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Pesticide interactions and risks of sperm chromosomal abnormalities

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Abstract

Disentangling the separate and synergistic effects of chemicals poses methodological challenges for accurate exposure assessment and for investigating epidemiologically how chemicals affect reproduction. We investigated combined exposures to ubiquitous contemporary use pesticides, specifically organophosphates (OP) and pyrethroids (PYR), and their association with germ cell abnormalities among adult men. Fluorescence *in situ* hybridization was used to determine disomy in sperm nuclei and urine was analyzed for concentrations of PYR metabolites (3-phenoxybenzoic acid; 3PBA) and OP dialkyl phosphate (DAP) metabolites. Incidence rate ratios using Poisson models were estimated for each disomy type by exposure quartile of DAP metabolites and 3PBA, controlling for confounders. The shape of the associations between PYRs, OPs and disomy were frequently nonmonotonic. There were consistent interactions between OP and PYR metabolite concentrations and the risk for sperm abnormalities. Taking both chemicals into account simultaneously resulted in quantitatively different associations than what was reported previously for OPs and PYRs separately, demonstrating the importance of modeling multiple concentrations simultaneously. Methods investigating interactions using Poisson models are needed to better quantify chemical interactions and their effects on count-based health outcomes, the importance of which was shown here for germ cell abnormalities.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2019.07.001.

Conflicts of interest

There is no conflict of interest.

Keywords

Aneuploidy; Endocrine disruptors; *In situ* hybridization; Pesticide; Reproduction; Interactions

1. Introduction

Investigating interactions between environmental chemicals and their effects on human reproduction pose methodological challenges for environmental health and reproductive biology (Woodruff et al., 2008; Taylor et al., 2016) and considerable environmental epidemiology, toxicology, and exposure assessment expertise is being invested in developing statistical approaches to disentangle the human health effects of combined exposure to chemicals (Carlin et al., 2013; Grandjean and Landrigan, 2014; Claus Henn et al., 2014; Goodson et al., 2015; Braun et al., 2016). Real-world exposures to a specific environmental contaminant do not occur as single discrete events, but rather in combination to several toxicants at once (US EPA 2001; WHO, 2009; Zeliger, 2011). Chemical compounds interact with each other and with biological systems and they can alter the toxicity of individual compounds even in low doses (Zeliger, 2011; Tsatsakis et al., 2009; Hernández et al., 2013). Observable adverse effect levels may be different for chemical mixtures, as different toxicants may affect the body at different points along the biological pathway (Ray and Forshaw, 2000). Environmental toxicants with endocrine disrupting properties can affect spermatogenesis (Schiffer et al., 2014) and congenital abnormalities and nonviable pregnancies are related to problems during spermatogenesis, including poor sperm DNA integrity and increased human sperm aneuploidy (Jacobs, 1992; Hassold and Hunt, 2001; Cheng et al., 2011; Nagaoka et al., 2012). Because sex chromosomes (X and Y) are particularly susceptible to aneuploidy, researchers have attempted to understand the paternal role in sex chromosome disomy, the most common type of aneuploidy (Hassold and Hunt, 2001; Martin et al., 1991).

Despite advances in studying germ cell abnormalities, the exact causes of aneuploidy and/or the specific critical windows of chemical susceptibility associated with aneuploidy risk remain unknown (Herrera et al., 2008; Axelsson et al., 2010; Ashton Acton, 2013).

Regulatory assessments do not consistently identify the interactive effects that can occur between chemicals (Teuschler et al., 2004; Hernández et al., 2013; US EPA 2013; NAS, 2014). Organophosphate (OP) and pyrethroid (PYR) insecticides account for a large share of all US insecticide use. OPs are most frequently used in agriculture, recreational and commercial areas, while PYRs are regularly used in homes and gardens. Approximately 82 million US households use insecticides (US EPA, 2017).

Urinary concentrations of OP (such as dialkyl phosphates or DAPs) and PYR (3-phenoxybenzoic acid or 3PBA) metabolites have been measured in the general population (CDC, 2019). Adverse effects related to OP and PYR metabolite concentrations have been demonstrated for hormone functions (Meeker et al. 2006, 2009; Lacasaña et al., 2010), semen parameters and sperm DNA damage/fragmentation (Meeker et al. 2004b, 2008; Lifeng et al., 2006; Perry et al., 2007b; Recio-Vega et al., 2008; Yucra et al., 2008; Xia et al., 2008; Hossain et al., 2010; Ji et al., 2011; Toshima et al., 2012), sperm chromatin structure

alteration (Sanchez-Pena et al., 2004) and sex chromosome disomy in human sperm (Padungtod et al., 1999; Recio et al., 2001; Young et al., 2013; Radwan et al., 2015).

To our knowledge, very limited information exists about the reproductive health effects of combined environmental exposures to pesticides and their association with sperm chromosomal abnormalities. In this study we examined interactions between OP and PYR metabolite concentrations and their association with the frequency of sperm sex chromosome disomy, using samples from adult men. We evaluated the hypothesis that OP and PYR interactions alter associations with sperm chromosomal abnormalities.

2. Methods

2.1. Study subjects

Participants (n = 159) were selected from a previous study of couples seeking infertility evaluation at Massachusetts General Hospital (MGH) Fertility Center. The parent study (n = 341) was conducted between January 2000–May 2003 and assessed the impact of environmental exposures on semen quality. A detailed description of the parent study has been provided elsewhere (Hauser et al., 2003). Approximately 65% of eligible subjects aged 20–54 agreed to participate in the parent study. Lack of participation was due to lack of time during their clinic visit. Men receiving treatment for infertility and/or scheduled for post-vasectomy semen analysis were excluded from the parent study. Information on demographics, medical and fertility history, and lifestyle factors were obtained by a self-administered questionnaire, and urine and semen samples were collected on the same day. Occupational exposure to pesticides or other agents was not reported (i.e., study population was selected without attention to specific occupational exposure). A prior retrospective review of anonymized non-participants' clinical records, who met the same eligibility found no differences between participants and non-participants in regards to age or semen parameters (Duty et al., 2005). Because urine and semen from the parent study (n = 341) had been used for other analyses, inclusion in this study was based on sample availability in the biorepository (n = 159 or 47%). Signed informed consent was obtained from each participant. The parent study was approved by the Massachusetts General Hospital Human Subjects Committees and the Harvard School of Public Health. This study was approved by the Office of Human Research of the George Washington University.

