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Investigation of Associations Between Exposures to Pesticides and Testosterone Levels in Thai Farmers

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Abstract

We conducted a cross-sectional study to assess the relationship between pesticide exposures and testosterone levels in 133 male Thai farmers. Urine and serum samples were collected concurrently from participants. Urine was analyzed for levels of specific- and non-specific metabolites of organophosphates (OPs), pyrethroids, select herbicides, and fungicides. Serum was analyzed for total and free testosterone. Linear regression analyses revealed significant negative relationships between total testosterone and the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) after controlling for covariates (e.g., age, BMI, smoking status). Positive significant associations were found between some OP pesticides and total testosterone. Due to the small sample size and the observational nature of the study, future investigation is needed to confirm our results and to elucidate the biological mechanisms.

Keywords

pesticides; exposures; farmers; testosterone; endocrine disruptor; reproductive health

1. Introduction

Thailand is a developing country that relies heavily on pesticide use in agriculture to sustain its economy. Approximately 45% of its total area is used as agricultural fields and about 9% of the gross domestic product is derived from the agricultural sector. Of the total export value, 25% consists of agricultural products¹. Thai farmers apply pesticides heavily to their crops to prevent damage from tropical pests and diseases. Data from the Department of Agriculture indicates that 259 active ingredients of pesticides were imported into the country

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for a total of 84.3 million kilograms of active ingredients in 2016. In the same year, glyphosate, paraquat, 2,4-D, chlorpyrifos, and mancozeb were among the top 10 imported pesticides (by quantity)³. Thai farmers generously apply pesticides with minimal or no use of appropriate protective equipment, resulting in a high magnitude of exposure to a wide variety of pesticides and pesticide classes. The exposure frequency is unpredictable, ranging from a few days to a few weeks, depending on crop type and geographical location^{4,5}.

Current-use pesticides in Thailand are considered, “non-persistent,” as they break down easily in the environment and do not tend to bioaccumulate in the human body⁶. Many of these pesticides have been screened by the U.S. Environmental Protection Agency for their potential effects on the human endocrine system. Examples of these pesticides are 2,4-D, chlorpyrifos, prothiofos, fenthion, permethrin, cypermethrin, and cyfluthrin⁷. Several studies have indicated that exposure to current-use, non-persistent pesticides, including organophosphate (OP) and pyrethroid (PYR) insecticides is associated with altered levels of gonadotropic pituitary hormones [e.g. follicle stimulating hormone (FSH), luteinizing hormone (LH)], steroid hormones (e.g. testosterone, estradiol), and the testicular hormone inhibin B^{8,9,10,11,12,13,14,15} in men. Other studies have addressed potential reproductive hormone suppression following exposure to herbicides (such as 2,4-D)^{16,17}. Still, only a few studies^{9,18} have attempted to assess the link between fungicide exposure and reproductive hormone alteration in men. Alterations of reproductive hormone levels may lead to a decline in semen quality (i.e. concentration, motility, and morphology) and sperm DNA damage/fragmentation. These alterations may not only impede fertility, but may also contribute to developmental disorders in offspring¹⁹. While reproductive health is a topic of considerable concern, inconsistent findings across studies highlight the need for further study.

Previously, in a biomonitoring study, we reported that male Thai farmers from different communities were exposed to several classes of pesticides^{4,5}. Some of these pesticides were reported to be associated with levels of testosterone^{1,2,3,5,7,8,9,13}. Therefore, we conducted a crosssectional study to look into dose-response relationships between exposures to these pesticides (as measured by levels of urinary pesticide metabolites) and levels of total and free testosterone in archived serum samples. Levels of total testosterone in males indicate the production capacity or efficiency of Leydig cells. Levels of free testosterone, the biologically active fraction of the hormone, contribute to the development of sexual characteristics^{26,27}. Alterations of total testosterone levels may indicate malfunction of the Leydig cells or inhibition of the enzymes responsible for testosterone production. Changes in free testosterone levels may lead to outcomes related to male sexual characteristics.

We hypothesized that there is a monotonic, dose-response relationship between urinary metabolites of OP insecticides or the herbicide 2,4-D and total and free testosterone levels. We also hypothesize that there is no relationship between the non-specific urinary pyrethroid metabolite 3-PBA and total and free testosterone levels. Because there is little to no information available in the literature for other pesticides, we decided to take an unbiased exploratory approach by including other metabolites in this study to observe their potential associations with total and free testosterone. This approach will provide us with insight into the development of hypotheses for future studies. We hope that our results will prompt the public health sector of Thailand to develop effective prevention strategies and intervention

programs to protect the health of farmers or to inform the scientific community with the information needed to design future investigations of the relationships between pesticide exposures and reproductive hormone alterations.

2. Methodology

2.1 Studied population and locations

The cross-sectional study observed a population from Chiang Mai province of Thailand. The province contains a diversity of elevations but suitable climates for intensive cultivation of tropical, subtropical, and temperate crops. These include rice, strawberries, tomatoes, bell peppers, lychees, tangerines, cut flowers, and others. Farmers work on their farms throughout the week and pesticide use in this area is highly diverse and subjective. Application of pesticides appeared to be irregular; it did not follow a fixed schedule. However, farmers reported that they usually applied pesticides in the field in the morning or in the evening when the temperature was cooler. More detailed information regarding the study sites, population, and pesticide usage, can be found in Panuwet et al. (2008)⁵.

In 2006, a total of 136 male farmers, aged 20 to 65 years old, volunteered to participate in the study. The participants consisted of native Thais residing in Inthakhin and ethnic Hmong minorities residing in Pong Yaeng districts of Chiang Mai province. Criteria for enrollment included having good physical health, working on farms and applying pesticides, and willingness to provide biological samples and survey data relevant to the study. A consent form was introduced and signed by participants prior to the collection of personal information and biological samples. The research protocol, including urine and blood collection, was reviewed and approved by the Human Experimentation Committee, Research Institute for Health Sciences (RIHES), Chiang Mai University. The reference number was 18/2006. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2013.

