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Exposure to non-persistent pesticides and sexual maturation of Spanish adolescent males

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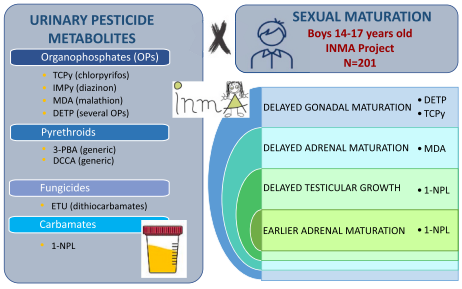
HIGHLIGHTS

- DETP, TCPy, and MDA were associated with delayed gonadal development in adolescent males.
- 1-NPL was associated with advanced adrenal development but delayed testicular growth.
- Exposure to certain pesticides was related to delayed sexual maturation in adolescent males.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Several non-persistent pesticides are endocrine disrupting chemicals and may impact on sexual maturation.
Objective: To examine the association between urinary biomarkers of non-persistent pesticides and sexual maturation in adolescent males in the Environment and Childhood (INMA) Project.
Methods: The metabolites of several pesticides were measured in spot urine samples collected from 201 boys aged 14–17 years, including: 3,5,6-trichloro-2-pyridinol (TCPy), metabolite of chlorpyrifos; 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMPy), metabolite of diazinon; malathion diacid (MDA), metabolite of malathion; diethyl thiophosphate (DETP) and diethyl dithiophosphate, non-specific metabolites of organophosphates; 3-phenoxy-benzoic acid (3-PBA) and dimethyl cyclopropane carboxylic acid, metabolites of pyrethroids; 1-naphthol (1-NPL), metabolite of carbaryl; and ethylene thiourea (ETU), metabolite of dithiocarbamate fungicides. Sexual maturation was assessed using Tanner stages, self-reported Pubertal Development Scale, and testicular volume (TV). Multivariate logistic regression was employed to examine associations between urinary pesticide

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metabolites and the odds of being in Tanner stage 5 of genital development (G5) or pubic hair growth (PH5); stage ≥4 of overall pubertal development, gonadarche, and adrenarche; or having mature TV (≥25 mL).
Results: DETP concentrations>75th percentile (P75) were associated with lower odds of being in stage G5 (OR = 0.27; 95% CI = 0.10–0.70), detectable TCPy with lower odds of gonadal stage≥4 (OR = 0.50; 95% CI = 0.26–0.96), and intermediate detectable MDA concentrations (<P75) with lower odds of adrenal stage≥4 (OR = 0.32; 95% CI = 0.11–0.94). Conversely, detectable concentrations of 1-NPL were associated with higher odds of adrenal stage≥4 (OR = 2.61; 95% CI = 1.30–5.24) but lower odds of mature TV (OR = 0.42; 95% CI = 0.19–0.90).
Conclusion: Exposure to certain pesticides may be associated with delayed sexual maturity in adolescent males.

1. Introduction

Pesticides are widely used in the food production chain and in urban and domestic settings. In the general population, diet is the main source of exposure to contemporary pesticides (Barr et al., 2010; Fernández et al., 2020a, 2020b), which are rapidly metabolized and mainly excreted through urine (Egeghy et al., 2011). Organophosphate (OP) and carbamate insecticides are used on crops, although some have been banned in the European Union (EU); for instance, the OP chlorpyrifos have not been approved since 2020 due to its possible adverse health effects (Dalsager et al., 2019; EU Pesticides Database (v.2.2) Active Substance, n.d.; Guo et al., 2019). Synthetic pyrethroids are relatively newer, becoming more available in industrial and domestic products (Tang et al., 2018). Among fungicides, ethylene-bis-dithiocarbamates (EBDC) such as maneb and mancozeb were banned in the EU in 2017 and 2021, respectively (EU Pesticides Database (v.2.2) Active Substance, n.d.). Human biomonitoring studies have shown comparable levels of dietary exposure to pesticides among adults and children worldwide (Holme et al., 2016; Li et al., 2019; Papadopoulou et al., 2019).

