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## Exploratory analysis of urinary metabolites of phosphorus-containing flame retardants in relation to markers of male reproductive health

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### Abstract

The use of phosphorus-containing flame retardants (PFRs) has increased over the past decade. Widespread human exposure has been reported, but information on the safety or potential health risks of PFRs is lacking. We assessed the relationship between urinary concentrations of two PFR metabolites [bis(1,3-dichloro-2-propyl) phosphate (BDCPP) and diphenyl phosphate (DPHP)] and semen quality, sperm motion parameters, and serum hormone levels in 33 men. BDCPP and DPHP concentrations were significantly greater in urine samples collected in the afternoon compared to those collected in the morning ( $p < 0.05$ ). In multivariable models, a number of statistically significant or suggestive associations were observed between the reproductive health measures and both PFR metabolites. While the study was limited by a small sample size, these results warrant further investigation in a larger study population. Additional studies on sources, pathways, and routes of PFR exposure, along with research on toxicokinetics and exposure measure utility, are also needed.

### Keywords

Biomarker; Epidemiology; Exposure; Fertility; Reproduction; Thyroid

### INTRODUCTION

Organophosphate chemicals have commonly been used as plasticizers and flame retardants in a range of materials and products over the past few decades. However, the use of phosphorus-containing as flame retardants (PFRs) has been increasing dramatically over the past decade with the recent bans or voluntary phase-outs of the widely used polybrominated

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diphenyl ethers (PBDE) and other persistent brominated flame retardants (BFRs).<sup>1, 2</sup> Recent studies have reported evidence of widespread human exposure to PFRs,<sup>3-8</sup> but information on the safety or potential health risks of common PFRs following human exposure is lacking. Studies of PFR exposure sources and pathways are also limited, but contact with dust in the home and other indoor environments is thought to be important as it is with the more intensely studied BFRs.<sup>9, 10</sup> We previously reported that concentrations of two PFRs, tris(1,3,-dichloro-propyl) phosphate (TDCPP) and triphenyl phosphate (TPHP; formerly abbreviated as TPP),<sup>11</sup> measured in house dust of men participating in a reproductive health study were similar in magnitude to those found for PBDEs, and that levels of these triesters in dust were associated with significant alterations in several reproductive health markers.<sup>6</sup> TDCPP was associated with increased serum prolactin levels and decreased free androgen index and free T4 levels, while TPHP was associated with increased prolactin and decreased sperm concentration.

We also recently reported urinary concentrations of metabolites of TDCPP [bis(1,3-dichloro-2-propyl) phosphate (BDCPP)] and TPHP [diphenyl phosphate (DHP)] in a subset of these men.<sup>7</sup> Greater than 90% of the men had detectable concentrations of both BDCPP and DHP, with concentrations spanning four orders of magnitude. However, TDCPP in house dust was only moderately correlated with urinary BDCPP from the same men (Spearman  $r = 0.3$  to  $0.5$ ), and there was no correlation between TPHP and DHP (Spearman  $r < 0.1$ ). In this brief report we conducted an exploratory analysis of the association between urinary PFR metabolite concentrations and semen quality, sperm motion parameters, and reproductive and thyroid hormone levels in men. The comparison of effect estimates when using PFR biomarker versus house dust concentrations as the exposure variable may also provide indirect evidence for which exposure assessment approach may be associated with less measurement error and thus potentially considered preferred in future epidemiology studies.<sup>12</sup>

## RESULTS

Data on urinary PFR metabolite concentrations and semen quality, sperm motion, and hormone measures were available for 33 men. Median (range) age and BMI were 35 (28 to 46) years and 25.8 (20.4 to 42.4) kg/m<sup>2</sup>, respectively. In preliminary bivariate analyses, there were no associations between urinary PFR metabolite concentrations and age or BMI. SG-corrected concentrations of BDCPP and DHP were significantly greater in urine samples collected in the afternoon (BDCPP geometric mean = 384 pg/ml; DHP geometric mean = 1090 pg/ml) compared to those collected in the morning (BDCPP geometric mean = 122 pg/ml; DHP geometric mean = 265 pg/ml;  $p$ -values  $< 0.05$ ).

