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Circulating estradiol in men is inversely related to urinary metabolites of nonpersistent insecticides☆

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Abstract

Background: Estradiol plays an important role in male reproductive health as a germ cell survival factor. Chlorpyrifos and carbaryl, nonpersistent insecticides to which the general population are commonly exposed, were recently shown to inhibit estradiol metabolism *in vitro* which could lead to altered hormone balance.
Methods: Subjects ($N = 322$) were the male partners in couples presenting to a Massachusetts infertility clinic from years 2000–2003. 3,5,6-Trichloro-2-pyridinol (TCPY), the major urinary metabolite of chlorpyrifos and chlorpyrifos-methyl, and 1- and 2-naphthol (1N and 2N), urinary metabolites of carbaryl and naphthalene, were measured in a spot urine sample from each subject. Estradiol, sex hormone binding globulin (SHBG), and prolactin were measured in serum collected from subjects during the same clinic visit.
Results: Using multiple linear regression, an interquartile range (IQR) increase in TCPY was associated with a 1.36 pg/mL decline (95% confidence interval = −2.91 to −0.22) in estradiol concentration. When estradiol and TCPY were divided into quintiles, there was a dose-dependent increase in the odds of being in the lowest estradiol quintile with increasing TCPY quintiles.
Conclusion: On a population level, these reductions in estradiol levels are of potential public health importance because of widespread exposure to TCPY and its parent insecticides.

Keywords: Carbaryl; Chlorpyrifos; Endocrine disruption; Estradiol; Hormone; Male reproduction; Pesticides; Prolactin

1. Introduction

Although estradiol is regarded as a female steroid hormone, it circulates in low but measurable levels in human males [1,2]. Estradiol has recently been reported to play an important role in male reproductive health. Studies of estrogen receptor α (ERα) knockout and aromatase knockout mice first suggested an indirect role of estrogens in male fertility [3–5]. Male ERα knockout mice were infertile, with post-pubertal degeneration of the testes and disrupted spermatogenesis [3,4]. Estradiol is produced in the testes from aromatized testosterone, and progressive disruption of spermatogenesis and infertility was also observed among aromatase knockout mice [5]. A direct role of estradiol as a germ cell survival factor was then demonstrated in the human testis *in vitro*, where estradiol was shown to inhibit testicular apoptosis much more effectively than testosterone [2]. A potential role of nonpersistent pesticides (e.g. organophosphates, pyrethroids and carbamates) in the alteration of circulating estradiol levels was recently reported in experimental studies [6,7], but to our knowledge these associations remain untested in humans.

Nonpersistent pesticides are widely used in agricultural, commercial, and residential settings. Due to the extensive use of these chemicals, a large proportion of the general population is

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exposed to low levels of nonpersistent pesticides or their environmental degradation products. The Second National Report on Human Exposure to Environmental Chemicals (NHANES 1999–2000) found that over 90% of males in the US population had urine samples with detectable levels of 3,5,6-trichloro-2-pyridinol (TCPY), the major urinary metabolite of chlorpyrifos and chlorpyrifos-methyl [8]. The report also found that over 75% of the US male population had detectable levels of 1-naphthol (1N), a urinary metabolite of carbaryl and naphthalene. The Third National Report (NHANES 2001–2002) did not report percentile concentrations below the median, but geometric means and upper percentiles remained consistent with those in the Second National Report [9].

Chlorpyrifos, a broad-spectrum organophosphate insecticide, was one of the most commonly used insecticides in homes until residential restrictions were placed on its use by the Environmental Protection Agency (EPA) in 2000 [10]. According to the most recent market estimates of pesticide sales and usage, between 11 and 16 million pounds of chlorpyrifos were applied in the US in 2001 [11]. This represents only a small decline in overall use following the 2000 restrictions (in 1999 between 13 and 19 million pounds were applied), as usage in the agricultural and industry/commercial/government market sectors remained stable between 1999 and 2001 [11]. The high rate of detection of TCPY in urine from the general population suggests ongoing environmental exposure to chlorpyrifos and/or TCPY. Though referred to as nonpersistent, contemporary use insecticides applied indoors or tracked in from outdoors may persist for extended periods when protected from breakdown reactions. Diet likely remains an important route of exposure as trace concentrations of chlorpyrifos continue to be found in many types of food [12,13]. In addition, chlorpyrifos-methyl is used as an insecticide on stored grains [14]. Carbaryl, a broad-spectrum insecticide known as Sevin®, is still commonly used to protect residential lawns and gardens from a variety of insects. It is estimated that between 2 and 4 million pounds of carbaryl were applied in the home and garden market sector in 2001 [11].

