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Gene expression profile of sex-linked metabolic enzyme in rats inhabiting dichlorodiphenyltrichloroethane (DDT)-sprayed areas of South Africa

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Abstract

Dichlorodiphenyltrichloroethane (DDT) is still used in some developing countries to control vectors such as malaria, despite being highly toxic to humans and animals. Cytochromes P450 (CYPs) are enzymes that break down xenobiotics such as DDT. However, only few studies have investigated DDT effects on biological organisms. We collected wild rats in DDT indoor sprayed (IRS) and non-IRS residential areas in the KwaZulu-Natal province in South Africa, and several organs, including the liver, kidney, brain, spleen, and lung, were collected. DDT levels were determined using GC-ECD, and their effects on 12 metabolic enzymes in the liver were assessed using qRT-PCR. The liver showed the highest total median concentrations of DDT and its metabolites. Sex-linked gene expression differences disappeared in the IRS region, but the same difference was seen in the rat livers in the non-IRS region, with female rats having low expression levels, suggesting that DDT may affect the expression of some CYP genes in different ways depending on the extent of exposure. As only a few studies have investigated gene expression patterns of metabolic enzymes in terrestrial mammals, the findings of this study will be useful for future *in-vivo* studies employing rats and field samples.

Key Words: CYP, DDT, nuclear receptor, sex-linked, South Africa

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Abbreviation List

AhR	aryl hydrocarbon receptor
CAR	constitutive androstane receptor
CYP	Cytochrome P450
DDD	dichlorodiphenyldichloroethane
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DDTs	DDT, DDD, and DDE
GC-ECD	gas chromatography-electron capture detector
IARC	International Agency for Research on Cancer
IRS	indoor residual spraying
NADH	nicotinamide adenine dinucleotide
PPARα	peroxisome proliferator-activated receptor alpha
PXR	pregnant X receptor
qRT-PCR	quantitative reverse transcription PCR
SULT	sulfotransferase
UGT	uridine 5'-diphospho-glucuronosyltransferase

Introduction

Dichlorodiphenyltrichloroethane (DDT) is a pesticide used in developing countries with certain restrictions owing to environmental persistence and its adverse effects on mammals, including humans, and the environment. The Republic of South Africa, where our study was conducted, still uses DDT in indoor residual spraying (IRS)[44]. However, various investigations have indicated DDT toxicity in mammals, and DDT has been classified as a carcinogen and an endocrine disruptor[36]. In 2015, the International Agency for Research on Cancer (IARC) re-evaluated the carcinogenicity of DDT and categorized it into Group 2A (probably carcinogenic to humans). Higher testosterone and estradiol levels and decreased follicle-stimulating hormone levels were found in the blood of highly DDT exposed males in a field study conducted in the Republic of South Africa[5]. Although DDT is required in some countries to control mosquito-borne diseases, the advantages and risks to human health and the environment should be carefully considered[36].

DDT can be metabolized into dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD), both of which have high potential to accumulate in animals, humans, and the environment over time and can travel vast distances in the upper atmosphere[1,21]. While microorganisms are responsible for the environmental DDT degradation, DDT has a long half-life and persists in the environment for a long period[1]. Previous studies have employed wild rodents to assess the likely exposure levels on individuals living in IRS areas in countries where DDT is used as a malaria vector control[30,46]. As rodents commonly share habitats with people, especially in houses, it is assumed that they have human-like exposure patterns, including exposure to high amounts of DDT in IRS areas. Previous studies have reported high concentrations of DDTs in house rats from IRS areas, and *para, para*-isomers (*p,p'*-DDT, *p,p'*-DDE, and *p,p'*-DDD) were found at higher liver tissue concentrations than *ortho, para*-isomers (*o,p'*-DDT, and *o,p'*-DDD)[30,46].