Numerous non-persistent pesticides have been found to exert endocrine-disrupting activity and interfere with the mechanisms that regulate the initiation and progression of puberty and sexual maturation by interacting with the receptors or metabolism of steroid hormones (Kojima et al., 2004; Orton et al., 2011). Thus, several OP insecticides (Archer and van Wyk, 2015; Kjeldsen et al., 2013; Kojima et al., 2004; Manabe et al., 2006; Yu et al., 2015), pyrethroids (Kjeldsen et al., 2013; Kojima et al., 2004; Manabe et al., 2006), carbamates (Kojima et al., 2004; Tange et al., 2016), and EBDC fungicides (Archer and van Wyk, 2015; Kjeldsen et al., 2013) bind to the estrogen receptors (ERα and/or -β) and/or the androgen receptor (AR) *in vitro*, and they can interfere with the metabolism of steroid hormones (Andersen et al., 2002). Nevertheless, few epidemiological studies have assessed their potential impact on pubertal development, with inconsistent results (Castiello and Freire, 2021). Thus, urinary concentrations of dialkyl phosphate (DAP) metabolites of OPs were associated with delayed genital development and lower testosterone levels in 14–15-year-old boys in Belgium (Croes et al., 2015), whereas urinary 3-phenoxinenzoic acid (3-PBA), metabolite of pyrethroids, were associated with earlier genital development in 9–16-year-old boys in China (Ye et al., 2017). In a Danish cohort, boys born to mothers who worked in greenhouses during pregnancy had smaller testicular size and shorter penis length at the age of 6–11 years compared to non-exposed boys (Wohlfahrt-Veje et al., 2012).

Within the framework of the INMA (Environment and Childhood) Project in Spain, out group recently reported that exposure to OP insecticides, pyrethroids, and EBDC fungicides was associated with earlier puberty development in a large sample of 7–11-year-old girls and boys (Castiello et al., 2023). We also previously reported that urinary metabolites of chlorpyrifos, diazinon, and carbaryl were associated with altered serum levels of sex hormones in 134 adolescent males from the INMA-Granada cohort at the age of 15–17 years (Freire et al., 2021; Suárez et al., 2021). The objective of the present study of a larger sample of late adolescent males from the INMA Project was to evaluate the association of urinary metabolites of OPs, pyrethroid, and carbamate insecticides and EBDC fungicides with their sexual maturation status.

2. Material and methods

2.1. Study population

The INMA Project is a multicenter population-based mother-child cohort study that investigates the effect of environmental exposures and diet during pregnancy on fetal and child development in different geographic areas of Spain (http://www.proyectoinma.org). Recruitment and general characteristics of the INMA cohorts are described elsewhere (Guxens et al., 2012). All cohorts included boys and girls except for Granada, which recruited only boys (Fernandez et al., 2007). Measurement of urinary metabolites of pesticides in adolescents (14–17 years) was performed in participants from urban and rural areas in Menorca (Eastern Spain) and Granada (Southern Spain) cohorts. The INMA-Granada cohort was established in 2000–2002 by recruiting 668 mother-son pairs at delivery. Randomly selected pairs from the baseline cohort were contacted to request their participation in different clinical follow-ups at 4–5 (N = 220, 32.9%) and 9–11 years (N = 300, 44.9%). Those who attended both follow-up sessions (N = 269) were re-contacted and asked to participate in the most recent follow-up at the age of 15–17 years (2017–2019), from which 151 agreed to participate and underwent physical examination (Castiello et al., 2020). In Menorca, 482 pregnant women were recruited during pregnancy in 1997–1998. Mother-child pairs underwent several follow-ups from birth to the child age of 7–8 years (participation rate of 97–100%). At 9–10 years, 88% of the children included in the original INMA-Menorca cohort participated in a follow up, and at 14–16 years, 72% of children (N = 345) participated in a new follow up. Of these 345 children, 139 males underwent pubertal assessment. Therefore, at 14–17 years of age, 290 boys in Menorca and Granada underwent assessment of pubertal status, of whom 201 (69.3%) had their urine analyzed for urinary pesticide metabolites. Study participants in the Granada cohort were more likely to reside in an urban area and their mothers were less likely to have a stable partner than those initially recruited in the cohort, while mothers of participants in the Menorca cohort were also less likely to have a stable partner than mothers of boys in the original cohort. However, there were no substantial differences in the general characteristics of participants between the current and the previous follow-up visits (Table S1). An informed consent form was signed by the parents of all participants before gathering personal information and biological samples. The research protocol, including urine collection, was approved by the Biomedical Research Ethics Committee of Granada and the Balearic Islands.

