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# Association between urinary biomarkers of exposure to organophosphate insecticides and serum reproductive hormones in men from NHANES 1999–2002

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## Abstract

Exposure to organophosphate (OP) insecticides may alter reproductive hormone levels in men and increase the risk for poor reductive health and other adverse health outcomes. However, relevant epidemiology studies in men are limited. We evaluated urinary concentrations of OP metabolites (3,5,6-trichloro-2-pyridinol and six dialkyl phosphates) in relation to serum concentrations of testosterone (T) and estradiol among 356 men aged 20–55 years old from the U.S. National Health and Nutrition Examination Survey. Biomarkers were detected in greater than 50% of the samples, except for diethyldithiophosphate, dimethylphosphate, and dimethyldithiophosphate. In adjusted regression models, we observed a statistically significant inverse relationship between diethyl phosphate (DEP) and T when DEP was modeled as either a continuous or categorical variable. These findings add to the limited evidence that exposure to certain OP insecticides is linked to altered T in men, which may have important implications for male health.

### Keywords

Biomarkers; epidemiology; exposure; testosterone; pesticides

## 1. INTRODUCTION

Organophosphate (OP) insecticides are a diverse group of pesticides that are characterized by their potent acetylcholinesterase inhibitor activity [1]. Although residential uses of OP insecticides have been largely phased out in the U.S. due in part to regulatory efforts [1], they are still widely used for insect control on food crops and in certain public health applications (e.g., malathion for mosquito control) [2]. The use of OP insecticides in the



**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**TRANSPARENCY DOCUMENT**

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U.S. exceeds 30 million pounds on an annual basis, and accounts for approximately one-third of all insecticides used in the U.S. [3]. Globally, the demand for OP insecticides is considerable, accounting for nearly 30% of the market share of all insecticides [4].

OP insecticides are notorious for their acute toxicity resulting from human poisonings, which have been reported in scenarios involving agricultural use, unintentional exposure, suicide, and, to a much lesser extent, homicide [5]. Such exposures lead to the overstimulation of the muscarinic and nicotinic receptors [6], and often result in death if a diagnosis is not made in a timely manner or if not managed properly [5]. In comparison, much less is known about the toxicity of chronic, low-level exposures to OP insecticides in humans [6]. There is growing concern that non-acute exposures may adversely impact human health as epidemiology studies have linked exposures to OP insecticides to detrimental child neurodevelopment [7–10], decreased gestational age [11–13], reduced birth weight [12], reduced semen quality [14], wheeze [15], and lung cancer [16].

Altered serum reproductive hormone levels have also been reported in epidemiology studies of OP insecticide exposure conducted in men [6,17,18]. For example, serum testosterone (T) levels were inversely related to urinary biomarkers of OP insecticide exposure in cohorts of men from a U.S. fertility clinic [17] and male floriculture workers from Mexico [6]. At the population level where exposure to OP insecticides is widespread [2], reductions in T may have important public health implications because low T concentrations may adversely impact semen quality parameters and resultant fertility [19]. Low T has also been linked to various additional health endpoints, including metabolic syndrome [20], diabetes [21], cardiovascular disease [22], fractures [23,24], neurodegenerative disorder [25,26], and mortality [27–29]. In addition to serum T, exposure to OP insecticides has been associated with reduced serum estradiol (E2) among men from the same U.S. fertility cohort [18], suggesting that population-wide OP exposures may impact male reproductive health through altered T, E2, or both. A decrease in serum E2 may also increase one's risk for prostate cancer as E2 may play a protective role in prostate cancer etiology [18]. Experimental animal studies suggest that OP insecticides may be hormonally-active [17], which supports the findings of altered T, E2, and other serum hormone levels in humans following exposure to OP insecticides.

The present study was designed to the examine relationships between levels of urinary biomarkers of OP insecticide exposure and serum T or E2 in men 20–55 years old from the U.S. population. This is the first analysis of its kind using data from the U.S. National Health and Nutrition Examination Survey (NHANES).

