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Thomas Dang April 21, 2022

Association of Prenatal Chlorpyrifos Exposure with Sexually Dimorphic Differences in Anogenital Distance Among Thai Farmworker Children

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An abstract of
a thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Environmental Health
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Abstract

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By Thomas Dang

Pesticides are used globally, yet prenatal exposure can unknowingly impact infants and their adulthood health. There needs to be a better understanding of adverse effects following prenatal exposure to endocrine-disrupting chemicals (EDCs). Research has focused on organophosphate insecticides and their effect on the reproductive health of newborns after maternal exposure. Anogenital Distance (AGD) measurements measured during infancy can provide a noninvasive measurement and accessible end-point marker for male and female reproductive health at birth and adulthood. AGD can be an early predictor to better treat for reproductive health complications early in life to allow these children a healthy adulthood.

We attempt to answer the overarching question of whether trimester-specific or mean prenatal exposure across pregnancy to chlorpyrifos in Thai farmworker children, as measured by its primary urinary metabolite (TCPy), is associated with sex-specific anogenital distance at 12 months of age. We hypothesized that chlorpyrifos exposure in the first trimester and averaged over pregnancy will be associated with shorter AGD in males and longer ADG in females. Identifying early markers of reproductive development through AGD could clarify the biological associations between maternal exposure and reproductive function without confounding by the many postnatal contributors to reproductive health.

Exposure was assessed by measuring its primary urinary metabolite (TCPy). Analysis included linear exposure-outcome models: trimester-specific and average exposure over pregnancy adding in covariates identified a priori. Differences in effect based upon sex were evaluated because literature demonstrates sexually dimorphic adverse effects. Focus was primarily on prenatal exposure as this critical time window of 12 months is hypothesized to drive environmental-related disease outcomes in children.

Findings were consistent with previous studies further supporting that prenatal exposure to pesticides is associated with AGD measurements. Models were produced unadjusted and then adjusted for creatinine levels to correct for urine dilution, consistent with the literature. We generally observed reductions in AGD in males and increases in females. Generally, this was observed in trimester-specific models and overall pregnancy, supported by previous studies. By understanding the effects of exposure to infants, better protective policies can protect mothers while giving their baby the best chance to a healthy and happy life.

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Acknowledgements

From Emory University, I would like to thank Drs. Dana Boyd Barr, Stephanie Eick, Melissa Smarr, supporting staff and graduate research assistants. From Chiang Mai University (CMU), I would like to thank Dr. Tippawan Prapamontol, Warangkana Narksen, and other staff and team members at the Research Institute of Health Sciences at CMU. I also like to thank the participants of the study for their invaluable contribution to this research.

I sincerely want to express deep admiration and gratitude to my thesis advisor and faculty mentor, Dr. Dana Boyd Barr for her unwavering moral support and insightful guidance on this project's conceptualization to its completion. Her passion and dedication to exposure science, biomarkers, environmental justice, and her students are truly inspiring, and I am thankful to have had this opportunity to work with her. Finally, I would like to extend a special thank you to my family, partner, and friends for their constant support, encouragement, and love throughout my pursuit of higher education and reminding me to do what is best for improving the environment and those in it.

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Introduction

I. Organophosphate Pesticides and Chlorpyrifos

Organophosphate (OP) pesticides are a class of insecticides used in numerous applications including agriculture, homes, gardens and veterinary practices (Environmental Protection Agency 2013). This class of insecticides has been used abundantly worldwide up until the 21st century, yet they are known to cause acute and subacute toxicity to human health (Environmental Protection Agency 2013). In the United States some organophosphate pesticides have been discontinued for use, such as parathion for any use and chlorpyrifos, which is no longer registered for home use (Environmental Protection Agency 2013). Although organophosphate pesticides have begun to be discontinued in the United States, they have continued to be produced and be imported worldwide with the Americas being the third largest exporter of total pesticides regionally (Food and Agriculture Organization of the United Nations 2020).

Organophosphate insecticides have become the most widely used pesticides globally with chlorpyrifos (CPF) being used extensively worldwide (John and Shaike 2015). Producers of chlorpyrifos find it easy to market in most developing and non-developing countries where low-economic price and easy access make it commonly used over other pesticides (John and Shaike 2015). Humans can be exposed through several pathways including dietary, occupational, paraoccupational, non-dietary ingestion and via multiple routs (oral, dermal and inhalation routes). Given the increasing usage of pesticides internationally there is growing evidence that attention should be given to both occupational and para-occupational exposure especially where many countries have agricultural workers, and their families live on or near farms (López-Gálvez et al. 2019).

II. Thailand Agriculture

Thailand agricultural activities have continued to grow and expand. The introduction to more mechanical equipment, heavy machinery, hybrid seeds and synthetic chemicals have made changes to the farming process yet present new challenges to the health and safety of agricultural farm workers and their families. The country relies heavily on pesticides to control insects, weeds and fungi while maintaining high crop yield. Thailand ranks fourth in annual pesticide consumption out of fifteen Asian countries. With increasing imports of pesticides to meet global demand, there continues to be gaps in understanding the health impacts of pesticide use on Thailand farmworkers (Laohaudomchok et al. 2021).