Following an introductory meeting with the chosen locations, volunteers were provided with instructions for self-collection of their morning urinary void and return of the samples to the clinic between 8--11 am on the appointment day, during which time blood samples were drawn using an evacuated blood collection kit (Becton, Dickinson and Company, New Jersey, U.S.A.), with heparin preservative. Of the 136 initial participants, only 133 provided both urine and blood samples. Serum was isolated from blood within 24 hours of collection. Samples were stored at -20 °C prior to international shipping on dry ice to laboratories in the United States. After delivery, urine samples were stored at -20 °C and analyzed in 2007 for a large suite of biomarkers of exposure to pesticides. Serum samples were stored at -70°C until analysis in 2012 for testosterone and in 2014 for cotinine. Since the study began, pesticide use patterns have remained similar in both study communities.

2.2 Sample Analysis

Urine samples were analyzed for a variety of urinary biomarkers of pesticides using two different analytical methods. The common metabolites of OP insecticides [dialkylphosphate metabolites (DAPs)] were analyzed using gas chromatography-tandem mass spectrometry

(GC-MS/MS)²⁰. The specific metabolites of OP and PYR insecticides, including some herbicides and fungicides, were analyzed using high performance liquid chromatography-atmospheric pressure ionization-tandem mass spectrometry (HPLC-APCI-MS/MS)²¹. Table 1 provides details on the urinary metabolites measured in this study^{20,21,22}. The urinary creatinine measurements were performed according to the Roche “Creatinine Plus Assay,” using a Roche Hitachi Automatic Analyzer Model 912 (Hitachi, Inc., Japan). Urinary creatinine levels were used to normalize the concentration of detectable metabolites based on the dilution of the urine. The creatinine-adjusted analyte concentrations were given in micrograms per gram creatinine ($\mu\text{g/g}$ creatinine).

Serum samples were analyzed for levels of total and free testosterone as well as cotinine using enzyme-linked immunosorbent assay kits (CalBiotech Prod. No. TE187S, No. FT014S, No. CO096D, respectively, California, U.S.A.)²³. These commercial kits were reported to have a cross reactivity of 100%. Based on our triplicate results, the precision, expressed as % relative standard deviation, was below 10%. For both total and free testosterone levels, 25 μL of sample were used and analyzed in triplicate. The calibration curve for total testosterone included concentrations from 0 to 18 ng/mL , while free testosterone concentrations ranged from 0 to 100 pg/mL . The LODs were 0.075 ng/mL for total testosterone and 0.002 pg/mL for free testosterone, respectively. For cotinine levels, 10 μL of sample was used and analyzed in triplicate in a 96-well plate. The calibration curve for cotinine included concentrations from 0 to 100 ng/mL . The LOD for the cotinine assay was 5 ng/mL .

All assays were conducted by a single analyst according to procedures outlined within the commercial kits. The assays were read for absorbance at 450 nm with path length correction on a BioTek Epoch Plate Reader (Biotek, Vermont, U.S.A.). The calibration curves were fit using non-linear, 4-parameter logistic regression. Data were collected via the Gen5 software package (v1.0) (Biotek). If any sample concentration was calculated above the calibration range, a repeat of the sample was conducted with dilutions as prescribed in each assay.

2.3 Statistical analyses

All statistical calculations were conducted in Stata/IC version 12.1 (StataCorpLP, College Station, TX). For DAP metabolites, their molar summed concentrations were calculated and then converted to nmol/g creatinine. ΣDMP was a molar sum of dimethylphosphate (DMP), dimethylthiophosphate (DMTP), and dimethyldithiophosphate (DMDTP). ΣDEAP was a molar sum of diethylphosphate (DEP), diethylthiophosphate (DETP), and diethyldithiophosphate (DEDTP). ΣDAP was a molar sum of all DAP metabolites. Detailed information regarding the conversion can be found in a previously reported procedure⁵. Cotinine concentrations were converted to a binary variable (smoker or non-smoker) prior to use. According to the U.S. Centers for Disease Control and Prevention, a cut point of 10 ng/mL was used to differentiate smokers (>10 ng/mL) from non-smokers (<10 ng/mL)²⁴.

For each urinary pesticide biomarker, its normality was assessed using the Kolmogorov-Smirnov test. In order to reduce skewness observed in these variables, log transformation was performed. Total and free testosterone levels were also log-transformed to reduce

skewness. No imputation was performed for the urinary metabolite values below the LODs to avoid introducing bias into the dataset²⁵.

Linear regression analyses were conducted using two different conditions. First, when the detection frequency was greater than or equal to 50% per location, each urinary pesticide metabolite (independent variable) was modeled separately with either the levels of total testosterone or free testosterone (dependent variable). All models were conducted with and without location stratification and controlled for the following covariates: age, BMI, smoking status (as indicated by cotinine level), record of pesticide use prior to sample collection, number of years applying pesticides, and crop type. Linear regression analysis was expressed as follows;

$$Y_{ik} = \left[\beta_0 + \sum_j^6 \beta_j X_{ij} \right]_k + \gamma_{it} C_{ik} + \epsilon_{ik}$$

where pesticide concentration, C, describes a testosterone outcome, Y, for each subject, i, adjusting for covariates, j. Residual error, ϵ , is $N(0, \theta)$. The covariates were age, body mass index (BMI), smoking status, years of pesticide application, pesticide use prior to sample collection, and crop type. The model was run separately for each pesticide-outcome pair, k.

Second, when the detection frequency was greater than 25% but lower than 50%, each urinary pesticide metabolite (independent variable) was converted into a categorical variable (detected vs. nondetected) and modeled separately with either the levels of total testosterone or free testosterone (dependent variable). All models were conducted with and without location stratification and controlled for the same covariates as mentioned above.