2.2. Semen analysis

Semen collection and analysis have been previously described (Hauser et al., 2003). Semen samples were collected at the clinic via masturbation. Participants were asked to abstain from ejaculation for at least 48 h prior to sample collection. Semen samples were liquefied at 37 °C for 20 min before analysis. Andrologists from the MGH Andrology Laboratory analyzed the samples; they were blinded to exposure status. Volume, pH, color, and viscosity properties were determined for each semen sample. A computer-aided sperm analysis (CASA) using the Hamilton-Thorn Motility Analyzer (10HTM-IVO) was used to determine sperm count and percent motility. Two slides per sample were prepared for a morphological assessment. An oil immersion microscope lens with a 100x objective was used for this analysis (Nikon Company, Tokyo, Japan). Sperm were scored normal or abnormal using the

Tygerberg Strict Criteria for morphology (< 4% normal morphology) described by Kruger et al., 1988.

2.3. Disomy analysis

In germ cells, failure of sex chromosomes (X or Y) to separate properly during meiosis results in extra or missing chromosomes, known as aneuploidy. Sex chromosome disomy, or an extra X or Y chromosome, is the most frequent form of aneuploidy observed in human sperm and was the primary outcome of interest in this study. Semen samples were stored in −80 °C without cryoprotectant until FISH analysis was performed. Disomy detection procedures are published elsewhere (McAuliffe et al., 2012). Laboratory technicians were blinded to exposure status. Fluorescence *in situ* hybridization (FISH) analysis was performed for chromosomes X, Y and 18 (autosomal control) to determine the presence of disomic sperm or XX18, YY18, XY18 and total sex chromosome disomy in sperm nuclei. For each FISH slide, a sequence of non-overlapping field images was taken. A fluorescence microscope was utilized to take images subsequently scored for size and shape using custom MATLAB (Mathworks Inc., Natick, MA) software. The software was designed to utilize scoring algorithms based on criteria for size and shape as previously described (Baumgartner et al., 1999). Details of the sperm FISH control procedures and validation of the semi-automated scoring method have been reported (Perry et al. 2007a, 2011).

2.4. Urinary OP and PYR measurements

Exposure to OP pesticides was estimated using six DAP urinary metabolites [dimethylphosphate (DMP); dimethylthiophosphate (DMTP); dimethyldithiophosphate (DMDTP); diethylphosphate (DEP); diethylthiophosphate (DETP); and diethyldithiophosphate (DEDTP)] according to methods described previously (Prapamontol et al., 2014). Briefly, urinary samples were analyzed using gas chromatography coupled with mass spectrometry with isotopic dilution quantification. Transformed metabolite concentrations (i.e., each DAP metabolite divided by its molecular weight) were summed and multiplied by 1,000 to obtain a total DAPs (ΣDAP) concentration in units of nmol/mL (Figueroa et al., 2015). Urinary 3PBA metabolite was used to estimate human exposure to PYR pesticides. Urine samples were analyzed using a small modification of the method described by Baker et al. (2004). The samples were spiked with an isotopically labeled analogue to enable isotope dilution quantification. The target analyte was isolated using solid phase extraction. The extract was concentrated prior to analysis by high performance liquid chromatography-tandem mass spectrometry using an Agilent 6460 triple quadruple mass spectrometer (Santa Clara, CA) with Jetstream electrospray ionization. Quality control and blank samples were analyzed jointly with unknown samples to ensure method stability and robustness. The limit of detection (LOD) for each metabolite was 0.6 ng/mL (DMP), 0.2 ng/mL (DMTP), 0.2 ng/mL (DEP), and 0.1 ng/mL (DETP, DMDTP, DEDTP, 3PBA).

Specific gravity and creatinine concentrations were measured in urine samples using a handheld refractometer (National Instrument Company, Inc., Baltimore, MD, USA) and kinetic colorimetric assay technology with a Hitachi 911 automated chemistry analyzer (Roche Diagnostics, Indianapolis, IN, USA), respectively.

2.5. Statistical analysis

Descriptive statistics were generated for demographic and semen parameters. Semen parameters were dichotomized using the World Health Organization reference values for sperm concentration (< 15 million sperm/mL) and motility (< 32% motile sperm), and the Tygerberg Strict Criteria for morphology (< 4% normal morphology) (Kruger et al., 1988; WHO, 2010). For metabolite values below the LOD, an imputed value equal to one-half the LOD was used (Helsel, 2005). Descriptive statistics for pesticide metabolite concentrations (ng/mL) in urine were summarized. Creatinine and specific gravity adjusted summaries as well as volume-based (unadjusted) urinary values were calculated and compared. The urinary concentration results were similar when using both creatinine and specific gravity adjusted methods. Specific gravity was used in the analysis as an independent variable and crude metabolite concentrations were used in the adjusted models. Urinary metabolite concentrations were not normally distributed and were log transformed prior to examining Pearson correlations to explore associations between individual urinary metabolites.

Poisson regression (SAS GENMOD procedure) was used to model the association between each specific DAP and 3PBA volume-based urinary metabolite concentrations and the disomy measure (i.e., total sex chromosome disomy) due to the large number of sperm being scored and the relatively low frequency of disomy. The number of sperm scored and the number of disomic nuclei were summed separately for each subject; the individual subject was treated as the unit of analysis. The natural logarithm of the number of sperm counted was used as the offset variable to standardize across subjects.

Models were fitted using a disomy measure as the outcome variable (i.e., as a count of disomic cells of total sex chromosome disomy) and the metabolites of interest as the independent variables. Variables considered to have biological plausibility based on prior studies and found to be associated with aneuploidy and/or pesticide exposure were included in the adjusted models. Age, body-mass index (BMI), motility, morphology, log of sperm concentration and specific gravity were included as continuous covariates, along with categoricals smoking and race. Because sperm concentration is generally non-normal and positively skewed (Berman et al., 1996), sperm concentration was log transformed. Incidence rate ratios (IRRs) and 95% confidence intervals (95% CI) were calculated for each model (i.e., for total sex chromosome disomy by quartiles of DAP metabolite (DMP, DMTP, DMDTP, DEP, DETP) and 3PBA quartiles), and the exposure variable was included as an ordinal variable to test for trend. IRRs and 95% CIs were also calculated for total disomy by tertiles of ∑DAP by 3PBA tertiles. Statistical significance was set at p-value < 0.05.

