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Urinary Glyphosate, 2,4-D and DEET Biomarkers in Relation to Neurobehavioral Performance in Ecuadorian Adolescents in the ESPINA Cohort

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BACKGROUND: Herbicides are the most used class of pesticides worldwide, and insect repellents are widely used globally. Yet, there is a dearth of studies characterizing the associations between these chemical groups and human neurobehavior. Experimental studies suggest that glyphosate and 2,4-dichlorophenoxyacetic acid (2,4-D) herbicides can affect neurobehavior and the cholinergic and glutamatergic pathways in the brain. We aim to assess whether herbicides and insect repellents are associated with neurobehavioral performance in adolescents.

METHODS: We assessed 519 participants (11–17 years of age) living in agricultural communities in Ecuador. We quantified urinary concentrations of glyphosate, 2,4-D, and two N,N-diethyl-meta-toluamide (DEET) insect repellent metabolites [3-(diethylcarbamoyl)benzoic acid (DCBA) and 3-(ethyl-carbamoyl)benzoic acid (ECBA)] using isotope-dilution mass spectrometry. We assessed neurobehavioral performance using 9 subtests across 5 domains (attention/inhibitory control, memory/learning, language, visuospatial processing, and social perception). We characterized the associations using generalized estimating equations and multiple imputation for metabolites below detection limits. Models were adjusted for demographic and anthropometric characteristics, urinary creatinine, and sexual maturation. Mediation by salivary cortisol, dehydroepiandrosterone, 17β-estradiol, and testosterone was assessed using structural equation modeling.

RESULTS: The mean of each neurobehavioral domain score was between 7.0 and 8.7 [standard deviation (SD) range: 2.0–2.3]. Glyphosate was detected in 98.3% of participants, 2,4-D in 66.2%, DCBA in 63.3%, and ECBA in 33.4%. 2,4-D was negatively associated with all neurobehavioral domains, but statistically significant associations were observed with attention/inhibition [score difference per 50% higher metabolite concentration (β) = -0.19 95% confidence interval (CI): -0.31, -0.07], language [$\beta = -0.12$ (95% CI: -0.23, -0.01)], and memory/learning [$\beta = -0.11$ (95% CI: -0.22, 0.01)]. Glyphosate had a statistically significant negative association only with social perception [$\beta = -0.08$ (95% CI: -0.14, -0.01)]. DEET metabolites were not associated with neurobehavioral performance. Mediation by gender and adrenal hormones was not observed.

CONCLUSION: This study describes worse neurobehavioral performance associated with herbicide exposures in adolescents, particularly with 2,4-D. Replication of these findings among other pediatric and adult populations is needed. https://doi.org/10.1289/EHP11383

Introduction

Following the introduction of N-(phosphonomethyl) glycine (glyphosate)-resistant "Roundup ready" crops in 1996 and 2,4-dichlorophenoxyacetic acid (2,4-D)-resistant crops in 2014, there has been a global 15-fold increase in glyphosate use and a substantial increase in 2,4-D use, making them the most widely used herbicides in the world.[1,2] 2,4-D is a broadleaf herbicide, which mimics auxin and kills dicotyledon (dicots) without affecting monocotyledon (monocots),[3] allowing agricultural users to selectively target weeds. Glyphosate is a nonselective, broad-spectrum herbicide that targets the enzyme 5-enolpyruvyl-3-shikimate phosphate synthase and is slow acting.[4–6] It is generally used in agriculture to control vegetation by damaging growth or by acting as a desiccant.[3,5] Insect repellants, like N,N-diethyl-meta-toluamide (DEET), are also widely used, as an estimated 2.6 to 4.5 million pounds of DEET are produced or imported annually in countries like the United States.[7] In a random sample of 40 homes in the Ecuadorian coastal region, 32% of homes used DEET-based repellents for vector control.[8] There is a considerable use of herbicides and insect repellants in South America due to the growing agricultural and floricultural industries,[9] which can result in increased exposure to these chemicals in both occupational and nonoccupational populations.

In mice models, subchronic and chronic exposure to glyphosate-based herbicides from *in utero* to adulthood resulted in neurobehavioral changes, such as decreased locomotor activity, impaired recognition memory, cognitive function alterations, and increased levels of anxiety and depression.[10–13] Adult zebrafish exposed to chronic, low environmentally relevant concentrations of glyphosate over 2 weeks showed increased anxiety and increased levels of lipid peroxidation, a marker of oxidative stress.[14] Doses of 2,4-D or 2,4-D esters have also been linked to behavioral changes and neurological toxicity (depressed locomotor activity, circling behavior, increased limb grip strength, increased landing foot splay) along with altered serotonin and dopamine levels in rats.[15–18] Furthermore, when zebrafish larvae are exposed to minimal doses of 2,4-D, it leads to changes in their swimming behaviors.[19] As for DEET, rats given a single oral dose of 500 mg/kg, a toxic level of exposure, developed increased thermal response time and increased exploratory behavior.[20] These neurobehavioral changes did not occur at lower exposures, even with consistent exposure across a 14-d period.[20] Additionally, dermal application of 400 mg/kg of DEET, a concentration comparable to a human exposure dose, on adult male rats led to reduced neurobehavioral performance of sensorimotor functions, such as beam-walking score and grip response assessments.[21,22] Worse performance was observed when coupled with permethrin or malathion exposure.[21,22]

Despite compelling data in animal models, epidemiological evidence in humans that correlates 2,4-D, glyphosate, or DEET

exposure with cognitive effects is sparse. For glyphosate, a cross-sectional study of 288 Ugandan farmers identified a positive association between exposure, assessed via pesticide-specific yearly exposure-intensity scores based on self-reported glyphosate use, and impaired visual memory [−0.103; 95% Bayesian credible interval (BCI): −0.24, 0]; units decrease in Benton visual retention test scores per interquartile range increase in annual glyphosate exposure.[23] Prenatal exposure to glyphosate, assessed by calculating pounds per acre applied within a 2,000-m radius of mothers' homes using data from the California Pesticide Use Reporting (CA-PUR) program, was associated with elevated risk of autism spectrum disorder (ASD) [odds ratio (OR) = 1.16; 95% confidence interval (CI): 1.06, 1.27] and ASD with intellectual disability [OR = 1.33 (95% CI: 1.05, 1.69)].[24,25] For 2,4-D, no associations were reported between urinary metabolites of 2,4-D and neurobehavioral performance in a representative sample of adults in the United States.[26] Per unit increase in log based 10 concentrations of 2,4-D, there was no statistically significant changes for simple reaction time ($\beta = 3.86$, $p = 0.62$), symbol digit substitution mean total latency ($\beta = -0.93$, $p = 0.44$), and number of errors ($\beta = 0.0007$, $p = 0.99$), or the serial digit learning trials to criterion ($\beta = -0.39$, $p = 0.19$) and total score ($\beta = -1.03$, $p = 0.10$). However, in a study of 232 infants from rural Southeast China, participants who had umbilical cord blood plasma concentrations of 2,4-D over 1.17 ng/mL had deficits in auditory processing measured by slower wave V latencies [0.12 milliseconds (95% CI: 0.03, 0.22)] and auditory brainstem response central conduction time [0.15 milliseconds (95% CI: 0.05, 0.25)] compared to infants with nondetectable concentrations.[27] Another study found that workers of a 2,4-D plant had decreased nerve conduction velocity compared to manufacturing plant workers that had no direct contact with 2,4-D (mean = 34.0 vs. 40.1 m/s, $p < 0.02$).[28] While nerve conduction velocity may not correlate with central nervous system (CNS) development, these findings highlight the neurotoxic potential of 2,4-D exposures. DEET exposure has been associated with neurological changes, including impaired cognitive functioning, agitation, and aggressive behavior in humans.[29] More severe neurological symptoms related to DEET exposure, such as seizures and encephalopathy, have been observed in children who experienced high exposures according to clinical reports and Poison Control Center records.[30,31] Currently, data on the effects of herbicide and DEET exposure on neurobehavioral or cognitive performance among adolescents are lacking.

Herbicide and insect repellent exposures could potentially lead to neurobehavioral and cognitive changes through multiple mechanisms. Glyphosate exposure may lead to membrane depolarization by increasing entry of sodium and calcium ions from the extracellular medium through overstimulation of N-methyl-D-aspartate glutamatergic receptors (NMDAR), inhibition of acetylcholinesterase (AChE), or increased expression of the Wnt-5a mRNA.[32] Glyphosate also increases intracellular reactive oxygen species concentrations that are not sufficiently counteracted by endogenous antioxidants.[32] Animal model studies have shown that 2,4-D exposure during sensitive periods of development may lead to lower myeline deposition and numbers.[27] Proper cortical myelination has been found to be important to cognitive development and brain plasticity.[33,34] In insects, DEET affects odorant receptors as well as olfactory and gustatory receptor neurons and can impact the peripheral nervous system by inducing neuroexcitation and toxicity and reversibly inhibiting AChE in insects and mammals.[31,35–38] DEET's effects may lead to adverse neurologic effects in humans through a similar mechanism.[31]

The endocrine system is crucial to the proper development of the CNS and human behavior.[39–41] Considering that glyphosate and 2,4-D have endocrine disrupting characteristics,[39,42–50] endocrine changes induced by 2,4-D or glyphosate may mediate neurobehavioral alterations associated with those exposures.[39,42] There has been no evidence of endocrine alterations in humans due to DEET. In animal studies, intermediate and chronic oral exposure to DEET did not result in alterations of the adrenal, pituitary, thyroid, or parathyroid glands.[29] Our prior work with adolescents in agricultural communities suggested that sex and adrenal hormones accounted for a portion of the effect modification seen by sex on the association between AChE activity, a marker of cholinesterase inhibitor pesticide exposure (i.e., organophosphate and carbamate insecticides), and mood symptoms in adolescents.[51] In the same cohort, elevated testosterone [OR = 1.78 (95% CI: 0.98, 3.23)], cortisol [OR = 1.69 (95% CI: 0.95, 2.99)], and estradiol [OR = 2.43 (95% CI: 1.01, 53.84)] concentrations were associated with altered anxiety, while elevated estradiol concentration [OR = 4.75 (95% CI: 1.95, 11.56)] was associated with depression symptoms.[52]