## 2. MATERIALS AND METHODS

### 2.1 Study population

This analysis utilized publicly-available data that was derived from NHANES 1999–2002, the survey years in which data on the exposure biomarkers and outcome measures of interest overlapped. NHANES is a cross-sectional survey that is administered by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention (CDC) that collects nationally representative data on the health and nutritional status of non-

institutionalized civilian residents, and conducts more detailed laboratory analyses on a subset of the participants [30]. We analyzed data from a subset of men that were 20–55 years old (n=2809). Men with missing data on the urinary exposure biomarkers of interest, serum T or E2, serum sex hormone binding globulin (SHBG), serum cotinine, urinary creatinine, body mass index (BMI), race/ethnicity, or education were excluded from the current analysis (n=2453), resulting in a final sample size of 356 men. The large number of men that were excluded had to do with the fact that only a subset was measured for the exposure biomarkers and hormones of interest. Missing data on the other variables was much less prevalent, accounting for only 0–12% of the eligible subjects. NHANES received approval from the NCHS Ethics Review Board, and informed consent was obtained for all participants.

### 2.2 Demographic data and body measurements

Information on race/ethnicity and education was obtained from the men in their homes by a Computer-Assisted Personal Interviewing system [31]. Height and weight were collected in a Mobile Examination Center (MEC) by trained technicians with the assistance of a recorder during body measures [32]. BMI was calculated by CDC as weight in kilograms divided by height in meters squared [32].

### 2.3 Laboratory measurements

Whole venous blood and urine were collected from participants at the MEC, which were then processed, stored, and shipped to laboratories at either the CDC or the University of Minnesota (urinary creatinine only) for analysis [33]. Samples were analyzed for urinary OP insecticide metabolites and creatinine, and serum T (total), E2, SHBG, and cotinine. We focused our current analysis on a urinary metabolite of chlorpyrifos and chlorpyrifos-methyl [3,5,6-trichloro-2-pyridinol (TCPY)], and six non-specific urinary metabolites [diethylphosphate (DEP), diethylthiophosphate (DETP), diethyldithiophosphate (DEDTP), dimethylphosphate (DMP), dimethylthiophosphate (DMTP), and dimethyldithiophosphate (DMDTP)] to be consistent with previous epidemiology studies conducted in men focusing on the potential relationship between urinary OP insecticide metabolites and serum T and serum E2 [6,17,18]. Urinary OP insecticide metabolites and urinary creatinine were measured using gas chromatography-tandem mass spectrometry [34] and the Jaffe rate reaction method [33], respectively. Serum T, E2, and SHBG were measured using an immunoassay method [35], and serum cotinine was measured using isotope dilution-high performance liquid chromatography/atmospheric pressure chemical ionization-tandem mass spectrometry [36]. Urinary metabolite concentrations below the limit of detection (LOD) were assigned a value of LOD divided by the square root of 2. LODs by NHANES cycle (1999–2000 and 2001–2002) are reported in Table 2.

### 2.4 Statistical analysis

Statistical analysis was performed using SAS version 9.3 for Windows (SAS Institute, Cary, NC, USA). Descriptive statistics of participant demographics and concentrations of the biomarkers were calculated.