As shown in Figures 1 and 2, a number of moderately strong and statistically significant correlations were observed between the reproductive health measures and BDCPP (decreased sperm motility, VSL and LIN; increased TSH) or DHP (sperm concentration, motility, VSL, LIN and inhibin b; increased prolactin). In multivariable models adjusted for age, BMI, time of day, and abstinence period (for semen quality and sperm motion parameter models only), many of these relationships remained (Table 1). Negative effect estimates for semen quality parameters were consistently large in relation to both urinary

metabolites, although confidence intervals were wide. For BDCPP, an IQR increase in urinary metabolite concentration was associated with a suggestive ( $p=0.06$ ) 15% decrease in sperm motility and a significant 37% decrease in sperm morphology. The negative relationship with LIN observed in the preliminary bivariate analysis was no longer statistically significant, though negative associations with sperm morphology and VCL, and a positive association with total T3, were found in the multivariable models. For DPHP, an IQR increase in urinary metabolite concentration was associated with a 57% decrease in sperm concentration and a 20% decrease in sperm motility. The positive association with prolactin observed in the preliminary analysis was confounded by time of day of sample collection and was no longer significant in the adjusted model. As with BDCPP, a positive association between DPHP and total T3 was observed in the multivariable analysis.

Since urinary DPHP and house dust TPHP concentrations<sup>6</sup> were both negatively associated with sperm concentration but not correlated with one another, a secondary analysis was conducted where both urinary DPHP concentrations and TPHP concentrations in house dust were included in the same model with the other covariates. Both exposure variables were statistically significant in this model, where the negative association with urinary DPHP was stronger than that for dust TPHP. An IQR increase in urinary DPHP was associated with a 56% (95% CI -76%, -22%;  $p=0.007$ ) decrease in sperm concentration, whereas an IQR increase in dust TPHP was associated with a 20% (95% CI -33%, -4%;  $p=0.02$ ) decrease in sperm concentration.

## DISCUSSION

In the present exploratory study, we report a number of statistically significant or suggestive relationships between urinary biomarkers of two PFRs and markers of male reproductive health. While experimental toxicology studies of these chemicals in the peer-reviewed literature are limited, there is some laboratory evidence to support our findings. High doses of TDCPP and TPHP have been associated with histopathologic abnormalities of the male reproductive tract and reduced male fertility in rats.<sup>13, 14</sup> More recently, both PFRs were found to cause changes in hormone levels through altered steroidogenesis or estrogen metabolism in zebrafish,<sup>15-17</sup> and have been shown to alter thyroid signaling mechanisms *in vitro* and *in vivo*.<sup>17-19</sup> We also found that PFR metabolite concentrations were much greater in urine samples collected in the afternoon compared to those collected in the morning, which may provide important clues for human exposure sources, patterns and toxicokinetics. For example, since the half-life of these chemicals is on the order of several hours, it may suggest that diet may be an important exposure pathway, that activity (e.g., leading to dust aerosolization) in the home may be an important contributor for exposure beyond simply spending time in the home (e.g., during sleep), or perhaps more likely that exposures outside the home (e.g., automobile or workplace) among these men may have been greater than those experienced while inside the home. However, more detailed time-activity data than were collected in this study would be needed in order to investigate these differences further.

Overall, the number, magnitude and strength of statistically significant or suggestive associations between urinary PFR metabolites and measures of male reproductive health were greater than our previous report assessing house dust PFR concentrations in relation to

these outcomes in the same cohort.<sup>6</sup> Despite the lack of correlation ( $r < 0.1$ ) we recently reported between TPHP concentrations in house dust and DPHP concentrations in urine,<sup>7</sup> in the present study we found that urinary DPHP was associated with decreased sperm concentration and increased serum prolactin (though the association with prolactin was confounded by time of day of sample collection since prolactin levels are also higher in samples collected in the afternoon<sup>20</sup>) in patterns consistent with our previous report on house dust concentrations of TPHP.<sup>6</sup> Although peculiar, there may be several possible explanations for consistent relationships between these endpoints and two exposure assessment approaches that were not correlated with one another: 1) the significant results for either house dust or urinary biomarker concentrations (or both) are due to chance findings; 2) Both TPHP and DPHP are bioactive, and there may be other uses (e.g., as a plasticizer) and sources of DPHP besides TPHP in house dust,<sup>21</sup> thus both measures may be associated with the same outcome measure but not with each other; 3) DPHP may be a metabolite of other chemicals in addition to TPHP (e.g., certain alkylated triphenyl phosphate isomers used as flame retardants, among others<sup>22</sup>) that may be associated with these endpoints; 4) Since TPHP exposure may come from sources or locations outside the home (e.g., offices, automobiles), perhaps the two measures reflect differing aspects of exposure and both were associated with substantial but differing error in relation to true exposure. The latter possible explanations may be supported by the observation that both house dust TPHP and urinary DPHP were significantly associated with sperm concentration when included in the same multivariable model. If possibility #4 were to in fact explain our observations, the true association between TPHP exposure and sperm concentration could be quite strong.