The aim of our study is to characterize the associations of urinary concentrations of glyphosate, 2,4-D, and metabolites of DEET [3-(diethylcarbamoyl) benzoic acid (DCBA) and 3-(ethylcarbamoyl) benzoic acid (ECBA)] with neurobehavioral performance in Ecuadorian adolescents and whether these associations are mediated by gonadal and adrenal hormones.
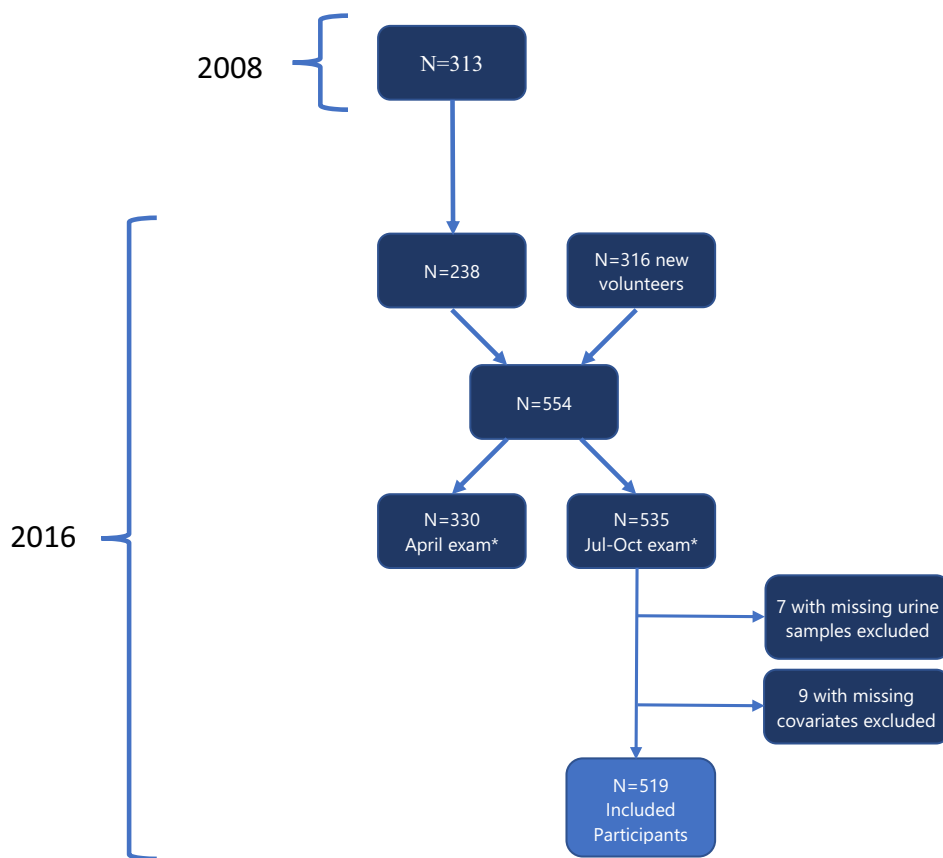
Methods

Participants

Established in 2008, the Secondary Exposures to Pesticides among Children and Adolescents (ESPINA) study is a prospective cohort study that examines the associations of subclinical pesticide exposures on human development. In 2008, 313 boys and girls 4–9 years of age living in Pedro Moncayo County, Pichincha province, Ecuador, were examined. Most participants (n = 228, 73%) were recruited using data from the 2004 Survey of Access and Demand of Health Services (SADHS), a representative survey of the County conducted by collaboration of Fundación Cimas del Ecuador, the Local Rural Governments of Pedro Moncayo, and community members. The remaining participants (n = 85, 27%) were enrolled through community announcements given by governing councils, leaders, and by word-of-mouth. Recruitment aimed to achieve a balanced distribution of participants who lived with a flower plantation worker and participants who did not live with any agricultural workers. Further details of the 2008 examination have been published.[53]

A participant flow chart can be found in Figure 1. In 2016, we conducted two follow-up examinations of participants 11–17 years of age: *a*) April, which included 330 participants (11–17 years of age) and *b*) July to October, which included 535 participants (comprising 238 participants examined in 2008, 330 examined in April 2016, and 316 new volunteers). As in 2008, new participants were recruited using the System of Local and Community Information (SILC), a large geospatial database that contains information of the 2016 Pedro Moncayo County Community Survey (formerly the SADHS). This community health survey was administered in-person at participants' homes and captures data from ∼ 50% of the population living in Pedro Moncayo County. The present analysis included participants examined in the July to October examination, of whom we included 519 who had information for all covariates of interest (528 participants had urinary pesticide metabolite measures, and 9 had missing covariate information).

We acquired informed consent for participation from parents and obtained parental permission and child assent for participating. This study was approved by the institutional review boards at the University of California San Diego (UCSD), Universidad

2008 — N=313

2016 — N=238, N=316 new volunteers → N=554 → N=330 April exam*, N=535 Jul-Oct exam*

7 with missing urine samples excluded

9 with missing covariates excluded

N=519 Included Participants

* 311 participants examined in both the April and July-October examinations in 2016

Figure 1. Participant flow chart with sample sizes between 2008 and 2016 of participants included in the present analyses of the ESPINA study. $n = 311$ of 330 participants examined in April 2016 also participated in the July to October 2016 examinations. Note: ESPINA, Secondary Exposures to Pesticides among Children and Adolescents.

San Francisco de Quito, and the Ministry of Public Health of Ecuador.

Setting

Pedro Moncayo County is located in the Ecuadorian Andes and houses a large floriculture industry that is vital to the County's economy. It employs 21% of all adults,[53] and its greenhouse floricultural crop land comprises 4.47% of the geographic area [1,495 hectares (ha)]. Flower crops in Pedro Moncayo are sprayed with over 50 different fungicides and over 20 different insecticides by workers using hand sprayers.[54,55] Other crops in the County exposed to either herbicides, insecticides, and/or fungicides include corn, wheat, barley, potatoes, strawberries, and leafed greens. In Ecuador in 2021, there were 130 glyphosate-based herbicides and 31 2,4-D-based herbicides registered.[56] In 2016, it was estimated that an average of 16.25 kg of pesticides are applied per hectare of agricultural land in Ecuador demonstrating its high use in the region.[57] Given the setting of Pedro Moncayo, potential pathways of pesticide exposure that may influence urinary pesticide concentrations include pesticide drift from pesticide-treated agricultural crops (flowers, wheat, corn, soybean, barley, etc.) onto populations nearby, cohabitation with an agricultural worker (para-occupational exposure), residential pesticide use, or contact with contaminated sources (i.e., dust, water, food).[58] All participants at the time of the ESPINA assessment reported not working in agriculture, thus occupational exposure of pesticides is not or rarely anticipated in this population.

Examination

In 2016, children were examined in schools twice, first in April and again between July and October during the summer closure or during weekends. Of those who were examined in April, 311 participants were also examined in the July to October exam. We identified whether participants completed the attention & inhibitory domain assessment from the April 2016 examination to assess for retest learning effect (described in Statistical Analysis), but otherwise only data collected during the July to October examination was included in the analysis. Examiners were kept blind to participants' pesticide exposure status. Interviews of participants' caregivers collected information on demographics, monthly salary, parental education, and participant education. Children's height was measured following recommended procedures to the nearest 1 mm,[59] using a height board, and weight was measured using a digital scale (Tanita model 0108 MC; Corporation of America, Arlington Heights, IL, USA). Sexual maturation rating (SMR) was assessed using Tanner Staging based on self-reported breast size and pubic hair growth/distribution for girls and pubic hair growth/distribution for boys, using modified Tanner drawings from Rasmussen et al. as a reference.[60–62] There was only one participant who identified as being White. Therefore, race and ethnicity were combined and dichotomized into White or Mestizo and Indigenous categories. Erythrocytic AChE activity and hemoglobin concentration were measured from a single finger stick sample using the

EQM Test-mate ChE Cholinesterase test system 400 and AChE Erythrocyte Cholinesterase assay kit 470 (EQM, Cincinnati, OH, USA).[53] The distance between each participant's home to the nearest flower plantation, and the surface area of flower plantations within a 150-m buffer of the participant's home was calculated using ArcGIS 9.3 (ESRI, Redlands, CA, USA).[53,63]

Neurobehavioral testing. Neurobehavioral performance was measured using the NEPSY-II test (NCS Pearson, San Antonio, TX).[64] During the July to October 2016 examination, trained psychologists blinded to participant exposure status tested participants in 9 subtests across five domains as follows: *a*) attention & inhibitory control (also known as attention and executive functioning, subtests: auditory attention & response set, inhibition); *b*) language (subtests: comprehension of instructions, speeded naming); *c*) memory & learning (subtests: immediate and delayed memory for faces); *d*) visuospatial processing (subtests: design copying, geometric puzzles); and *e*) social perception (subtest: affect recognition). Two subtests required translation into Spanish using terminology appropriate for the local population (auditory attention and response set and comprehension of instructions). The translation was approved by NCS Pearson. Participants were examined alone in a quiet room by the examiner. Attention & inhibitory control were also assessed in April 2016 among 303 participants included in the present analyses. NEPSY-II scaled scores for each subtest can range from 1 to 19, and have a mean of 10 [standard deviation (SD) = 3] that is based on a U.S. normative sample.[65,66]

NEPSY scaled scores for each subtest were used in analyses; these values are age-standardized based on a national sample of children in the United States.[66] Scaled scores for the NEPSY subtest were calculated using the NEPSY-II scoring assistant software (NCS Pearson, Inc., San Antonio, TX), and higher scores indicate better performance for all subtests. Domain scores were calculated by averaging primary scaled score from all subtests within each domain. For subtests that included either correct and error components (i.e., auditory attention and response set) or time and error components (i.e., inhibition, speeded naming, visuomotor precision), the combined scaled scores representing the combination of both components were used as primary scaled scores. Affect recognition was the only subtest in the social perception domain and, as such, the social perception domain is equivalent to the affect recognition scaled score. Additional details of subtest scoring have been published elsewhere.[66–68]