The toxicological literature suggests that chlorpyrifos, chlorpyrifos-methyl, and carbaryl may be hormonally active [7,15–20]. One potential mechanism whereby these pesticides may affect male reproductive function is through disruption of the endocrine axis, manifest as altered levels of reproductive hormones. We recently reported statistically significant relationships between urinary levels of TCPY and 1N in relation to testosterone and sperm DNA damage [21,22]. Urinary TCPY and 1N levels were inversely associated with circulating testosterone [21], and positively associated with DNA damage in sperm cells measured by the neutral comet assay [22]. Although estradiol is produced by testosterone aromatization, individual differences in aromatase activity results in varying agreement between the two hormones across a population. In addition, estradiol in the human male may have important functions independent of those of testosterone. For example, low estradiol levels serve as a better predictor than testosterone of bone loss and bone density among elderly men [23,24] and of carotid artery intima-media thickness in middle-aged men [1]. Thus, in light of recent research on the importance of estradiol in normal testis function [2] and the potential impact of nonpersistent pesticides on estradiol levels [6], the present study was performed to investigate associations between nonpersistent insecticide metabolites and circulating estradiol levels among adult men.

Data on prolactin levels were also available among men in the study and were included in the present analysis due to the potential importance of prolactin in male reproduction [25,26] and limited animal evidence of decreased prolactin following exposure to organophosphates [27].

2. Methods

Details of subject recruitment have been previously described [28]. Briefly, consecutive eligible men who were partners in subfertile couples being evaluated for infertility at the Massachusetts General Hospital Vincent Burnham Obstetrics and Gynecology Service Fertility Center between January 2000 and April 2003 were recruited to participate. Subjects signed informed consent after the study procedures were explained and any questions they had were answered. Of those approached, approximately 65% consented. Most men who declined to participate in the study cited lack of time on the day of their clinic visit as the reason for not participating. The study was approved by the Human Studies Institutional Review Boards of the Massachusetts General Hospital, Harvard School of Public Health, and the University of Michigan.

2.1. Reproductive hormones

One non-fasting blood sample was drawn between the hours of 9 a.m. and 4 p.m. on the same day that the urine sample was collected. Despite variability in serum hormone levels over time, a single blood sample likely provides a relatively reliable measure for estradiol and prolactin [29,30]. Blood samples were centrifuged and serum stored at −80 °C until analysis. Serum estradiol and prolactin concentrations were determined by microparticle enzyme immunoassay using an automated Abbott AxSYM system (Abbott Laboratories, Abbott Park, IL, USA). A recent study measuring estradiol levels in men using both immunoassay and gas chromatography–mass spectrometry reported strong Pearson and Spearman correlations near one (both $p < 0.001$) for results produced by the two methods [31]. In the present study the assay sensitivity for estradiol and prolactin were 20 pg/mL and 0.6 ng/mL, respectively. For estradiol the within-run coefficient of variation (CV) was between 3 and 11%, and the total CV was between 5 and 15%. For prolactin the within-run CV was ≤3% and the total CV was ≤6%. Sex hormone binding globulin (SHBG) was measured using a fully automated system (Immulite: DPC Inc.) which uses a solid-phase two-site chemiluminescent enzyme immunometric assay and has an interassay CV of less than 8%.