Likewise, for TDCPP/BDCPP, the significant associations we previously reported between house dust concentrations and free T4, FAI and prolactin, but lack of relationship between these measures and urinary metabolite concentrations reported here, may signify either chance associations with house dust or that house dust may be associated with less exposure measurement error when considering the exposure window of importance for these hormones (i.e., dust may represent a more stable, long-term measure of exposure if house dust is a primary source of exposure and dust may not be as susceptible to confounding by time of day of sample collection compared to urinary PFR metabolites). On the other hand, in the present study we report associations between urinary BDCPP and semen quality parameters, sperm motion parameters, total T3, and TSH that were not evident in our previous analysis of house dust. This too may reflect either chance findings, evidence that urinary biomarkers of PFRs may be associated with less error compared to dust when studying these outcome variables, or some combination of these and other possible explanations. As suggested above, it may also be possible that the two exposure measures capture different aspects of exposure and measurement error in consideration of overall health effects associated with true TDCPP/BDCPP exposure or biologically effective dose.

In conclusion, this brief report describes, as far as we are aware, the first human study of relationships between biomarkers of exposure to PFRs and male reproductive health. While the study was limited by a small sample size, our compelling results warrant further investigation in a larger study population. Additional studies on sources, pathways, and

routes of PFR exposure, along with research on toxicokinetics and exposure measure utility, are also needed.

## MATERIALS & METHODS

The present analysis was conducted among a subset of men participating in an ongoing study of environmental factors in reproductive health. Subject recruitment and participation has been described previously.<sup>4, 23</sup> Briefly, men between 18 and 54 years of age were recruited from the Andrology Laboratory of Massachusetts General Hospital. Men included in this sub-study were enrolled in years 2003 and 2004. Exclusion criteria included prior vasectomy or current use of exogenous hormones. All men participating in the study provided written informed consent, and institutional review board approval was obtained from each participating institution.

A urine, serum and semen sample was collected from each participant at their enrollment clinic visit. Following the clinic visit, the men were given detailed instructions to send the collection bag from their home vacuum cleaner to study team in a pre-paid mailer. In the laboratory, after dust was obtained from the vacuum bags and passed through a 150  $\mu\text{m}$  screen sieve, TDCPP and TPHP concentrations were measured using gas chromatography-mass spectrometry (GC/MS).<sup>4, 6</sup> DBCPP and DPHP, metabolites of TDCPP and TPHP, respectively, were measured in the urine samples via atmospheric pressure chemical ionization liquid chromatography-tandem mass spectrometry (ACPI-LC/MS/MS).<sup>7, 24</sup> Specific gravity (SG) was also measured in each urine sample using a digital handheld refractometer.

Semen samples were analyzed for sperm concentration and motion parameters [sperm motility, straight line velocity (VSL), curvilinear velocity (VCL), and linearity (LIN)] by a computer-aided semen analyzer as described previously (Meeker and Stapleton 2010; Meeker et al. 2011).<sup>6, 25</sup> Sperm morphology was assessed using on two slides per specimen via microscope using an oil-immersion 100x objective.<sup>6, 25</sup> Serum samples were analyzed for follicle-stimulating hormone (FSH), luteinizing hormone (LH), inhibin b, testosterone, estradiol, sex hormone binding globulin (SHBG), prolactin, free T4, total T3, and thyrotropin (TSH) using commercially available immunoassays, also described previously.<sup>6, 23</sup> Finally, the free androgen index (FAI) was calculated as the molar ratio of total testosterone to SHBG.