Quantification of urinary creatinine, pesticide biomarkers, and hormones. Urine samples used for measuring creatinine and pesticide biomarkers and saliva samples used for measuring hormones were collected on the same day for each participant during the July to October examination. Urinary concentrations of creatinine and pesticide biomarkers were measured in samples collected upon awakening. Participants brought the urine samples to the examination site in the morning where they were aliquoted and frozen at −20°C. At the end of each day, samples were transported to Quito for storage at −80°C. Samples were then transported overnight to UCSD at −20°C using a courier and stored at −80°C at UCSD. Samples were then shipped overnight at −20°C from UCSD to the National Center for Environmental Health, Division of Laboratory Sciences of the CDC (Atlanta, GA) for quantification of 2,4-D, 3,5,6-trichloro-2-pyridinol (TCPy) and para-nitrophenol (PNP), DCBA, and ECBA, and to the Laboratory for Exposure Assessment and Development in Environmental Research at Emory University (Atlanta, GA) for quantification of glyphosate and creatinine. Quality control/quality assurance protocols were followed to ensure data accuracy and reliability of the analytical measurements. All of the study samples were reextracted if quality control failed the statistical evaluation.[69]

Glyphosate. Urine aliquots (250 μL) were spiked with isotopically labeled glyphosate, diluted to 1 mL with doubly deionized water, and extracted using a C18 solid phase extraction (SPE). Glyphosate was derivatized to create its heptafluorobutyl analogue then concentrated for analysis. To measure urinary glyphosate, all urine samples (aliquots) were randomized using a Fisher-Yates shuffling algorithm prior to analysis to reduce potential batch effects.[70,71] Concentrated extracts were analyzed using gas chromatography–mass spectrometry using electron impact ionization in the multiple ion monitoring mode. The limit of detection (LOD) was 0.25 μg/L with a relative standard deviation (RSD) of 3%.

DEET metabolites. ECBA and DCBA were quantified in urine using a method described in detail elsewhere.[69] In brief, the analytical method is based on enzymatic hydrolysis of 0.2 mL of urine and online SPE to release, extract, and concentrate the target biomarkers, followed reverse-phase high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) using electrospray ionization (ESI). The LOD for ECBA and DCBA was 0.2 μg/L. The precision of the measurements, expressed as the percent RSD of multiple measures of two urine-based quality control (QC) materials, was below 6%, depending on the biomarker and concentration.

2,4-D, TCPy, and PNP. These biomarkers were extracted from 1.0 mL of urine and concentrated using a semi-automated SPE system, followed by HPLC-MS/MS as described in detail.[72] The method LOD for 2,4-D, TCPy and PNP were 0.15 μg/L, 0.1 μg/L, and 0.1 μg/L, respectively, and the precision, calculated as described above, was below 7% RSD. PNP and TCPy concentrations were only used to improve the multiple imputation estimates for biomarker concentrations that were below the LOD (see below).

Creatinine. Urinary creatinine was quantified using HPLC-MS/MS with ESI. A 10-μL aliquot of urine was diluted prior to analysis.[73] No further sample preparation was performed prior to analysis. The LOD was 5 mg/dL with an RSD of 7%.

Hormones. Salivary concentrations of estradiol, testosterone, cortisol, and dehydroepiandrosterone (DHEA) were measured using enzyme-linked immunosorbent assays (ELISA) (Salimetrics, Carlsbad, CA) at the UCSD Integrative Health and Mind-Body Biomarker Laboratory.[74–77] Participants collected saliva samples through passive-drool upon awakening the same day they provided urine samples for metabolite collection. The saliva samples were stored at −80°C until assayed. Levels of cortisol, testosterone, and DHEA were measured in both girls and boys, while estradiol was only measured in boys, as the levels of estradiol in women vary according to the stage of the menstrual cycle.

Imputation for values below the LOD. We used two imputation methods for pesticide metabolite concentrations below the LOD as follows: imputation using a constant (LOD/$\sqrt{2}$) and multiple imputation. Imputation was needed for 1.7% ($n = 9$) of samples for glyphosate, 33.8% ($n = 174$) of samples for 2,4-D, 36.4% ($n = 192$) of samples for DCBA, and 66.6% ($n = 342$) of samples for ECBA. The multiple imputation method was built as a log-logistic regression model that was fitted using backward elimination selection where variables were retained if they had a significance level of $p < 0.10$. We used the backward elimination since the forward or stepwise selection generally yields incorrect results.[78] The initial model used for backwards stepwise regression included the following 15 variables defined *a priori* that were considered to be associated with pesticide exposure: age, gender, race, body mass index (BMI)-for-age *z*-score (*z*-BMI-for-age), height-for-age *z*-score (*z*-height-for-age), monthly family income, tanner maturation score, creatinine concentration, AChE activity, urinary concentrations of TCPy and PNP, flower crop

Table 1. Participant characteristics of the July to October 2016 ESPINA study examination ($n = 519$) based in Pedro Moncayo, Ecuador.

	Overall		Tertile 1 (0.17–0.75)		Tertile 2 (0.75–1.32)		Tertile 3 (1.32–9.09)			
	n	%	n	%	n	%	n	%	p-Trend	Detectable
Cohort	519	100.0%	173	33.3%	173	33.3%	173	33.3%	—	—
Gender									0.02	—
Female	266	51.3%	99	57.2%	90	52.0%	77	44.5%	—	—
Male	253	48.7%	74	42.8%	83	48.0%	96	55.5%	—	—
Race									0.26	—
Indigenous	114	22.0%	33	19.1%	39	22.5%	42	24.3%	—	—
Mestizo or White	405	78.0%	140	80.9%	134	77.5%	131	75.7%	—	—
Lives with an agricultural worker									0.49	—
No	169	32.6%	59	34.1%	54	31.2%	56	32.4%	—	—
Yes	350	67.4%	114	65.9%	119	68.8%	117	67.6%	—	—
Examination period									0.56	—
July through September	492	94.8%	165	95.4%	161	93.1%	166	96.0%	—	—
October	27	5.2%	8	4.6%	12	6.9%	7	4.0%	—	—
Completed attention & inhibitory domain in April examination									0.40	—
Yes	303	58.4%	94	54.3%	106	61.3%	103	59.5%	—	—
No	216	41.6%	79	46%	67	38.7%	70	40.5%	—	—
Mother worked in agriculture while pregnant									0.77	—
Yes	222	43.1%	82	48.0%	69	40.0%	71	41.3%	—	—
No	287	56.9%	89	52.0%	101	60.0%	97	58.7%	—	—
Missing	10	—	2	—	3	—	5	—	—	—
Mother lived with agricultural worker while pregnant									0.36	—
Yes	221	43.4%	70	40.9%	71	41.8%	80	47.6%	—	—
No	288	56.6%	101	59.1%	99	58.2%	88	52.4%	—	—
Missing	10	—	2	—	3	—	5	—	—	—

	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	—	—
Age (y)	519	14.46 ± 1.76	173	14.69 ± 1.78	173	14.26 ± 1.75	173	14.44 ± 1.73	0.14	—
Z-score BMI for age (SD)	519	0.38 ± 0.84	173	0.50 ± 0.87	173	0.35 ± 0.84	173	0.30 ± 0.80	0.05	—
Z-score height for age (SD)	519	−1.49 ± 0.90	173	−1.51 ± 0.83	173	−1.43 ± 0.88	173	−1.54 ± 0.99	0.99	—
Tanner score	519	2.89 ± 0.95	173	2.98 ± 0.93	173	2.80 ± 1.03	172	2.88 ± 0.89	0.63	—
Crop areas within 150 m of homes (m²)	519	2,054 ± 4,823	173	2,187 ± 4,900	173	1,971 ± 4,745	173	2,003 ± 4,848	0.50	—
Acetylcholinesterase (U/mL)	519	3.70 ± 0.55	173	3.69 ± 0.56	173	3.69 ± 0.54	173	3.73 ± 0.54	0.92	—
Hemoglobin (mg/dL)	519	12.95 ± 1.18	173	12.93 ± 1.30	173	12.96 ± 1.01	173	12.96 ± 1.20	0.91	—

	n	Median (25th–75th percentile)	n	Median (25th–75th percentile)	n	Median (25th–75th percentile)	n	Median (25th–75th percentile)	—	—
Testosterone (pg/mL)	515	39.63 (26.04–67.25)	172	37.22 (25.24–58.38)	170	40.26 (24.87–62.08)	173	44.24 (27.58–81.23)	0.02	—
17β-Estradiol (pg/mL)	247	0.43 (0.30–0.59)	72	0.45 (0.30–0.60)	81	0.42 (0.25–0.55)	94	0.43 (0.32–0.59)	0.42	—
Cortisol, μg/dL	518	0.21 (0.14–0.30)	173	0.20 (0.14–0.29)	172	0.18 (0.11–0.29)	173	0.23 (0.15–0.32)	0.45	—
Dehydroepiandrosterone (pg/mL)	498	61.21 (30.24–105.91)	168	70.27 (34.09–112.83)	165	51.36 (26.92–100.60)	165	63.36 (34.00–103.02)	0.61	—
Monthly income (imputed) (USD)[a]	519	$500 ($372–$720)	173	$500 ($370–$720)	173	$500 ($375–$700)	173	$560 ($370–$732)	0.65	—
Distance to nearest floriculture field (m)	519	329 (111–659)	173	332 (107–645)	173	334 (111–611)	173	307 (118–685)	0.08	—
Average parental education (imputed) (y)[b]	519	7.00 (6.00–10.50)	173	8.00 (6.00–10.50)	173	7.00 (6.00–10.50)	173	7.00 (6.00–9.50)	0.45	—
Participant education level (y)	519	9.00 (8.00–11.00)	173	10.0 (9.00–11.00)	173	9.00 (8.00–10.00)	173	9.00 (8.00–10.00)	0.04	—

	n	Geo Mean (95% CI)	n	Geo Mean (95% CI)	n	Geo Mean (95% CI)	n	Geo Mean (95% CI)	—	Detectable
Creatinine (mg/dL)	519	87.7 (83.3, 92.3)	173	64.7 (59.2, 70.7)	173	85.1 (78.7, 92.0)	173	122.7 (114.0, 132.0)	<0.001	—
2,4-D (μg/L)	344	0.35 (0.33, 0.38)	63	0.22 (0.21, 0.24)	127	0.27 (0.25, 0.29)	154	0.53 (0.46, 0.60)	<0.001	—
Multiple imputation	518	0.25 (0.24, 0.27)	173	0.19 (0.17, 0.21)	172	0.24 (0.22, 0.27)	173	0.36 (0.31, 0.42)	<0.001	—
2,4-D (μg/g of creatinine)[c]	518	0.27 (0.25, 0.29)	173	0.21 (0.20, 0.23)	172	0.25 (0.22, 0.27)	173	0.36 (0.31, 0.42)	<0.001	66.2%
Missing	1	—	0	—	1	—	0	—	—	—
Above LOD	343	0.34 (0.31, 0.37)	63	0.28 (0.24, 0.31)	127	0.29 (0.27, 0.33)	154	0.41 (0.35, 0.48)	<0.001	—
Below LOD	174	—	110	—	45	—	19	—	—	—
Interfering substances	0	—	0	—	0	—	0	—	—	—
Glyphosate (μg/L)	510	0.87 (0.79, 0.95)	165	0.29 (0.26, 0.32)	173	0.91 (0.84, 0.98)	172	2.40 (2.15, 2.68)	<0.001	—
Glyphosate (μg/g of creatinine)[c]	519	0.92 (0.83, 1.01)	173	0.38 (0.32, 0.45)	173	1.07 (0.96, 1.20)	173	1.90 (1.67, 2.17)	<0.001	98.3%