2.2. Insecticide metabolites in urine

A single spot urine sample was collected from each subject on the day of their clinic visit. Although TCPY and 1N are not persistent in the body, we recently found that a single urine sample may adequately predict exposure groups over several months [32]. Urine samples were frozen at −20 °C and sent to the US Centers for Disease Control and Prevention (CDC) where TCPY, 1N (a metabolite of both carbaryl and naphthalene), and 2-naphthol (2N; a metabolite of naphthalene but not carbaryl) were measured [33]. Samples were fortified with stable isotope analogues of the target analytes and glucuronide or sulfate-bound metabolites were liberated using an enzyme hydrolysis. The target metabolites were isolated using liquid–liquid extraction, chemically derivatized, and measured using gas chromatography–chemical ionization-tandem mass spectrometry. Specific gravity (SG) was used as the primary method to adjust metabolite concentrations for urine dilution. However, in addition to SG-adjusted results, volume-based (unadjusted) and creatinine-adjusted TCPY and 1N concentrations were also determined for comparison with exposure distributions from other studies. SG was measured using a handheld refractometer (National Instrument Company Inc., Baltimore, MD, USA). Creatinine was measured photometrically using kinetic colorimetric assay technology with a Hitachi 911 automated chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA).

2.3. Statistical analysis

Data analysis was performed using SAS Version 9.1 (SAS Institute Inc., Cary, NC, USA). Descriptive statistics on subject demographics were tabulated, along with the distributions of insecticide metabolite levels and hormones. For insecticide metabolite and hormone values below the limit of detection (LOD), corresponding to 0.25 μg/L for TCPY, 0.40 μg/L for 1N, and 20 pg/mL for estradiol, an imputed value equal to one-half the LOD was used. All samples were quantifiable for prolactin and SHBG. Hormone and insecticide metabolite levels were stratified by demographic categories to investigate the potential for confounding. Spearman correlation coefficients were used to determine correlations among insecticide metabolites, among hormones, and between insecticide metabolite and hormone levels.

Relationships between SG-adjusted urinary insecticide concentrations and hormone levels were explored using multivariate linear regression. Concentrations of estradiol closely approximated normality and were used in statistical models untransformed, while the distributions of prolactin and SHBG were skewed right and transformed to the natural log(ln) for statistical analyses. SG-adjusted TCPY and 1N concentrations were also both ln-transformed. Inclusion of covariates was based on statistical and biologic considerations [34]. Age and BMI were modeled as a continuous variable, smoking status was dichotomized by current smoker versus never smoked or former smoker, and race was categorized into four groups: white, African American, Hispanic, and other. Timing of blood sample by season (winter vs. spring, summer or fall) and time of day (09:00–12:59 h vs. 13:00–16:00 h) were considered for inclusion in the models as dichotomous variables. To improve interpretability, the regression coefficients were back transformed and expressed as a change in the dependent variable (i.e., hormone levels) for an interquartile range (IQR) increase in SG-adjusted metabolite levels. Because 1N is a metabolite of both carbaryl and naphthalene, while 2N is a metabolite of naphthalene but not carbaryl, models involving 1N were reanalyzed when stratifying 1N/2N ratio above or below 2.0 to explore differential effects of 1N by exposure to the two separate parent compounds [35].

In secondary analyses, the multivariate models were rerun for SG-adjusted metabolite levels after excluding men with SG outside the acceptable range. The analyses were also tested for robustness by performing analyses using both unadjusted and creatinine-adjusted insecticide metabolite levels. We also assessed non-linear relationships between metabolite levels and hormones by regressing the hormone concentrations on tertiles and quintiles of insecticide metabolites. Thirty-five serum samples (10.9%) had estradiol concentrations below the limit of detection, which may have introduced bias in the regression results. Thus, analysis was also conducted when dividing estradiol concentration into quintiles to eliminate the influence of non-detect values. In this analysis, multivariate logistic regression was used to calculate odds ratios for having estradiol concentration in the lowest quintile.