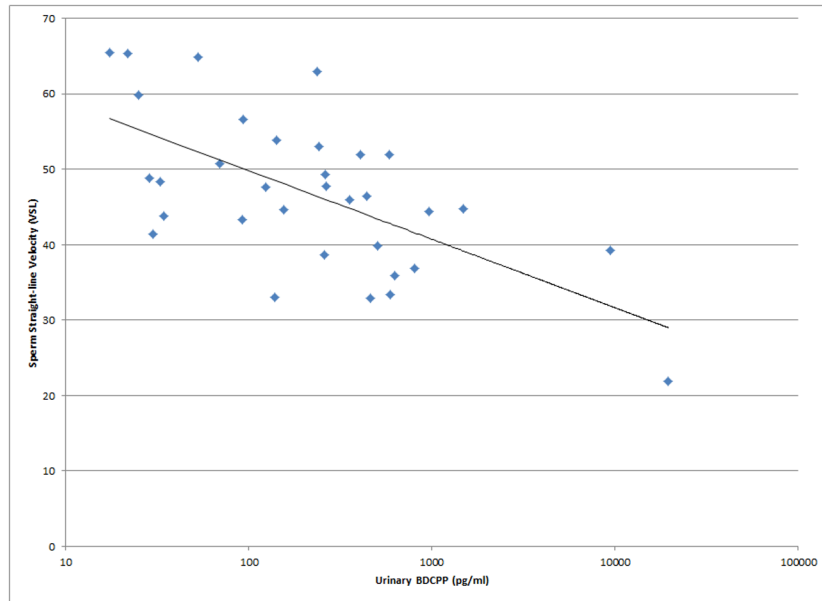
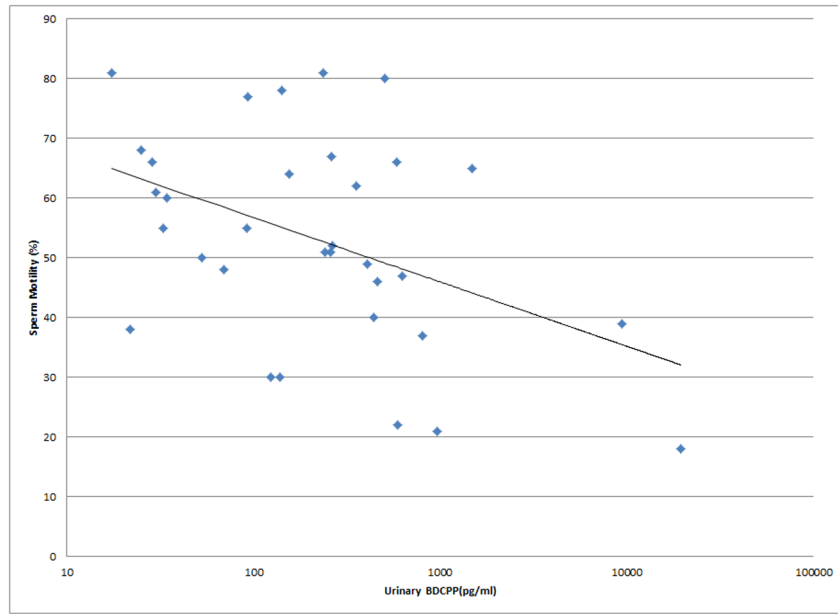
Statistical analysis was conducted using SAS version 9.2 (SAS Institute, Inc., Cary, NC) in a manner consistent with our previous report on relationships between PFRs in dust and male reproductive health measures.<sup>6</sup> Briefly, continuous variables were assessed for normality and proper transformations [e.g., natural log (ln)] were carried out prior to further statistical analysis. Preliminary bivariate relationships between variables were assessed by constructing scatter plots and calculating Spearman rank correlation coefficients ( $r_s$ ). We then utilized multivariable linear regression to assess relationships between urinary PFR metabolite concentrations and semen quality/sperm motion parameters and serum levels of reproductive/thyroid hormones while accounting for potential confounding variables (age, BMI, time of day of sample collection). To improve interpretability and to allow for

comparisons with our earlier dust analysis, regression coefficients were expressed as a percent change in semen or hormone measure (relative to the population median) associated with an interquartile range (IQR) increase in urine PFR metabolite concentration.