Although Thailand's industrial sector has grown rapidly, agriculture is still considered a foundation of its economy (Panuwet et al. 2008). Rural areas are populated with 70% of the Thai population who rely heavily on agriculture as their main source of income (Laohaudomchok et al. 2021). As of 2019, about 38% of Thailand's agricultural, forestry and fishing employees are female (Food and Agriculture Organization of the United Nations 2021). The health effects of environmental and occupational pesticide exposure in Thailand are only gaining importance despite the increasing reliance and continuous use of pesticides. According the Food and Agriculture Organization of the United Nations (Food and Agriculture Organization of the United Nations 2020), Thailand was among the top five importers of hazardous pesticides in 2018. Although pesticides are known to protect crops and ensure plentiful supply of food, the potentially adverse reproductive effects induced by EDCs is of concern.

III. Chlorpyrifos Exposure and Toxicity

Once exposure occurs via any pathway and route, chlorpyrifos biodegrades by enzymatic or hydrolytic oxidative desulfuration to its oxon and then undergoes hydrolysis to yield 3,5,6-

trichloro-2-pyridinol (TCPy) (Ubaid Ur Rahman et al. 2021) and diethylphosphate metabolites. Chlorpyrifos' low biological persistence allows its primary urinary metabolite TCPy to function as a biomarker for exposure in blood and when these metabolites or their glucuronide- or sulfate-bound conjugates excreted in urine (Muñoz-Quezada et al. 2013; Smegal 2000). The hydrolysis of chlorpyrifos into TCPy is mediated by the paraoxonase 1 (PON1) enzyme, which can phenotypically include low, normal, and high metabolism (Naksen et al. 2015). PON1, the gene responsible for transcribing PON, is an important biomarker of susceptibility studied for organophosphates has a polymorphism at amino acid 192 (R → Q) that can alter expression and activity of PON, thus altering a person's ability to detoxify organophosphate pesticides (Muñoz-Quezada et al. 2013).

The primary mechanism of toxicity of chlorpyrifos is inhibition of the enzyme acetylcholinesterase (AChE) by phosphorylation at nerve endings, a characteristic shared among the class of organophosphates (Environmental Protection Agency 2013). With a loss of available AChE, an excess of acetylcholine (ACh), the impulse transmitting substance) is obtained which causes overstimulation (Environmental Protection Agency 2013). AChE is an enzyme critical for normal control of nerve impulse transmission from nerve fibers to smooth and skeletal muscles, secretory cells and autonomic ganglia and the central nervous system (CNS) (Environmental Protection Agency 2013). Although chlorpyrifos is effective in controlling pests and improving crop yield, this primary mechanism of toxicity is also present in humans and other animals (Ambali et al. 2009; Ubaid Ur Rahman et al. 2021). Other studies have linked non-AChE inhibiting levels to neurological effects in animals and humans (Muñoz-Quezada et al. 2013; Rice and Barone 2000).

Non-AChE inhibiting effects have been observed after exposure to chlorpyrifos. Studies have shown that the chlorpyrifos oxon impairs axonal transport in embryonic neurons further supporting possible effects on various other neuronal targets not directly related with AChE (Ambali et al. 2009; Gao et al. 2017). People exposed to chlorpyrifos may be at greater risk of chromosomal mutations, sister-chromatid exchanges and formation of micronuclei compared to those not exposed (Ubaid Ur Rahman et al. 2021)

Prenatal exposure to organophosphates is of particular concern since they can cross the placenta as the developing brain is more vulnerable to injury than adult brains (Zhang et al. 2014). Assessing this exposure is crucial as there are critical periods of development of the nervous system that are sensitive to environmental insults leading to long-term consequences later in life in both animals and humans (Rice and Barone 2000). Reviews have provided consistent evidence that prenatal exposure to organophosphates is strongly linked to poor neurodevelopment and neurocognition (Burke et al. 2017; Cal 2008; Muñoz-Quezada et al. 2013).

As a fetus is developing another crucial window of development includes androgen-induced masculinization in which a fetus becomes phenotypically male or female based on androgen induced programming and androgen biosynthesis (Welsh et al. 2008). Chlorpyrifos, as an endocrine disrupting chemical (EDC), exhibits anti-androgenic activities and can decrease testosterone thus posing a threat to male and female reproductive systems as they alter sexual functions (Viswanath et al. 2010). Viswanath et al. (2010) was able to characterize chlorpyrifos anti-androgenic activity by screening it with the NIH3T3 cell line stably expressing human androgen receptor (hAR) and luciferase reporter gene for their

ability to stimulate luciferase activity or inhibit the response that was evoked by 0.4nM testosterone and being the most potent in anti-androgenic activity.

IV. Anogenital Distance

Although evidence exists for the effects of EDCs in experimental animals and wildlife, a challenge remains in defining a biomarker in humans that reflects EDC exposure (Thankamony et al. 2009). Anogenital distance (AGD) is the distance from the anus to the genitalia and is a sensitive marker used as a measure for fetal androgen action in animal and recently some human studies (Vafeiadi et al. 2013). Across many species, including humans, AGD is longer in males than in females (Barrett et al. 2013).

V. Purpose

As part to the Study of Asian Women and their Offspring's Development and Environmental Exposures (SAWASDEE study), we sought to investigate reproductive effects associated with prenatal chlorpyrifos exposure. Understanding exposure pathways and reproductive health effects of pesticide exposures in women, particularly pregnant women and their babies, is critical in informing global occupational and paraoccupational health and safety efforts while predicting reproductive health in adulthood.