3. Results

Of the 370 men with pesticide metabolites measured in urine, 336 also had hormone levels measured in serum. An additional 14 subjects taking hormonal medications (e.g. propecia, finasteride, cabergoline, clomid, GnRH, testosterone, or prednisone taper) were excluded. Demographic data on the study population have been presented elsewhere [21]. Briefly, among the remaining 322 subjects, the majority was white (83%) and had never smoked (71%). Thirty-five percent of the men had a previous examination for infertility and 40% had proven fertility (had previously made a partner pregnant). The mean (S.D.) age and BMI were 36 (5.4) years and 28 (4.6), respectively. Concentration distributions of hormone levels and urinary insecticide metabolites are presented in Table 1. TCPY and 1N were detected in 95.0 and 99.7% of subjects, respectively, with specific gravity-adjusted geometric means of 2.59 and 3.23 μg/L (Table 1). SG-adjusted TCPY and 1N levels were moderately correlated (Spearman correlation coefficient = 0.3; p-value <0.0001). The arithmetic mean (S.D.) concentrations of estradiol and prolactin were 31.8 (11.5) pg/mL and 13.1 (6.8) ng/mL, respectively.

Estradiol was inversely associated with age and positively associated with BMI in bivariate analyses. Blood samples collected in the morning (between 9:00 a.m. and 12:59 p.m.) had lower prolactin levels compared to those collected in the afternoon (between 1:00 p.m. and 4:00 p.m.; median = 10.0 and 12.6 ng/mL, respectively; p-value <0.05). Median estradiol levels did not differ between blood samples collected in the morning and afternoon (median = 31 and 32 ng/mL, respectively; p-value >0.05). For the specific gravity-adjusted insecticide metabolites, none of the demographic variables (i.e., age, BMI, race, smoking, season) were strongly associated with urinary TCPY levels. Current smokers had higher median levels of SG-adjusted 1N (8.4 μg/mL) than never (3.6 μg/mL) and former (2.8 μg/mL) smokers, which suggests naphthalene exposure via cigarette smoke. Median SG-adjusted 1N concentration was also higher among men whose urine samples were collected in the winter (4.3 μg/mL) versus samples collected in spring, summer or fall (2.8 μg/mL).

Results from the univariate and multivariate linear regression analyses are presented in Table 2. The final estradiol multivariate models were adjusted for (natural log-transformed) SHBG, age, BMI, and season. The final prolactin models were adjusted for age and time of day that the blood sample was collected. There was an inverse association between urinary TCPY concentration and serum estradiol levels. An IQR increase in SG-adjusted TCPY was associated with a 4.6% decrease in the median estradiol level (95% CI −8.8 to −0.5%). There was a suggestive inverse association between 1N and estradiol (p = 0.09), and no

Table 1
Concentration distributions of serum hormones and specific gravity-adjusted urinary insecticide metabolites among 322 men

	Geometric mean	Selected percentiles						
		10th	25th	50th	75th	90th	95th	Max
Hormones								
Estradiol (pg/mL)	29.3	<20	25	32	38	45	50	71
Prolactin (ng/mL)	11.7	6.55	8.35	11.5	16.1	21.5	25.4	52.2
Insecticide metabolites[a,b]								
TCPY (μg/L)	2.59	0.58	1.54	3.16	5.11	8.27	10.6	40.7
1N (μg/L)	3.23	0.90	1.70	3.20	5.64	11.7	16.9	160

[a] TCPY = 3,5,6-trichloro-2-pyridinol; 1N = 1-naphthol.
[b] Limit of detection (LOD) for TCPY = 0.25 μg/L; LOD for 1N = 0.40 μg/L; 95.0% of TCPY samples above LOD; 99.7% of 1N samples above LOD.

Table 2
Regression coefficients for a change in serum hormone levels[a] associated with an interquartile range (IQR) increase in SG-adjusted insecticide metabolite levels[b]; $N = 322$

	Estradiol[c]				Prolactin[d]			
	Crude (95% CI)	*p*-Value	Adjusted[e] (95% CI)	*p*-Value	Crude (95% CI)	*p*-Value	Adjusted[f] (95% CI)	*p*-Value
TCPY	−1.45 (−2.79, −0.10)	0.04	−1.49 (−2.82, −0.17)	0.03	1.03 (0.98, 1.10)	0.21	1.04 (0.98, 1.09)	0.23
1N	−1.12 (−2.66, 0.43)	0.16	−1.36 (−2.91, 0.22)	0.09	0.96 (0.90, 1.02)	0.22	0.96 (0.91, 1.02)	0.25