Phase 1 metabolic enzymes, such as cytochrome P450 (CYP), and phase 2 metabolic enzymes, such as uridine 5'-diphospho-glucuronosyltransferase (UGT) and sulfotransferase (SULT), are involved in the metabolism and excretion of numerous exogenous chemicals[11]. These enzymes also play roles in homeostasis, such as regulating the levels of activated hormone concentration in mammalians[48]. However, several chemical compounds can easily and significantly upregulate these enzymes. Evidence from various studies implies that DDT activates metabolic enzymes and their activity[8,13,31]. By activating these metabolic enzymes, human or animal bodies can oxidize or conjugate exogenous chemicals and excrete them from their bodies. However, it has been noted that inducing these enzymes can have negative

health consequences. For example, overexpression of CYP2B can produce reactive oxygen species that potentially exacerbate tumors in the liver[16]. These biological processes, in turn, are mediated by nuclear receptors such as the constitutive androstane receptor (CAR), pregnant X receptor (PXR), peroxisome proliferator-activated receptor alpha (PPARα), and transcription factor like aryl hydrocarbon receptor (AhR)[12,27].

Male rats or mice have mostly been used for toxicity tests as the sexual cycle can cause larger variations in female rats. However, one study found that after exposure to 100 mg/kg DDT, males and females had distinct reactions, such as a larger induction of CYP3A1 in females (31.9-fold) than in males (1.3-fold). However, the trend of CYP2B1 upregulation was the same for females and males at 19.3- and 19.4-fold induction, respectively[38]. These disparities highlight the importance of considering toxicity for both sexes. This study focuses on wild rats living in DDT-sprayed areas. It aims to investigate if there are links between DDT pollution levels and gene expression of nuclear receptors and metabolic enzymes in both males and females.

Materials and Methods

1. Sample collection and preparation

Rats from a sample set collected in South Africa that has been described in a previous paper were used[46]. Briefly, eight and ten rats were captured using gauze traps with food as bait in residential areas in the KwaZulu-Natal province from IRS and non-IRS areas in April 2014, respectively. The sampling area is provided in the previous paper[46]. The application of DDT in the IRS program areas was within the target range set by the IRS program: 75% DDT wettable powder was applied at a dosage of 2 g active ingredient per square meter. Following capture, the rodents were euthanized, their body weight and sex were determined, and were subsequently dissected to collect the brain, lung, spleen, liver, and kidney, which were stored at –20ºC until DDT analysis. For RNA extraction, a small portion of the liver was excised and kept in RNAlater (Sigma-Aldrich, Saint Louis, MO, USA). The eyes were recovered and preserved in formalin to determine age.

2. Extraction and analysis of DDT and its metabolites

Analytical methodology, including the use of chemicals and reagents, instrumental analysis, and quality control procedures, are described in detail in Yohannes *et al.*[47]. Approximately 1–5 g of each tissue was mixed with anhydrous sodium sulfate, spiked with 10 ng of PCB77, and extracted with hexane/acetone (3:1, v/v) using a Soxtherm extractor (S306AK, Gerhardt, Germany). To obtain DDT and its metabolites (DDE and DDD), the extracts were concentrated and subjected to a Florisil (5% deactivated) glass column, with elution carried out by hexane:dichloromethane (7:3, v/v). The eluate was then concentrated to near dryness under a gentle nitrogen stream and redissolved in 100 µL n-decane. Before instrumental analysis, all extracts were treated with 2,4,5,6-tetrachloro-m-xylene (TC*m*X). DDTs (DDT, DDE, and DDD) were analyzed using a Shimadzu 2014 gas chromatography model equipped with a 63 Nickel µ−electron capture detector (ECD) and an ENV-8MS capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness). The DDTs were identified by comparing their retention time with the corresponding standard. The correlation coefficients (r^2) of the calibration curves were all greater than 0.990. The mean DDT recovery for the spiked blanks was 60%. Thus, the DDT concentrations were calculated and corrected based on the recovery rate. Concentrations of DDTs were expressed as nanogram per gram of wet weight (ng/g ww).