Interactions between each DAP metabolite and 3PBA in association with each disomy outcome were examined. If an interaction was significant, the model IRRs for a specific DAP metabolite were calculated within each quartile of 3PBA. Linear tests for trend for most DAP metabolites and ∑DAP were also performed by 3PBA strata to further investigate the interaction. The interaction term was removed from the model if non-significant. Statistical analysis was performed using SAS version 9.3 (SAS Institute Inc., Cary, NC).

3. Results

Table 1 shows the demographic and semen parameters of the study subjects (n = 159). The men had an average age of 35 years and a mean BMI of 28 kg/m^2. The majority of the men were white (86%) and non-Hispanic (94%). Most men (74%) had never smoked and 7% were current smokers. Of the 159 men, 10% (n = 16) had sperm concentrations < 15 million/mL, 21% (n = 33) had < 32% motile sperm, and 18% (n = 28) had < 4% normally shaped sperm (Table 1). A median of 6,848 sperm nuclei were scored per subject (Table 2). The observed median percentages of XX18, YY18, XY18, and total sex chromosome disomy were 0.4%, 0.4%, 1.1%, and 1.9%, respectively. Table 3 summarizes the unadjusted urinary OP and PYR metabolite concentrations as well as the specific gravity and creatinine adjusted concentrations. The percent of samples above the LOD ranged from 57 to 89% for most metabolites. All DAP metabolites (r = 0.13–0.60) were weakly to moderately positive correlated. 3PBA was weakly correlated to DAP metabolites (r = −0.009–0.08) (Table 4). There were moderate positive correlations between Total DAPs and individual DAP metabolites (r = 0.20–0.87) and a weak correlation with 3PBA (r = 0.04).

In most cases, increased disomy rates were observed only in Q1 and/or Q2 and declined in Q3 and Q4 (Table 5). DMDTP and DMTP showed U-shaped association patterns whereas the patterns for DETP showed reverse U-shapes across the quartiles of 3PBA. The highest significant associations for total disomy were observed between the third exposure quartile of DETP and second 3PBA exposure quartile for an IRR = 2.31 (95% CI: 2.02, 2.64). Statistically significant inverse associations were also observed for total disomy by concentrations of DMP and 3PBA. Increase in disomy rates occurred mainly between the second and third exposure quartiles and without substantial additional increases or decreases between the third and fourth exposure quartile, demonstrating nonmonotonic dose-response curves. Statistically significant interactions were observed for total disomy between all DAP metabolites and 3PBA in the adjusted models. The significance of all the interaction terms persisted when variables were modeled continuously (data not shown).

To further investigate interactions between OP and PYR metabolites, graphs of the adjusted IRRs and 95% confidence intervals were examined for total sex chromosome disomy by quartiles of DAP metabolite (DMP, DMTP, DMDTP, DEP, DETP) and 3PBA quartiles. Dose-response relationships appeared nonmonotonic across most quartiles of 3PBA with increasing individual DAP exposure. Both U-shaped and inverted-U shaped relationships were observed across 3PBA quartiles with increasing DAP metabolites. Increased risks were consistently detected for total disomy in the second DMDTP exposure quartile across all 3PBA quartiles; adjusted IRRs ranged from 1.36 (95%CI: 1.11, 1.67) to 1.96 (95%CI: 1.59, 2.40) (Fig. 1). Fig. 2 shows adjusted IRRs and 95% confidence intervals for total disomy by tertiles of ∑DAP by 3PBA tertiles. Adjusted IRRs showed mainly null associations; small or no visible changes were observed in the first and third tertiles of 3PBA with increasing ∑DAPs exposure.

4. Discussion

Our results showed clear evidence of interactions, resulting in complex nonmonotonic relationships changing direction within the range of exposure categories. The highest disomy rates were seen either 1) at the intermediate exposure concentration (i.e., quartiles 2 or 3) with null or no significant association observed at low and high exposures (inverse U-shaped relationship); or 2) with the highest rates observed at low and high exposures (U-shaped relationship).

Increased risk associations higher than the rates previously reported for each individual chemical class (see Supplemental Tables 1–2), were observed when assessing total disomy and OP/PYR interactions. Strong and significant interactions were observed for total disomy across all OP metabolites and PYR concentrations. The risk estimates previously reported showed inverse associations between all disomy types and 3PBA without an interaction term for OP concentrations (see Supplemental Table 1). The quantification of interactions between simultaneous pesticide exposures shown here demonstrate that main exposure effects are different from results that take interactions into account. Previously reported results showed that DAP concentrations were associated with increased disomy rates without an interaction term (see Supplemental Table 2; Figueroa et al., 2015). Notably, nonmonotonic dose-response relationships were observed between the outcomes and exposure categories for all main effects.

Adjusted IRRs for total sex chromosome disomy by ∑DAPs and 3PBA exposures showed mainly null associations when compared to the reference group. These results confirmed our previous observation that aggregating all six DAP metabolites into a composite variable of ∑DAPs conceals their separate and distinct associations with sperm disomy (see Supplemental Table 2). Similar relationships were observed for other disomy types (XX18, YY18 and XY18) when modeled by concentrations of DAP metabolites at various 3PBA concentrations. These findings suggest that modeling metabolites separately are likely to produce distinct and potentially more informative findings than using an aggregate measure (Figueroa et al., 2015).

Unadjusted 95th percentile DAP urinary concentrations were slightly higher (0.39–38.71 ng/mL) in this study when compared to the unadjusted 95th percentile DAP urinary concentrations of men surveyed in the United States (US) general population for 2007–2008 (< LOD-36.10 ng/mL) (CDC Fourth National Report on Human Exposure to Environmental Chemicals, Updated Tables 2019). The unadjusted 95th percentile 3PBA urinary concentrations were lower in this study (2.27 ng/mL) than the concentrations reported for 2009–2010 (6.50 ng/mL).