[a] Estradiol was modeled untransformed, prolactin was adjusted to the natural logarithm prior to modeling.
[b] In all models ln-transformations of insecticide metabolite concentrations were used.
[c] Coefficient represents the change in hormone level for an IQR change in insecticide metabolite concentration after back-transformation of the insecticide metabolite concentrations. For an IQR change in insecticide metabolite concentration, a coefficient equal to 0 indicates no change in hormone level, a coefficient <0 indicates a decrease in hormone level, and a coefficient >0 indicates an increase in hormone level.
[d] Coefficient represents a multiplicative change in hormone level for an IQR change in insecticide metabolite concentration after back-transformation of both hormone and insecticide metabolite concentrations. For an IQR change in insecticide metabolite concentration, a coefficient equal to 1.0 indicates no change in hormone level, a coefficient <1.0 indicates a multiplicative decrease in hormone level, and a coefficient >1.0 indicates a multiplicative increase in hormone level.
[e] Adjusted for (ln) SGBG, age, BMI, and season.
[f] Adjusted for age and time of day blood sample was collected.

statistically significant associations between TCPY or 1N and prolactin. In further analyses there were no differences in 1N models when stratified by 1N/2N ratio to separate likely sources of urinary 1N [35].

Regression results for unadjusted or creatinine-adjusted TCPY and 1N concentrations were comparable to those using the SG-adjusted levels (data not shown). Results also remained largely unchanged when regression models were repeated after excluding 55 men with SG less than 1.01 or greater than 1.03 ($N = 268$; data not shown), though the point estimate became slightly smaller. An IQR increase in SG-adjusted TCPY was associated with a 3.9% decrease in estradiol (95% CI −8.3 to 0.6%).

To explore non-linear relationships we also performed analyses in which serum hormone levels were regressed on quintiles of insecticide metabolites. TCPY was associated with a decreasing (though non-monotonic) trend in estradiol [coefficients (95% CI) for quintiles 2, 3, 4, and 5 were −1.30 pg/mL (−5.15 to 2.55), −1.84 pg/mL (−5.71 to 2.03), −5.08 pg/mL (−8.93 to −1.23) and −2.98 pg/mL (−6.83 to 0.87) (Fig. 1)]. For 1N, the highest four quintiles were associated with declined estradiol but not in a dose-related manner (Fig. 2). When (ln-transformed) prolactin was modeled against SG-adjusted TCPY or 1N quintiles, no clear evidence of dose–response patterns emerged. However, the highest TCPY quintile was associated with significantly higher prolactin levels compared to the lowest TCPY quintile (Fig. 3).

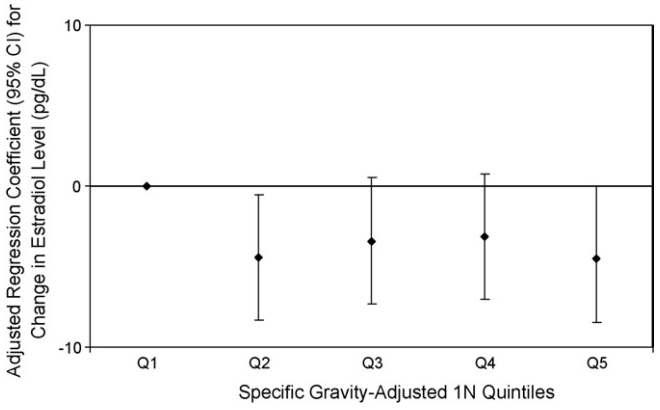

Fig. 2. Adjusted (adjusted for ln-transformed SHBG, age, BMI, and season) regression coefficients for a change in estradiol level associated with increasing quintiles of SG-adjusted 1-naphthol ($N = 322$). *p*-Value for trend = 0.09.