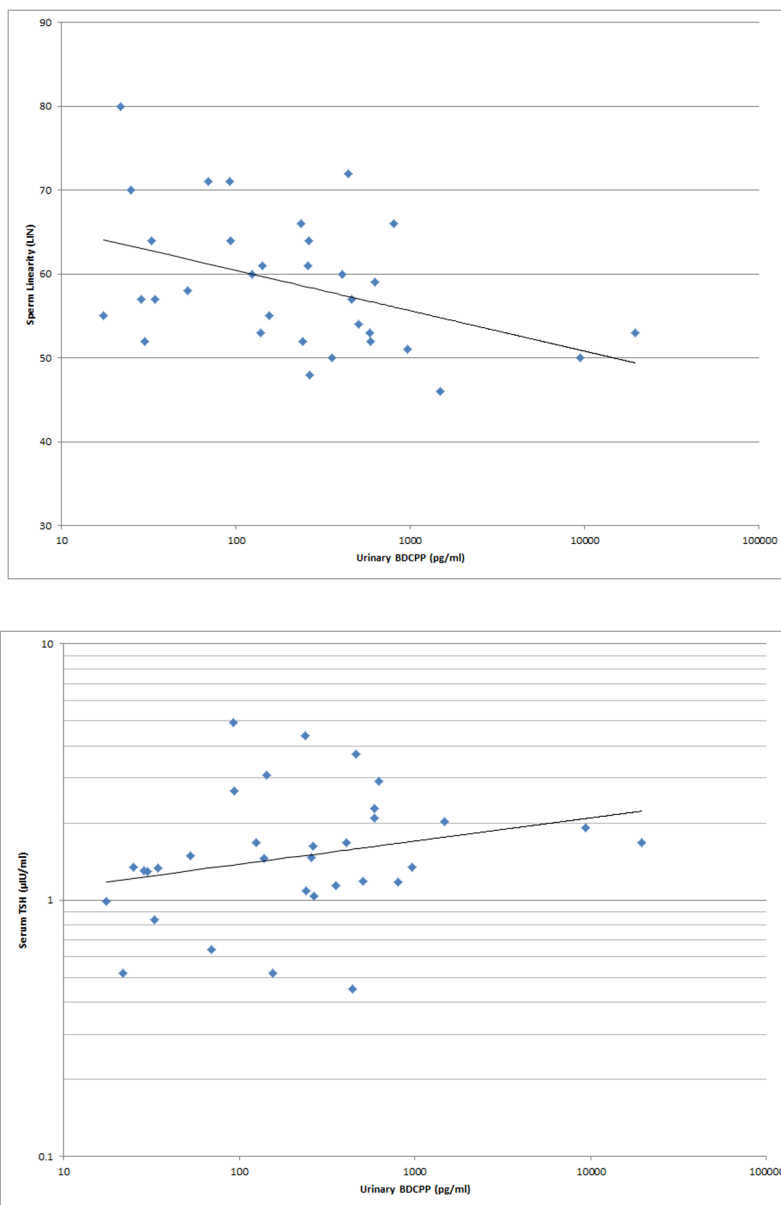
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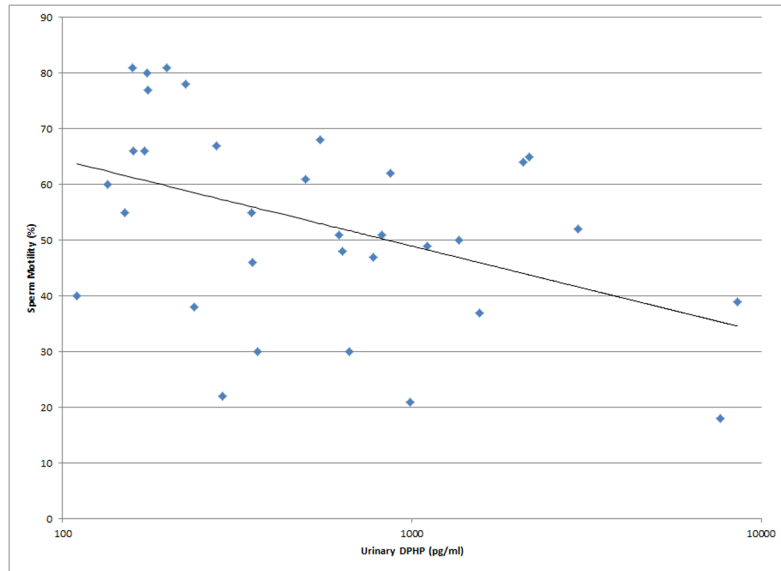
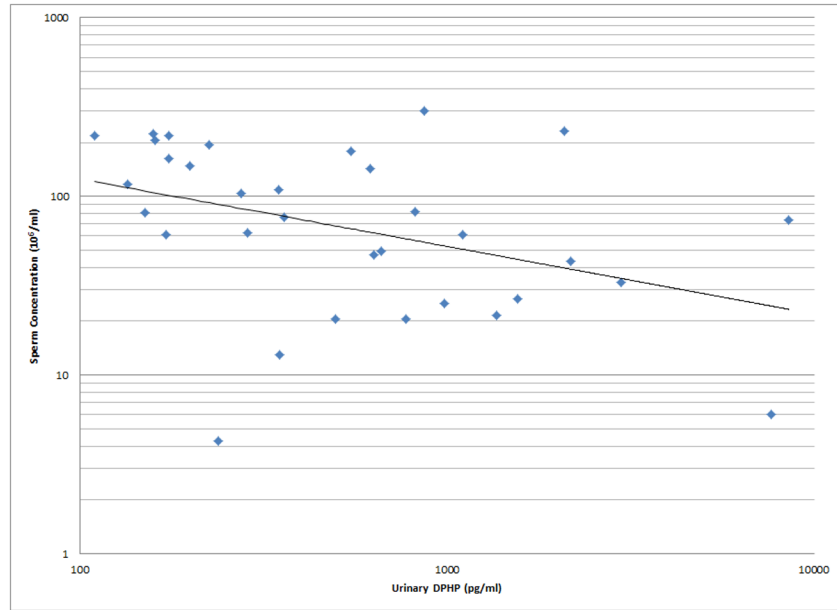