Table 1. (*Continued.*)

	n	Geo Mean (95% CI)	n	Geo Mean (95% CI)	n	Geo Mean (95% CI)	n	Geo Mean (95% CI)	—	Detectable
Above LOD	510	—	165	—	173	—	172	—	—	—
Below LOD (n)	9	—	8	—	0	—	1	—	—	—
Interfering substances (n)	0	—	0	—	0	—	0	—	—	—
ECBA (μg/L)	176	1.54 (1.20, 1.99)	52	0.24 (0.74, 1.72)	60	1.80 (1.11, 2.92)	109	1.72 (1.13, 2.61)	0.62	—
Multiple imputation	518	0.17 (0.14, 0.20)	173	0.17 (0.13, 0.22)	172	0.19 (0.14, 0.26)	173	0.15 (0.31, 0.42)	0.72	—
ECBA (μg/g of creatinine)[c]	518	0.36 (0.32, 0.41)	173	0.41 (0.34, 0.50)	172	0.39 (0.31, 0.51)	173	0.29 (0.23, 0.37)	0.33	33.4%
Missing	1	—	0	—	1	—	0	—	—	—
Above LOD	176	1.49 (1.15, 1.93)	52	1.58 (1.02, 2.44)	60	1.74 (1.07, 2.84)	64	1.23 (0.81, 1.89)	0.33	—
Below LOD	342	—	121	—	112	—	109	—	—	—
Interfering substances	0	—	0	—	0	—	0	—	—	—
DCBA (μg/L)	319	1.25 (1.04, 1.50)	104	0.98 (0.73, 1.32)	103	1.44 (1.02, 2.05)	112	1.37 (1.00, 1.88)	0.64	—
Multiple imputation	511	0.38 (0.31, 0.45)	172	0.40 (0.29, 0.53)	171	0.39 (0.29, 0.54)	168	0.34 (0.24, 0.47)	0.73	—
DCBA (μg/g of creatinine)[c]	511	0.63 (0.55, 0.73)	172	0.71 (0.57, 0.89)	171	0.66 (0.51, 0.85)	168	0.53 (0.41, 0.68)	0.28	63.3%
Missing	1	—	0	—	1	—	0	—	—	—
Above LOD	319	1.22 (1.01, 1.47)	104	1.34 (0.98, 1.81)	103	1.40 (0.99, 1.99)	112	0.99 (0.72, 1.36)	0.28	—
Below LOD	192	—	68	—	68	—	56	—	—	—
Interfering substances	7	—	1	—	1	—	5	—	—	—

Note: Values presented are *n* (percent), mean ± SD, median (25th percentile to 75th percentile), or geometric mean (95% CI). The herbicide summary score was calculated using the following steps. For each biomarker (2,4-D and glyphosate), observations below the LOD were imputed with a constant (LOD divided by the square root of two). One was added to the variable and was natural log-transformed. The log-transformed concentrations were divided by the group's standard deviation and then averaged with biomarkers of the same classification. We calculated the *p*-value for trend (*p*-trend) for participant characteristics across tertiles of the herbicide summary score using unadjusted linear regression for continuous variables (continuous participant characteristic = herbicide summary score) and unadjusted logistic regression for categorical variables (categorical participant characteristic = herbicide summary score). —, no data; 2,4-D, 2,4-dichlorophenoxyacetic acid; BMI, body mass index; CI, confidence interval; DCBA, 3-(diethylcarbamoyl)benzoic acid; DEET, N,N-diethyl-meta-toluamide; dL, deciliter; ECBA, 3-(ethylcarbamoyl)benzoic acid; ESPINA, Secondary Exposures to Pesticides among Children and Adolescents; g, gram; Geo mean, geometric mean; L, liter; LOD, limit of detection; m, meter; mL, mililiter; pg, picogram; SD, standard deviation; U, units; μg, microgram; USD, U.S. dollars; y, years.

[a]Household income was imputed for 11 adolescents. Five of these adolescents had household income imputed from what was reported from the 2008 examination. For the remaining 6 adolescents, income was imputed based on the average parental education (years). We calculated the cohort's mean ± SD income in 2016 for each year of average parental education. For those with missing income, we imputed the value based on a random normal distribution of the corresponding parental education.

[b]Average parental education was imputed using the reported education from 2008 to impute father's education for four participants and to impute mother's education for five participants. For the remaining four participants with missing paternal education and three participants with missing maternal education, we used a random imputation based on a normal distribution of paternal education and maternal education, respectively. The average parental education was then calculated from the imputed values.

[c]Creatinine adjusted. Values below the LOD were imputed using a constant (LOD divided by the square root of two). If a variable had missing observations, the sample size was provided. Missing was not used to calculate percentages.

area within 150 m of the participant's home, distance from the house to the contour of the nearest flower crop, and cohabitation with a floricultural or agricultural worker. Prediction models using multiple imputation were run 1,000 times (see Statistical Analysis). The observed pesticide biomarker concentrations (concentrations above LOD) and the multiple imputed concentrations for samples with censored data were included in the generalized estimated equation (GEE) models as separate variables. This maintained the robustness of observed variables, while allowing us to improve observations and power of the overall analysis. Given the high detectability of glyphosate, multiple imputation was not conducted for this biomarker.

Imputation for missing variables. We imputed missing information for parental education and household income to maximize the observations included in this study. Average parental education was imputed for eight adolescents. First, we used the reported education from 2008 to impute father's education for four participants and to impute mother's education for five participants. For the remaining four participants with missing paternal education, and three participants with missing maternal education, we used a random imputation based on a normal distribution of paternal education and maternal education, respectively. The average parental education was then calculated from the imputed values. Household income was imputed for 11 adolescents. Five of these adolescents had household income imputed from what was reported from the 2008 examination. For the remaining 6 adolescents, income was imputed based on the average parental education (years). We calculated the cohort's mean ± SD income in 2016 for each year of average parental education. For those with missing income, we imputed the value based on a random normal distribution of the corresponding parental education. Methods used for parental education and income imputation have been previously reported.[52]

Statistical analysis. This is a cross-sectional study that used data from the ESPINA July to October 2016 examination. Descriptive statistics were calculated across tertiles of the herbicide summary score (Table 1). We calculated means and SD for normally distributed variables, medians, and interquartile ranges (IQR) for nonnormally distributed variables, or column percentage for categorical variables for model covariates. All variables identified to be nonnormally distributed were natural log (ln)-transformed. We calculated the *p*-value for trend (*p*-trend) for participant characteristics across tertiles of the herbicide summary score using unadjusted linear regression for continuous variables (continuous participant characteristic = herbicide summary score) and unadjusted logistic regression for categorical variables (categorical participant characteristic = herbicide summary score). Characteristics were identified to be statistically significantly different across the herbicide summary score if it had a $p < 0.05$. For characteristics that differed across herbicide summary score, we ran an adjusted GEE to further assess whether there were still differences. To evaluate how representative of the population of Pedro Moncayo our study sample was in terms of age, gender, and cohabitation with agricultural workers, we compared ESPINA distributions with those of the 2016 Community Health Survey described above. We calculated geometric means of creatinine adjusted concentrations for 2,4-D, glyphosate, DCBA, and ECBA concentrations (μg/g) that were imputed using a constant (LOD/√2). We compared the biomarker concentrations to those that are presented in the National Health and Nutrition Examination Survey (NHANES) biomonitoring data to determine how exposure patterns differed from our cohort. 2,4-D, glyphosate, and DEET biomarkers were measured in the general population of adolescents in the United States from 2013 to 2014 or 2015 to 2016.[79] NHANES biomarker concentration was measured in 428 individuals for 2,4-D (2013–2014), 309 individuals

for glyphosate (2015–2016), 400 individuals for DCBA (2015–2016), and 399 individuals for ECBA (2015–2016). Methods used to measure 2,4-D,[72] glyphosate,[80] ECBA,[69] and DCBA[69] biomarkers in the NHANES study have been previously reported. Biomarker concentrations were compared to NHANES participants 12–19 years of age. In order to assess whether results are influenced by co-exposures to other pesticides, we conducted a Pearson correlation matrix of 2,4-D, glyphosate, DCBA, and ECBA with acetamiprid-N-desmethyl (AND) (neonicotinoid), PNP (organophosphate), TCPy (organophosphate), and 3-phenoxybenzoic acid (3-PBA) (pyrethroid).

Summary scores for herbicide and DEET were calculated using the following steps. For each biomarker, observations below the LOD were imputed with a constant (LOD divided by the square root of two[81]). One was added to the variable and was natural log-transformed. The log-transformed concentrations were divided by the group's standard deviation and then averaged with biomarkers of the same classification. For the DEET summary score, ECBA and DCBA were combined while for the herbicide summary score, 2,4-D, and glyphosate were combined.