We chose to conduct regression analyses for urinary biomarkers detected in >50% of the samples (i.e., DEP, DETP, DMTP, and TCPY). The associations were initially assessed using simple linear regression in unadjusted models. These same associations were then examined using multiple linear regression in statistical models that were adjusted for the following variables: age, BMI, race/ethnicity, education, serum SHBG, and serum cotinine. These additional variables were considered and included in final models based on methods employed in previous studies [17,18], and because when most were individually added to unadjusted models, the beta estimate for the exposure biomarker changed by >10%. Urinary creatinine concentration was also added to adjusted models as a covariate to account for variability in urinary output [37]. In both unadjusted and adjusted models, serum T and serum E2 were log-transformed because they were right-skewed and more closely followed a log-normal distribution. All independent variables were modeled as continuous variables, except for race/ethnicity and education, which were both modeled as categorical variables. In particular, race was categorized as Mexican American, other Hispanic, non-Hispanic Black, non-Hispanic White, and other/multi-racial. Education was categorized as less than $9^{th}$ grade, $9^{th}$–$11^{th}$ grade, high school graduate or equivalent, some college or associate degree, and college graduate or greater. As a sensitivity analysis, we also ran these models where the exposure biomarker was log-transformed to minimize the potential for associations that may have been driven by influential or extreme values. In addition, we assessed non-linear relationships by regressing serum hormones on categories of exposure biomarkers. We chose to categorize bins of exposure as less than $50^{th}$ percentile, $50^{th}$–$75^{th}$ percentile, $75^{th}$–$90^{th}$ percentile, and greater than $90^{th}$ percentile based on the percentage of non-detects. We also chose not to use sampling weights in our analysis because when variables employed in the calculation of sampling weights are also included in statistical models, such as race/ethnicity and age groups [38], a weighted analysis can decrease the precision of effect estimates [39]. This approach has been employed in other recent studies using data from NHANES [40,41]. To facilitate interpretation of the beta coefficients, when biomarker concentration was modeled as a continuous variable our results were expressed as percent change in serum T or E2 concentration associated with a doubling (i.e., 100% increase) in urinary biomarker concentration (equation: % change = [(exp(2*beta)) − 1]*100). When biomarker concentration was modeled as a categorical variable out results were expressed as percent change in serum T or E2 associated with each bin of exposure relative to the referent group (equation: % change = [(exp(beta)) − 1]*100).

## 3. RESULTS

Table 1 shows the demographic characteristics of the subset of men (n=356) from NHANES 1999–2002 included in our study. Overall, these men had a median age of 37 years, were predominantly overweight (67%), moderately educated (some college/associate degree, 34%), and non-Hispanic White (44%), and had no to little secondhand tobacco smoke exposure (59%). The breakdown of these characteristics was similar to those of the overall sample (n=2809) of 20–55 year-old men (data not shown).

Table 2 presents the distributions of the urinary biomarker concentrations. All urinary biomarkers were detected in greater than 50% of the samples, except for DEDTP (29%), DMP (48%), and DMDTP (33%).

Table 3 shows the percent change in T or E2 associated with a doubling (100 % increase) in biomarker concentration where the biomarker was modeled as a continuous variable. In unadjusted models, there was a statistically significant inverse association between DEP and T (β: −2.4%; 95% confidence interval (CI): −3.7, −1.2%), and a suggestively significant positive association between TCPY and E2 (β: 1.8%; 95% CI: −0.2, 3.8). When the covariates were included in adjusted models, the inverse association between DEP and T remained statistically significant as in the unadjusted model, but a suggestive relationship between TCPY and E2 was no longer observed. Similar findings were also observed when the biomarker was log-transformed (data not shown).

As shown in Figure 1, there appears to be a monotonic but non-linear inverse relationship between DEP and T (trend p=0.01). A similar relationship was also noted in the unadjusted model between DEP and T (data not shown). Using the $50^{th}$ percentile as the reference, on average men in this age range are expected to have a decrement of −3.2% (95% CI: −11.1, 5.3%), −2.5% (95% CI: −12.0, 8.1%), or −17.1% (95% CI: −26.7, −6.3%) in T concentration if their DEP concentration is in the $50^{th}$–$75^{th}$, in the $75^{th}$–$90^{th}$, or greater than the $90^{th}$ percentile, respectively. Like the continuous measures analysis in Table 3, there were no statistically significant associations between DETP, DMTP, or TCPY and T or E2 in both unadjusted and adjusted regression models when the biomarker was modeled as a categorical variable (data not shown).