3. Results

Descriptive statistics of demographic and urinary pesticide biomarkers of male participants in this study are shown in Table 2. After accounting for the number of participants who provided complete data on demographic and biological variables, the total was 133 (the n for Pong Yeang was 65, while the n for Inthakhin was 68). The study participants had a mean age of 40 ± 9 (range 20--64). Farmers from Pong Yaeng appeared to be, on average, younger than farmers from Inthakhin. Farmers from both locations showed no significant difference in their BMI values. The geometric mean BMI values were 22.3 for farmers from Pong Yeang (75th and 95th percentiles = 23.8 and 26.7) and 22.9 for farmers from Inthakhin (75th and 95th percentiles = 24.7 and 28.7).

Overall geometric mean concentrations of total and free testosterone in the study population were 7.36 ng/mL and 6.57 pg/mL, respectively. Farmers from Pong Yaeng had, on average, higher concentrations of total and free testosterone compared to farmers from Inthakhin (9.75 vs 5.63 ng/mL for total testosterone; 7.22 vs 6.00 pg/mL for free testosterone). These differences were statistically significant (Mann-Whitney test, $p < 0.05$). Age was not significantly correlated with log total testosterone levels (Pearson test, $R^2 = 0.0014$, $p =$

0.67), but was with log-free testosterone (Pearson test, $R^2 = 0.0387$, $p = 0.02$). In each location, 50% of the participants were smokers.

Dialkylphosphate metabolites were detected in urine samples of participants with detection frequencies ranging from 13.0% to 95.6% with the widest concentration range of <LOD-815 $\mu\text{g/g}$ creatinine for dialkylmethylphosphate (DMP). For urinary specific-pesticide metabolites, malathion diacid (MDA, a metabolite of malathion) was detected in the range of <LOD-558 $\mu\text{g/g}$ creatinine (the overall detection frequency of 18.4%) as well as 2,4-dichlorophenoxyacetic acid (2,4-D) which was detected in the range of <LOD-939 $\mu\text{g/g}$ creatinine (the overall detection frequency of 37.5%). The detection frequency of alachlor mercapturate (ALA), a metabolite of alachlor, was as low as <10% per location. On the other hand, para-nitrophenol (PNP), a metabolite of parathion and EPN, had a detection frequency of 100% in one of the two locations.

Linear regression results (controlled for age, BMI, smoking status, years of pesticide application, record of pesticide use prior to sample collection, and crop type) showing the associations between urinary pesticide metabolites and both total and free testosterone are shown in Table 3. Note that associations between log-transformed levels of urinary 2,4-D (treated as a continuous variable) and total testosterone were observed among farmers from Inthakhin ($n = 41$) (coef. = -0.084 , 95% CI: -0.167 , -0.001 , $p = 0.047$). No associations were found in the regression models for the remaining urinary metabolites and both total and free testosterone levels, with or without location stratification and control of covariates.

Linear regression results (controlled for the same covariates) showing the associations between urinary pesticide metabolites (converted into a categorical variable) and both total and free testosterone are shown in Table 4. The association was observed between detection of urinary 2,4-D and total testosterone among all participating farmers (coef. = -0.128 , 95% CI: -0.223 , -0.033 , $p = 0.009$). Among all participating farmers, other associations were also found between detection of DEP and total testosterone (coef. = 0.107 , 95% CI: 0.021 , 0.193 , $p = 0.016$) and between DEDTP and total testosterone (coef. = 0.105 , 95% CI: 0.021 , 0.190 , $p = 0.016$).

4. Discussion

This study aimed to investigate the linear associations between levels of urinary pesticide metabolites and total and free serum testosterone in male northern Thai farmers. The urinary biomarkers measured in this study are derived from pesticides that are metabolized quickly; therefore, the measured concentrations indicate recent exposures. Testosterone in men acts in all organs and systems and has a significant influence on physical appearance, behavior, mentality, abilities, sexuality, and social status²⁶. Alterations of both total and free testosterone levels may change these aspects. Moreover, reduction of testosterone may lead to impaired spermatogenesis and infertility or sterility²⁷. In this study, the associations between pesticide metabolites and levels of total and free testosterone were evaluated. Levels of total testosterone are related to the production capacity of Leydig cells. Alteration of total testosterone levels may indicate exposure-induced malfunction of the Leydig cells or exposure-induced inhibition of the enzymes responsible for testosterone production.

Changes in levels of free testosterone, the biologically active fraction of the hormone, is of interest, as it may be indicative of exposure-related outcomes on male sexual characteristics.

Participants in the present study were male farmers who had applied pesticides themselves. All participants appeared to be physically healthy. The high physical demands of agricultural work might be the primary reason why few participants were overweight. A lack of reference testosterone values for Thai people prevented further comparison. However, when compared with Mexican farmers who were also reported to be exposed to a high volume of pesticides⁹, the levels of total testosterone reported in Thai farmers were, on average, higher. In addition, the average and range of testosterone levels found in our participants were similar to those found among Chinese males¹⁰.