VI. Hypothesis and Main Question

We hypothesized that higher levels of urinary TCPy in each trimester and across pregnancy would result in EDC-related changes in AGD in children indicative of reproductive toxicity and that this relation may be modified by PON activity phenotype.

Methods

I. Study Population and Sample Collection

This project uses extant data derived from the SAWASDEE Study which focuses on collecting highly refined maternal exposure assessments data related to pregnancy and health

outcomes of the children. This thesis will focus specifically on the health outcome AGD as a biomarker for reproductive health outcomes that is predictive of adulthood reproductive health.

SAWASDEE is a birth cohort study in an agricultural region of northern Thailand. The longitudinal birth cohort study was established in 2017 to examine pesticide biomarker concentrations in pregnant farmworker mothers and their newborn children. All women enrolled in the SAWASDEE birth cohort work in agriculture as tangerine, rice, fruit, vegetable or flower farmworkers in Fang and Chom Thong Districts in Chiang Mai Province of Northern Thailand and are occupationally exposed to organophosphate pesticides. Some paraoccupational exposure may also occur through residential use of pesticides. The routes of exposure of primary concern for these women are dermal exposure that occurs from picking or thinning of fruit from trees shortly after they have been sprayed with pesticides and inhalational exposure from re-volatilized pesticide residues. Data collection included extensive exposure and demographic questionaries along with biological sample collection including maternal blood and urine. The SAWASDEE cohort improves upon previous studies by measuring pesticide exposure at multiple time points throughout pregnancy. All study protocols were reviewed and approved by the Institutional Review Board of Emory University and the Ethic Board of Chiang Mai University.

II. TCPy, Creatinine and PON Phenotpying Analysis

TCPy and Creatinine Analysis

Urine samples were composited using equal volumes to create early, mid and late pregnancy samples that roughly correspond to trimester. All samples were randomized using a Fisher-Yates shuffling algorithm prior to analysis to reduce any potential batch effects (Fisher 1948; Knuth 1969). Samples were analyzed for 3,5,6-trichloro-2-pyridinol (TCPY), a “specific” metabolite of organophosphate insecticides chlorpyrifos and chlorpyrifos methyl using a modification of a

previously validated method (Olsson et al. 2004). Briefly, samples are spiked with stable isotopic analogues of the target analytes then are enzymatically digested using purified β-glucuronidase and sulfatase enzymes (derived from H. pomatia) to liberate bound metabolites. The hydrolysates are centrifuged and transferred to autosampler vials. To facilitate on-line solid phase extraction, samples are injected into a column switching system for concentration of the target analytes on a Strata RP on-line SPE column (2.1 x 20 mm). The on-line extraction column is washed with the acetonitrile:Milli-Q water (10:90, V/V) solution to remove undesired matrix interferences. The target analytes are then eluted from the on-line extraction column to a Poroshell 120 EC-C18 analytical column (3.0 x 100 mm, 2.7 um) for chromatographic separation. The target analytes are measured using negative mode electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) with isotope dilution quantification. During mass spectrometric analysis, the target analytes are monitored using the multiple reaction monitoring (MRM) mode. One quantitation ion and one confirmation ion are monitored for the native analytes, and one quantitation ion is monitored for the labeled analogues. Concentrations of the target analytes are determined from the relative response (per volume of sample injected) of native to labeled standards in the samples, using an equation derived from a matrix-matched standard calibration curve. For each analytical run of 44 unknown samples, 2 blank samples (negative control) and four positive quality control samples at 2 different levels were analyzed concurrently. The quality control information is provided in Table 1. Successful participation in the German External Quality Assessment Scheme (GEQUAS) served as an additional quality assurance parameter of the method. The limit of detection (LOD) was 0.31 ng/mL for TCPy and the relative recoveries ranged from 90-99%. For statistical analysis, the LOD divided by the square root 2 was imputed for all values below the LOD.

Creatinine was measured by diluting urine samples 1000-fold with water after spiking with its isotopically labeled analogue. Diluted samples were analyzed by liquid chromatography electrospray ionization coupled with tandem mass spectrometry. For creatinine, two ion transition were monitored (m/z 113.9 → m/z 44.2 and m/z 113.9 → 86) and only one ion transition was monitored for labeled creatinine (m/z 116.9 → m/z 47.2) (Kwon et al. 2012). Quantification was achieved using isotope dilution calibration. Quality control/assurance included the concurrent measurement of calibrants, blanks and quality control materials and semi-annual certification by the GEQUAS program. The LOD was 5 mg/dL with a relative standard deviation of 5%.