2.2. Urinary pesticide metabolites

A first morning spot urine sample was collected from each study participant the same day as the clinical examination and was kept at −80 °C until analysis. Urine samples were analyzed for the following metabolites: diethyl-thiophosphate (DETP) and diethyl-dithiophosphate (DEDTP), non-specific metabolites of OP insecticides; 3,5,6-trichlo-2-pyridinol (TCPy), specific metabolite of chlorpyrifos and chlorpyrifos-

methyl; 2-isopropyl-6-methyl-4-pyrimidinol (IMPy), specific metabolite of the OP diazinon; 3-phenoxybenzoic acid (3-PBA), generic metabolite of pyrethroids; 1-naphthol (1-NPL), metabolite of the carbamate insecticide carbaryl; and ethylene thiourea (ETU), major metabolite of EBDC fungicides. It was not possible to measure DEP or DMP metabolites because reference standards were not available. Malathion dicarboxylic acid (MDA), a specific metabolite of malathion, and 2,2-dimethylcyclopropane carboxylic acid (DCCA) (sum of *cis*- and *trans*-isomers), a metabolite of *cis*- and *trans*-isomers of the pyrethroids permethrin, cypermethrin, and cyfluthrin, were measured in the urine of a subsample of 161 boys.

Ultra-high-performance liquid chromatography coupled to mass spectrometry was employed to analyze DETP, DEDTP, TCPy, IMPy, 3-PBA, 1-NPL, and ETU, and liquid chromatography coupled to mass spectrometry was used to analyze the acid metabolites MDA and DCCA, as previously described (Freire et al., 2021; Rodríguez-Carrillo et al., 2022; Suárez et al., 2021). All metabolites were calibrated and extracted according to Suárez et al. (2021). Flow rates for chromatographic separation of MDA and DCCA were set at 0.3 mL/min. Limits of detection (LOD) and quantification (LOQ), retention times, analytical parameters of calibration curves, mean accuracy, selected reaction monitoring (SRM), and relative standard deviation (RSD) values are reported in Supplementary Material (Table S2). Urine dilution was considered by using a commercial kit (CREJ2) to measure urine creatinine concentrations in a Roche Cobas C-311 system, following the Jaffe method.

2.3. Assessment of sexual maturity

Tanner stages of genital development (G) and pubic hair growth (PH) were assessed in 175 and 181 boys, respectively, and Petersen's Pubertal Development Scale (PDS) score (Petersen et al., 1988) was obtained in 197 boys. The Tanner scale for boys classifies G and PH in one of five stages, ranging from prepubertal/absence of development to adult stage/complete maturation (Marshall and Tanner, 1970). Tanner stages were assessed by a pediatric endocrinologist in the Granada cohort and were self-rated in the Menorca cohort. Testicular volume (TV) was measured in 139 boys from Granada by comparison with the Prader orchidometer, a chain of 12 numbered beads of increasing size from 1 to 25 mL (Prader, 1966). When the size differed between testicles, the larger volume was recorded. All TV measurements were performed in duplicate, considering the arithmetic mean of the two measurements if they differed. Both self-reported Tanner and PDS questionnaires were completed by study participants on the same day as the clinical visit and urine collection.

The PDS contains five items: three for both males and females (growth spurt in height, pubic hair, and skin changes/pimples) and two solely for males (facial hair and voice deepening) (Petersen et al., 1988). Responses are on a four-point scale from 1 (development not commenced) to 4 (complete development). The continuous PDS score was transformed into five ordinal stages using the algorithm of Petersen et al. (Petersen et al. (1988) and Carskadon and Acebo (1993) as follows: 1-prepubertal, 2-early pubertal, 3-midpubertal, 4-late pubertal, and 5-postpubertal. The PDS has proven to be a reliable and valid instrument to assess pubertal development in children (Carskadon and Acebo, 1993; Koopman-Verhoeff et al., 2020), and both self-reported and parent-reported PDS have shown strong internal consistency and test-retest reliability (Koopman-Verhoeff et al., 2020). The PDS score was also categorized according to adrenal and gonadal development scales following the algorithm of Shirtcliff et al. (2009) using all five PDS indicators and differentially gathering gonadal and adrenal signs of physical development. In males, growth spurt, voice deepening, and facial hair growth are associated with gonadal development and pubic/body hair and skin changes with adrenal development (Shirtcliff et al., 2009).