Earlier work from our group suggests that exposures to pesticides among Thai farmers are nearly inevitable due to a pervasive lack of awareness on pesticide toxicity^{4,5,28} and the limited availability of protective equipment suitable for tropical and sub-tropical climates. In addition, economic constraints in crop production act as a major driving force for farmers to disregard proper use of pesticides. A previous report⁴ suggested that Thai farmers used several classes of pesticides in their fields to prevent damages from a variety of pests. To save time, farmers often mixed different classes of pesticides together and applied this mixture on their crops. Some of the commercial products that are ready to use, are also available as a mixture of different classes of pesticides²⁸. Thus, in order to assess and characterize the farmers' exposure patterns, a series of analytical methods capable of measuring as many urinary pesticide metabolites as possible must be employed. In this study, it was found that that these groups of farmers were exposed to varieties of pesticides including OP and PYR insecticides, dithiocarbamate fungicides, and chlorophenoxy herbicides. Generally, farmers from both locations were exposed similarly to OP and PYR insecticides. However, farmers from Pong Yaeng were exposed more to dithiocarbamate fungicides such as mancozeb, maneb, and zineb compared to farmers from Inthakhin. On the other hand, farmers from Inthaknin were exposed more to chlorophenoxy herbicides (i.e., 2,4-D and alachlor) compared to Pong Yaeng' farmers. These location-specific exposure patterns can be explained by different crop production. Farmers from Inthakhin used open fields to plant their crops (rice, vegetables, legumes, and fruits). Open fields required frequent weeding, leading to increased use of herbicides. Pong Yaeng farmers typically planted their crops (mainly cut flowers, tomatoes, and bell peppers) in greenhouses. The high humidity in greenhouses leads to fungal vulnerability and leads to increased fungicidal use.

In this study, due to a wide range of detection frequencies across analytes, we chose not to impute urinary pesticide metabolite values below the LODs in order to avoid the introduction of possible biases²⁵. However, this resulted in a reduction in sample size, contributing to lack of statistical power. In the linear regression analyses, location stratification was performed because the two populations differed in characteristics that may or may not be associated with the biological endpoints and pesticide exposures. They were different in race (Thai vs ethnic Hmong), age, total and free testosterone levels, and pesticide use patterns. After controlling for covariates, regression results revealed dose-dependent, negative relationships between urinary 2,4-D (modeled as either a continuous or categorical

variable) and total testosterone. Although the relationships are weak, they are statistically significant with p -values <0.05 . Significant positive associations were also found between detection of DEP and DEDTP with total testosterone. However, because of the small sample size, we recommend using caution while interpreting our findings. It is important we note here that the statistically significant associations found in this study may be random.

In some aspects, our results were both similar to and different from previous studies reported in the literature. One, this is the first time an association between urinary 2,4-D levels and total testosterone levels was shown in a human population. Garry et al. reported no direct relationship between urinary 2,4-D and testosterone levels among select applicators in the United States^{16,17}. However, they reported potential suppression of testosterone levels during high season application by comparing testosterone levels of these applicators during low- and high-use seasons^{16,17}. Two, when DEP and DEDTP were modeled separately as categorical variables, evidence of positive relationships was found for total testosterone levels. This is contrary to the study by Omoike et al. where they reported a statistically significant negative relationship between DEP and testosterone when DEP was modeled as either a continuous or categorical variable²⁹. However, the study by Blanco-Munoz et al. (2010) documented a marginally positive association between urinary DEP and testosterone levels⁹. Three, we found no relationships between 3-PBA, a non-specific metabolite of PYR insecticides, and testosterone levels. This is similar to the studies of Han et al. (2008)¹⁰, Yoshinaga et al. (2014)³⁰, and Meeker et al. (2009)³¹. Four, we found no negative relationship between TCPY and testosterone levels, unlike the study reported by Meeker et al.¹¹. For more information, Table S1 (supplemental material) provides a summary of existing human studies designed to investigate the potential impact of pesticide exposures on reproductive hormone levels. These studies were primarily selected because they focused on testosterone.

Animal and *in vitro* studies in the literature, unfortunately, were as inconsistent as the human epidemiological studies in drawing associations between pesticide exposure and testosterone. For 2,4-D, some studies reported evidence of testosterone reduction following exposure (oral administration)³². While Oakes et al. (2002)³³, reported no significant difference in the serum concentration of testosterone in control animals when compared to treated animals, they noticed a severe reduction in testicular weight in some high dose animals with histology showing shrunk tubules with germ cell depletion. In addition, Sun et al. (2012)³⁴, conducted gene assays and found that 2,4-D showed no agonist or antagonist activity but it significantly enhanced the activity of testosterone through androgen receptors. Liu et al. (1996)³⁵ also reported no effects of 2,4-D on testosterone levels in rat Leydig cells when conducting the experiment *in vitro*.

There have been several animal studies that reported links between exposure to other pesticides and testosterone levels^{36,37,38,39,40}. For these studies, pesticides of interest included methyl- parathion, chlorpyrifos, chlorpyrifos-methyl, diazinon, profenofos, glyphosate, piperophos, methoxychlor, cypermethrin, and malathion. The majority of these studies were in agreement that exposures to these pesticides contributed to a reduction in testosterone levels in experimental settings. Still, there are other interesting results. For instance, exposure to metolachlor, another chlorophenoxy herbicide, has been shown to

increase serum testosterone levels in rats⁴¹. Table S2 (supplemental material) summarizes the key findings of animal and *in vitro* studies. Note that several studies have provided information about plausible mechanisms of testosterone reduction following exposure to some pesticides. For instance, Viswanath et al. (2010)⁴², were able to demonstrate, using the NIH3T3 cell line, that piperophos and chlorpyrifos inhibited the biosynthesis of testosterone by disrupting CYP11A1, HSD3B, HSD17 B3, and decreasing StAR protein expression. In addition, they found that chlorpyrifos could decrease LH receptor- stimulated cAMP production. Other pesticides that could inhibit biosynthesis of testosterone by disruption of its associated enzymes included methoxychlor and its bioactive metabolite 2,2- bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE)²⁷.

We offer two hypotheses that need further testing. One is that testosterone production is robust and not vulnerable to small changes in biological condition induced by exposures to pesticides such as PYR insecticides, dithiocarbamate fungicides, and the herbicide alachlor. Even with advancing age, which could cause a decrease in testosterone secretion, a majority of older men still have a circulating total testosterone concentration well within the accepted reference intervals established for younger men⁴³. This might be the result of a high level of adaptability of both mitochondria and Leydig cells. Two is that different endocrine endpoints associated with OP insecticide exposures among Thais and Mexicans, as well as in U.S. populations, are observed due to susceptibilities mediated by paraoxonase (PON1) polymorphisms and activity.