GEE models were used to analyze the relationship between each pesticide biomarker and summary score with the 5 NEPSY-II domains: attention & inhibitory control, language, memory & learning, visuospatial processing, and social perception. Confounders were identified *a priori* and were first assessed using a directed acyclic graph (DAG), and visualized using Daggity.[82] Confounders that were considered included age (continuous in years), gender [male, female (reference)], race [Indigenous, White, or Mestizo (reference)], z-BMI-for-age (continuous), z-height-for-age (continuous), hemoglobin concentration (continuous), urinary creatinine concentration (continuous), tanner score (continuous), monthly salary (continuous in United States dollar), average parental education (continuous in years), participant education level (continuous in years), examination date, and living with an agricultural worker [yes, no (reference)]. In addition to using a DAG to identify potential confounders, and to limit parameters of our model, additional testing was conducted to determine if confounders were necessary such as using a generalized linear model or 10% change in estimate criteria. Further details are described below.

Using a directed acyclic graph (Figure S1), we achieved confounding control by adjusting for included age, gender, race, z-BMI-for-age, urinary creatinine concentration, monthly salary, and parental education. Age and gender helped to control for social determinants of pesticide exposure and neurobehavior, as pesticide exposure and performance on NEPSY-II may vary by age and gender. Differences in pesticide exposure and neurobehavior have been observed across different racial groups.[83] Z-BMI-for-age is included as a marker of chronic and subacute nutritional status, and has been associated with neurobehavioral deficits.[54] Z-BMI-for-age may also influence herbicide and DEET exposure, as adipose tissue is a known site of pesticide accumulation.[84] Creatinine concentration helps to adjust for urine dilution when measuring pesticide metabolite concentrations.[85] Monthly salary was adjusted in the model to control for confounding by economic factors.

Variables that were considered to be confounders but were identified to be unnecessary (Figure S1) include hemoglobin concentration, z-height-for-age, tanner staging, participant education level, living with an agricultural worker, examination date, and prenatal pesticide exposure. For hemoglobin concentration, iron deficiency has been inversely related to cognitive function and may have a negative impact on the developing brain[86] but has not been known to influence pesticide exposure. Tanner staging was identified to be a mediator in our DAG, as pesticide exposure may influence puberty development[87] and hormonal changes caused by puberty can affect neurobehavior.[88] Participant education level was found to be highly

correlated with age ($r = 0.81$, $p < 0.0001$). We only included age in our models to avoid collinearity with participant education; however, age in this study population can control for confounding by education, although with some amount of residual confounding. Living with an agricultural worker is a proxy pesticide exposure measure from take-home exposure. However, as this is an alternative pesticide exposure construct, it would introduce bias by partially adjusting for our exposure of interest. There was concern that participants would perform differently on neurobehavioral tests if they were evaluated during the summer vacation (July–August) than during the school year (October). We ran a general linear model (GLM) to determine whether there were performance differences between examination in October vs. July to August [1 = October assessment, 0 = July–August examination (reference)] and each NEPSY-II domain score, adjusting for age, gender, race, and parental education. We did not find a statistically significant difference between the two time periods for attention & inhibitory control [0.70 (−0.12, 1.53), $p = 0.09$], language [0.22 (−0.53, 0.98), $p = 0.56$], memory & learning [−0.69 (−1.57, 0.20), $p = 0.13$], visuospatial processing [0.72 (−0.15, 1.61), $p = 0.11$], or social perception [−0.14 (−1.05, 0.75), $p = 0.75$]. Thus, the final model does not adjust for examination period (October vs. summer). There has been evidence that prenatal pesticide exposure may influence neurobehavioral performance in childhood.[89] We used two constructs for prenatal pesticide exposure: *a)* mother worked in agriculture while pregnant with participant and *b)* mother cohabitated with an agricultural worker while pregnant with participant. After limiting the sample size to only include participants with the prenatal exposure constructs and adjusting our model for both prenatal pesticide exposure constructs, independently and together, we did not observe a 10% change in our beta estimates for the herbicide-neurobehavioral associations of interest for any of the domains (Table S1–S5). There were >10% changes for some of the associations between DEET metabolites and all domains (Table S1–S5). As the estimates for these associations were very small (close to null), and to maintain consistency across the models, we decided not to include it as a covariate.

Any models that had attention & inhibitory control as the outcome were also adjusted for a learning effect (test-retest),[90] as participants were also examined for this domain in April 2016. For associations between biomarker and summary score concentrations with NEPSY-II scores, clustered the bootstrap method was used to obtain each *p*-value.[91] This was done by running a GEE model for each ln-transformed biomarker or summary score concentration with each NEPSY-II outcome 1,000 times. We then obtained asymptotic estimates and *p*-values. Curvilinearity was assessed by testing squared (quadratic) terms of the pesticide biomarkers measured and summary scores in the adjusted linear models and were reported if they reached a threshold significance level of $p < 0.10$.

To assess whether there were additive effects between both herbicides, interaction terms of continuous metabolite concentrations (i.e., 2,4-D × glyphosate) were introduced in the model and were considered if they had a $p < 0.10$. If these effects were found to be statistically significant, effect modification on the multiplicative and additive scales were assessed. For the multiplicative scale, the association of one biomarker with the neurobehavioral domain was stratified across median splits of the second pesticide. To assess effect modification on the additive scale, we examined joint indicators using median splits of both biomarkers on the NEPSY-II domain [lower median of biomarker 1 and 2 (reference); upper/lower; lower/upper; upper/upper]. We then calculated the interaction contrast ratios (ICRs) and 95% CI to determine risk of having NEPSY-II scores below the expected level (score ≤5).[65] Effect modification by gender was assessed using a multiplicative interaction term for the herbicide or DEET metabolite concentrations associations with

neurobehavioral domain scores. If the multiplicative term had a significance of $p < 0.10$, the association was stratified by gender. Given that our exposure was log-transformed, we multiplied the β estimates by log(1.5) to determine the change in NEPSY-II score per 50% higher herbicide or DEET metabolite concentrations. Reported squared terms, however, were not back transformed.

Mediation analyses. Using structural equation modeling (SEM), we assessed whether testosterone, 17β-estradiol, cortisol, or DHEA mediated the herbicides or DEET metabolites associated with neurobehavioral domain scores that were either statistically significant or borderline statistically significant ($p < 0.10$) using the R lavaan package.[92–94]

The SEM consisted of two regression modules, one relates the mediator (Z) to the predictor (X) and the other relates the outcome (Y) to the mediator (Z) and predictor (X). The modules are as follows:

$$\text{Module 1:} \ \ Z = \beta_0 + \beta_1 X,$$

$$\text{Module 2:} \ \ Y = r_0 + r_1 Z + r_2 X.$$

Module 1 regressed the herbicide or DEET metabolite concentration (predictor, X) on hormone concentration (mediator, Z) and Module 2 regressed the herbicide or DEET metabolite concentration (predictor, X) and hormone concentration (mediator, Z) with NEPSY outcomes (outcome, Y).[95]

The primary hypothesis concerns full mediation, $r_2 = 0$, in which case Z fully mediates the effect of X on Y. Generally, Z mediates some (not all) of the effects of X on Y, in which case r_2 is not zero. In such partial mediation cases, we compute the direct, indirect, and total effects, often expressed in percentages to indicate the degree to which Z mediates the effects of X on Y. Thus, in the case of full mediation, the direct effect is 0 and the indirect effect is 100%. The direct effect of pesticide exposure was estimated by the effect of the pesticide biomarkers on the outcome in Module 2. The indirect effect was estimated by cumulating the effect of the mediator on the outcome in the second module and the effect of pesticide biomarkers on mediator in the first module. Model fit was assessed by chi-square test, the comparative fit index, the index of Tucker and Lewis, and root mean square error of approximation.[96,97] Mediation was determined to be present if the indirect effect was statistically significant. Mediation models were adjusted for retest learning effect, age, gender, race, creatinine, z-BMI-for-age, monthly salary, saliva collection time minus awakening time, and average parental education for the attention & inhibitory control outcome. All other mediation models were adjusted for the same covariates, minus retest learning effect. We found that our data met assumptions of SEM, which include normality of observations, no systematic missing data, no measurement or sampling and has a good model fit.[98]

Locally weighted polynomial regression (LOESS) curves graphs were created to visualize associations and to assess the presence of a threshold effect between 2,4-D and all 5 neurobehavioral domains and between glyphosate and social perception. The LOESS graphs plotted the associations and 95% CIs (smoothness factor: 0.9) using the fully adjusted least squares means of each domain score for 300 ranks of observed pesticide metabolite concentrations and ranks of imputed concentrations. All analyses were performed using R statistical programming software.

Results

Participant Characteristics

Participants had a balanced gender distribution (51.3% female) and 78.0% were Mestizo or White (Table 1). The average age of participants was 14.46 y old (SD = 1.76), and the average tanner score

was 2.89 (SD = 0.95). In unadjusted analyses, participants with higher herbicide summary scores were more likely to be male, have higher urinary creatinine levels, higher education levels and have lower z-BMI-for-age (Table 1). The differences in gender [β = 0.10 (−0.04, 0.24), $p = 0.16$] and z-BMI-for-age score [β = −0.08 (−0.17, 0.002), $p = 0.06$] were no longer present after adjusting the GEE models for age, z-Height-for-age, and creatinine concentration. The differences in education level were not present when adjusted for age and gender [β = −0.05 (−0.13, 0.02), $p = 0.16$]. The mean of each neurobehavioral domain score was between 7.0 and 8.7 (SD range: 2.0–2.3) (Table 2). Our study population is relatively representative of the population of Pedro Moncayo. Compared to the adolescents in the 2016 Community Health Survey of Pedro Moncayo, the ESPINA cohort had an equivalent gender distribution (ESPINA: 51.3% female vs. 2016 Survey: 50.8% female) and mean age [ESPINA: 14.47 (SD: 1.77) vs. 2016 Community Health Survey: 14.45 (SD: 2.27)]. However, there was a higher proportion of agricultural workers who were parents within ESPINA (67.5%) than adults between the ages of 26 and 74 years in the 2016 Community Health Survey (42.6%). There were low positive correlations ($0 < r \leq 0.32$) with glyphosate, 2,4-D, DCBA, and ECBA with AND, PNP, TCPy, and 3-PBA (Table S6).