2.4. Covariates

Information on potential confounders was obtained from follow-up questionnaires administered to the adolescents and parents and included: cohort (Granada/Menorca), age (years), body mass index (BMI) z-score, weight status (normal weight/overweight/obese), passive smoking, area of residence (urban/sub-urban/rural), maternal age (years), maternal education (up to primary/secondary/university), and maternal stable partner (yes/no). The weight and height of boys were measured using standardized procedures. The BMI was calculated and converted to a z-score for age and sex based on World Health Organization reference curves for children (5–19 years) (WHO, 2007), classifying the boys as normal weight (±1 standard deviation [SD]) or overweight/obese (>+1 SD). Covariates also included the timing of urine sampling (spring/summer/fall/winter), given its potential influence on dietary patterns and pesticide exposure (Pontual et al., 2021).

2.5. Statistical analysis

Urinary concentrations of pesticide metabolites were expressed as detection frequencies and as 25th, 50th, 75th, and 95th percentiles. DEDTP and 3-PBA were excluded from the association analysis because they were only detected in 1 sample and 35 samples, respectively. Pesticide metabolites detected in <50% of urine samples (TCPy, 3-PBA, and 1-NPL) were converted to dichotomous variables (detected/undetected) before regression analysis, and the concentration of metabolites detected in ≥50% of samples was categorized as low (<LOD), moderate (LOD-75th percentile), or high (>75th percentile). Tanner stage and TV were categorized as reaching sexual maturity (Tanner G = 5, Tanner PH = 5, TV ≥ 25) or not. PDS stages were categorized as late/postpuberty (stage≥4) or pre/early/midpuberty (stage <4) for overall pubertal, adrenal, and gonadal development. Data were missing on passive smoking and marital status for nine participants, respectively, and on BMI for one. Missing data on covariates were imputed by using the mode for categorical covariates (passive smoking and marital status) and the median for BMI.

The association between pesticide exposure and the odds of sexual maturity was assessed using logistic regression, modeling each pesticide metabolite (independent variable) separately with each outcome (dependent variable). Adjusted models included the following covariates as independent variables: 1) model 1 (basic model): child age, cohort, and urinary creatinine (log-transformed) (included *a priori*). Unadjusted urinary pesticide metabolites and urinary creatinine concentrations were considered as separate independent variables, considered a better approach for controlling measurement error bias caused by variability in urine concentrations (Barr et al., 2005; O'Brien et al., 2016); 2) model 2: additionally adjusted for maternal education as a proxy of socioeconomic status; and model 3 (fully-adjusted model): additionally adjusted for child BMI z-score. BMI was introduced in a third step to examine whether results changed from those of model 2, possibly reflecting mediation, since pesticide exposure could have an impact on obesity (Pinos et al., 2021), which is strongly associated with pubertal development (Reinehr and Roth, 2019). The remaining covariates were tested for potential confounding following the 10% change-in-estimate criterion, but none of them confounded the association between exposures and outcomes.

Since Tanner stages were self-reported in the Menorca cohort, a sensitivity analysis was performed by excluding boys in this cohort from the fully-adjusted model with Tanner outcomes. In addition, given that individuals are typically exposed to multiple pesticides simultaneously, further analyses were performed in the total sample of boys to assess potential confounding by co-exposure by adjusting regression models simultaneously for all pesticide metabolites (except for DETP to avoid overestimating exposure as it is an unspecific metabolite that shares parent compounds with other OP metabolites included in the model). Results are presented as odds ratios (ORs) with 95% confidence interval

(CI) for reaching sexual maturity with detected versus undetected concentrations, moderate/high versus low concentrations, or with each log-unit increase in urinary metabolite concentrations. The significance level was set at $p < 0.05$. IBM SPSS Statistics v.26 was used for data analyses.