Generally, PON1 plays an important role in the mechanism of some OP toxicity, influencing susceptibility or protecting humans and other mammals from toxicity or disease⁴⁴. PON1, which is produced by the cytochrome P-450 enzyme in the liver, is an enzyme that detoxifies the active forms (oxon metabolites)⁴⁵ of a number of OP insecticides⁴⁴. The three distinct PON1*192 genotypes (QQ, QR, and RR) were reported to have different influences on the rates of hydrolysis of, for example, paraoxon and chlorpyrifos oxon, in which the RR genotype contributes the highest enzymatic activity⁴⁵. The existence of the PON1*55 polymorphism, on the other hand, controls the extent of PON1 expression. In most cases, the expression of PON1*55M has shown to decrease serum enzyme concentrations⁴⁴. Thais, Mexicans, Chinese, Japanese, and Caucasian Americans all have the *192R allele available in different degrees of abundance⁴⁶. For the *192R allele, Thais were reported to have approximately 29--61% frequency^{47,48}, while about 49--60% was found in Mexicans^{49,50}. Approximately 29--31% of Caucasian Americans were reported to carry the *192R allele⁴⁶. For the *55M allele, Thais had a 5--6% frequency^{47,48} compared to 6--16% in Mexicans^{49,50}. The Caucasian Americans had 36% of their population carrying the *55M allele⁴⁶.

These differences in the distribution of PON1 genotypes may have contributed to the different observed toxicological endpoints of OP insecticides. We can assume that those whose PON1 is primarily made of the abundant *192R allele, would have reduced susceptibility to OP insecticides reaching and exerting their toxicity on the target cells (i.e. Leydig cells that produce testosterone). Thus, Thais and Mexicans may be less vulnerable to OP insecticide toxicity than the Caucasian Americans. This might explain the contradictory results between our study and the study of Meeker, et al.¹¹ which reported an inverse

association between testosterone levels and urinary metabolite levels of the OP insecticide chlorpyrifos¹¹. Also, a recent report on thyroid function suggested PON1 may mediate the effects of OP insecticides on hormone levels. Lacasana et al. reported that increased PON1 activity resulted in a decrease in the percentage of variation of thyroid stimulating hormone levels for each increment in one logarithmic unit of the Σ DAP levels⁵¹.

Although our study added new evidence to the existing body of literature on associations between pesticide exposure and reproductive hormone levels, because of the cross sectional design of this study, our findings must be interpreted carefully, as the results do not imply causation. In addition, some other limitations must be mentioned. Unlike blood, urine is often chosen as a matrix to investigate exposure to pesticides because of the ease of sample collection and the high concentration of analytes and sample volume available for analysis. However, it is necessary to understand the kinetics of metabolite excretion in order to infer the internal dose of the parent compounds and to allow the optimum time for sample collection⁵². In our study, a morning void was collected from each participant, the concentrations of urinary metabolites only represent a fraction of the total dose excreted and accumulated overnight. Because urinary concentrations may vary across individuals due to differing metabolic rates, dose, and times of exposure; they provide limited surrogate measures of exposures. Several known confounders (age, smoking status, and BMI) and their effects on testosterone were included in the regression analysis. Still, a lack of access to other confounders such as serum DDT levels, sexual abstinence behavior, alcohol consumption^{9,11} and genetic polymorphism data indicating PON1 status, may have contributed to errors in our findings. Last, relying on the results generated from the dataset containing only urinary pesticide metabolite data above their LODs, had both advantages and disadvantages: 1) the reduced sample size contributed to diminished statistical power to detect any relationships, 2) significant associations found in this study may have occurred by chance, and 3) the observed relationships are free from biases derived from imputation.

Further study is needed to confirm our findings, as they suggest that occupational exposure to 2,4-D and some OP insecticides (as inferred by non-specific urinary OP metabolites DEP and DEDTP) may be able to alter total serum testosterone levels in male farmers. In Thailand, 2,4-D is primarily used in agriculture to control broad-leaf weeds in rice, corn, sugarcane, and legume fields. It is also used to control aquatic weeds. The herbicide 2,4-D and its derivatives (2,4-D dimethylamine salt; 2,4-D sodium salt; 2,4-D butyl ester; and 2,4-D isobutyl ester) have been imported into Thailand in large quantities annually. Data from 2008 onwards indicates that 2,4-D dimethylamine salt and 2,4-D sodium salt are among the top 10 imported pesticides, by amount of active ingredient. With a total imported amount of 8.3 million kilograms of active ingredients in 2016, this herbicide ranked 3rd, only below glyphosate and paraquat. Therefore, Thai farmers continue to be at risk for exposure to this herbicide, which may contribute to health outcomes.

5. Conclusion

We employed biomonitoring through the measurement of urinary metabolites of OP and PYR insecticides, chlorophenoxy herbicides, as well as dithiocarbamate fungicides in Thai farmers who were exposed to these pesticides, in order to investigate any links between

exposure levels (as inferred by urinary pesticide metabolite levels) and both total and free serum testosterone levels. It appeared that urinary 2,4-D levels were negatively associated with total serum testosterone levels. On the contrary, urinary DEP and DEDTP were positively associated with total testosterone levels. Due to a small sample size and other limitations, these results should be interpreted with caution. Although statistically significant results were revealed, this finding has yet to be confirmed by larger epidemiological studies with longitudinal data collection and that are specifically designed to handle multiple confounding effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.