The urinary concentrations of herbicides and DEET metabolites, overall and across categories of the herbicide summary score, are listed in Table 1. The 2,4-D and DEET biomarkers were measured in the general population of adolescents in the United States in 2013–2014 or 2015–2016, respectively, as part of the NHANES. Compared to NHANES, our cohort's geometric mean concentrations were higher for glyphosate [ESPINA: 0.85 μg/g of creatinine (0.74, 0.97) vs. NHANES 2015–2016: 0.32 μg/g (0.29, 0.36)], equivalent for 2,4-D [ESPINA: 0.27 μg/g (95% CI: 0.25, 0.29) vs. NHANES 2013–2014: 0.29 μg/g (0.26, 0.32)], but lower for DCBA [ESPINA: 0.63 μg/g (0.55, 0.73) vs. NHANES 2015–2016: 4.42 μg/g (3.48, 5.61)] and ECBA [ESPINA: 0.36 μg/g (0.32, 0.41) vs. NHANES 2015–2016: 1.77 μg/g (1.40, 2.23)] concentrations.[79] Average NEPSY-II domain scores can be found in Table 2. Compared to the U.S. normative sample of the NEPSY-II, scaled scores among ESPINA participants were lower.

Urinary Pesticide Biomarker Concentrations and Neurobehavioral Performance

The DEET summary score and DCBA and ECBA concentrations were not found to be associated with any of the domains (Table 3). Herbicide metabolites, however, were found to be negatively associated with neurobehavior. Higher observed urinary concentrations of

Table 2. Unadjusted mean neurobehavioral domain scores: overall and stratified by herbicide summary score medians of adolescent participants of the ESPINA study examination in the July to October 2016 examination ($n = 519$).

		Herbicide summary score	
	Overall	Lower median (0.17–0.99)	Upper median (1.00–9.09)
Attention & inhibition	8.38 ± 2.15	8.42 ± 2.08	8.31 ± 2.23
Language	7.04 ± 2.04	7.16 ± 1.95	6.91 ± 2.13
Memory & learning	8.30 ± 2.28	8.43 ± 2.30	8.19 ± 2.27
Visuospatial processing	8.69 ± 2.28	8.66 ± 2.22	8.71 ± 2.35
Social perception	8.23 ± 2.34	8.44 ± 2.30	8.02 ± 2.36

Note: Estimates are mean ± SD. The herbicide summary score was calculated using values for 2,4-D and glyphosate. Observations below the LOD were imputed, one was added, and the variable was natural log-transformed. The log-transformed concentrations were divided by the group's standard deviation, and then the two biomarkers were averaged. 2,4-D, 2,4-dichlorophenoxyacetic acid; ESPINA, Secondary Exposures to Pesticides among Children and Adolescents; LOD, limit of detection; SD, standard deviation.

2,4-D were associated with worse performance across the 5 neurobehavioral domains assessed. These associations were statistically significant for attention & inhibitory control [score difference per 50% higher metabolite concentration $(\beta_{per50\%}) = -0.19$ (95% CI: -0.31, -0.07)] and language [$\beta_{per50\%} = -0.12$ (-0.23, -0.01)]. The associations were also negative, albeit borderline nonstatistically significant, for memory & learning [$\beta_{per50\%} = -0.11$ (-0.22, 0.01)] and nonstatistically significant for social perception [-0.11 (-0.27, 0.06)]; however, social perception had a statistically significant curvilinear association ($p_{quadratic} < 0.01$) (Table S7; Table 3, Figure 2). The numeric data for Figure 2 can be found in the supplemental excel file. Urinary glyphosate concentrations were negatively associated with social perception [$\beta_{per50\%} = -0.08$ (-0.14, -0.01)]. However, it was not associated with any other domain.

The herbicide summary score had negative associations with language [$\beta_{per50\%} = -0.12$ (-0.25, -0.01)] and social perception [$\beta_{per50\%} = -0.24$ (-0.39, -0.10)], and borderline statistically significant negative associations with memory & learning [$\beta_{per50\%} = -0.12$ (-0.25, 0.02)] and visuospatial processing [$\beta_{per50\%} = -0.14$ (-0.29, 0.004)] (Table 3). The interaction terms between glyphosate and 2,4-D were statistically significant for memory & learning [$\beta = 0.14$ (0.01, 0.27), $p = 0.04$], but not for attention & inhibitory control [0.01 (-0.14, 0.16), $p = 0.89$], language [0.04 (-0.05, 0.14), $p = 0.37$], visuospatial processing [-0.07 (-0.23, 0.09), $p = 0.39$], or social perception [0.10 (-0.04, 0.24), $p = 0.15$]. For the median splits, the range for creatinine adjusted 2,4-D with imputation using a constant was 0.04 µg/L to 0.253 µg/L in the lower median, and 0.254 µg/L to 57.60 µg/L for the upper median. While for creatinine adjusted glyphosate with imputation using a constant, the range was 0.001 µg/L to 1.001 µg/L in the lower median, and 1.002 µg/L to 21.94 µg/L in the upper median (Table 4). The association between glyphosate and memory & learning was stronger in the upper median of 2,4-D [$\beta_{per50\%} = -0.06$ (-0.19, 0.07), $p = 0.38$] compared to the lower median [$\beta_{per50\%} = -0.001$ (-0.07, 0.07), $p = 0.99$]. The association between 2,4-D and memory & learning was stronger and more negative in the upper median of glyphosate concentration [$\beta_{per50\%} = -0.10$ (-0.21, 0.02), $p = 0.10$] compared to concentrations in the lower median [$\beta_{per50\%} = -0.06$ (-0.23, 0.10), $p = 0.45$]. Participants who had concentrations in the upper median of both 2,4-D and glyphosate had lower scores in memory & learning by 0.55 (95% CI: -1.11, 0.01; $p = 0.05$) compared to those in the lower median of both 2,4-D and glyphosate concentration (Table 5); the ICR for a below-expected memory & learning score was not statistically significant (1.52; 95% CI: -1.94, 4.98). No other interactions between metabolites were observed.

Additionally, no effect modification by gender was observed on any of the associations between any of the metabolites and neurobehavioral performance (Table S8).

Mediation by Gender and Adrenal Hormones

We investigated mediation by DHEA, testosterone, estradiol (measured in boys only) and cortisol on the observed statistically significant associations between pesticide biomarker concentrations with neurobehavioral outcomes, namely, glyphosate with social perception and 2,4-D with attention & inhibitory control, language, memory & learning, and social perception. There was no evidence of mediation in any of the models (Table S9).

Discussion

To our knowledge, this is the first study to examine the association between urinary glyphosate concentration and neurobehavioral performance in humans. In our study of adolescents growing up in agricultural settings, we observed that greater concentrations

Table 3. Adjusted associations of pesticide metabolite concentrations or summary scores with neurobehavioral performance for participants of the July to October 2016 ESPINA study examination ($n = 519$).

|	n	Attention & inhibitory control[a]		Language[b]		Memory & learning[b]		Visuospatial processing[b]		Social perception[b]	
		β (95% CI)	p-Value	β (95% CI)	p-Value	β (95% CI)	p-Value	β (95% CI)	p-Value	β (95% CI)	p-Value
Herbicide summary score	519	-0.09 (-0.22, 0.05)[c]	0.21	-0.12 (-0.25, -0.01)	0.04	-0.12 (-0.25, 0.02)	0.09	-0.14 (-0.29, 0.004)	0.06	-0.24 (-0.39, -0.10)	0.001
2,4-D, observed	518	-0.19 (-0.31, -0.07)	0.002	-0.12 (-0.23, -0.01)	0.03	-0.11 (-0.22, 0.01)	0.08	-0.05 (-0.16, 0.06)	0.40	-0.11 (-0.27, 0.06)[d]	0.19
2,4-D, imputed		0.003 (-0.16, 0.17)	0.98	-0.03 (-0.20, 0.14)	0.73	0.05 (-0.14, 0.25)	0.60	-0.01 (-0.18, 0.18)	0.95	0.03 (-0.17, 0.22)	0.80
Glyphosate	519	0.01 (-0.05, 0.07)	0.67	-0.01 (-0.07, 0.04)	0.62	-0.02 (-0.09, 0.05)	0.54	-0.02 (-0.09, 0.05)	0.64	-0.08 (-0.14, -0.01)	0.02
DEET summary score	518	0.01 (-0.07, 0.08)	0.86	0.01 (-0.06, 0.09)	0.72	0.01 (-0.08, 0.10)	0.78	-0.06 (-0.14, 0.02)	0.13	-0.08 (-0.14, 0.01)	0.86
ECBA, observed	518	0.01 (-0.06, 0.08)	0.80	-0.03 (-0.11, 0.06)	0.55	-0.01 (-0.10, 0.08)	0.90	-0.01 (-0.08, 0.07)	0.87	0.01 (-0.09, 0.11)	0.38
ECBA, imputed		0.01 (-0.05, 0.06)	0.84	0.003 (-0.05, 0.05)	0.90	-0.01 (-0.07, 0.05)	0.75	0.001 (-0.06, 0.06)	0.98	-0.04 (-0.13, 0.05)	0.98
DCBA, observed	518	0.01 (-0.05, 0.06)	0.72	-0.01 (-0.07, 0.05)	0.75	0.01 (-0.06, 0.07)	0.75	-0.04 (-0.09, 0.02)	0.23	-0.001 (-0.06, 0.06)	0.61
DCBA, imputed		0.01 (-0.06, 0.08)	0.83	0.01 (-0.06, 0.08)	0.79	0.01 (-0.07, 0.09)	0.77	0.02 (-0.06, 0.09)	0.70	0.02 (-0.06, 0.10)	0.68