3. Species identification and age estimation of wild rodents

Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) was used to recover genomic DNA from wild rodent liver tissues. Species were identified

Table 1. Primer and amplicon information of target and reference genes for qPCR

Gene	5'-forward primer-3'	5'-reverse primer-3'	Size (bp)
Beta-actin	GGTCCACACCCGCCACCAGTT	ACCCATACCCACCATCACACCCTG	169
AhR	AAACCAAAGACACGGGATAAACTC	TCGGACTCTGAAACTTGCTTAGG	179
PXR	GCGTCATCAACTTCGCCAAA	CCAGGTTCCTGTTTCCGTGT	140
PPARα	ACTAGCAACAATCCGCCTTTTG	GGACCTCTGCCTCCTTGTTTTC	115
Cyp2a1	GAGGCGAACAGGCTACCTACA	CGCTCCCCACTGCTGAAT	67
Cyp2a2	TACCTGAGCAAAACAGTCTCCA	TCGATCATCTCCAACAGTGACAA	101
Cyp2b1	GCTCAAGTACCCCCATGTCG	ATCAGTGTATGGCATTTTACTGCGG	109
Cyp2c11	CGCACGGAGCTGTTTTTGTT	GCAAATGGCCAAATCCACTG	115
Cyp3a1	GATTCTGTGCAGAAGCATCGA	ATAGGGCTGTATGAGATTCTT	91
Cyp3a2	CGATTCCAACATATGCTCTTCATCA	TTCTCCTTGCTAAACCTTTCTGGAT	85
Cyp3a18	AAGCACCTCCATTTCCTTCATA	TCTCATTCTGGAGTTTCTTTTG	74
Cyp4a1	TCCACCCGCTTCACGGGCAGC	AGCCTTGAGTAGCCATTGCC	120
Cyp7a1	TGACCGGTACCTTGATGAAAG	AAGAGTCTTCCAGGACATATT	120
Ugt1a6	AGTTCTAGGTGACAAGCTGC	CACTAGCACCACAATGTCGT	112
Ugt2b1	CAACCATTTAAAGAGAAGTCCTG	GGTAAGAATGGGTGTGGAAA	143
Sult1e1	GATGAAGAACAATCCATGCACC	CTCCTCAAATCTCTCCCTCAGG	142

following the method of Robins *et al.*[35], with some modifications. The cytochrome b-containing genome region was amplified using PrimeSTAR Max (Takara Bio Inc., Shiga, Japan) and the following primers (forward primer 5′-GGTGAAGGCTTCAACGCCAACCCTA-3′ and reverse primer 5′-TAGAATATCAGCTTTGGGTG TTGATGG-3′). Molecular Evolutionary Genetics Analysis (MEGA) 7 software was used to perform maximum-likelihood analyses on these sequences. *Rattus tanezumi* was the subject of this study.

Measuring the lens weight of rodents has provided researchers with a reliable indicator of their age. Thus, the formalin-preserved lens from each eye was dried for 48 hr at 50ºC and then weighed in pairs to estimate the rat's age using the equations [log Y = 1.00 + 0.023X] for male and [log Y = 1.05 + 0.023X] for female animals, where Y is the age in days and X is the total weight of both lenses in milligrams based on Tanikawa's protocol[41]. This protocol was developed for *Rattus rattus*, a species closely related to *R. tanezumi*, aged 20 to 1121 days. Commonly, both species are referred to as "black rats"[23]. We thus classified *R. tanezumi* as a "black rat" in our data analysis.

4. RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA was extracted from the liver samples of *R. tanezumi* using the Nucleospin RNA kit (Macherey-Nagel Düren, Germany) and the quality and amount of the extracted RNAs were evaluated using a Nanodrop-1000A spectrophotometer (Delaware, USA). The ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) was used to synthesize first-strand cDNA from RNA according to the manufacturer's protocol. Quantitative real-time PCR analysis of 12 metabolic enzymes and three nuclear receptor genes was performed on StepOne Plus Real-Time PCR system (Applied Biosystems) using cDNA and Fast SYBR Green master mix (Applied Biosystems, CA, USA). The primers used for the amplification are summarized in Table 1. Some of the primers used in this study were those used in previous studies[3,20,25,26,28,29,33,39]. A 10 µL reaction mixture containing 5 µL Fast SYBR Green master mix, 300 nM forward and reverse primers, and 20 ng of cDNA was used for the PCR. The PCR protocol was 95°C for 20 s, followed by 40 cycles of 95°C for 3 s, and 60°C (for all genes) for 30 s. The specificity of the reaction was verified by melt curve analysis.