3. Results

Study participants had a mean age of 16.2 years (range = 14.3–17.9), one-quarter were overweight/obese, one-third were passive smokers, and approximately half of them resided in urban areas. Their mothers had a mean age of 46.3 years; and more than three-quarters had a stable partner and low/medium education, respectively (Table 1).

Table 2 and Table S3 exhibit the distribution of urinary pesticide metabolite concentrations. The metabolite detected in the highest proportion of samples was MDA (87.6%, median = 0.31 μg/L), followed by DCCA (68.3%, median = 1.35 μg/L), IMPy (67.2%, median = 0.27 μg/L), ETU (63.2%, median = 0.15 μg/L), DETP (52.2%, median = 0.25 μg/L), TCPy (31.3%, 75th percentile = 0.04 μg/L), 1-NPL (29.4%, 75th percentile = 0.20 μg/L), and 3-PBA (17.4%, 95th percentile = 0.31 μg/L) (Table S3).

The sexual maturity status of participants is reported in Table 3 and Table S4. Regarding Tanner stage, 38.9% and 55.2% of boys had completed genital and pubic hair development, respectively. Regarding the PDS, 50.0% of boys were in stage≥4 for gonadarche and 46.7% for adrenarche. Among 139 boys with available data on TV, 46.8% had a TV ≥ 25 mL (Table 3).

Results of logistic regression analysis are exhibited in Tables 4 and 5. In general, estimates obtained from the three models were similar; however, inclusion of BMI z-score in the model led to a slightly stronger association in some cases. In the fully-adjusted model, higher concentrations of DETP (>75th percentile) were associated with lower odds of being in Tanner stage G5 (OR = 0.27; 95% CI = 0.10–0.71) (Table 4), while detectable concentrations of TCPy were associated with lower odds of being in gonadal stage≥4 (OR = 0.51; 95% CI = 0.27–0.99) (Table 5). In addition, intermediate (LOD-75th percentile) but not higher concentrations of MDA were associated with lower odds of being in adrenal stage≥4 (OR = 0.30; 95% CI = 0.10–0.90) (Table 4) and marginally associated with lower odds of mature TV (OR = 0.36, 95% CI = 0.12–1.09) (Table 4). Conversely, intermediate ETU concentrations were marginally associated with higher odds of mature TV (fully-adjusted model: OR = 2.34; 95% CI = 0.95–5.81) (Table 4), and detectable 1-NPL was significantly associated with higher odds of adrenal stage≥4 (fully-adjusted model: OR = 2.67; 95% CI = 1.32–5.40) (Table 5) but lower odds of mature TV (fully-adjusted model: OR = 0.41; 95% CI = 0.19–0.89) (Table 4).

Exclusion of boys from Menorca did not change the association between DETP and genital development observed in the pooled analysis (Table S5), and results of regression models adjusted for co-exposure were not substantially different from the results of single-exposure models (Table S6).

Table 1
Characteristics of study participants (N = 201).

Characteristic	n (%) or mean ± SD	Granada (N = 150)	Menorca (N = 51)
Cohort			
Granada (age range = 15.9–17.9 years)	150 (74.6)	–	–
Menorca (age range = 14.3–14.8 years)	51 (25.4)	–	–
Age (years)	16.2 ± 0.9	16.6 ± 0.4	14.6 ± 0.1
Season of follow up visit			
Spring	69 (34.3)	34 (22.7)	35 (68.6)
Summer	35 (17.4)	21 (14.0)	14 (27.5)
Autumn	64 (31.8)	63 (42.0)	1 (2.0)
Winter	33 (16.4)	32 (21.3)	1 (2.0)
BMI (kg/m^2)	22.75 ± 4.66	23.23 ± 4.84	21.34 ± 3.79
BMI z-score (kg/m^2)	0.48 ± 1.24	0.48 ± 1.29	0.47 ± 1.04
Overweight/obese (BMI ≥ 25 kg/m^2)	50 (25.0)	43 (28.9)	7 (13.7)
Passive smoking	64 (33.3)	58 (38.7)	6 (11.8)
Area of residence			
Urban	105 (52.2)	105 (70.0)	0 (0.0)
Sub-urban/rural	96 (47.8)	45 (30.0)	51 (100)
Urinary creatinine (mg/dL)	184.7 ± 58.3	184.1 ± 57.5	186.3 ± 60.9
Maternal age	46.3 ± 4.7	46.9 ± 4.7	44.4 ± 4.1
Maternal education			
Primary	93 (47.9)	68 (45.3)	25 (49.0)
Secondary	59 (30.4)	49 (32.7)	18 (35.3)
Univeristy	41 (21.1)	33 (22.0)	8 (15.7)
Maternal stable partner (yes)	181 (90.0)	10 (7.1)	10 (19.6)

SD: Standard deviation; BMI: Body mass index.