Several human studies have reported individual exposures of OP or PYR pesticides associated with decreased sperm volume and decreased sperm count, higher abnormal morphology and decreased sperm motility, sperm damage, sperm chromatin alteration, increased luteinizing hormone and decreased testosterone, and sperm aneuploidy (Padungtod et al., 1998, 1999; Recio et al., 2001; Tan et al., 2002; Kamijima et al., 2004; Xia et al., 2004; Sanchez-Pena et al., 2004; Meeker et al. 2004a, 2008; Lifeng et al., 2006; Yucra et al.,

2008; Recio-Vega et al., 2008; Hossain et al., 2010; Ji et al., 2011; Young et al., 2013; Radwan et al., 2015). Less is known about the combined effects of OP/PYR pesticides and their association with human sperm parameters. Perry et al. conducted a pilot biomonitoring study to examine the relationship between environmental OP/PYR exposures and sperm concentration among Chinese men living in rural areas (Perry et al., 2007b). Results showed a high prevalence of exposure to OP/PYR pesticides and suggested that the higher exposure group had lower sperm concentration.

These findings are consistent with many other studies showing the potential low dose effects of endocrine disrupting chemicals (EDCs) affecting hormone expression and affecting the risk for adult disease (Vandenberg et al., 2012). Our findings showed complex nonmonotonic dynamics between OP/PYR exposures and disomy. Even though the effects of low doses cannot be predicted by the effects observed at high doses and sometimes low environmental exposures cannot be identified (Vandenberg et al., 2012), this study was able to detect significant associations between OP/PYR pesticides and disomy. It remains unclear how combined OP/PYR exposures could be protective for sex chromosome disomy when specific DAP metabolites are modeled within differing quartiles of 3PBA. Our results suggest that pesticide toxicity estimates may underestimate the risks associated with reproductive outcomes because they do not account for the possibility of co-occurring synergistic chemical interactions.

Acute OP toxicity involves the inhibition of the enzyme acetylcholinesterase (AChE) leading to neurotoxicity in the central and peripheral nervous system, while PYRs act via the activation and inactivation of voltage-gated sodium channels (VGSCs), causing neuronal excitability (Mandhane and Chopde, 1997; Tyler et al., 2000; Nasuti et al., 2003; US EPA, 1999; 2009; Barr et al., 2010). Because the use of PYRs in the US, particularly in residential settings, has increased dramatically over the past decade, it is important to consider their effects when combined with other pesticides that have higher mammalian toxicity such as the OPs (Williams et al., 2008; US EPA 2009; 2013; Horton et al., 2011). Exposure to CYP450-activated OP insecticides or their toxic metabolites (such as chlorpyrifos oxon) can enhance PYR toxicity by inhibiting carboxylesterases, enzymes used by the body to detoxify PYRs (Ray and Forshaw, 2000). With metabolic detoxification inhibited and sometimes irreversible even at low concentrations, PYR toxicity potency pesticides in the presence of OPs can increase due to greater tissue concentration and lower excretion rate (Wielgomas and Krechniak, 2007). Both classes of insecticides are often used simultaneously in the US and are formulated in combined products on the global market, posing the risk of unintended synergistic or potentiation effects (He et al., 2002).

Because chemical mixtures are multidimensional, there is not a standard methodology to investigate the effects of real-world exposures to multiple compounds with different modes of action (Teuschler et al., 2004, 2007; Perobelli et al., 2010; Woodruff, 2011). This highlights the difficulties with treating combined environmental exposures observed in epidemiological studies as single entities (EEA-JRC, 2013). In general, environmental exposures are highly correlated, introducing methodological issues. Several methodological advances have been made to account for issues such as collinearity, high dimensionality, and synergistic, potentiation or inhibitory effects. Advanced methods have been designed to

identify subsets of mixtures, assess mixtures that are also affected by a limit of detection, accommodate joint analysis of high-dimensional biomarker data, and to model interactions (Charles et al., 2002; Moser et al., 2005, 2006; Gennings et al., 2010; Herring, 2010; Yeatts et al., 2010; Zhang et al., 2012; VanderWeele and Tchetgen, 2014). Some of these methods are relatively new and have mainly used linear and logistic regression. Methods for treating interactions are needed in non-logistic models, such as Poisson regression which is best suited to the nature of the disomy rate data examined here.

This analysis was limited by the number of men available in each quartile when stratifying by exposure category. Commonly used trend tests available to evaluate monotonic dose-response relationships present a disadvantage when evaluating nonmonotonic responses. Those tests may not fully describe the nonmonotonicity patterns often observed for EDCs. In addition, multiple statistical tests may increase the probability of finding a statistically significant association and the role of chance in these significant findings cannot be completely ruled out.

DAPs and 3PBA are non-specific urinary metabolites of OPs and PYRs insecticides. These metabolites do not retain the structure from which they were derived, and cannot be attributed to a specific original parent compound (Bravo et al., 2004). Although the use of biological measurements has been criticized due to the inability to distinguish parent compound exposures from exposures to other pre-formed breakdown products, these urinary biomarkers are still the most widely used method to estimate the internal dose of a wide range of pesticides due to their relatively low costs and their utility for interpreting health outcomes of interest (Lu et al., 2005; Sudakin, 2006, 2011; Angerer et al., 2007; Starr et al., 2008; Krieger et al., 2012).

Even though the existing multi-analyte methods are highly sensitive and able to measure low concentrations of exposure biomarkers in urine, detailed time-specific information on windows of exposure vulnerability are still needed to improve exposure assessment (Needham et al., 2007). The spermatogenic cycle lasts from 75 to 90 days and a single urinary measurement is not likely to be a robust exposure measurement as these analyses assumed that these urinary measurements are reflective of longer-term exposure. Spot urine samples may not accurately reflect cumulative pesticide exposures when the parent compounds are rapidly metabolized (Martenies and Perry, 2013). Biological half-lives of OP and PYR pesticides vary and a single urine sample may not accurately reflect exposure due to the potential changes in the concentration of chemicals from void to void and the urine volume variability (Barr et al., 2005).