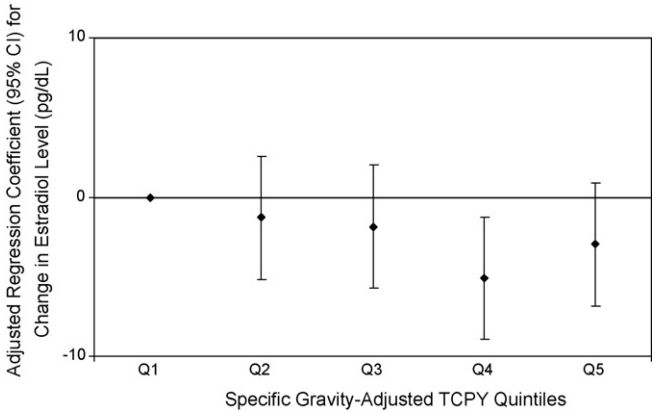

Fig. 1. Adjusted (adjusted for ln-transformed SHBG, age, BMI, and season) regression coefficients for a change in estradiol level associated with increasing quintiles of SG-adjusted TCPY ($N = 322$). *p*-Value for trend = 0.03.

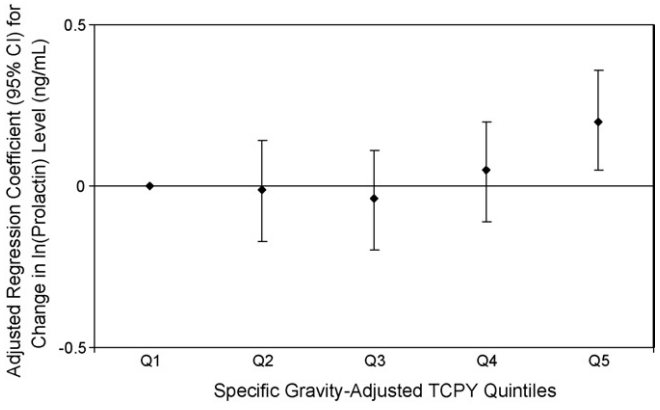

Fig. 3. Adjusted (adjusted for age and time of day blood sample was collected) regression coefficients for a change in ln-adjusted prolactin level associated with increasing quintiles of SG-adjusted TCPY ($N = 322$). *p*-Value for trend = 0.01.

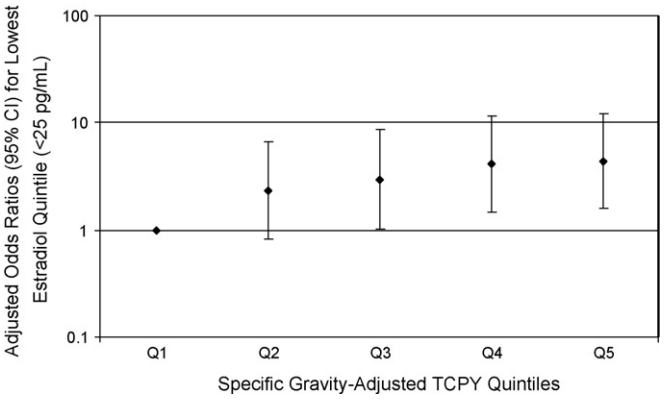

Fig. 4. Adjusted (adjusted for ln-transformed SHBG, age, BMI, and season) odds ratios for having estradiol level in the lowest quintile (<25 pg/mL) with increasing SG-adjusted TCPY quintiles ($N = 322$). p-Value for trend = 0.002.

Since a proportion (10.8%) of men had estradiol concentrations below the detection limit, multivariate logistic regression was also conducted where estradiol levels were divided into quintiles. A dose-dependent increase in odds ratios for being in the lowest estradiol quintile was observed for increasing TCPY quintiles [odds ratios (95% CI) for quintiles 2–5 were 2.3 (0.8–6.6), 3.0 (1.0–8.5), 4.1 (1.5–11.4) and 4.4 (1.6–12.1) (Fig. 4)]. There was a suggestive though non-monotonic trend between 1N quintiles and lowest estradiol quintile (data not shown; p-value for trend = 0.05).