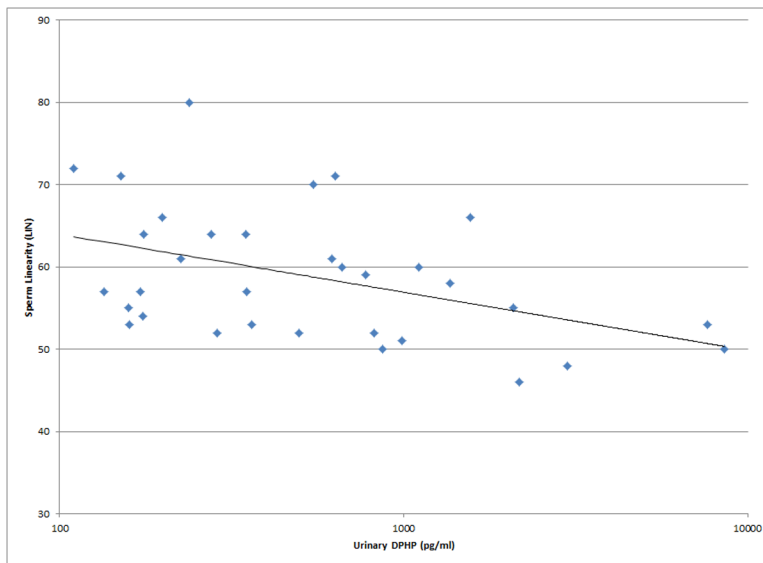
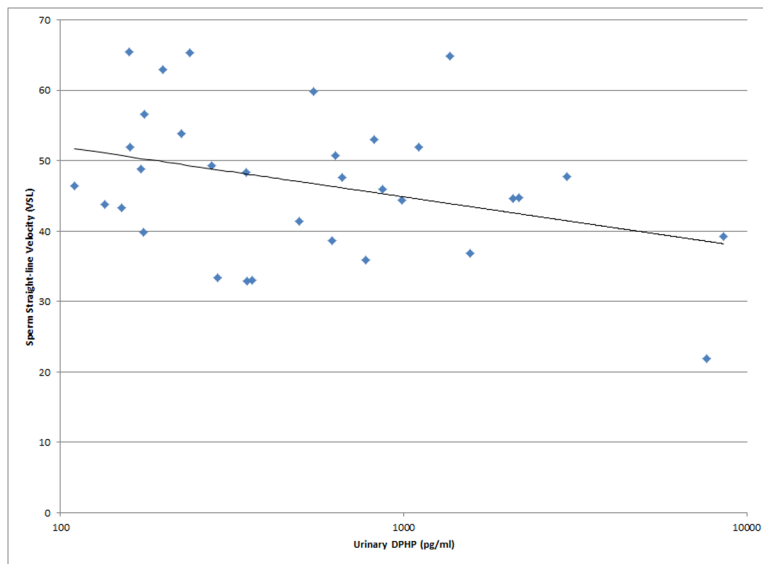