Although our study population was recruited from a fertility clinic, their response to pesticide exposures is not expected to differ from men in the general population. Our sample included men with a range of semen parameters, the majority whom were above the WHO lower reference values for sperm concentration and motility, and the Tygerberg Strict Criteria for morphology. Factors specific to the male partner are thought to be responsible for 30% of infertility cases (Agarwal et al., 2015) and the majority of men in this sample were likely to have normal reproductive profiles. Extensive information was collected about potential confounders. Semen measurements reflect recent spermatogenic cycles (75–90

days), and DAP and PYR urinary metabolites reflect recent exposure to pesticides. A validated semi-automated method was used to determine disomy frequency in this study, allowing for a reliable and an objective counting of disomic sperm in a large number of samples (Perry et al., 2007b, 2011). In addition, the technicians who performed semen and disomy analyses were blinded to exposure status, preventing bias in the analysis of the outcome.

5. Conclusions

To our knowledge, this is the first epidemiologic study to explore the relationship between contemporary use pesticide interactions and germ cell abnormalities. Testing for interactions revealed new relationships, mainly showing that PYRs and OPs acted not independently, but rather interdependently in increasing the risk for germ cell abnormalities. These results demonstrate that single chemical main effect analyses miss the reality that people are exposed to multiple chemicals simultaneously and likely underestimate the synergistic and/or potentiation effects of multiple exposures.

The frequency of sperm aneuploidy is best modeled assuming a Poisson distribution, and we found few prior studies that modeled interactions in Poisson models. Methods to disentangle the combined effects of pesticides and their interactions need further development to best model outcomes that do not routinely fit linear or logistic distributions. Specific attention is needed to the methodologies assessing the combined effects of simultaneous low-concentration pesticide exposures (with different modes of action) to better quantify the reproductive impacts of environmental contaminants. Because this is the first analysis examining this combination of chemicals for this health outcome, replication of these findings is needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

3-PBA	3-phenoxybenzoic acid
AChE	Acetylcholinesterase
BMI	Body-mass index
CASA	Computer-aided sperm analysis

DAP	Dialkyl phosphate
DEP	Diethylphosphate
DETP	Diethylthiophosphate
DEDTP	Diethyldithiophosphate
DMP	Dimethylphosphate
DMTP	Dimethylthiophosphate
DMDTP	Dimethyldithiophosphate
DNA	Deoxyribonucleic acid
FISH	Fluorescence *in situ* hybridization
GENMOD	Generalized linear model procedure
IRR	Incidence rate ratios
LOD	Level of detection
MATLAB	Multi-paradigm numerical computing
MGH	Massachusetts General Hospital
OP	Organophosphate
PYR	Pyrethroid
SAS	Statistical Analysis System
VGSC	Voltage-gated sodium channels