Table 2. Spearman's correlation analysis between nuclear receptors and metabolic enzymes gene expression level

	PXR	*PPARα*	*Cyp2a1*	*Cyp2a2*	*Cyp2b1*	*Cyp2c11*	*Cyp3a1*	*Cyp3a2*	*Cyp3a18*	*Cyp4a1*	*Cyp7a1*	*Ugt1a6*	*Ugt2b1*	*Sult1e1*
AhR	0.63*	0.49*	0.33	0.42	0.70*	0.31	0.29	0.19	0.34	0.08	0.02	0.49*	0.77*	0.08
PXR		0.78*	0.47*	0.64*	0.55*	0.16	0.37	0.50*	0.59*	0.43	0.01	0.38	0.57*	0.24
PPARα			0.39	0.61*	0.44	0.16	0.3	0.56*	0.67*	0.49*	0.29	0.47	0.56*	0.23

[Male and female rats in IRS and non-IRS areas were combined in this analysis. Each value is the Spearman's rank correlation coefficient between each pair. * Significant correlation ($P < 0.05$).]

For each sample, the amount of gene expression was normalized against the housekeeping gene, *beta-actin*, and relative expression was calculated using the comparative CT method (ΔΔCT method). The lowest expressions of each gene in all rats were assigned a value of one and served as reference samples for calculation. Expression levels not identified and out of the range of primer efficiency were set to 0.5.

5. Statistics

JMP 16 (SAS Institute, NC, USA) software was used for all the statistical analyses, with a significant threshold of $P < 0.05$. Both parametric and non-parametric approaches were used according to the sample size and normality of the data. Wilcoxon's test was used to compare DDT concentrations between sexes. For age differences between areas and sexes, both parametric (Tukey-Kramer's HSD test) and non-parametric (Steel-Dwass test) statistical analyses were performed. To bring the distribution of gene expression closer to normal, the natural logarithm was applied. Both parametric (Welch's t-test) and non-parametric (Wilcoxon's exact test) statistical analyses were used to compare gene expression between sexes. We used $P < 0.05$ from both analyses as significance. The significance of the correlative analysis between nuclear receptor and metabolic enzymes gene expression was tested using Spearman's correlation test.

Results

The information on the sex, weight, age, and collected area for each *R. tanezumi* rat is summarized in Supplementary Table 1. There were eight male and ten female rats in total and their age ranged from 39–356 days. Ten rats were collected from the IRS area and eight from the non-IRS area. Tukey-Kramer's HSD and Steel-Dwass tests revealed that the ages did not differ significantly among all the pairs of sexes and areas with the lowest *P*-value 0.26 detected between male rats in non-IRS and IRS area by Tukey-Kramer's HSD test.

The bio-accumulation of DDTs in tissues of wild rodents by sampling area is depicted in Figure 1 and Supplementary Table 2. The levels of DDTs in all tissues in the IRS area were significantly higher than those in the non-IRS area ($P < 0.05$). The median concentration of sum of *p,p'*-DDE, *p,p'*-DDD, and *p,p'*-DDT ranged from 723.9 ng/g ww in the brain to 5053.1 ng/g ww in the liver in the IRS area and from 9.7 ng/g ww in the spleen to 189.3 ng/g ww in the liver in the non-IRS area. DDTs residues were found almost in all samples, indicating that distribution is ubiquitous in the study area, even in the non-IRS areas. In this study, no sex differences were observed in any of the DDT concentration levels.

The composition profiles of DDT and its metabolites in each of the tissues analyzed in the present study are shown in Figure 2. Except for the liver, all organs from the same site had similar patterns. The brain, lung, spleen, and kidney had the highest abundance of *p,p'*-DDT in the IRS area, with ratios of 62%, 76%, 78%, and 76%, respectively, compared to only 12% in the liver. The liver had a substantial amount of *p,p'*-DDD, a metabolite of *p,p'*-DDT, with a ratio of 73%. The accumulation profile of DDTs in the IRS area was DDT > DDD ≈ DDE, reflecting that *p,p'*-DDT was