Detailed information on pesticide metabolites measured in this study

Specificity	Metabolite	Abbreviation	Parent Compound	LOD (µg/L)	Technique	References
non-specific	dimethyl/phosphate	DMP	azinphos methyl, chlorpyrifos methyl, dichlorvos (DDVP), dicromiddlehos, dimethoate, fenitrothion, fenthion, isazaphos-methyl, malathion, methidathion, methyl parathion, naled, oxydemeton- methyl, phosmet, pirimiphos-methyl, temephos, tetrachlorviphos, trichlorfon	0.6	GC	1
non-specific	dimethyl/thiophosphate	DMTP	azinphos methyl, chlorpyrifos methyl, dimethoate, fenitrothion, fenthion, isazaphos-methyl, malathion, methidathion, methyl parathion, oxydemeton-methyl, phosmet, pirimiphos-methyl, temephos	0.2		
non-specific	dimethyl/dithiophosphate	DMDTP	azinphos methyl, dimethoate, malathion, methidathion, phosmet	0.1		
non-specific	diethyl/phosphate	DEP	chlorethoxyphos, chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, parathion, phorate, sulfotepp, terbufos	0.2		
non-specific	diethyl/thiophosphate	DETP	chlorethoxyphos, chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, parathion, phorate, sulfotepp, terbufos	0.1		
non-specific	diethyl/dithiophosphate	DEDTP	disulfoton, ethion, phorate, terbufos	0.1		
non-specific	para-nitrophenol	PNP	methyl parathion, ethyl parathion, EPN	0.1		
specific	3,5,6-trichloro-2-pyridinol	TCPy	chlorpyrifos	0.2		
specific	2-diethylamino-6-methyl pyrimidin-4-ol	DEAMPY	pirimiphos	0.2		
specific	5-chloro-1-isopropyl-[3 H]– 1,2,4-triazol-3-one	CTT	isazophos	1.5	LC	3
specific	3-chloro-4-methyl-7-hydroxycoumarin	CMHC	coumaphos	0.2		
specific	[(dimethoxyphosphorothioyl) sulfanyl] succinic acid	MDA	malathion	0.3		
specific	2-isopropyl-6-methyl-4-pyrimidol	IMPY	diazinon	0.7		
non-specific	3-phenoxybenzoic acid	3-PBA	cypermethrin, cyhalothrin, deltamethrin, esfenvalerate, fenpropathrin, permethrin, phenothrin	0.1		
non-specific	cis and trans-3-(2,2- dichlorovinyl)-2,2- dimethylcyclopropane-1 - carboxylic acids	cis- and trans-DCCA	cyfluthrin, permethrin, cypermethrin	0.20/0.40		
specific	4-fluoro-3-phenoxybenzoic acid	4-F-3-BPA	cyfluthrin	0.2		
specific	cis-3-(2,2-dibromovinyl)- 2,2- dimethylcyclopropane-1 - carboxylic acid	DBCA	deltamethrin	0.1		
specific	atrazine mercapturate	ATZ	atrazine	0.3		
specific	acetochlor mercapturate	ACE	acetochlor	0.1		
specific	alachlor mercapturate	ALA	alachlor	0.3		3
specific	metolachlor mercapturate	MET	metolachlor	0.2		

Specificity	Metabolite	Abbreviation	Parent Compound	LOD (µg/L)	Technique	References
specific	2,4,5-trichlorophenoxyacetic acid	2,3,4-T	2,4,5-T	0.1		
specific	2,4-dichlorophenoxyacetic acid	2,4-D	2,4-D	0.2		
specific	acephate	AP	acephate	0.023		
specific	methamidophos	MMP	methamidophos	0.001		
specific	omethoate	Omet	omethoate	0.025		
specific	dimethoate	Dmet	dimethoate	0.004		
non-specific	ethy/lenethiourea	ETU	mancozeb, maneb, metriam, zineb	0.16		
specific	propy/lenethiourea	PTU	propineb	0.004		

Note: The last column provides references to support the parent-metabolite relationships of the listed pesticides.

Table 2.

Descriptive statistics of the demographic and urinary pesticide biomarker variables

Classification	Variable	Mean ⁽¹⁾ (range) or % Detection [n]		
		Pong Yaeng	Inthakhin	Overall
Demographics	Average Age (yr)	37±10* ^(a) (20–64) [n = 65]	43±6* ^(a) (20–52) [n = 68]	40±9 (20–64) [n = 133]
	20–29 yr	13 [20.0%]	4 [5.9%]	17 [12.8%]
	30–39 yr	26 [40.0%]	11 [16.2%]	37 [27.8%]
	40–49 yr	20 [30.8%]	45 [66.2%]	65 [48.9%]
	50–59 yr	5 [7.7%]	8 [11.8%]	13 [9.8%]
	>60 yr	1 [1.5%]	–	1 [0.7%]
	Average BMI	22.3, (18.4–29.4) [n = 66]	22.9, (16.2–52.3) [n = 64]	22.6, (16.2–52.3) [n = 130]
	<18.5	1 [1.5%]	5 [7.8%]	6 [4.6%]
	18.5–24.9	52 [78.8%]	43 [67.2%]	95 [73.1%]
	25.0–29.9	13 [19.7%]	14 [21.9%]	27 [20.8%]
	>30.0	–	2 [3.1%]	2 [1.5%]
	Smoking	50% [n = 64]	50% [n = 68]	50% [n = 132]
Testosterone ⁽²⁾	Total-T estosterone	9.75* ^(b) , (1.97–30.1) [n = 65]	5.63* ^(b) , (2.00–24.0) [n = 68]	7.36, (1.97–30.1) [n = 133]
	Free-Testosterone	7.22* ^(b) , (2.28–48.3) [n = 64]	6.00* ^(b) , (2.68–20.2) [n = 68]	6.57, (2.28–48.3) [n = 132]
		Range and [% detection frequency]		
		Pong Yaeng [n = 67]	Inthakhin [n = 69]	Overall [n = 136]
Nonspecific urinary metabolite of OPs ⁽³⁾	DMP	<LOD-815, [28.4%]	<LOD-90, [13.0%]	<LOD-815, [20.6%]
	DMTP	<LOD-13.4, [74.6%]	<LOD-380, [76.8%]	<LOD-380, [75.7%]
	DMDTP	<LOD-8.70, [68.7%]	<LOD-46.9, [46.4%]	<LOD-46.9, [57.4%]
	ΣDMAP	<LOD-6472, [61.0%]	<LOD-3690, [82.6%]	<LOD-6472, [86.8%]
	DEP	<LOD-74.1, [61.2%]	<LOD-60.5, [20.3%]	<LOD-74.1, [40.4%]
	DETP	<LOD-14.8, [94.0%]	<LOD-25.6, [97.1%]	<LOD-25.6, [95.6%]
	DEDTP	<LOD-2.10, [50.7%]	<LOD-2.00, [39.1%]	<LOD-2.10, [37.5%]
	ΣDEAP	<LOD-524, [95.5%]	<LOD-546, [65.7%]	<LOD-546, [95.6%]
	ΣDAP	<LOD-6476, [98.5%]	<LOD-3698, [95.7%]	<LOD-6476, [97.1%]
Specific urinary metabolite ⁽⁴⁾	TCPY	<LOD-53.5, [71.6%]	<LOD-123, [81.2%]	<LOD-123, [76.5%]
	MDA	<LOD-24.3, [10.4%]	<LOD-939, [26.1%]	<LOD-939, [18.4%]
	PNP	0.60–6.4, [100%]	<LOD-56.7, [98.6%]	<LOD-56.70, [99.3%]
	3-PBA	<LOD-21.0, [77.6%]	<LOD-9.1, [89.9%]	<LOD-21.0, [86.8%]
	t-DDCA	<LOD-49.2, [44.8%]	<LOD-11.0, [30.4%]	<LOD-49.2, [37.5%]
	2,4-D	<LOD-14.5, [9.1%]	<LOD-558, [65.2%]	<LOD-558, [37.5%]
	ALA	<LOD-4.2, [4.5%]	<LOD-221, [65.2%]	<LOD-221, [35.3%]