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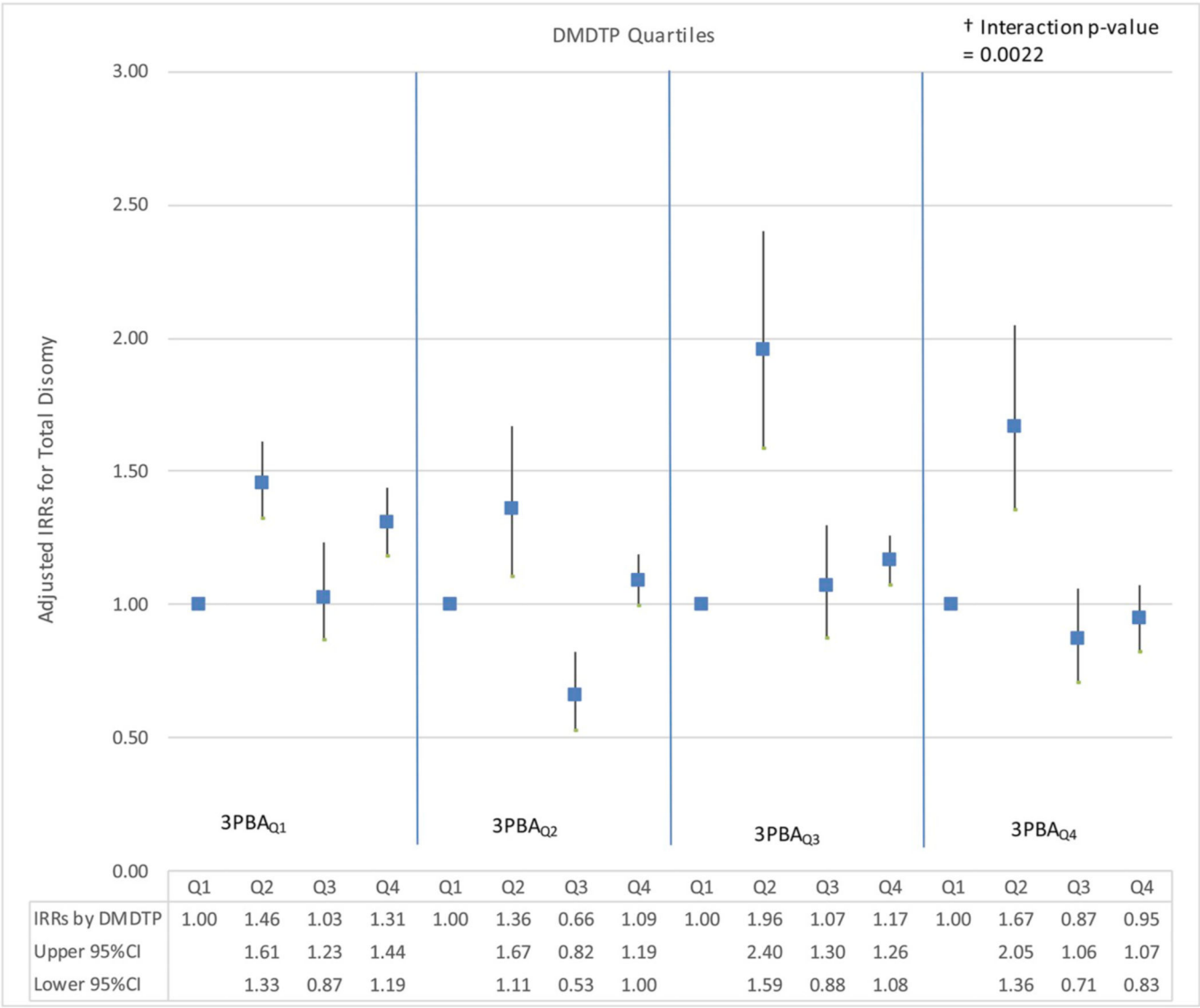
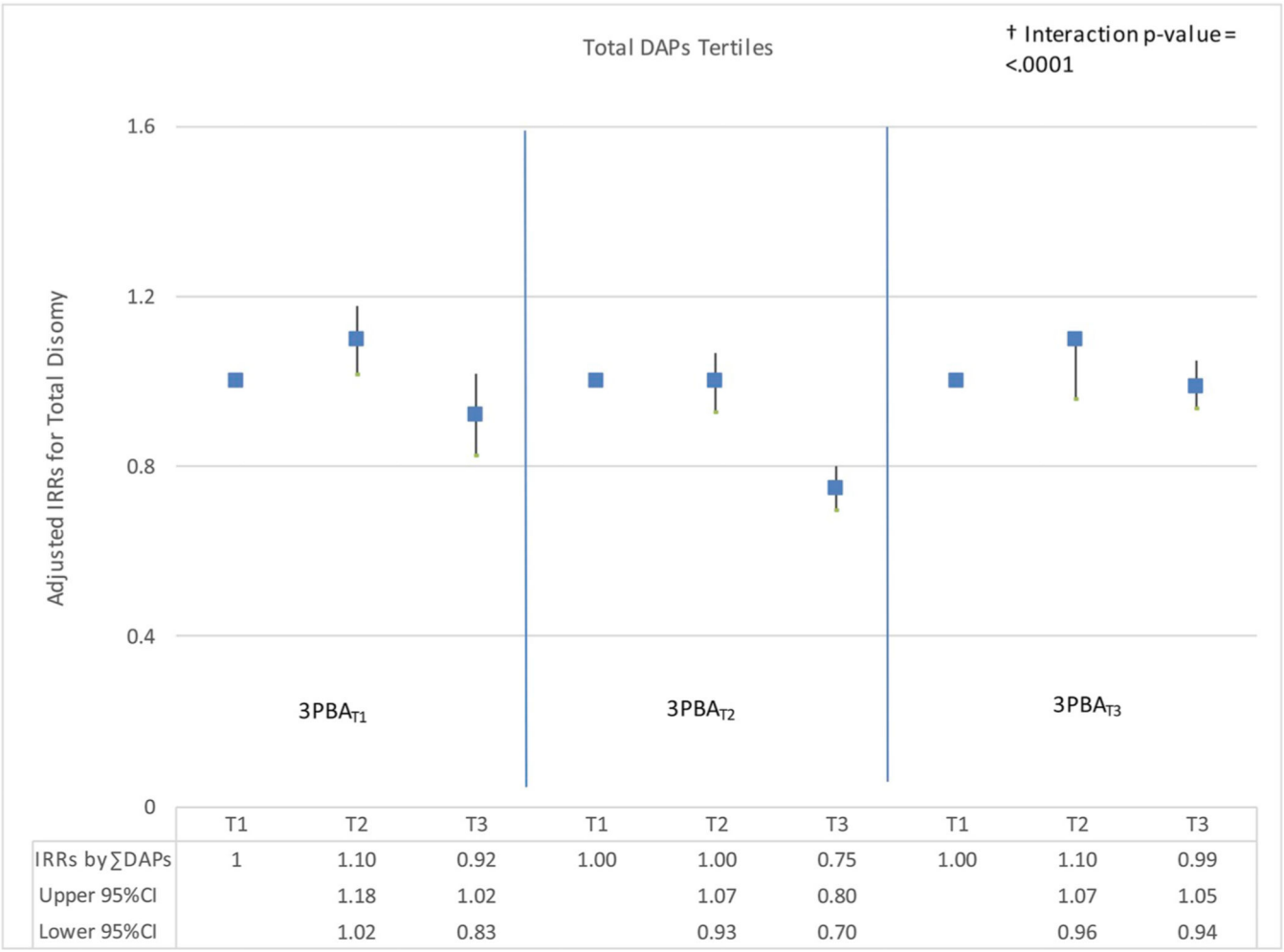


	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
IRRs by DMDTP	1.00	1.46	1.03	1.31	1.00	1.36	0.66	1.09	1.00	1.96	1.07	1.17	1.00	1.67	0.87	0.95
Upper 95%CI		1.61	1.23	1.44		1.67	0.82	1.19		2.40	1.30	1.26		2.05	1.06	1.07
Lower 95%CI		1.33	0.87	1.19		1.11	0.53	1.00		1.59	0.88	1.08		1.36	0.71	0.83

Fig. 1. Adjusted IRRs for Total Disomy by Quartiles of DMDTP by 3PBA Quartiles.

Adjusted IRRs (95% CI) for Total Disomy by Quartiles of DMDTP[a] (Exposure 1 changing) by 3PBA[b] Quartiles (Exposure 2 constant). [a] DMDTP Exposure Quartiles: Q1 = X ≤ LOD (n = 69), Q2 = 0.10 < X ≤ 0.73 ng/mL (n = 30), Q3 = 0.73 < X ≤ 1.86 ng/ mL (n = 30), Q4 = X > 1.86 ng/mL (n=30). [b] 3PBA Exposure Quartiles: Q1 = X ≤ LOD (n = 33), Q2 = 0.10 < X ≤ 0.61 ng/mL (n = 46), Q3 = 0.61 < X ≤ 0.83 ng/mL (n = 39), Q4 = X > 0.83 ng/mL (n = 41).† P-value of the interaction between DAP metabolites and 3PBA in adjusted models. IRRs were adjusted for specific gravity, age, race, BMI, smoking, sperm total concentration, motility, and morphology.