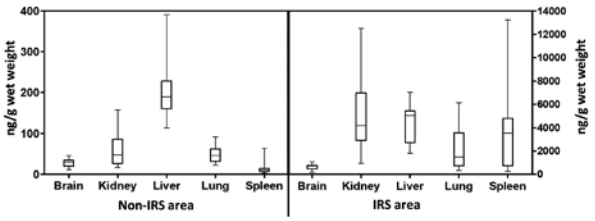

Fig. 1. Sum of DDTs concentration (ng/g wet weight) in each tissue in IRS area and non-IRS area

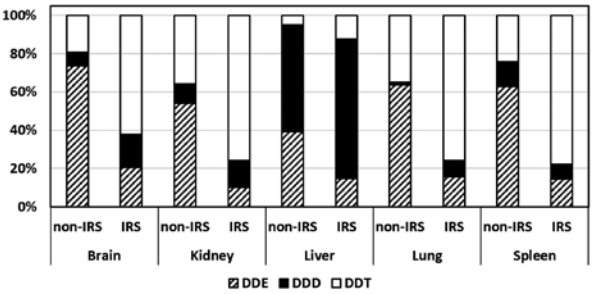

Fig. 2. Percentage composition of DDT metabolites in tissues of rats per area

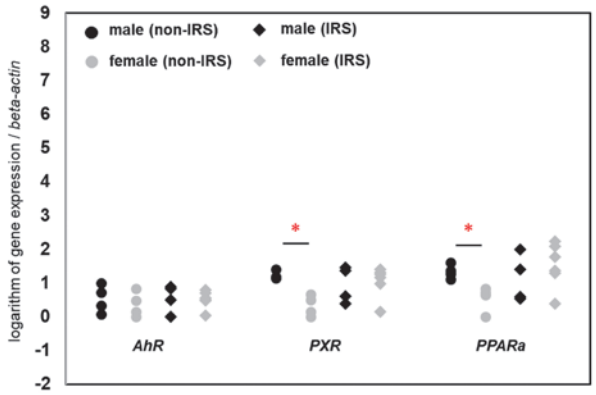

Fig. 3. Gene expression of nuclear receptors. Scatter plots show gene expression of the nuclear receptor in the rat liver. Gene expressions were normalized by beta-actin and calculated using the comparative CT method. * Significant differences ($P < 0.05$) between males and females in each group was calculated using both Welch's t test and Wilcoxon's exact test.

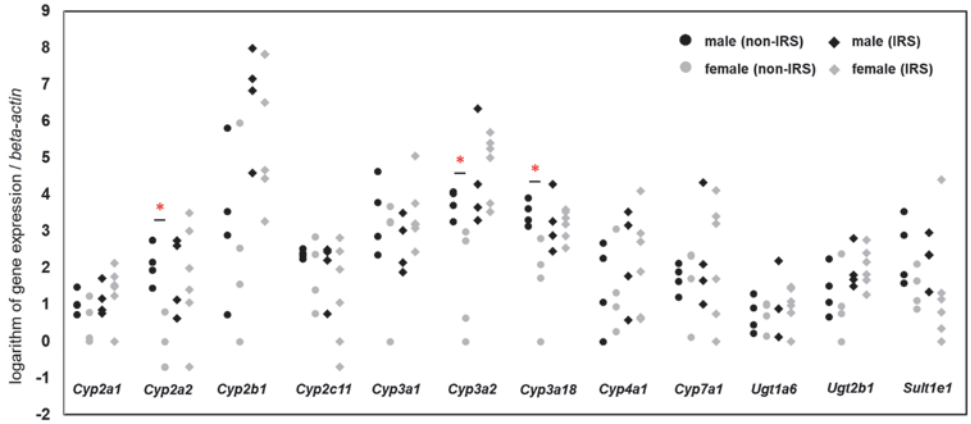

Fig. 4. Gene expression of metabolic enzymes. Scatter plots show gene expression of metabolic enzymes in rat liver. Gene expressions were normalized by beta-actin and calculated using the comparative CT method. The lowest expressions of each gene in all rats were defined as 1 and used as a reference sample for calculation. Undetected samples were not included for calculation but defined as 0.5 and added on the figure. * Significant differences ($P < 0.05$) between males and females in each group was calculated using both Welch's t test and Wilcoxon's exact test.

still used. The most common DDT metabolite, *p,p'*-DDE, was predominantly detected in most tissues from the non-IRS area (Figure 2). The brain, lung, spleen, kidney, and liver all had high levels of *p,p'*-DDE, at 74%, 64%, 63%, 54%, and 39%, respectively. The parent chemical *p,p'*-DDT was also detected in the non-IRS area.