Note: Results in the table are from linear exposure models except for associations from models with both linear and quadratic terms for exposure, such as those seen in footnote c and d. The p-values are from general estimating equations that were used to analyze the relationship between each pesticide biomarker or summary score with each NEPSY-II domain, adjusting for confounders. A p-value <0.05 indicates a statistically significant association between the tested biomarker and NEPSY-II domain. Summary scores for herbicide and DEET were calculated using the following steps: For each biomarker, observations below the LOD were imputed with a constant (LOD$/\sqrt{2}$). One was added to the variable and was natural log-transformed. The log-transformed concentrations were divided by the group's standard deviation and then averaged with biomarkers of the same classification. For the DEET summary score, ECBA and DCBA were combined. For the herbicide summary score, 2,4-D and glyphosate were combined. All curvilinear terms can be found in Table S7. 2,4-D, DCBA, and ECBA concentrations below the LOD were imputed using multiple imputation. Imputation using a constant (LOD$/\sqrt{2}$) was done for glyphosate. 2,4-D, 2,4-dichlorophenoxyacetic acid; BMI, body mass index; CI, confidence interval; DCBA, 3-(diethylcarbamoyl)benzoic acid; DEET, N,N-diethyl-meta-toluamide; ECBA, 3-(ethylcarbamoyl)benzoic acid; ESPINA, Secondary Exposures to Pesticides among Children and Adolescents; LOD, limit of detection; SD, standard deviation.
[a]Model adjusts for retest learning effect, age, gender, race, creatinine, z-BMI-for-age, monthly salary, and average parental education.
[b]Model adjusts for covariates of model A except for retest learning effect.
[c]Curvilinear associations: $\beta^2 = -0.12$ [(-0.25, 0.01), $p = 0.07$], $\beta = -0.36$ ($p = 0.03$). β and β^2 listed are part of the same model.
[d]Curvilinear associations: $\beta^2 = 0.21$ [(0.09, 0.34), $p < 0.01$], $\beta = -0.21$ [(-0.47, 0.05), $p = 0.11$]. β and β^2 listed are part of the same model.
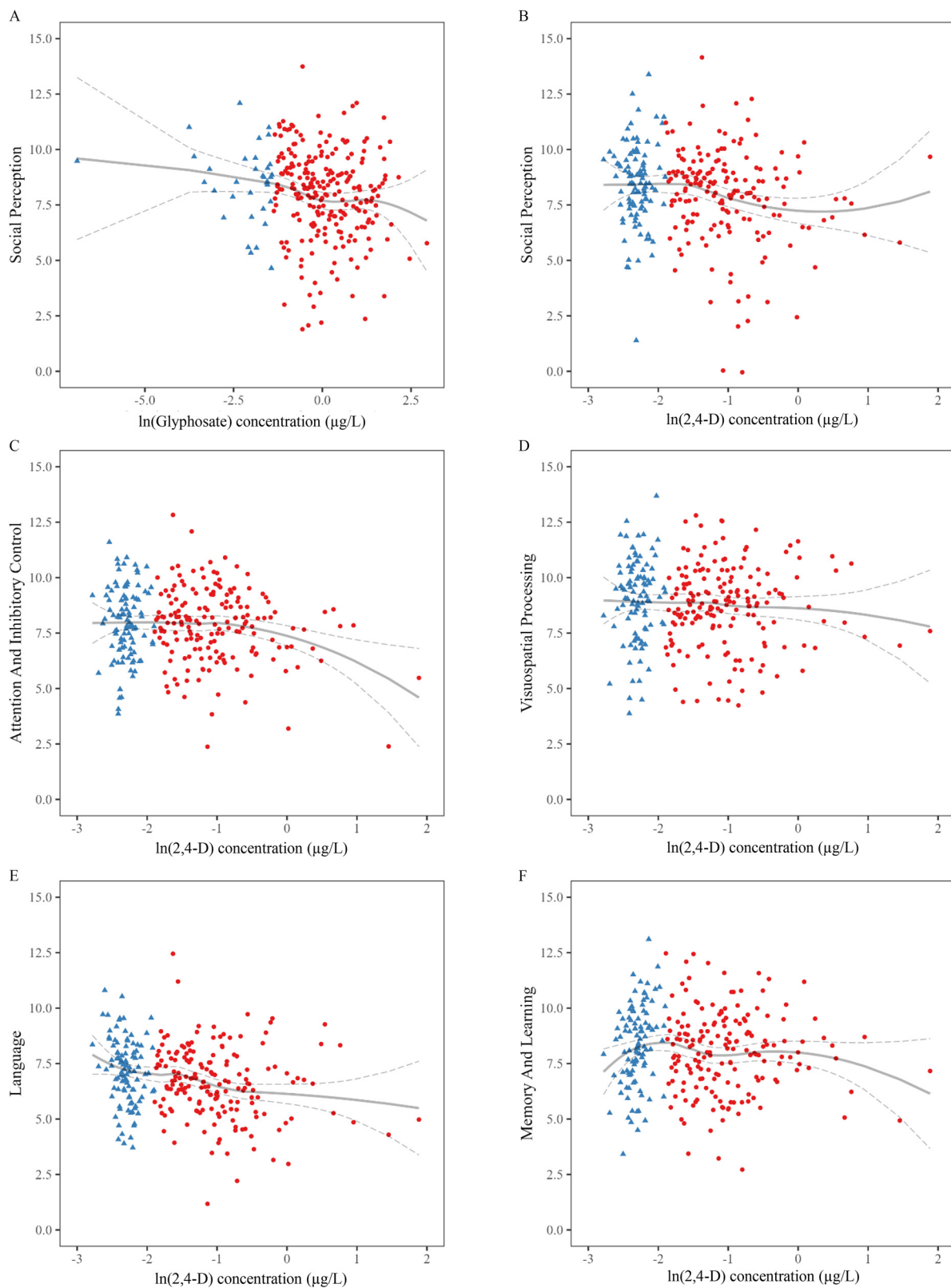
Figure 2. Adjusted associations of 2,4-D concentrations with neurobehavioral domains, and glyphosate concentration with social perception of participants of the July to October 2016 ESPINA study examination ($n = 519$). Each data point represents the adjusted least squares of NEPSY-II domain for 300 ranks of ln (metabolite). Blue triangles represent imputed values, while red dots represent observed values. Attention & inhibitory control adjusts for retest learning effect, age, gender, race, creatinine, z-BMI-for-age, monthly salary, and average parental education. All additional models adjust for the aforementioned model covariates except for retest learning effect. The gray line represents the LOESS line, while the outer bands are the 95% confidence intervals across each value of the *x*-axis. Numeric data for this figure can be found in the Supplemental Excel File. Note: 2,4-D, 2,4-dichlorophenoxyacetic acid; BMI, body mass index; ESPINA, Secondary Exposures to Pesticides among Children and Adolescents; L, liter; LOESS, locally weighted polynomial regression; μg, microgram.

Table 4. Adjusted associations of pesticide metabolite concentrations with memory & learning performance across strata of a secondary pesticide metabolite median split for participants of the July to October 2016 ESPINA study examination ($n = 518$).

	Difference in domain scores per 50% higher biomarker concentration, β (95% CI)							
	2,4-D				Glyphosate			
	Lower median (0.04–0.253 µg/L)		Upper median (0.254–57.60 µg/L)		Lower median (0.001–1.001 µg/L)		Upper median (1.002–21.94 µg/L)	
	β (95% CI)	*p*-Value	β (95% CI)	*p*-Value	β (95% CI)	*p*-Value	β (95% CI)	*p*-Value
2,4-D	—	—	—	—	−0.06 (−0.23, 0.10)	0.45	−0.10 (−0.21, 0.02)	0.10
Glyphosate	−0.001 (−0.07, 0.07)	0.99	0.06 (−0.19, 0.07)	0.38	—	—	—	—

Note: β estimates were obtained by running generalized estimating equations to test the association of one biomarker with the neurobehavioral domain stratified across median splits of the second pesticide. A $p < 0.05$ indicates a statistically significant association. Models adjusts for age, gender, race, creatinine, z-BMI-for-age, monthly salary, and average parental education. 2,4-D and glyphosate concentrations below the LOD were imputed using a constant (LOD/$\sqrt{2}$). 2,4-D, 2,4-dichlorophenoxyacetic acid; BMI, body mass index; CI, confidence interval; ESPINA, Secondary Exposures to Pesticides among Children and Adolescents; L, liter; LOD, limit of detection; µg, microgram.

of the herbicide 2,4-D were associated with lower performance on four of the five neurobehavioral domains assessed: attention & inhibitory control, language, memory & learning, and social perception. Additionally, urinary glyphosate concentration was only inversely associated with social perception, whereas the two DEET metabolites were not associated with neurobehavioral outcomes. Combined concentrations of 2,4-D and glyphosate, as assessed by an herbicide summary score, was associated with worse performance on all five neurobehavioral domains assessed. These findings suggest that exposure to these two herbicides may negatively impact neurobehavior in adolescents. Within our cohort, concentrations of 2,4-D are equivalent to those of a general population of adolescents in the United States,[79] and concentrations of glyphosate were higher than those reported in NHANES.[79] Although we identified differences in herbicide exposure by gender in crude analysis (Table 1), this difference disappeared after further adjustment was made.

Few epidemiological studies have investigated the association between 2,4-D exposure and cognitive development; none of the studies were conducted in adolescents. Two studies reported negative effects of 2,4-D on neurobehavior. In a study of 232 infants in Southeast China, prenatal exposure to 2,4-D was assessed by measuring its concentration in umbilical cord blood plasma, and they reported deficits in auditory processing among infants with prenatal exposure [−0.103 (95% BCI: −0.24, 0)].[27] A study of workers in Jacksonville, Florida found that active, retired and former workers of a plant that manufactured 2,4-D had a statistically significant decrease in nerve conduction velocity compared to nonherbicide factory workers (mean = 34.0 vs. 40.1 m/s, $p < 0.02$).[28] However, among 1,338 adult participants of the NHANES III conducted in the United States, there were no associations from adjusted linear regression models between 2,4-D urinary concentrations and

Table 5. The memory & learning score differences for the joint effects of 2,4-D and glyphosate biomarker concentrations in adolescent participants of the ESPINA study, July to October 2016 examination ($n = 518$).