Measurements of paraoxonase (PONase) and arylesterase (AREase)

Plasma from maternal venous blood were assayed for PONase and AREase activities using Huen's method (Huen et al. 2009). For the PONase assay, 20 μL of plasma (dilution: 1:10) was added to 200 μL of 1.2 mM paraoxon (0.1 M Tris-HCl, pH 8.5, 2 mM CaCl2, 2 M NaCl). The concentration of ρ-nitrophenol as the paraoxon hydrolytic product was determined at 405 nm every 15 s over 2 min. For AREase assay, 20 μL of plasma (dilution: 1:80) was added to 200 μL of 3.26 mM phenyl acetate solution (9 mM Tris–HCl pH 8.0 with 0.9 mM CaCl2) in each well of a 96-well UV transparent plate. The concentration of phenol as the hydrolytic product of phenyl acetate was determined at 270 nm every 15 s over 2 min. The concentrations of ρ-nitrophenol and phenol were calculated using Lambert–Beer's law (ερ-nitrophenol = 18,000 M−1 cm−1 and εphenol =1,310 M−1cm−1, respectively). All assays were performed in triplicate. Internal quality control aliquots using pooled samples were used for every assay. The inter-assay relative standard deviations (RSDs) of PONase and AREase assays were 4.2% and 1.6%, respectively.

III. Statistical Analyses

Statistical analysis was conducted using R (Team 2019). TCPy metabolite concentrations for trimesters 1, 2, and 3 were averaged to determine the mean concentration of TCPy across a mother's entire pregnancy. A univariate analysis was performed to examine TCPy metabolite distributions and obtain descriptive statistics and summary measures (i.e., frequency of metabolite, 95% confidence intervals and selected distribution percentiles).

Demographic variables, including study site location (Chom Thong or Fang), maternal age, household income, maternal ethnicity [Thai, ThaiYai, Hill Tribes i.e. (Hmong, Karen, Akha, Pa-Long, Lahu Na, Lahu Nyi), Other (Burmese, Other, NA)], maternal educational attainment (never attended school, primary school, junior high/high school, some high school, or high school/technical school and beyond), and marital status (married or living as married, widowed, separated or other) were selected a priori to be included in each full multivariable regression model. A household person adjusted income variable was created using the reported household income in Thai baht and reported as "greater than or equal to 6,000 baht". Marital status was categorized as "Married or Living as Married" and "Widowed, Separated, or Others". Natural log-transformed creatinine was also included in all full models to adjust for urinary dilution (Barr et al. 2005). Bivariate analyses were performed to assess associations between TCPy concentrations for each pregnancy period and mean concentration across pregnancy and potential exposure predictors. The potential predictors included maternal ethnicity, household income, maternal educational attainment, gestational age, and maternal age. Infant weight (kg) was included along with adjusted Z-score birthweight. In order prevent confounding variables and issues within the model, birthweight was standardized since it is confounded by gestational age.

With the use of the PediTools online software, Z-scores for birthweight were calculated to account for the confounding gestational age (Chou et al. 2020). Summed variable levels were collapsed as needed to generate levels containing at least 10% of respondents including income, marital status, and maternal educational attainment. Predictors were excluded from analyses if either binary variable level contained less than 10% of respondents.

A stepwise selection approach was used to identify covariates to include in the final models by entering and removing predictors based on p values of 0.10 in a stepwise manner. The selected model included all candidate predictor variables. Multivariable linear regression models were constructed to evaluate determinants of TCPy metabolite exposure if an effect with $p<0.10$ was observed. Separate models were built for TCPy concentrations in each trimester and the mean concentration across pregnancy. Multivariable model associations were considered significant if $p<0.05$. After selection we evaluated potential covariates implicated in fetal growth etiology including creatinine, maternal age at intake (years), maternal height (cm), maternal weight gain(kg), infant sex (male or female), birthweight (kg), gestational age z-score, PON status, maternal ethnicity, and household income. The variant inflation factor (VIF) was calculated to check for multicollinearity then models included site location (Chom Thong or Fang). We ultimately removed location because of collinearity issues. All initial models contained the *a priori* selected variables and natural log transformed creatinine. Final models included AGD measurement at 12 months as the outcome, log transformed TCPy metabolite exposure, infant sex, maternal ethnicity, marital status, maternal educational attainment, gestation age Z-scores, and infant weight (kg) at 12 months.

Furthermore, we investigated whether different methods of adjustment for urinary dilution altered our results. We performed analyses without adjustment for urinary dilution in our main

models, but also explored the consistency of findings when using creatinine-corrected the TCPy metabolite as the independent variable. All models produced similar results, so we ultimately decided to include creatinine as a separate adjusted variable in our main models to remain consistent with other studies that correct for urine dilution (Barr et al. 2005). Lastly, sex-specific models were constructed to compare sex-specific differences in AGD measurement associations.

Results

I. Demographics and Study Population

Participants were recruited from Chom Thong and Fang, two districts in Chiang Mai Province in Northern Thailand. These districts had two hospitals at which a sufficient number of farmworker women who were exposed to insecticides would be delivering their babies and best represented exposure.

Of the 333 pregnant women in the SAWASDEE study with urinary TCPy metabolite concentration measurements, 104 participants (n=54 from Chom Thong, n=50 from Fang) had AGD measurements and complete covariates including creatinine concentrations, and therefore were included in the analyses. Participants were predominantly of Thai ethnicity (56.7%), while other groups categorized were Thai Yai (7.7%), Hill Tribes (Hmong, Karen, Akha, Pa-Long, Lahu Na, Lahu Nyi) (28.8%), and other (Burmese, Other, NA) (6.7%). Average intake age of pregnant women was 24.3 years of age. Maternal measurements at birth included averages of 65.1kg weight and 152.61 cm height. Married or living as married (98.1%) was the most prevalent marital status while being widowed, separated, or others consisted of two participants (1.9%). Education levels varied among participants with levels including never attended school (22.1%), primary school (20.2%), junior high/high school (32.7%), some high school (13.5%), and high school/ technical school and beyond (11.5%). A majority (47.1%) of participants had high expression of PON1, thus having more protection from the TCPy metabolite, while 39.4%

had normal levels and 13.5% had low expression of the PON phenotype. At birth infant weight averaged 3.01 (kg) and height averaged 48.62 (cm), with adjusted average gestation z-score of -0.4. AGD measurements at 12 months of age for the infant included 8.65 (kg) weight, 70.78 (cm) height and average AGD of 58.67 (mm).