Table 2
Urinary concentrations (μg/L) of pesticide metabolites in first morning voids.

Metabolites	LOD	% >LOD	Percentiles				Max
			25	50	75	95	
OP insecticides							
TCPy	0.039	31.3	<LOD	<LOD	0.04	0.18	1.21
IMPy	0.117	67.2	<LOD	0.27	0.76	4.28	27.11
MDA (N = 161)	0.052	87.6	0.15	0.31	0.55	1.12	1.83
DETP	0.116	52.2	<LOD	<LOD	0.74	5.42	54.95
Pyrethroids							
3-PBA	0.117	17.4	<LOD	<LOD	<LOD	0.31	0.72
DCCA (cis + trans) (N = 161)	0.172	68.3	<LOD	1.35	3.56	6.74	8.95
Carbamates							
1-NPL	0.156	29.4	<LOD	<LOD	0.20	0.82	2.88
Dithiocarbamate fungicides							
ETU	0.072	63.2	<LOD	0.15	0.60	2.55	19.37

LOD: Limit of detection.

Table 3
Sexual maturity status of study participants.

Outcome	N	n (%)
Genital Tanner stage	175	
G = 3		11 (6.3)
G = 4		96 (54.9)
G = 5		68 (38.8)
Pubic hair Tanner stage	181	
PH = 2		3 (1.7)
PH = 3		20 (11.1)
PH = 4		58 (32.0)
PH = 5		100 (55.2)
Overall pubertal development[a]	197	
Pre + early + mid-pubertal		135 (68.5)
Late + post-puberty (PDS≥4)		62 (31.5)
Gonadal development[b]	196	
Pre + early + mid-pubertal		98 (50.0)
Late + post-puberty (PDS≥4)		98 (50.0)
Adrenal development[b]	197	
Pre + early + mid-pubertal		105 (53.3)
Late + post-puberty (PDS≥4)		92 (46.7)
TV ≥ 25 mL	139	65 (46.8)

[a] Based on PDS-Carskadon and Acebo algorithm.
[b] Based on PDS-Shirtcliff et al. algorithm.

4. Discussion

In this sample of adolescent males from the general population, urinary metabolites of OP insecticides and 1-NPL were associated with delayed sexual maturation, especially delayed genital development. Regarding the adrenal axis, malathion exposure was associated with delayed and 1-NPL with accelerated adrenarche. These associations appear to be independent of socioeconomic status, BMI, and pesticide

Table 4
Association between urinary pesticide metabolites, Tanner stage, and testicular volume (TV).