2,4-D, µg/L	Glyphosate, µg/L	Score difference (95% CI), *p*-Value
≤median: 0.04–0.253	≤median: 0.001–1.001	Reference
>median: 0.254–57.60	≤median: 0.001–1.001	−0.15 (−0.73, 0.43), 0.61
≤median: 0.04–0.253	>median: 1.002–21.94	−0.16 (−0.73, 0.41). 0.58
>median: 0.254–57.60	>median: 1.002–21.94	−0.55 (−1.11, 0.01), 0.05

Note: The model adjusted for retest learning effect, age, gender, race, creatinine, z-BMI-for-age, monthly salary, and average parental education. The score difference estimates were obtained by conducting generalized estimating equations to assess the association between an indicator variable based on dichotomous variables for 2,4-D and glyphosate concentrations, and memory & learning scores. A *p*-value <0.05 indicates that the respective category (e.g., upper median of both 2,4-D and glyphosate) is statistically significantly different than the reference group (lower median of 2,4-D and glyphosate). 2,4-D and glyphosate concentrations below the LOD were imputed using a constant (LOD/$\sqrt{2}$). 2,4-D, 2,4-dichlorophenoxyacetic acid; BMI, body mass index; CI, confidence interval; ESPINA, Secondary Exposures to Pesticides among Children and Adolescents; LOD, limit of detection.

concurrent performance on three neurobehavioral tests: simple reaction time (β = 3.86, $p = 0.62$), symbol digit substitution [mean total latency (β = −0.93, $p = 0.44$) and number of errors (β = 0.0007, $p = 0.99$)], and serial digit learning [trials to criterion (β = −0.39, $p = 0.19$) and total score (β = −1.03, $p = 0.10$)].[26] The differing findings between ESPINA and NHANES may be due to multiple factors. Compared to adults, adolescents have increased susceptibility to insecticide neurotoxicity,[99] which may also be true for 2,4-D. Brain development and performance on neurobehavioral tests can vary across different stages of life. For these two reasons, adults and adolescents may not be the most comparable groups. Furthermore, the neurobehavioral domains evaluated in NHANES III[26] (simple reaction time, symbol digit substitution, and serial digit learning) are not comparable to those measured in this study (attention & inhibitory control, language, memory & learning, visuospatial processing, and social perception). It is plausible that 2,4-D may affect certain neurodevelopmental domains more than others.

To our knowledge, there are no studies which focus on the effects of 2,4-D and its effects on the nervous system in adolescents. However, it is possible that the associations that we observed between 2,4-D exposure and cognition may be due to its neurotoxic effects. 2,4-D has been found to induce neuronal and glial cell death and neuronal necrosis in rodents[100,101] due to an increase in reactive oxygen species and free radicals.[102] Additionally, rats that were exposed orally and via inhalation to 2,4-D for 6 months had increased expression of BAX, a B-2 cell lymphoma pro-apoptotic molecule, and had a greater incidence of individual neuron necrosis and lower cerebral cortex thickness compared to the rat control group.[103] The rats exposed to 2,4-D had reduced cognitive ability, measured using object recognition and impaired exploration behaviors.[104] Each of these neurotoxic events have been shown to negatively affect behaviors, such as locomotion among animal subjects.[105,106]

We observed a statistically significant inverse association between glyphosate and facial affect recognition (social perception). This is the first time this association has been characterized in an epidemiologic study, to our knowledge. Recent evidence from epidemiological and animal studies suggests potential links between glyphosate exposure and ASD.[24,25] ASD is a condition that is associated with reduced emotion recognition and is evidenced by lower scores on the Affect recognition subtest (social perception domain, NEPSY-2).[107–111] In a systematic review based on two epidemiological and 15 *in vivo* studies, glyphosate showed a moderate level of evidence with an increased risk of ASD in children.[25] Prenatal exposures to pesticides (measured as pounds of pesticides applied per acre/month within 2,000 m from the maternal residence) was associated with increased risk of ASD when compared to the offspring of women from the same agricultural region in California without exposure [OR = 1.16 (95% CI: 1.06, 1.27) for glyphosate].[24] Elevated intracellular chloride levels of neurons and neocortical tissue have been observed in

autism.[112,113] Glycine binding of the glycine receptor of neurons can influence the rate of calcium influx into neurons during neuro-development.[110,114] Given glyphosate's potential to be a glycine mimetic, it could cause similar patterns of overconcentrated chloride within immature neurons.[110] We could not identify any other published epidemiologic studies that evaluated the associations between glyphosate urinary concentration and neurobehavioral outcomes in a human population.

Mediation by Gender and Adrenal Hormones

Although there is evidence of the endocrine disrupting potential of 2,4-D and glyphosate,[44,45,115] we did not observe evidence of mediation by the gonadal or adrenal hormones assessed on the herbicide-neurobehavior associations. There is biological evidence in animal and *in vivo* studies suggesting that pesticide exposure may influence endocrine alterations. Glyphosate has been found to have 8 of 10 key characteristics of being an endocrine disrupting chemical, including, but not limited to, interacting with hormone receptors and altering hormone receptor expression.[46] 2,4-D has been considered a potential endocrine disrupting chemical through its effects on gonadal morphology and testosterone production.[116] Factors that may have influenced our inability to detect mediating effects of hormones are the notable variability of sex hormones across puberty stages and our limited ability to evaluate temporality (the pesticide exposure leading to an endocrine change and then to a neurobehavioral alteration). It is plausible that mediation effects may be most detectable and valid if they are assessed longitudinally. Studies conducting longitudinal measures of pesticides, neurobehavior, and hormone concentrations during adolescence are warranted. Likewise, incorporating biological markers of puberty stage, like luteinizing or follicle stimulating hormones could have improved our models. Additionally, we did not observe any effect modification by gender among these adolescent participants, unlike previous findings in this cohort, when participants were children.[67]

Multiple Exposure

We saw evidence of effect modification on both the additive and multiplicative scales between both 2,4-D and glyphosate herbicides with memory & learning. In the lower median of 2,4-D concentrations, higher glyphosate concentration had a stronger and more negative association with memory & learning than in the upper median, while in the lower median of glyphosate concentration, 2,4-D had a more negative association with memory & learning. No studies currently exist that have examined the effect measure modification of these herbicides on neurobehavioral outcomes; however, some studies have looked into the joint effects of glyphosate and 2,4-D in phytoplankton and periphyton and genotoxicity in fish. Lozano et al. found that there were additive effects of the combined herbicides, as the effects of the mixture did not exceed the estimated combined effects from the individual chemicals in terms of toxicity.[117] while a synergistic effect was observed among a combination of glyphosate and 2,4-D in terms of genotoxicity in a certain species of fish (*Cnesterodon decemmaculatus*).[118]

Strengths and Limitations

This is among the largest studies of adolescents to have assessed the effects of pesticide exposures and neurobehavior, using both urinary biomarker concentrations of pesticide exposures and neurobehavioral performance testing. A limitation is that the quantification of exposure biomarkers occurred at a single point in time. Urinary biomarker concentrations reflect recent exposure to the herbicides and insect repellants measured, as glyphosate has a half-life of 3.5–14.5 h,[119] 2,4-D has a half-life of 11.6 h,[120] and DEET has a half-life of 4 h.[121] However, it is plausible that these

spot concentrations may be correlated with the yearly pesticide exposures given that agricultural production in this equatorial location occurs year-round. A prior analysis of this cohort assessed the relationship between home proximity to the nearest agricultural greenhouse and AChE exposure longitudinally (2008, spring 2016, and summer 2016 assessments).[122] Using repeated measure regression, home proximity was negatively associated with AChE concentration, suggesting that there was potential pesticide exposure across all three time points, which indicates that there is year-round exposure to pesticides.[122] This exposure may vary, as floricultural production does fluctuate seasonally depending on the demand for flowers for holidays like Thanksgiving, Christmas, Valentine's day, and Mother's day. Thus, periods of high pesticide use generally ranges from October to May, while June to September has lower pesticide use. Although the summer assessment occurred in a period of lower pesticide applications in floriculture, we still saw associates between 2,4-D and glyphosate with neurobehavioral outcomes. Another consideration is that herbicides are used sparingly in floriculture, as they can also lead to the destruction of rose crops, but are more commonly used in agriculture for crops like corn, wheat, and beans.[55,123] Pedro Moncayo has year-round agriculture and subsequent pesticide application due to its tempered climate, equatorial location, and adequate irrigation water supply. This association needs to be further evaluated in this population using multiple measures of exposure. An additional limitation is our inability to distinguish whether the effects that we observed are due to short-term or long-term exposure to pesticides due to the cross-sectional analyses of this study. Lastly, it is possible that some of the results are influenced by co-exposures to other pesticides. However, we found low positive correlations ($0 < r \leq 0.32$) across all 4 metabolites presented in this study with urinary metabolite concentrations of AND (neonicotinoid), PNP (organophosphate), TCPy (organophosphate), and 3-PBA (pyrethroid).

To improve causal inference, future studies should analyze changes in neurobehavioral development over time based on initial or time-varying exposures. Finally, we believe that our results for 2,4-D may be generalizable to other adolescent, nonoccupational populations given that we observed similar 2,4-D urinary concentrations in ESPINA as in the U.S. general population (Centers for Disease Control and Prevention, 2022). Glyphosate concentrations were higher in our cohort compared to reported concentrations of adolescents in the United States.[79] Therefore, additional research is needed to assess whether a similar association with social perception would still be detectable in adolescent populations with lower glyphosate exposure. Differences between glyphosate concentrations observed in ESPINA vs. NHANES may be due to differential patterns of herbicide use between the two regions that may be specific to the types of crops grown. DEET concentrations were lower in our cohort compared to NHANES, which may be why we did not detect any associations with the NEPSY-II domains. The lower DEET concentrations may be due to reduced use of insect repellents in the Ecuadorian highlands, where ESPINA participants live ($\sim 2,800$ m above sea level), since there is a lower prevalence of mosquitoes and lower concern of insect-borne diseases compared to coastal regions.[124] Individuals living in the highlands, such as Pedro Moncayo, may use less insect repellents compared to the U.S. NHANES population, which contains regions of lower altitude and higher risk of insect-borne diseases.

Conclusion

Urinary concentrations of 2,4-D and glyphosate were associated with lower scores in attention & inhibitory control, language, visuospatial processing, memory & learning, and social perception among adolescents in rural Ecuador. Independently, urinary 2,4-D concentration was negatively (statistically significantly or borderline

statistically significantly) associated with attention & inhibitory control, language, visuospatial processing, and memory & learning, whereas urinary glyphosate concentration was inversely associated only with social perception. We did not identify any mediating effects by gonadal and adrenal hormones on the associations between herbicide or DEET exposure on neurobehavioral outcomes. These are among the first population-based findings to describe lower neurobehavioral performance associated with urinary concentrations of 2,4-D and glyphosate. Replication of these findings is needed, including longitudinal studies and assessments of joint effects of herbicide mixtures among various pediatric and adult populations.

Acknowledgments

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