	T1	T2	T3	T1	T2	T3	T1	T2	T3
IRRs by ∑DAPs	1	1.10	0.92	1.00	1.00	0.75	1.00	1.10	0.99
Upper 95%CI		1.18	1.02		1.07	0.80		1.07	1.05
Lower 95%CI		1.02	0.83		0.93	0.70		0.96	0.94

Fig. 2. Adjusted IRRs for Total Disomy by Tertiles of DAPs by 3PBA Tertiles.

Adjusted IRRs (95% CI) for Total Disomy by Tertiles of DAPs[a] (Exposure 1 changing) by 3PBA[b] Tertiles (Exposure 2 constant). [a] ∑DAPs Exposure Tertiles: T1 = 2.76 ≤ X ≤ 35.00 (n = 53), T2 = 35.00 < X ≤ 155.00 (n = 52), T3 = X > 155.01 (n = 54). ∑DAPs is the sum of all six individual metabolites. [b] 3PBA Exposure Tertiles: T1 = X ≤ LOD (n = 33), T2 = 0.10 < X ≤ 0.69 (n = 68), T3 = X > 0.69 (n = 58). † P-value of the interaction between DAP metabolites and 3PBA in adjusted models. IRRs were adjusted for specific gravity, age, race, BMI, smoking, sperm total concentration, motility, and morphology.

Table 1

Characteristics of MGH men (n =159).

Variable	Mean ± SD
Age	35 ± 5
BMI (kg/m^2)	28 ± 5
Race	**N (%)**
White	137 (86)
Black	5 (3)
Other	17 (11)
Hispanic ethnicity No	149 (94)
Yes	10 (6)
Semen Concentration	
< 15 million/mL	16 (10)
Semen Morphology	
< 4% normal	28 (18)
Semen Motility	
< 32% motile	33 (21)
Abstinence time	
< =2 days	35 (22)
3–4 days	74 (47)
> =5 days	50 (32)
Smoking (n = 2 missing)	
No	116 (74)
Current smoker	11 (7)
Former smoker	30 (19)

Table 2

Number of sperm nuclei scored and percent disomy of men seeking infertility evaluation (n =159).

Variable	Mean ± SD	Median	25th	75th
Nuclei (n)	6,848 ± 4,815	5,503	2,939	9,976
%X18	37.6 ± 9.1	39.8	33.3	44.8
%Y18	36.5 ± 8.8	39.2	32.8	43.3
% XX18	0.4 ± 0.4	0.3	0.2	0.5
% YY18	0.4 ± 0.3	0.3	0.2	0.5
% XY18	1.1 ± 0.8	0.9	0.6	1.5
Total Disomy %	1.9 ± 1.3	1.6	1.1	2.5

Table 3

Distribution of individual pesticide metabolite concentrations in urine of men seeking infertility evaluation (n= 159).

Metabolite[a] (ng/mL)	Mean ± SD	Percentile					Range
		25th	50th	75th	90th	95th	
Unadjusted							
DMP	10.77 ± 25.30	0.30	4.34	12.48	29.42	38.60	0.30–270.75
DMTP	8.62 ± 17.42	0.68	3.13	7.28	21.11	38.71	0.10–148.12
DMDTP	1.18 ± 2.03	0.05	0.43	1.16	3.51	5.96	0.05–10.32
DEP	3.68 ± 8.24	0.10	1.00	3.65	8.87	14.82	0.10–63.56
DETP	1.70 ± 3.48	0.05	0.60	1.46	3.83	9.27	0.05–20.08
DEDTP	0.09 ± 0.12	0.05	0.05	0.05	0.26	0.39	0.05–0.66
3PBA	0.89 ± 1.51	0.51	0.62	0.87	1.57	2.27	0.05–15.18
SG-adjusted							
DMP	11.35 ± 24.04	0.51	5.17	15.39	27.51	43.11	0.20–258.98
DMTP	9.76 ± 18.07	0.85	3.73	9.13	28.62	44.19	0.05–121.76
DMDTP	1.41 ± 2.52	0.08	0.46	1.30	4.46	8.36	0.03–15.13
DEP	3.72 ± 7.48	0.22	1.33	4.14	9.33	13.70	0.05–53.78
DETP	1.77 ± 2.92	0.16	0.76	1.71	4.51	7.04	0.03–16.98
DEDTP	0.11 ± 0.13	0.05	0.06	0.11	0.29	0.43	0.03–0.72
3PBA	1.15 ± 2.64	0.44	0.69	1.17	1.99	2.55	0.04–27.83
CR-adjusted (missing = 2)							
DMP	7.87 ± 16.26	0.47	2.57	7.24	19.80	27.86	0.12–147.95
DMTP	7.22 ± 16.21	0.61	2.59	6.07	16.14	24.91	0.02–121.93
DMDTP	1.02 ± 2.14	0.07	0.29	0.96	2.64	5.32	0.01–18.89
DEP	2.47 ± 4.71	0.19	0.78	2.30	7.07	11.22	0.03–33.77
DETP	1.29 ± 2.20	0.12	0.51	1.40	3.80	5.87	0.01–15.60
DEDTP	0.08 ± 0.09	0.03	0.05	0.10	0.19	0.48	0.01–0.56
3PBA	1.08 ± 3.92	0.28	0.47	1.01	1.72	2.24	0.02–46.56

[a]Unadjusted urinary metabolite concentration (units) =ng/mL. LOD= 0.6 ng/mL (DMP), 0.2ng/mL (DMTP), 0.2 ng/mL (DEP), and 0.1 ng/mL (DETP, DMDTP, DEDTP, 3PBA). Percent of metabolite samples above the LOD: DMP =57% (n= 91); DMTP = 87% (n =139); DMDTP = 57% (n =90); DEP =64% (n= 101); DETP = 72% (n =114); and DEDTP =10% (n=16); 3PBA= 79% (n =126).

Table 4

Pearson Correlation Coefficients (p-value) between individual urinary metabolite concentrations (N = 159).