Quantitative PCR was used to assess changes in gene expression between males and females in both areas. Both Welch's t-test and Wilcoxon's exact test were used for statistical analyses as the sample number in this study was small and it was impossible to ensure that the samples followed a normal distribution. In this study, the results of significance from both parametric and non-parametric analyses were taken as significant ($P < 0.05$) to prevent loose significance. Figures 3 and 4 show the natural logarithm of calculated

expressions using the ΔΔCT method. The expression of nuclear receptor genes in both sexes and sampling areas is shown in Figure 3. There was a significant difference ($P < 0.05$) in *PXR* and *PPARα* gene expression between the sexes in the non-IRS area. Compared to female rats (*PXR* range: 1.0–1.96; median: 1.40 and *PPARα* range: 1–2.29; median: 1.98), male rats had higher expression levels of *PXR* (3.14–4.05; 3.23) and *PPARα* genes (3.04–4.98; 3.75). However, this significant difference was not seen in the IRS area, where female rats had slightly higher median *PXR* and *PPARα* expression than male rats. Female rats showed a modest difference in gene expression between areas ($P = 0.07$ for both genes) for *PXR* and *PPARα*. No significant differences were found in both comparisons for *AhR*.

The mRNA expression profiles of 12 metabolic enzymes are shown in Figure 4. Significant sexual differences ($P < 0.05$) in mRNA expression levels were seen in the non-IRS area for *Cyp2a2*, *Cyp3a2*, and *Cyp3a18*. Male rats had significantly higher medians for *Cyp2a2*, *Cyp3a2*, and *Cyp3a18* than female rats. In the IRS area, however, the median of *Cyp3a18* expression in female rats (26.5) was slightly higher than that in male rats (22.2), indicating that high levels of DDTs seemed to eliminate the sexual difference in this gene. This difference seemed to be derived from the high expression of *Cyp3a18* in females rather than the change of expression in males. For the other genes, in both areas, there were no significant differences in gene expression between males and females. Many genes, such as *Cyp2c11* and *Cyp7a1*, had similar patterns in both areas, indicating that male or female dominance in the non-IRS area was maintained in the IRS area. The dominance revealed in the non-IRS area of some genes such as *Cyp2a1*, *Cyp3a1*, and *Ugt2b1* was reversed in the IRS area.

Table 2 shows the correlation between nuclear receptors and metabolic enzymes as determined by Spearman's correlation test using entire group (both males and females). Even though sex specific Spearman's correlation tests were conducted, the tendency in highly associated gene pairs remained unchanged. The highest Spearman's rank correlation coefficient ($\rho = 0.77$) was found in *AhR* and *Ugt2b1*. There were many gene expression pairs with significant correlations ($P < 0.05$), indicating interactions between nuclear receptors and metabolic enzymes such as *Cyp2b1*, *Cyp3a2*, *Cyp3a18*, and *Ugt2b1*. In contrast, *Cyp2c11*, *Cyp3a1*, *Cyp7a1*, and *Sult1e1* showed no strong correlation with nuclear receptors. The correlation between gene expression and DDTs concentration was also investigated and is shown in Supplementary Table 3. Some pairs showed rather high correlations, for example, the correlation coefficient between *p,p'*-DDT and *Cyp3a2* in females was 0.9.

Discussion

The levels of DDTs measured in different tissues of the captured rodents reflect the widespread occurrence of these compounds in the environment. This study found that the concentrations of DDTs in each organ of wild rodents from the IRS area were significantly higher than in those from the non-IRS area. The liver was the primary organ for DDTs accumulation, followed by the kidney. This result revealed that the IRS program in residential areas for malaria vector control routinely exposed rodents to high concentrations of DDT. However, the accumulation of DDTs was low in the brain, which could be due to the protective role of the blood-brain barrier, which prevents xenobiotics from entering the cerebral compartment[2].