II. TCPy Metabolite

After stepwise selection was performed on overall pregnancy (all trimesters), models could be conducted for trimester specific models and overall pregnancy for concentrations of TCPy urinary metabolites. Preliminary descriptive statistics showed a mean natural log TCPy concentration of 1.50 (ng/mL) across pregnancy. Next trimester specific TCPy concentrations were examined for first, second and third trimesters were 1.61, 1.58, 1.30 (ng/mL), respectively. Creatinine levels were calculated to correct urine dilution for overall pregnancy then trimester specific mean levels of 92.6, 115.40, 85.2, 77.8 mg/dL, respectively.

III. PON1 Phenotyping

An individual's PON1 phenotype was determined by using the ratio of salt-stimulated PONase activity to AREase activity according to Eckerson's method (Eckerson et al. 1983). Intra-person activities of each enzyme from three collections were averaged before calculating the ratio. The cumulative distribution were plotted and cutpoints determined (Figure 1). The obvious gaps from the plot were used to separate into three PON1 phenotypes of low (AA), middle (AB) and high (BB) activity which are related to QQ, QR and RR genotypes, respectively, at codon192 of PON1 (Humbert et al. 1993). One of the PON1polymorphisms is the Q192R polymorphism. The change of codon in exon 6 of PON1 gene results in substitution of amino acid glutamine with arginine at the position 192. The activities of these two alloenzymes towards different OP pesticides were different because the amino acid at the

position 192 is an important residue of PON1 active site (Harel et al. 2004). In an *in vivo* study, R192 alloenzyme provides more protection against chlorpyrifos oxon (Li et al. 2000).

IV. Associations between TCPy metabolite and AGD

Multivariate analyses were created overall pregnancy then for trimester specific associations. These models were adjusted for exposure without creatinine then including creatinine. Generally, there was reduction in AGD in these models. For a log unit increase in the mean urinary metabolite levels of TCPy there was a -1.33 mm (CI -3.25, 0.58) reduction in AGD across pregnancy without adjustment for creatinine. Trimester one and three showed a -0.53 mm (CI -2.17, 1.12) reduction and -1.59 mm (CI -3.09, -0.08) reduction without creatinine adjustment, respectively. However, in trimester two there was a 0.05 mm (CI -1.62, 1.73) increase in AGD for a log unit increase of TCPy urinary metabolite levels. To stay consistent with past studies evaluating the appropriateness of creatinine adjustment, we generally observed a narrower confidence interval in the models after adjustment. For a log unit increase in the mean urinary metabolite levels of TCPy there was a -0.03 mm (CI -2.17, 2.11) reduction in AGD across pregnancy without adjustment for mean creatinine levels. Trimester one presented -0.15 mm (CI -1.99,1.70) and three showed -0.36 mm (CI -2.06,1.35) consistent decreases in AGD for a log unit increase in the mean urinary metabolite levels of TCPy after adjusting for creatinine. There was a greater increase in AGD of 1.15 mm (CI -0.86, 3.16) increase for a unit increase of urinary metabolite levels TCPy within trimester two.

For the final sex-specific models, all covariates were included from the original selected models; including log-transformed TCPy urinary metabolite levels, mean creatinine levels, infant sex, maternal ethnicity, maternal marital status, maternal educational attainment, gestational age Z-score, and infant weight at 12 months.

First the original models were stratified by males. Again, multivariate analyses were created overall pregnancy then for trimester specific associations. These male-specific models were adjusted for exposure without creatinine then including creatinine. However, marital status had be removed in male stratified models since all male infants had married or living as married mothers. This allowed the male models to run. All unadjusted models had consistent reductions in AGD for every log unit increase in the mean urinary metabolite levels of TCPy [-1.59 mm (CI -4.02, 0.84), -0.64 mm (CI -3.46, 2.19), -1.23 mm (CI -3.84, 1.39)], respectively), including overall pregnancy reduction of -2.02 mm (CI -5.24, 1.19). After adjusting for creatinine there were reductions in AGD with narrower confidence intervals for trimester one reduction of -0.94 mm (CI -3.77, 1.89) and trimester two reduction of -0.06 mm (CI -3.74, 3.62) and overall pregnancy reduction of -0.46 mm (CI -4.18, 3.25). However, there was in increase in AGD after adjustment for creatinine of 0.92 mm (CI -2.35, 4.19) observed in trimester three.