Metabolites[a]	DMP	DMTP	DMDTP	DEP	DETP	DEDTP	∑DAPs	3PBA
DMP	1.00							
DMTP	0.57 (< 0.001)	1.00						
DMDTP	0.48 (< 0.001)	0.59 (< 0.001)	1.00					
DEP	0.60 (< 0.001)	0.57 (< 0.001)	0.50 (< 0.001)	1.00				
DETP	0.33 (< 0.001)	0.39 (< 0.001)	0.35 (< 0.001)	0.54 (< 0.001)	1.00			
DEDTP	0.17 (0.0340)	0.14 (0.0807)	0.13 (0.1032)	0.20 (0.0107)	0.22 (0.0055)	1.00		
∑DAPs	0.87 (< 0.001)	0.82 (< 0.001)	0.63 (< 0.001)	0.74 (< 0.001)	0.49 (< 0.001)	0.20 (0.0098)	1.00	
3PBA	0.07 (0.3799)	0.08 (0.2875)	0.07 (0.3660)	0.07 (0.3974)	0.07 (0.3942)	−0.009 (0.9089)	0.04 (0.5784)	1.00

[a]Unadjusted urinary metabolite concentration (units) = ng/mL. The unadjusted urinary metabolite concentrations were log-transformed prior to examined Pearson correlations.

Table 5

Adjusted IRRs (95% CI, p-value) for total sex-chromosome disomy by Quartiles of DAP Metabolite Concentrations (Exposure 1) by 3PBA Concentrations Quartile (Exposure 2) of men seeking infertility evaluation (n= 159).

3PBA Quartile[a]	DAP Quartile[b-f]	Total Disomy				
		DMP IRR (95% CI)	DMTP IRR (95% CI)	DMDTP IRR(95% CI)	DEP IRR (95% CI)	DETP IRR (95% CI)
Q1	Q1	1	1	1	1	1
	Q2	0.75 (0.68, 0.82)	1.29 (1.18, 1.41)	1.46 (1.33, 1.61)	1.33 (1.23, 1.45)	1.04 (0.94, 1.15)
	Q3	0.43 (0.33, 0.58)	1.08 (0.97, 1.19)	1.03 (0.87, 1.23)	1.24 (1.13, 1.37)	2.00 (1.83, 2.19)
	Q4	0.70 (0.64, 0.77)	1.13 (0.97, 1.30)	1.31 (1.19, 1.44)	1.03 (0.94, 1.13)	0.96 (0.87, 1.07)
Q2	Q1	1	1	1	1	1
	Q2	0.69 (0.64, 0.75)	1.05 (0.89, 1.24)	1.36 (1.11, 1.67)	1.16 (1.00, 1.33)	0.39 (0.34, 0.44)
	Q3	0.67 (0.60, 0.74)	0.76 (0.66, 0.88)	0.66 (0.53, 0.82)	1.07 (0.93, 1.23)	2.31 (2.02, 2.64)
	Q4	0.67 (0.61, 0.74)	1.31 (1.09, 1.57)	1.09 (1.00, 1.19)	0.62 (0.53, 0.71)	0.76 (0.69, 0.84)
Q3	Q1	1	1	1	1	1
	Q2	1.13 (1.06, 1.21)	1.75 (1.48, 2.06)	1.96 (1.59, 2.40)	1.68 (1.48, 1.91)	0.60 (0.53, 0.68)
	Q3	1.09 (1.02, 1.16)	0.95 (0.83, 1.07)	1.07 (0.88, 1.30)	0.95 (0.84, 1.09)	1.89 (1.65, 2.16)
	Q4	0.98 (0.89, 1.07)	1.26 (1.06, 1.49)	1.17 (1.08, 1.26)	0.92 (0.80, 1.06)	0.87 (0.80, 0.95)
Q4	Q1	1	1	1	1	1
	Q2	0.53 (0.48, 0.59)	1.12 (0.96, 1.32)	1.67 (1.36, 2.05)	1.27 (1.11, 1.46)	0.53 (0.46, 0.61)
	Q3	0.83 (0.76, 0.91)	0.94 (0.83, 1.08)	0.87 (0.71, 1.06)	1.24 (1.07, 1.43)	1.28 (1.08, 1.52)
	Q4	0.92 (0.84, 1.01)	0.72 (0.61, 0.86)	0.95 (0.83, 1.07)	0.57 (0.49, 0.67)	0.84 (0.76, 0.93)
Interaction p-value[g]		0.0015	< 0.0001	0.0022	< 0.0001	< 0.0001

[a] 3PBA Exposure Quartiles: Q1 = X ≤ LOD (n = 33), Q2 = 0.10 < X ≤ 0.61 ng/mL (n = 46), Q3 = 0.61 < X ≤ 0.83 ng/mL (n = 39), Q4 = X > 0.83 ng/mL (n = 41).

[b] DMP Exposure Quartiles: Q1 = X ≤ LOD (n = 68), Q2 = 0.60 < X≤ 7.95 ng/mL (n = 30), Q3 = 7.95 < X ≤ 13.39 ng/mL (n = 30), Q4 = X > 13.39 ng/mL (n = 31).

[c] DMTP Exposure Quartiles: Q1 = X ≤ LOD (n = 30), Q2 = 0.20 < X ≤ 2.21 ng/mL (n = 43), Q3 = 2.21 < X ≤ 6.47 ng/mL (n = 42), Q4 = X > 6.47 ng/mL (n = 44).

[d] DMDTP Exposure Quartiles: Q1 = X ≤ LOD (n = 69), Q2 = 0.10 < X ≤ 0.73 ng/mL (n = 30), Q3 =0.73 < X ≤ 1.86 ng/mL (n = 30), Q4 = X > 1.86 ng/mL (n = 30).

[e] DEP Exposure Quartiles: Q1 = X ≤ LOD (n = 58), Q2 = 0.20 < X ≤ 1.46 ng/mL (n = 34), Q3 = 1.46 < X ≤ 3.96 ng/mL (n = 33), Q4 = X > 3.96 ng/mL (n = 34).

[f] DETP Exposure Quartiles: Q1 = X ≤ LOD (n = 45), Q2 = 0.10 < X ≤ 0.62 ng/mL (n = 39), Q3 = 0.62 < X ≤ 1.51 ng/mL (n = 37), Q4 = X > 1.51 ng/mL (n = 38).

[g] P-value of the interaction between DAP metabolites and 3PBA in adjusted models. IRRs were adjusted for specific gravity, age, race, BMI, smoking, sperm total concentration, motility, and morphology.