The composition analysis of DDT showed that *p,p'*-DDD accumulated predominantly in the liver, both in IRS and non-IRS areas. This could reflect the liver's high metabolic capacity or the direct ingestion of DDD from the field. In the presence of nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH), *p,p'*-DDT is generally converted to *p,p'*-DDD in rat liver microsomes[21]. Furthermore, non-

enzymatic metabolism involving the heme group of hemoglobin produces *p,p'*-DDD in the blood[21,40]. The high level of *p,p'*-DDD accumulation in the liver was unique, as *p,p'*-DDT and *p,p'*-DDE were the most abundant compounds in the other organs. Our findings agree with previous study[13]. DDE has higher persistency in the environment and living organisms than DDT and is more resistant to degradation and elimination from the body[10]. All investigated organs had a larger DDE to total DDT ratio in non-IRS areas as compared with IRS areas in this study. This result could derive from the high persistency of DDE. Various effects of DDE as endocrine disruptor have been reported[7]. Thus, even if the use of DDTs has been banned, DDE effects, especially on endocrine disruption, persist in the environment for a long time in areas that were sprayed with DDT in the past.

In the IRS area, female rats lost their sexual differences in some gene expressions of metabolic enzymes and transcription factors. In the non-IRS area, the studied nuclear receptors, *PXR* and *PPARα*, were significantly different between male and female rats. These nuclear receptors are responsible for the induction of CYP450 gene expression, such as CYP2, CYP3, and CYP4 families[6,12]. Previous studies have shown a difference by sex in nuclear receptor activity or CYP enzyme induction in response to chemical exposure[15,24,45]. There have only been a few studies on nuclear receptor mRNA levels that focused on sexual differences. Nuclear receptor gene expression in mouse liver was studied extensively, and many of them, including *PXR* and *PPARα*, exhibited no sexual differences[34]. However in the case of rats, *PPARα* showed sexual differences[18].

Unlike *Rattus norvegicus*, which is a commonly used species in experimental trials, the metabolic ability of *R. tanezumi* has yet to be studied. As a result, it was not possible to determine whether the variation in the non-IRS area was due to the effects of pollutants or natural species-specific characteristics. The specificity of *R. tanezumi* could explain some mismatched gene expression patterns between sexes, such as higher *Cyp2a1* expression in males than females in the non-IRS area and a modest sexual difference in *Cyp2c11*. Therefore, an *in vivo* investigation including *R. tanezumi* and *R. norvegicus* is required to understand the process of sexual differentiation elimination in the IRS area.

In rats, distinct sexual differences in metabolic enzymes have been reported[3,19]. For example, Asaoka *et al.*[3] reported that for Sprague-Dawley rats, juvenile males have a higher expression of *Cyp3a1*, *Cyp3a2* and *Cyp3a18* than female juveniles. An *in vivo* study by Sierra-Santoyo *et al.*[38] found higher expression of CYP3A1 and CYP3A2 in basal males than females, as well as a notable induction of CYP3A1 and CYP3A2 in female rats after a single DDT exposure. In this study, the female-specific response to DDT exposure was also observed in some metabolic enzymes. One of the key mechanisms involved in CYP sexual morphism is thought to be the growth hormone. In rats, episodic growth hormone influences male-specific CYP expression, and many studies have reported a relationship between growth hormone and CYP expression in rat liver, occasionally with a hypophysectomized operation[42,43]. Hypophysectomized female rats, for example, had a high level of *Cyp3a2* mRNA, similar to male rats, but growth hormone restoration fully repressed *Cyp3a2* induction and restored female expression[32].