## 4. DISCUSSION

In the present study, we examined the potential association between urinary biomarkers of OP insecticide exposure and serum reproductive hormones in men of reproductive age from NHANES. We observed a statistically significant inverse relationship between DEP and T in adjusted models when exposure was modeled as either a continuous or a categorical variable. Our findings suggest that the relationship may occur primarily at the high end of the exposure distribution. Since DEP is a common metabolite of several OP insecticides, exposure to one or more OP insecticides may increase a man's risk for fertility issues and also a variety of other adverse health endpoints through altered T. Aside from chlorpyrifos, one the parent compounds of TCPY, there are 9 other OP insecticides that are metabolized to DEP [42]. However, for the men in our analysis, chlorpyrifos does not appear to be the OP insecticide driving this relationship as TCPY was not associated with T and TCPY was not strongly correlated with DEP (Spearman's rho: 0.29). This analysis represents a unique contribution to the state-of-the-science on this matter as it is the first of its kind to use data from NHANES.

Men in our study had similar urinary concentrations of TCPY detected at comparable frequencies relative to those presented in a study of men from a U.S. fertility clinic (uncorrected geometric mean: 1.7 ng/ml vs. 2.1 ng/ml; detection: 83% vs. 95%) [17,18,43]. In that population, statistically significant inverse dose-dependent relationships between TCPY and T [17] and TCPY and E2 [18] were observed, which is contrary to our findings of no observed association for TCPY and T or E2. Akin to Aguilar-Garduño et al. [6], which focused on exposures to OP insecticides in Mexican male floriculture workers, detection frequencies of urinary concentrations of dimethyl moieties (median: 54%) were higher

relative to diethyl moieties (median: 48%) in our analysis. In addition, the authors observed a statistically significant inverse relationship between sum DEPs and T. The observed inverse association between DEP and T in our study, and similar findings of other studies, could be the result of a dose-dependent inhibition of cholinesterase in the hypothalamus, which, in turn, could alter the rate of gonadotropin-releasing hormone (GnRH) secretion [17, 44,45]. The neuroendocrine axis and specifically GnRh neurons in the brain could also mediate the effects of reproductive and neurologic toxicants, such as OP insecticides [17,46].

Aside from T and E2, several epidemiology studies involving cohorts of men from the U.S. [43], Mexico [6,47,48], Venezuela [49], and China [50] have reported altered levels of thyroid stimulating hormone, follicle stimulating hormone, and/or luteinizing hormone in relation to exposure to OP insecticides, which provides additional support for the potential endocrine disrupting capabilities of this class of insecticide. Studies conducted in rodent species have also demonstrated decreases in T following exposure to the OP insecticides chlorpyrifos [51], methamidophos [52], and propetamphos [53], corroborating our findings. In addition, these laboratory studies observed reductions in testicular size and seminal vesicle weight, decreases in sperm count and motility, and increases in sperm morphological abnormalities, lending support for the potential role of OP insecticides as male reproductive toxicants.

This analysis represents a unique contribution to the state-of-the-science on the potential link between exposure to OP insecticides and male reproductive hormones as it is the first of its kind to use data from NHANES. While our analysis had a reasonably large sample size (n=356), it was not without limitations. The cross-sectional study design limited conclusions of causality due to temporal ambiguity. Nowadays, liquid chromatography-tandem mass spectrometry is available for measuring serum levels of reproductive hormones, which can provide greater sensitivity than the immunoassays used by CDC during the 1999–2002 cycles [54]. In addition, a single measurement of biomarkers of OP insecticides may lead to appreciable exposure measurement error [55], which, if non-differential, would have biased our effect estimates towards the null and underestimated the true associations.

## 5. CONCLUSIONS

In conclusion, DEP was associated with reduced T in men of reproductive age from NHANES. Because altered T has been linked to a wide variety of adverse health effects, additional epidemiology studies are needed in other populations from the U.S. and other countries to confirm the results of our analysis.