The same calculations and models were run for female specific models along with previous variables, including marital status as there were no conflicts in running these models. Unadjusted female-specific models all showed increases in AGD for every log unit increase in the mean urinary metabolite levels of TCPy trimester with 0.52 mm (CI -1.94, 2.98) increase in trimester one, 0.97 mm (CI -1.37, 3.32) increase for trimester two. There was a 0.68 mm (CI -2.38, 3.74) increase in AGD after adjustment for overall pregnancy. This pattern of increase did not occur for trimester 3 unadjusted with an AGD creatinine reduction of -1.27 mm (CI -3.45, 0.92) and creatinine adjusted AGD reduction of -0.40 mm (CI -2.79,1.99). The female stratified overall pregnancy without adjustment for creatinine also produced a reduction in AGD of -0.29 mm (CI -3.00, 2.41).

Discussion

I. Important Findings & Comparison to Other Studies

This study investigated trimester-specific period associations and overall chlorpyrifos exposure during pregnancy with AGD measurements in infants at 12 months who were born to a cohort of female agricultural workers form northern Thailand. We observed exposure through the primary urinary metabolite of the OP pesticide chlorpyrifos, TCPy as a biomarker for AGD measurements in infants. We suggest that AGD is a better biomarker than more invasive measurements, as it reflects both exposure to and effects of the endocrine disrupting capabilities of OP pesticides, specifically chlorpyrifos. It is well documented that humans and wildlife have suffered adverse health effects after exposure to chemicals that interact with the endocrine system (Kavlock et al. 1996). This study aims to support the research need of establishing an association between these concerning exposures during pregnancy and health outcomes of children. More specifically, this study attempts to delineate how chlorpyrifos exposure influences these changes in AGD.

Previous studies document neurodevelopmental effects in children associated with OP pesticides (Muñoz-Quezada et al. 2013) and phalates. The association of chlorpyrifos induced toxicity in reproductive organs has also been well studied in non-human animals, specifically female rats (Nishi and Hundal 2013) and male rats (Sai et al. 2014). These studies examined biological mechanisms within the reproductive organs, showing endocrine disruption through estrogenic activity inhibition. Further studies have shown that maternal characteristics can affect reproductive health of offspring. For example in Dean and Sharpe (2013) found that, after prenatal exposure, AGD did appear to provide a reliable guide to fetal androgen exposure, which may be predictive of adult-onset reproductive health (Dean and Sharpe 2013). Building on this finding, our study establishes the biological plausibility of sex-hormone reactions in shaping

changes in AGD. Our findings that AGD is associated are supported from a previous study of chlorpyrifos and pyrethroid exposure showing associations of shorter AGD in males and elongation in females, there were weak associations in females as predicted by other studies (Dalsager et al. 2018). Generally, our findings matched other studies done globally measuring AGD, since we observed reduction in AGD for males and an increase in females (Barrett et al. 2018; Romano-Riquer et al. 2007; Schwartz et al. 2019). While other studies in the region have extensively studied OP levels in pregnant women, our study further illustrates the birth outcome implications of this work (Kongtip et al. 2014; Panuwet et al. 2008).

II. Strengths and Limitations

Although our study has many strengths it also had some limitations. For example, this study measured specifically an association of prenatal chlorpyrifos exposure, but we did not account for postnatal exposure, nutrition, other metabolites, and other environmental factors that could possibly be covariates. We also could not account for other endocrine disrupters that have been studied to affect androgen exposure after chemical exposure. The inconsistent weak associations and changes in stratified models were limited by the knowledge gap of how physiologic changes in pregnancy affect creatinine so it is unpredictable for both sexes. Another limitation would be sample size since AGD measurements were only used at 12 months since this measurement was the most complete. Additionally urinary TCPy metabolite levels cannot represent all pesticides exposures, but we know that chlorpyrifos was the most abundantly used in Thailand during data collection. Despite its limitations, our study had many strengths which included refined, highly resolved exposure assessments, interrater variability was low and there was a range of exposures. In-depth analysis of dietary data, endocrine-disruption measurements, more AGD measurements within the first years of life could be explored to make this study stronger.

III. Policy Implications

Chlorpyrifos, an organophosphate insecticide, acaricide and miticide, has been used primarily to control foliage and soil-borne insect pests widely in the United States and globally. The United States EPA issued a regulatory final rule revoking all tolerances of chlorpyrifos in 202, meaning there is now no amount of chlorpyrifos residue on food or feed that would be considered safe (Environmental Protection Agency 2021). The final rule of revocation was debated greatly after many objections from other parties, which was complicated more after the agency concluded that despite several years of study, the science addressing neurodevelopmental effects remains unresolved and further evaluation of the science during the remaining time for completion of registration review is warranted, causing more confusion and prolonging of action (Environmental Protection Agency 2021). This legislation has been complicated given that chlorpyrifos insecticides have yet to be canceled by the EPA and farmers are still using them, while there are food products containing residues still being shipped globally and do directly protect workers handling these pesticides. Thailand took similar action when the Ministry of Public Health published the Notification on Food Containing Pesticide Residues in the Royal Gazette to ban paraquat and chlorpyrifos residues on imported food products, in which the maximum residue limits (MRLs) of paraquat and chlorpyrifos will be zero on all products starting on June 1, 2021 (Prasertsri and Chanikornpradit 2020). The ban affected imports of several agricultural commodities whose imports were valued at U.S. $3.1 billion in 2019 (Prasertsri and Chanikornpradit 2020). It was found Thailand came under immense pressure from the U.S after Thailand's ban, even after the U.S administration was already sued for records detailing evidence that the U.S. Department of Agriculture and U.S. trade officials worked closely with the pesticide and processed-food industries to pressure Thailand into reversing its ban

on glyphosate (Donley 2020). The United States government interference acting for the most wealthy, and powerful corporate interests is a concern not only for the future of pesticide regulatory legislation, but for the protection of our health and environment.