Many previous reports have revealed female-specific responses to CYP induction by chemical exposure. In fetal female rats, prenatal monocrotaline exposure significantly increased *Cyp3a* gene expression and protein levels[45]. A prenatal-retrorsine-exposure animal experiment significantly upregulated CYP3 protein and *Cyp3a1* expression in female rat liver[9]. Sex-dependent upregulation patterns were also seen in the CYP2B family in rodents after exposure to nicardipine, nifedipine, and TCPOBOP[22,24]. However, this study did not reveal the mechanism of the female-specific impact of DDTs. It is probable that the endocrine disturbance produced by DDTs, which targets growth hormone, affects

the gene expression of CYP. More hormone profiles could reveal the mechanism through which DDTs affect sex-linked CYP expression. Another possible mechanism is that DDTs could disrupt CYP expression via up- or down-regulation of nuclear receptors' mRNA levels. The same tendencies were observed when comparing nuclear receptors and some CYPs, such as *PXR* and *Cyp3a18* from IRS and non-IRS areas. The overexpression of nuclear receptors by DDTs in females may have induced some CYP expressions as shown by the significant correlation between nuclear receptors and some metabolic enzymes in this study. DDT-induced CYP induction has already been reported and CYPs are involved in xenobiotic metabolism and maintaining homeostasis[14,48]. For a better understanding of the effect of DDTs on animals, the toxicity of DDTs on metabolic enzymes should be carefully monitored. The human residents in the current research area may be contaminated with the same level of DDTs, but the effect of DDTs on metabolic enzymes in humans in this area is still unclear. Several studies have reported alterations in the blood metabolome of humans and experimental animals due to DDT exposure[17,37]. Discovery of an effective biomarker that can be applied in sprayed areas and used across various mammals is desirable in addition to molecular analyses.

The small number of rats in the sample was a weakness of this study. The main issue with this sample size was that it was difficult to use only one type of statistical analyses. For some gene expressions, even natural log-transformed relative gene expression showed a non-normal distribution. Moreover, at such a small sample size, non-parametric statistical tests were inappropriate. Thus, to ensure the statistical power of our results, we performed both parametric and non-parametric analyses. In addition, our sample size did not allow us to analyze the age groups separately. Thus, determining the precise effect of DDT on gene expression based on sex and age was difficult. Moreover, previous studies have suggested that other environmental pollutants such as chlordane and polychlorinated biphenyls contaminate the environment in KwaZulu-Natal Province[4,44]. Wild rats in the area are likely to be also exposed to these various other toxic chemicals; thus, exposure experiments using experimental rats need to be performed to evaluate the individual effect of DDT. However, this type of research, particularly with wild animals, is rare. Therefore, despite the small sample size, the findings of this field study are relevant for augmenting *in vivo* studies of DDT.

In conclusion, even though global usage of DDTs is declining, some developing countries still use DDTs for vector control of mosquito-borne diseases. Our results showed that gene expression varied between sexes in the non-IRS area but not in the IRS area, indicating that high DDT levels might disrupt CYP genes expression levels in both sexes. As replicating DDT-sprayed environments in animal experiments is difficult, this study will encourage further field studies in a DDT-sprayed areas and the use of wild rodents to assess DDT toxicity. The data from this study may open up the environment-based evaluation of DDT toxicity in this area and help researchers in assessing the toxicity of chemicals in the field.

Statements and Declarations

Ethics approval and consent to participate: This study used tissue samples from a previous study (Yohannes *et al*. 2017). The field sampling and dissection was done with the supervision of veterinarian for the proper euthanasia. Ethics on animal welfare were mentioned in Yohannes *et al*. 2017.

Consent for publication: All coauthors have read the manuscript and agreed to publication.

Availability of data and materials: The datasets used in the study are available in the Supplementary Data and the corresponding author on reasonable request. Supplementary

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Authors' contribution

KM: responsible for conceptualization, methodology, data curation, formal analysis, funding acquisition, writing – original draft, and writing – review & editing. **YBY**: responsible for methodology, investigation, writing – review & editing. **YI**: responsible for funding acquisition, supervision, and writing – review & editing. **TO**: responsible for methodology and data curation. **SMMN**: responsible for supervision, funding acquisition and writing – review & editing. **VW**, **NJS**, and **JHJV**: responsible for methodology (sampling) and supervision. **MI**: responsible for supervision, writing – review & editing, funding acquisition and for acting as the corresponding author.

All authors read and approved the final manuscript.

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