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## ABBREVIATIONS

| | |
|---|---|
| **BMI** | body mass index |
| **CDC** | Centers for Disease Control and Prevention |
| **CI** | confidence interval |
| **DEP** | diethylphosphate |
| **DEDTP** | diethyldithiophosphate |
| **DETP** | diethylthiophosphate |
| **DMP** | dimethylphosphate |
| **DMDTP** | dimethyldithiophosphate |
| **DMTP** | dimethylthiophosphate |
| **E2** | estradiol |
| **GnRH** | gonadotropin-releasing hormone |
| **LOD** | limit of detection |
| **NCHS** | National Center for Health Statistics |
| **NHANES** | National Health and Nutrition Examination Survey |
| **OP** | organophosphate |
| **SHBG** | sex hormone binding globulin |
| **T** | testosterone |
| **TCPY** | 3,5,6-trichloro-2-pyridinol |

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## HIGHLIGHTS

- We studied 7 urinary OP insecticide metabolites in men 20–55 years old from NHANES
- Biomarkers were detected in >50% of the samples, except for DEDTP, DMP, and DMDTP
- There was a statistically significant inverse association between DEP and T
- Exposure to certain OP insecticides may have important implications for male health

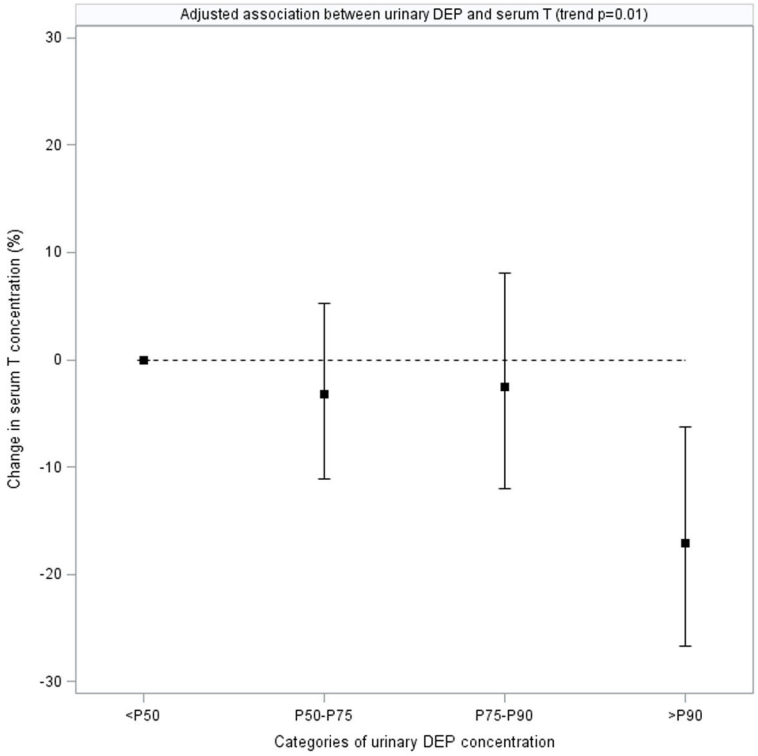


**Figure 1.**

Percent change in serum T concentration associated with increasing urinary DEP concentration in men aged 20–55 years from NHANES 1999–2002 (n=356). Results were adjusted for age, BMI, serum cotinine, urinary creatinine, serum SHBG, race/ethnicity, and education.

## Table 1

Characteristics of men aged 20–55 years from NHANES 1999–2002 (n=356)

| Variable | Median (IQR)/n (%) |
|---|---|
| Age (years) | 37 (29, 46) |
| BMI (kg/m$^2$)[a] | |
| <18.50 (underweight) | 5 (1) |
| 18.50–24.99 (normal weight) | 114 (32) |
| ≥25.00 (overweight) | 237 (67) |
| Education | |
| <9$^{th}$ grade | 43 (12) |
| 9$^{th}$–11$^{th}$ grade | 58 (16) |
| High school grad/GED | 67 (19) |
| Some college/AA degree | 120 (34) |
| College graduate or greater | 68 (19) |
| Race/ethnicity | |
| Mexican American | 88 (25) |
| Other Hispanic | 19 (5) |
| Non-Hispanic Black | 75 (21) |
| Non-Hispanic White | 158 (44) |
| Other/multi-racial | 16 (5) |
| Serum cotinine (ng/ml)[b] | |
| <1 (no or little STS exposure) | 209 (59) |
| 1–10 (high STS exposure) | 25 (7) |
| >10 (likely smoker) | 122 (34) |

Abbreviations: AA, associate degree; BMI, body mass index; GED, general education development; IQR, interquartile range; STS, secondhand tobacco smoke.