Finally, since there was a significant inverse association between TCPY and estradiol and a suggestive inverse association between 1N and estradiol we explored the potential for interaction between the two metabolites. To avoid sparse strata, tertiles (instead of quintiles) for both TCPY and 1N were formed and men in the highest tertile for both metabolites ($N = 51$) were compared to men in the lowest tertile for both metabolites ($N = 62$). In multiple linear regression men in the highest tertiles for both metabolites had lower estradiol levels than men in the lowest tertiles for both metabolites (coefficient = −2.38 pg/dL; 95% CI −5.75 to 0.10 pg/dL), and in multiple logistic regression men in the highest metabolite tertiles were more likely to have an estradiol level in the lowest quintile (OR = 2.03; 95% CI 0.88–4.71). However, there was no evidence of interaction as these effect estimates were smaller and weaker than those for the highest TCPY tertile alone compared to the lowest TCPY tertile.

4. Discussion

We found an inverse association between urinary TCPY concentration and serum estradiol in men, and a suggestive inverse association between 1N and estradiol. With increasing TCPY quintiles there was also an increasing trend in odds of having estradiol in the lowest quintile. The urinary concentrations of TCPY and 1N in this study are environmentally relevant, as they were comparable to the distributions reported from the US general population [8,9]. To our knowledge this is the first human study exploring the association between the urinary metabolites TCPY and 1N and circulating levels of estradiol and prolactin.

Our results are consistent with a recent study of Mexican agricultural workers, where Recio et al. [36] measured dialkylphosphate metabolite concentrations in urine and sex hormone levels in serum from 64 men. They found an inverse association between diethylphosphate (DEP), a non-specific metabolite of chlorpyrifos, and estradiol (p-value = 0.04). However, a number of other organophosphate pesticides are also metabolized to DEP [9] which limited the interpretation of results. In addition, diethylthiophosphate (DETP), also a non-specific metabolite of chlorpyrifos, was not inversely associated with estradiol in the study but there appeared to be a suggestively large yet unstable inverse association between estradiol and dimethylphosphate (DMP), a non-specific metabolite of chorpyrifos-methyl and several other organophosphates (p-value = 0.19).

Potential mechanisms for the inverse association between estradiol and TCPY or its parent compounds, chlorpyrifos and chlorpyrifos-methyl, remain unclear. Several studies suggest chlorpyrifos or chlorpyrifos-methyl may be hormonally active but specific findings have not been fully consistent. Chlorpyrifos and other organophosphates have been shown to inhibit steroidogenesis in adrenal cells [37,38], and chlorpyrifos potently affected GnRH gene expression and biosynthesis *in vitro* [18]. Weak responses to chlorpyrifos were reported in two *in vitro* estrogenicity assays [19], whereas chlorpyrifos-methyl exhibited antiandrogenic activity, but not estrogenic or estrogen-like activity, in immature rats [20]. In addition, male rats exposed to chlorpyrifos-methyl *in utero* and postnatally had dose-dependent declines in serum estradiol, testosterone and thyroxin, though these associations were not found in rats exposed in adulthood [7]. Finally, chlorpyrifos potently and irreversibly inhibited estradiol metabolism by pooled human liver microsomes and human cytochrome P450 isoforms CYP1A2 and CYP3A4, as did carbaryl and its metabolite 1-naphthol to a lesser extent [6].

In an *in vitro* study conducted to investigate the protein expression of estrogen receptors α and β in the human seminiferous epithelium, estradiol was found to be a germ cell survival factor in the human testis [2]. Germ cell apoptosis was induced *in vitro* by incubating segments of seminiferous tubules under serum and hormone-free conditions without survival factors, and estradiol was found to effectively inhibit the induced apoptosis. The authors concluded that i*n vitro* estradiol is a more potent inhibitor of male germ cell death than androgens, as the concentration of testosterone needed for the same effective apoptosis inhibition was 100–1000 times the effective concentrations of estradiol [2]. Thus, it is possible that a decrease in estradiol associated with urinary TCPY concentrations may act as an intermediate step in our previous observation of a significant increase in sperm DNA damage associated with increased urinary TCPY [22].