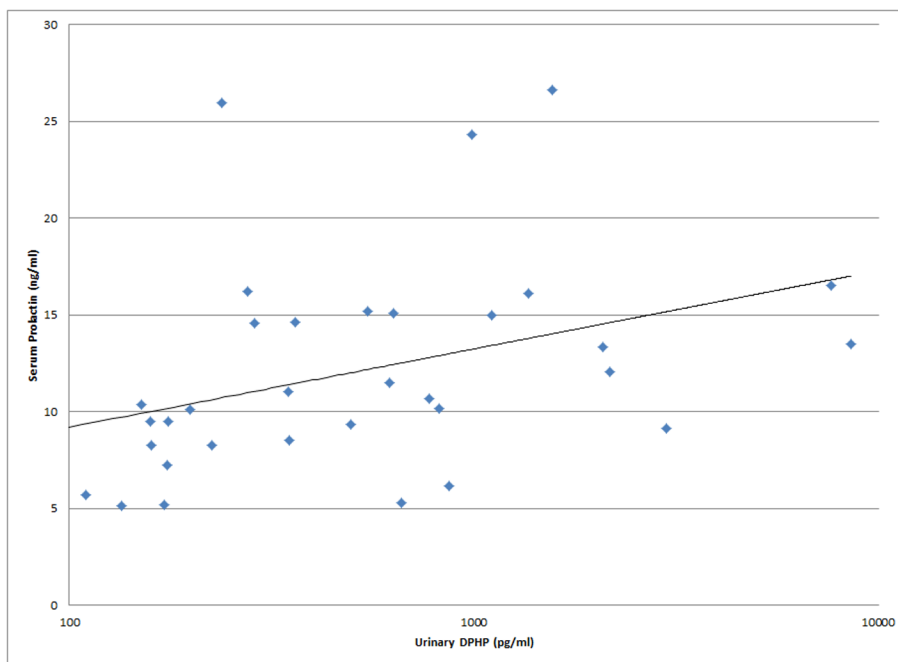
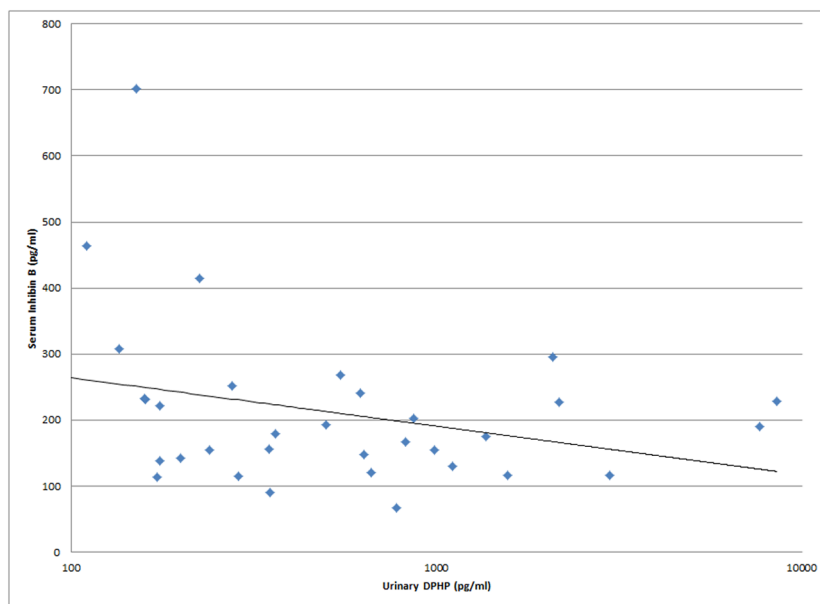


**Figure 1.**

Selected scatterplots of SG-corrected urinary BDCPP concentration in relation to a) sperm motility ( $r_s = -0.38$ ,  $p < 0.05$ ); b) sperm straight line velocity ( $r_s = -0.57$ ,  $p < 0.01$ ); c) sperm linearity ( $r_s = -0.43$ ,  $p < 0.05$ ); d) serum TSH ( $r_s = 0.33$ ,  $p < 0.1$ ).