[a] Categories defined based on World Health Organization BMI classification criteria [56].

[b] Categories defined based on Hukkanen et al. [57].

**Table 2**

Urinary concentrations of organophosphate insecticide metabolites (ng/ml, uncorrected for urinary creatinine) in men aged 20–55 years from NHANES 1999–2002 (n=356)

| | LOD by cycle | | | | Percentiles | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Biomarker** | **99–00** | **01–02** | **n (%) ≥LOD** | **GM** | **10th** | **25th** | **50th** | **75th** | **90th** | **95th** | **Max** |
| DEP | 0.2 | 0.2 | 204 (57) | 0.8 | <LOD | <LOD | 0.8 | 3.5 | 8.4 | 15.0 | 51.9 |
| DETP | 0.09 | 0.1 | 226 (64) | 0.4 | <LOD | <LOD | 0.5 | 1.2 | 2.3 | 3.2 | 26.4 |
| DEDTP | 0.05 | 0.1 | 104 (29) | 0.1 | <LOD | <LOD | <LOD | 0.1 | 0.6 | 0.7 | 8.4 |
| DMP | 0.58 | 0.5 | 170(48) | 1.0 | <LOD | <LOD | <LOD | 2.7 | 7.1 | 12.3 | 67.0 |
| DMTP | 0.18 | 0.4 | 187 (53) | 1.3 | <LOD | <LOD | 0.6 | 6.0 | 29.5 | 40.8 | 900 |
| DMDTP | 0.08 | 0.1 | 118 (33) | 0.2 | <LOD | <LOD | <LOD | 0.9 | 3.0 | 7.8 | 33.0 |
| TCPY | 0.4 | 0.4 | 296 (83) | 1.7 | <LOD | 0.9 | 1.9 | 3.8 | 7.3 | 11.1 | 42.0 |

Abbreviations: GM, geometric mean; LOD, limit of detection.

**Table 3**

Percent change in serum hormone concentration associated with a doubling (100% increase) in urinary organophosphate insecticide metabolite concentration in men aged 20–55 years from NHANES 1999–2002 (n=356)

| | T [% change (95% CI)] | | E2 [% change (95% CI)] | |
|---|---|---|---|---|
| **Biomarker** | **Unadjusted** | **Adjusted[a]** | **Unadjusted** | **Adjusted[a]** |
| DEP | −2.4 (−3.7, −1.2)** | −2.5 (−3.5, −1.4)** | 0.1 (−1.4, 1.7) | −0.2 (−1.7, 1.4) |
| DETP | 0.4 (−3.6, 4.6) | 1.3 (−2.0, 4.6) | 1.3 (−3.5, 6.3) | 1.4 (−3.2, 6.2) |
| DMTP | −0.1 (−0.2, 0.1) | −0.02 (−0.2, 0.1) | −0.1 (−0.3, 0.1) | −0.1 (−0.3, 0.1) |
| TCPY | 0.2 (−1.4, 1.9) | 0.3 (−1.1, 1.7) | 1.8 (−0.2, 3.8)* | 1.5 (−0.5, 3.5) |

Abbreviations: CI, confidence interval.

[a]Models were adjusted for age (continuous), BMI (continuous), serum cotinine concentration (continuous), urinary creatinine concentration (continuous), serum SHBG concentration (continuous), race/ethnicity (categorical), and education (categorical).

*$0.05 \leq p < 0.10$.

**$p < 0.05$.