Besides potential reproductive effects, altered male estradiol levels in relation to environmental exposures may also be associated with other health consequences. There have been consistent reports of increased risk of prostate cancer among farmers [39,40], and among male pesticide applicators an increased risk of cancers potentially associated with endocrine disruption

(testicular, prostate) has been reported [41–43]. In the US Agricultural Health Study, where data on the application of specific pesticides was collected by questionnaire, there was evidence for an increase in the risk for prostate cancer through an interaction between exposure to chlorpyrifos or other organophosphate insecticides (fonofos, phorate) and having a family history of prostate cancer [44–46]. Exogenous estrogen/estradiol is used in the treatment of certain prostate cancers [47] and a decreased risk of prostate cancer has been reported among men with higher circulating estradiol levels, suggesting a potential protective role [48–51]. Thus, results from the present study may provide evidence for a biologic mechanism in the association between exposure to pesticides and prostate cancer that involves altered circulating estradiol levels. Of particular note may be the suggestion of a threshold whereby only men with the lowest estradiol levels were at increased risk for prostate cancer [48], making our observation of a dose–response increase in the odds of having low estradiol with increasing TCPY of potential concern (Fig. 4).

Strengths of the present study include its size and the use of sensitive hormone and urinary insecticide metabolite measures. A limitation of the study is that only a single urine sample was collected to measure urinary insecticide metabolite concentrations, and only a single serum sample was collected to estimate hormone levels. Despite the diurnal and pulsatile fluctuations in serum hormone levels a single blood sample may be able to provide a reliable measure of estradiol and prolactin over both short and long time periods in population studies [29,30]. We also collected data on time of day and season in which the blood sample was collected to include in the multivariate analysis where appropriate. In addition, requiring multiple blood samples may limit participation rates in epidemiologic studies [52]. Measuring insecticide metabolite levels in urine provides a measure of individual internal dose, but nonpersistent insecticides are metabolized and excreted rapidly so levels of both TCPY and 1N measured in urine reflect insecticide exposure in the previous 24–48 h [53]. Although insecticide metabolite levels in urine can vary considerably over time, suggesting that a single urine sample may not be a reliable surrogate for longer-term exposure [54], we recently showed that a single urine sample was predictive of 3-month average urinary insecticide metabolite levels [32]. A single urine sample correctly classified men in the highest 3-month exposure tertile with a sensitivity of 0.6 and specificity of 0.9 for SG-adjusted 1N and, for SG-adjusted TCPY, a sensitivity of 0.5 and specificity of 0.8.

Recruitment of subjects through an infertility clinic is not likely to introduce selection bias in the present study. A recent study among a cohort of men that overlaps with the men in the present study reported no differences when semen characteristics were compared between participants and non-participants, suggesting that men did not participate based on semen quality [55]. Likewise, we believe it is unlikely that men participated based on their hormone status or based on exposure to nonpersistent insecticides. In addition, the participation rate in the present study (65%) was higher than other male reproductive health study designs, which reduces the potential for selection bias and increases the internal validity of a study.

The study included both fertile and infertile men, since the female partner's infertility may be the cause of some couples' infertility and subsequent evaluation. Furthermore, pesticide metabolite levels in the present study were similar to those found in the general population, suggesting that men from the infertility clinic did not have widely different levels of exposure. For these reasons, we believe the results are generalizable to men in the general population. In order for generalizablity to be limited, men in the present study would need to be differentially affected by exposure (i.e., more susceptible to exposure) compared to other men. There is currently no evidence suggesting that reproductive hormone levels in men visiting an infertility clinic are more sensitive to nonpersistent insecticide exposure than in other men.

In conclusion, the present study found that urinary TCPY levels that are representative of those found among the general US population may be associated with altered estradiol levels in adult men. On a population level these reductions are of potential public health importance because of widespread exposure to TCPY and its parent insecticides and the potential for other health consequences stemming from alterations in circulating estradiol. We also found suggestive evidence for an inverse association between exposure to 1N or its parent compounds and circulating estradiol. More research is needed to confirm these associations in humans and to elucidate potential biological mechanisms.

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