Classification	Variable	Mean ⁽¹⁾ (range) or % Detection [n]		
		Pong Yaeng	Inthakhin	Overall
	ETU	<LOD-13.2, [58.2%]	<LOD-2.2, [26.1%]	<LOD-13.2, [41.9%]

Note: DMP = Dimethylphosphate; DMTP = Dimethylthiophosphate; DMDTP = Dimethyldithiophosphate; DEP = Diethylphosphate; DETP = Diethylthiophosphate; and DEDTP = Diethyldithiophosphate. These are non-specific metabolites of OP insecticides and were analyzed using GC-MS/MS.

Note: TCPY = 3,5,6-Trichloro-2-pyridinol (a metabolite of chlorpyrifos and chlorpyrifos ethyl); MDA = 2-[(Dimethoxyphosphorothioyl) sulfanyl] succinic acid (a metabolite of malathion); PNP = Paranitrophenol (a metabolite of methyl parathion, ethyl parathion, and EPN); 3-PBA = 3-Phenoxybenzoic acid (a common metabolite of pyrethroid insecticides); t-DCCA = trans-3- (2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acids (a metabolite of cyfluthrin, permethrin, cypermethrin); 2,4-D = 2,4-Dichlorophenoxyacetic acid (a metabolite of 2,4-D); ALA = Alachlor mercapturate (a metabolite of alachlor); ETU = Ethylenethiourea (a metabolite of mancozeb, maneb, metiram, and zineb); and PTU = Propylenethiourea (a metabolite of propineb). These metabolites were analyzed using LC-MS/MS.

⁽¹⁾ **Note:** Geometric means were reported for BMI, total-, and free-testosterone levels, while arithmetic means were reported for age;

⁽²⁾ Total-testosterone levels were reported in ng/mL, while free-testosterone levels were reported in pg/mL;

⁽³⁾ Concentrations of individual DAPs presented in units of µg/g creatinine while concentrations of molar summed metabolites (ΣDMP, ΣDEAP, and ΣDAP) were presented in units of nmol/g creatinine;

⁽⁴⁾ Concentrations of specific urinary pesticides were presented in units of µg/g creatinine.

^(a) **Note:**Significantly different at $p < 0.05$ using ANOVA;

^(b) Significantly different at $p < 0.05$ using Mann-Whitney test.

Note: The study subjects consisted of native Thais residing in Inthakhin and ethnic Hmong minorities residing in Pong Yaeng districts of Chiang Mai province.

Table 3.

Adjusted¹ regression coefficients (95%CI) (*p value*) for change in hormone levels associated with a log-unit change in urinary pesticide concentration (non-imputed data)