**Figure 2.**

Selected scatterplots of SG-corrected urinary DPHP concentration in relation to a) sperm concentration ( $r_s = -0.44$ ,  $p < 0.1$ ); b) sperm motility ( $r_s = -0.43$ ,  $p < 0.05$ ); c) sperm straight line velocity ( $r_s = -0.57$ ,  $p < 0.01$ ); d) sperm linearity ( $r_s = -0.44$ ,  $p < 0.05$ ); e) serum inhibin b ( $r_s = -0.28$ ,  $p = 0.2$ ); f) serum prolactin ( $r_s = 0.50$ ,  $p < 0.01$ ).

**Table 1**

Adjusted<sup>a</sup> regression coefficients (95% confidence intervals) for percent change (relative to population median) in semen quality parameter, sperm motion parameter, reproductive hormone level, or thyroid hormone level associated with an interquartile range (IQR) increase in specific gravity corrected BDCPP or DPHP concentration in urine (N=33).

	BDCPP <sup>b</sup>		DPHP <sup>b</sup>	
	% Change (95% CI)	p-value	% Change (95% CI)	p-value
Sperm concentration <sup>b</sup>	-27.3 (-57.5, 22.0)	0.22	-57.2 (-77.8, -18.8)	0.01
Sperm motility	-14.9 (-30.5, 0.8)	0.06	-20.3 (-41.1, 0.5)	0.055
Sperm morphology	-36.7 (-70.6, -3.1)	0.03	-28.1 (-76.1, 19.9)	0.24
Straight-line velocity (VSL)	-18.7 (-27.8, -9.7)	0.0002	-19.0 (-32.7, -5.3)	0.009
Curvilinear velocity (VCL)	-14.1 (-24.1, -4.2)	0.007	-7.2 (-22.2, -7.8)	0.33
Linearity (LIN)	-5.1 (-11.5, 1.3)	0.11	-12.6 (-20.1, -5.2)	0.002
FSH <sup>b</sup>	7.2 (-15.8, 36.7)	0.55	15.5 (-16.1, 59.0)	0.35
LH <sup>b</sup>	-2.4 (-20.0, 19.4)	0.81	21.1 (-6.2, 56.5)	0.14
Inhibin B	-5.4 (-40.2, 29.5)	0.76	-37.6 (-82.2, 7.0)	0.095
Testosterone <sup>c</sup>	-2.2 (-13.2, 8.8)	0.68	9.8 (-4.6, 24.3)	0.18
Estradiol	5.0 (-18.2, 28.3)	0.66	5.4 (-25.9, 36.6)	0.73
SHBG <sup>b</sup>	-9.5 (-25.8, 10.5)	0.34	-16.1 (-36.1, 10.1)	0.20
FAI <sup>b</sup>	4.1 (-14.7, 27.0)	0.68	27.1 (-0.6, 61.6)	0.06
Prolactin <sup>b</sup>	6.2 (-13.0, 29.6)	0.53	19.2 (-7.7, 56.5)	0.18
Free T4	0.7 (-3.2, 4.7)	0.70	3.9 (-1.0, 8.9)	0.12
Total T3	6.6 (1.6, 12.8)	0.02	7.8 (-0.2, 15.8)	0.054
TSH <sup>b</sup>	40.3 (11.4, 77.1)	0.006	25.1 (-11.2, 77.6)	0.19

<sup>a</sup> Adjusted for age, BMI, and time of sample collection. Models for semen quality and sperm motion parameters additionally adjusted for abstinence period.

<sup>b</sup> Variable ln-transformed in statistical analysis

<sup>c</sup> Models for testosterone also adjusted for ln-transformed SHBG