonsson, B.A.; Rylander, L.; Hagmar, L.; Bjorsvik, C.; Henrichsen, T.; Saether, T.; Figenschau, Y.; Giwercman, A., 2011. Differences in serum levels of CB-153 and p,p'-DDE, and reproductive parameters between men living south and north in Norway. Reprod Toxicol. 32, 261-267.

Holloway, A.C.; Stys, K.A.; Foster, W.G., 2005. DDE-induced changes in aromatase activity in endometrial stromal cells in culture. Endocr J 27, 45-50.

Kaufman, J.M.; Vermeulen, A., 2005. The decline of androgen levels in elderly men and its clinical and therapeutic implications. Endocr Rev. 26, 833-876.

Kelce, W.; Stone, C.; Laws, S.; Gray, L.; Kemppainen, J.; Wilson, E., 1995. Persistent DDT metabolite p, p′-DDE is a potent androgen receptor antagonist. Find this article online: Nature. 581-585.

Kelce, W.R.; Wilson, E.M., 1997. Environmental antiandrogens: developmental effects, molecular mechanisms, and clinical implications. J Mol Med (Berl). 75, 198-207.

Krause, W., 1977. Influence of DDT, DDVP and malathion on FSH, LH and testosterone serum levels and testosterone concentration in testis. Bull Environ Contam Toxicol. 18, 231-242.

Lemaire, G.; Mnif, W.; Mauvais, P.; Balaguer, P.; Rahmani, R., 2006. Activation of alpha- and beta-estrogen receptors by persistent pesticides in reporter cell lines. Life Sci. 79, 1160-1169.

Mabaso, M.L.H.; Sharp, B.; Lengeler, C., 2004. Historical review of malarial control in southern African with emphasis on the use of indoor residual house-spraying. Tropical Medicine & International Health. 9, 846-856.

Martin Jr, S.A.; Harlow, S.D.; Sowers, M.F.; Longnecker, M.P.; Garabrant, D.; Shore, D.L.; Sandler, D.P., 2002. DDT metabolite and androgens in African-American farmers. J Epidemiol. 13, 454-458.

Metcalf, D.; Nicola, N. The hemopoietic colony-stimulating factors: from biology to clinical applications ed^eds: Cambridge University Press; 1995

Moline, J.M.; Golden, A.L.; Bar-Chama, N.; Smith, E.; Rauch, M.E.; Chapin, R.E.; Perreault, S.D.; Schrader, S.M.; Suk, W.A.; Landrigan, P.J., 2000. Exposure to hazardous substances and male reproductive health: a research framework. Environ Health Perspect. 108, 803-813.

Mortimer, D.; Barratt, L.R.; Björndahl, L.; de Jager, C.; Jequier, A.M.; Muller, C.H., 2013. What should it take to describe a substance or product as "sperm-safe"? Human Reproduction Update. 19 (Suppl 1), i1-i45.

O'Connor, J.C.; Frame, S.R.; Ladics, G.S., 2002. Evaluation of a 15-day screening assay using intact male rats for identifying antiandrogens. Toxicol Sci. 69, 92-108.

Ottoboni, A., 1969. Effect of DDT on reproduction in the rat. Toxicol Appl Pharmacol. 14, 74-81.

Ottoboni, A., 1972. Effect of DDT on the reproductive life-span in the female rat. Toxicol Appl Pharmacol. 22, 497-502.

Ottoboni, A.; Bissell, G.D.; Hexter, A.C., 1977. Effects of DDT on reproduction in multiple generations of beagle dogs. Arch Environ Contam Toxicol. 6, 83-101.

Ramaswamy, S.; Weinbauer, G.F., 2014. Endocrine control of spermatogenesis: Role of FSH and LH/ testosterone. Spermatogenesis. 4,

Rhouma, K.B.; Tebourbi, O.; Krichah, R.; Sakly, M., 2001. Reproductive toxicity of DDT in adult male rats. Hum Exp Toxicol. 20, 393-397.

Rignell-Hydbom, A.; Rylander, L.; Giwercman, A.; Jönsson, B.; Nilsson-Ehle, P.; Hagmar, L., 2004. Exposure to CB-153 and p, p′-DDE and male reproductive function. Hum Reprod. 19, 2066-2075.

Robaire, B.; Seenundun, S.; Hamzeh, M.; Lamour, S.A., 2007. Androgenic regulation of novel genes in the epididymis. Asian J Androl. 9, 545-553.

Rylander, L.; Wallin, E.; Jönssson, B.A.; Stridsberg, M.; Erfurth, E.M.; Hagmar, L., 2006. Associations between CB-153 and p, p′-DDE and hormone levels in serum in middle-aged and elderly men. Chemosphere. 65, 375-381.

Smith, A. Chlorinated hydrocarbon Insecticides. Handbook of Pesticide Toxicology. Vol. 2 Classes of Pesticides, Chapter 15. Academic Press. New York, NY; 1991

Tarjan, R.; Kemeny, T., 1969. Multigeneration studies on DDT in mice. Food Cosmet Toxicol. 7, 215-222.

Tavares, R.S.; Amaral, S.; Paiva, C.; Baptista, M.; Ramalho-Santos, J., 2015. In vitro exposure to the organochlorine p,p′-DDE affects functional human sperm parameters. Chemosphere. 120, 443-446.

Turek, P. Male reproductive physiology. in: McDougal W.S., Wein A.J., Kavoussi L.R., Partin A.W., Peters C.A., eds. Campbell-Walsh Urology Elsevier Health Sciences; 2012

Turusov, V.; Day, N.; Tomatis, L.; Gati, E.; Charles, R., 1973. Tumors in CF-1 mice exposed for six consecutive generations to DDT. J Natl Cancer Inst. 51, 983-997.

Van Dyk, J.C.; Bouwman, H.; Barnhoorn, I.; Bornman, M., 2010. DDT contamination from indoor residual spraying for malaria control. Sci Total Environ. 408, 2745-2752.

WHO. DDT in indoor residual spraying: human health aspects. World Health Organization; 2011

WHO. World Malaria Report. Geneva: World Health Organization; 2016

Younglai, E.V.; Holloway, A.C.; Lim, G.E.; Foster, W.G., 2004. Synergistic effects between FSH and 1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethylene (p, p′-DDE) on human granulosa cell aromatase activity. Hum Reprod. 19, 1089-1093.