Urinary Metabolite	Total-testosterone			Free-testosterone		
	Pong Yaeng	Inthakhin	Total	Pong Yaeng	Inthakhin	Total
DMTP	0.084 (−0.032, 0.201) (0.153)	0.007 (−0.103, 0.117) (0.900)	0.034 (−0.056, 0.125) (0.450)	−0.015 (−0.200, 0.169) (0.859)	0.067 (−0.009, 0.143) (0.081)	0.037 (−0.041, 0.114) (0.350)
DMDTP	0.042 (−0.104, 0.187) (0.565)	NE	−0.037 (−0.143, 0.069) (0.488)	−0.063 (−0.265, 0.138) (0.526)	NE	−0.023 (−0.130, 0.083) (0.665)
ΣDMAP	0.014 (−0.063, 0.091) (0.718)	−0.005 (−0.098, 0.089) (0.918)	0.032 (−0.036, 0.099) (0.352)	0.001 (−0.102, 0.105) (0.977)	0.046 (−0.020, 0.112) (0.164)	0.034 (−0.024, 0.093) (0.246)
DEP	−0.060 (−0.175, 0.055) (0.293)	NE	NE	−0.189 (−0.415, 0.038) (0.099)	NE	NE
DETP	0.049 (−0.057, 0.156) (0.357)	0.072 (−0.045, 0.190) (0.221)	0.059 (−0.031, 0.149) (0.199)	0.009 (−0.140, 0.158) (0.901)	−0.025 (−0.115, 0.066) (0.585)	−0.020 (−0.100, 0.060) (0.625)
DEDTP	0.007 (−0.117, 0.132) (0.907)	NE	NE	−0.045 (−0.317, 0.227) (0.736)	NE	NE
ΣDEAP	0.034 (−0.056, 0.124) (0.455)	−0.014 (−0.116, 0.087) (0.774)	0.067 (−0.005, 0.139) (0.068)	0.022 (−0.104, 0.148) (0.727)	−0.047 (−0.123, 0.029) (0.223)	−0.004 (−0.069, 0.060) (0.890)
ΣDAP	0.031 (−0.063, 0.125) (0.508)	−0.024 (−0.114, 0.066) (0.591)	0.059 (−0.011, 0.128) (0.099)	0.006 (−0.125, 0.137) (0.926)	−0.020 (−0.088, 0.049) (0.567)	0.006 (−0.056, 0.068) (0.846)
TCPY	−0.021 (−0.174, 0.133) (0.786)	0.027 (−0.097, 0.152) (0.658)	0.060 (−0.039, 0.159) (0.234)	−0.038 (−0.255, 0.178) (0.720)	−0.028 (−0.117, 0.062) (0.536)	−0.011 (−0.100, 0.077) (0.804)
PNP	0.098 (−0.107, 0.302) (0.342)	0.104 (−0.098, 0.306) (0.305)	0.021 (−0.138, 0.181) (0.789)	−0.061 (−0.340, 0.218) (0.662)	0.042 (−0.115, 0.199) (0.592)	−0.039 (−0.177, 0.099) (0.579)
3-PBA	0.016 (−0.091, 0.122) (0.766)	0.049 (−0.103, 0.200) (0.521)	0.032 (−0.071, 0.134) (0.540)	0.057 (−0.085, 0.199) (0.424)	−0.065 (−0.178, 0.047) (0.248)	0.001 (−0.088, 0.089) (0.986)
2,4-D	NE	−0.084 (−0.167, −0.001) (0.047)	NE	NE	−0.047 (−0.111, 0.017) (0.144)	NE
ALA	NE	0.012 (−0.119, 0.143) (0.852)	NE	NE	−0.103 (−0.207, 0.002) (0.054)	NE
ETU	0.096 (−0.061, 0.253) (0.219)	NE	NE	0.104 (−0.125, 0.333) (0.362)	NE	NE

¹⁾ Note: Each metabolite was modelled individually. The models were adjusted for age, BMI, smoking status, years of pesticide application, record of pesticide use prior to sample collection, and crop type.

2) Hormone levels were log-transformed.

3) Numbers of subjects varied per location per metabolite measured. Total of 65 subjects were from Pong Yaeng, while a total of 68 subjects were from Inthakhin. The grand total n was = 133.

4) NE referred to “Not Estimated” because the detection frequency was lower than 50% per location.

5) Cells in bold typeface highlight significant associations at $\alpha < 0.05$

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Table 4.

Adjusted¹ regression coefficients (95%CI) (*p value*) for change in hormone levels associated with detection of urinary pesticide metabolites (detection vs non-detection)

Urinary Metabolite	T total-testosterone			Free-testosterone		
	Pong Yaeng	Inthakhin	Total	Pong Yaeng	Inthakhin	Total
DMP	0.011 (−0.092, 0.115) (0.831)	NE	NE	0.050 (−0.090, 0.190) (0.486)	NE	NE
DMDTP	NE	−0.021 (−0.147, 0.106) (0.752)	NE	NE	0.019 (−0.074, 0.112) (0.694)	NE
DEP	NE	NE	0.107 (0.021, 0.193) (0.016)	NE	NE	0.026 (−0.049, 0.100) (0.505)
DEDTP	NE	0.091 (−0.033, 0.215) (0.156)	0.105 (0.021, 0.190) (0.016)	NE	0.037 (−0.055, 0.130) (0.434)	0.053 (−0.020, 0.126) (0.156)
MDA	NE	−0.086 (−0.219, 0.048) (0.216)	NE	NE	−0.026 (−0.125, 0.074) (0.617)	NE
t-DDCA	−0.019 (−0.111, 0.073) (0.690)	−0.100 (−0.230, 0.030) (0.137)	−0.039 (−0.130, 0.052) (0.406)	−0.066 (−0.189, 0.058) (0.301)	−0.025 (−0.122, 0.072) (0.613)	−0.041 (−0.118, 0.037) (0.303)
2,4-D	NE	NE	−0.128 (−0.223, −0.033) (0.009)	NE	NE	−0.061 (−0.143, 0.021) (0.148)
ALA	NE	NE	−0.092 (−0.186, 0.0002) (0.053)	NE	NE	0.027 (−0.054, 0.107) (0.517)
ETU	NE	−0.016 (−0.150, 0.118) (0.814)	0.057 (−0.031, 0.145) (0.207)	NE	−0.077 (−0.174, 0.019) (0.121)	−0.010 (−0.086, 0.065) (0.789)

¹)Note: Each metabolite was modelled individually. The models were adjusted for age, BMI, smoking status, years of pesticide application, record of pesticide use prior to sample collection, and crop type.

²)Hormone levels were log-transformed.

³)Numbers of subjects varied per location per metabolite measured. Total of 65 subjects were from Pong Yaeng, while a total of 68 subjects were from Inthakhin. The grand total n was = 133.

⁴)NE referred to “Not Estimated” because the detection frequency for the metabolite was less than 25% or greater than 50% per location.

⁵)Cells in bold typeface highlight significant associations at $\alpha < 0.05$