Currently, Thailand's farming consists of many informal sectors, there lacks government enforcement and support, especially with the variations to bans and enforcement (Kongtip et al. 2018). These informal sectors allow gaps in the regulations covering pesticide sales allow farmers to purchase and import pesticides without adequate training in their safe use, thus making workers vulnerable to unsafe working conditions. Studies conducted in Thailand addressed knowledge gaps, attitudes, and practices among pregnant women in Northern Thailand, further showing that interventions early on not only can protect these pregnant women, but all agricultural workers exposed to pesticides (Lorenz et al. 2012).

There is a need for more health protective policies related to pesticides and EDCs especially in cases where paraoccipital and prenatal exposure may occur such as agricultural reliant regions such as Thailand or California. It is important to protect people from exposure since do not even know the implications of exposure and the adverse health effects. Understanding the association of pesticide exposure among pregnant women is critical in reducing exposures and mitigating potentially harmful impacts on a developing fetus. Even with this understanding being widely known, there still must be established protection of these workers, considering they often experience pesticide exposure at greater rates due to occupation. Although many agricultural workers know the paraoccupational risk of pesticides, there is still reporting of engaging in risky behaviors, pushing the urgent need for policy interventions in this and similar populations of pregnant women worldwide.

Conclusion

Chlorpyrifos prenatal exposure, as measured by its primary urinary metabolite TCPy, was associated with AGD measurement of infants at 12 months of age. As hypothesized, we noticed a general reduction of AGD in males and a general increase measured in females. These findings suggest that prenatal exposure during these critical development windows may be critical in predicting birth outcomes. Our findings were consistent and aligned with previous studies. By further refining our analysis to account for potential covariates and predictors along with these exposure covariates, such as postnatal exposure and nutrition, we hope to further elucidate chlorpyrifos exposure and AGD as a biomarker of adulthood reproductive health. Long-term research can help further establish and launch preventive programs in the future to protect all agricultural workers.

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Tables and Figures

Table 1. Target Analyte Quality Control Pool Data

Target Analyte	%RSD*	Low Pool		High Pool	
		Mean (ng/mL)	RSD (%)	Mean (ng/mL)	RSD (%)
TCPY	98±7	4.7	3.5	12.1	6.4
RSD=relative standard deviation; *across entire calibration range; TCPY=3,5,6-trichloro-2-pyridinol					

Table 2. Descriptive Characteristics of 104 pregnant farmworker women and their infants, SAWASDEE birth cohort, Chiang Mai, Thailand (2017-2019)

Variable	SAWASDEE (N=104)
Location (n (%))	
Chom Thong	54 (51.9)
Fang	50 (48.1)
Maternal Age at intake (years, mean ± SD)	24.31 ± 5.04
Maternal Height at birth (cm**,** mean ± SD)	152.61 ± 5.40
Infant Sex (n(%))	
Female	56 (56.7)
Male	45 (43.3)
Gestational age Z-Score (Z-scores, mean ± SD)	-0.48 ± 0.78
Household income (Thai Baht[a],) (n(%))	
Greater than or Equal to 6,000 Baht	62 (59.6)
Less than 6,000 Baht	42 (40.4)
Ethnicity (n (%))	
Thai	59 (56.7)
Thai Yai	8 (7.7)
Hill Tribe	30 (28.8)
Other[c]	7 (6.7)
Highest education level (n (%))	
Never attended school	23 (22.1)
Primary school	21 (20.2)
Junior high/High school	34 (32.7)
Some high school	14 (13.5)
High school/technical school and beyond[d]	12 (11.5)
Marital status (n (%))	
Married or living as married[e]	102 (98.1)
Widowed, Separated or Others[f]	2 (1.9)
PON1 Phenotype (n (%))[g]	
Low	14 (13.5)
Normal	41 (39.4)
High	49 (48.1)

Abbreviations: SD, standard deviation.
[a]About 30 Thai Baht is equivalent to 1 United States Dollar
[b]Hill Tribe ethnicities include Hmong, Karen, Akha, Pa-Long, Lahu Na, Lahu Nyi
[c]Other ethnicities include Burmese, Other or NA
[d]High school/technical school & beyond included diploma/technical school equivalent, attended college but did not graduate, college graduate or more
[e]Included Legally married and living as married
[f]Included Widowed, Divorced, Separated, Single

[g]PON1 Phenotype signifies low as having lower protection, normal levels, and high showing more protection

Table 3. Associations between natural log-transformed urinary TCPy concentrations and AGD potential covariates by trimester, SAWASDEE birth cohort (2017-2019).

Coefficient	β estimate (95% Confidence Interval)	P-value	β estimate (95% Confidence Interval)[f]	P-value
Trimester 1[a] (n=99)				
Intercept	-0.53 (-2.17, 1.12)	0.528	-0.15 (-1.99, 1.70)	0.875
Trimester 2[b] (n=103)				
Intercept	0.05 (-1.62, 1.73)	0.950	-1.15 (-0.86, -3.16)	0.258
Trimester 3[c] (n=104)				
Intercept	-1.59 (-3.09, -0.08)	0.039*	-0.36 (-2.06, 1.35)	0.680*
Mean Across Pregnancy Model[d] (n=104)				
Intercept	-1.33 (-3.25, 0.58)	0.170	-0.03 (-2.17, 2.11)	0.979

*Significant at the $p < 0.05$ level.
[a]Adjusted R^2= 0.837 unadjusted & adjusted for creatinine
[b]Adjusted R^2=0.828 unadjusted for creatinine, 0.833 adjusted for creatinine
[c]Adjusted R^2=0.836 unadjusted for creatinine, 0.847 adjusted for creatinine
[d]Adjusted R^2=0.831 unadjusted for creatinine, 0.840 adjusted for creatinine
[f]Adjusted for Natural log-transformed creatinine

Table 4. Associations between natural log-transformed urinary TCPy concentrations and AGD potential covariates by sex, SAWASDEE birth cohort (2017-2019).

Coefficient	**Female** β estimate (95% Confidence Interval)	P-value	**Male** β estimate (95% Confidence Interval)	P-value
Trimester 1[a] (F)n=55)) (M)n=44))				
Intercept	0.52 (-1.94, 2.98)	0.671	-1.59 (-4.02, 0.84)	0.191
Natural log-transformed creatinine	0.64 (-2.16, 3.44)	0.648	-0.94 (-3.77, 1.89)	0.504
Trimester 2[b] (F)n=59)) (M)n=44))				
Intercept	0.97 (-1.37, 3.32)	0.408	-0.64 (-3.46, 2.19)	0.649
Natural log-transformed creatinine	2.28 (-0.48, 5.04)	0.103	-0.06 (-3.74, 3.62)	0.973
Trimester 3[c] (F)n=59)) (M)n=45))				
Intercept	-1.27 (-3.45, 0.92)	0.250	-1.23 (-3.84, 1.39)	0.347
Natural Log-Transformed Creatinine	-0.40 (-2.79, 1.99)	0.739	0.92 (-2.35, 4.19)	0.571
Mean Across Pregnancy Model[d] (F)n=59)) (M)n=45))				
Intercept	-0.29 (-3.00, 2.41)	0.830	-2.02 (-5.24, 1.19)	0.210
Mean natural log-transformed creatinine	0.68 (-2.38, 3.74)	0.657	-0.46 (-4.18, 3.25)	0.801

*Significant at the $p < 0.05$ level.
[a]Adjusted Female R^2= 0.467 unadjusted for creatine, 0.455 adjusted for creatinine; Male Adjusted R^2= 0.480 unadjusted for creatine, 0.477 adjusted for creatinine

[b]Adjusted Female R^2=0.439 unadjusted for creatinine, 0.461 adjusted for creatinine; Male Adjusted R^2= 0.457 unadjusted for creatine, 0.445 adjusted for creatinine

[c]Adjusted Female R^2=0.446 unadjusted for creatinine, 0.466 adjusted for creatinine; Male Adjusted R^2= 0.479 unadjusted for creatine, 0.525 adjusted for creatinine

[d]Adjusted Female R^2=0.431 unadjusted for creatinine, 0.440 adjusted for creatinine; Male Adjusted R^2= 0.490 unadjusted for creatine, 0.512 adjusted for creatinine

Figure 1.

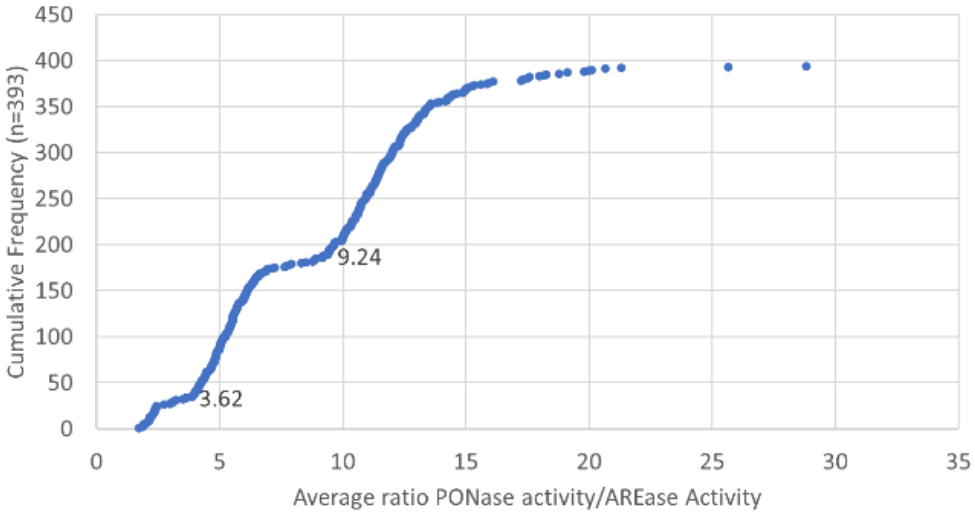

Figure 1. The cumulative frequency of the mean ratio of paraoxonase to arylesterase for each sample (n=3 replicate analyses, total n=393 samples. Cutpoints established for low, medium and high metabolizers were <3.62, ≥ 3.62-<9.24, and ≥9.24, respectively.