Outcomes	Pesticide metabolites	n	OR (95% CI)	OR (95% CI)	OR (95% CI)
Tanner G = 5 (N = 175)[a]	**TCPy: ≥ *vs.* <LOD**	55	1.57 (0.81–3.04)	1.52 (0.78–2.97)	1.39 (0.70–2.75)
	IMPy (ref: <LOD)				
	LOD-P75	80	1.33 (0.63–2.79)	1.43 (0.67–3.06)	1.50 (0.69–3.25
	>P75	40	1.08 (0.44–2.64)	1.12 (0.45–2.74)	1.16 (0.46–2.91)
	p-trend		0.91	0.97	0.97
	MDA (ref: <LOD)				
	LOD-P75	92	1.00 (0.34–2.93)	0.97 (0.33–2.87)	0.93 (0.31–2.80)
	>P75	38	0.67 (0.19–2.34)	0.66 (0.19–2.31)	0.64 (0.15–2.28)
	p-trend		0.39	0.32	0.35
	DETP (ref: <LOD)				
	LOD-P75	49	0.77 (0.35–1.68)	0.76 (0.34–1.65)	0.91 (0.40–2.05)
	>P75	41	0.28 (0.11–0.70) *	0.25 (0.10–0.65) *	0.27 (0.10–0.71) *
	p-trend		0.006	0.004	0.005
	DCCA (ref: <LOD)				
	LOD-P75	66	0.83 (0.38–1.82)	1.79 (0.79–4.04)	1.67 (0.73–3.85)
	>P75	34	0.78 (0.32–1.86)	0.91 (0.34–2.48)	0.76 (0.27–2.14)
	p-trend		0.58	0.61	0.41
	ETU (ref: <LOD)				
	LOD-P75	68	0.84 (0.38–1.82)	0.80 (0.36–1.75)	0.80 (0.36–1.78)
	>P75	46	0.78 (0.32–1.86)	0.75 (0.31–1.80)	0.81 (0.33–1.99)
	p-trend		0.65	0.62	0.74
	1-NPL: ≥ vs. <LOD	51	1.00 (0.49–2.03)	0.97 (0.48–1.99)	0.89 (0.43–1.86)
Tanner PH = 5 (N = 181)[b]	**TCPy: ≥ *vs.* <LOD**	57	1.09 (0.54–2.21)	1.12 (0.55–2.29)	1.04 (0.50–2.18)
	IMPy (ref: <LOD)				
	LOD-P75	81	0.49 (0.21–1.12)	0.46 (0.20–1.07)	0.51 (0.22–1.22)
	>P75	43	0.48 (0.18–1.25)	0.47 (0.18–1.23)	0.55 (0.50–1.46)
	p-trend		0.77	0.32	0.40
	MDA (ref: <LOD)				
	LOD-P75	92	1.37 (0.46–4.05)	1.45 (0.48–4.35)	1.33 (0.42–4.23)
	>P75	45	0.94 (0.27–3.24)	0.97 (0.28–3.37)	0.94 (0.25–3.53)
	p-trend		0.63	0.63	0.61
	DETP (ref: <LOD)				
	LOD-P75	51	0.78 (0.34–1.77)	0.78 (0.34–1.77)	1.00 (0.42–2.36)
	>P75	45	0.64 (0.27–1.54)	0.65 (0.27–1.58)	0.79 (0.31–1.97)
	p-trend		0.37	0.39	0.50
	DCCA (ref: <LOD)				
	LOD-P75	66	1.78 (0.79–4.04)	1.76 (0.77–4.01)	1.64 (0.70–3.78)
	>P75	36	2.14 (0.77–5.97)	2.11 (0.74–5.95)	1.81 (0.60–5.43)
	p-trend		0.18	0.20	0.34
	ETU (ref: <LOD)				
	LOD-P75	72	0.52 (0.22–1.22)	0.52 (0.22–1.24)	0.54 (0.22–1.32)
	>P75	46	0.56 (0.21–1.47)	0.57 (0.22–1.49)	0.68 (0.25–1.85)
	p-trend		0.52	0.53	0.41
	1-NPL: ≥ vs. <LOD	52	1.49 (0.70–3.19)	1.52 (0.71–3.26)	1.49 (0.68–3.28)
TV ≥ 25 mL (N = 139)	**TCPy: ≥ *vs.* <LOD**	44	1.12 (0.54–2.32)	1.05 (0.05–2.20)	1.03 (0.49–2.16)
	IMPy (ref: <LOD)				
	LOD-P75	68	1.58 (0.68–3.66)	1.76 (0.74–4.17)	1.73 (0.72–4.13)
	>P75	36	1.02 (0.38–2.69)	1.03 (0.38–2.78)	1.03 (0.38–2.77)
	p-trend		0.62	0.58	0.59
	MDA (ref: <LOD)				
	LOD-P75	91	0.39 (0.13–1.16)	0.36 (0.12–1.09)	0.36 (0.12–1.09)
	>P75	31	0.92 (0.27–3.18)	0.89 (0.26–3.10)	0.89 (0.26–3.13)
	p-trend		0.37	0.37	0.38
	DETP (ref: <LOD)				
	LOD-P75	42	1.32 (0.56–3.10)	1.34 (0.57–3.18)	1.41 (0.58–3.41)
	>P75	34	0.93 (0.36–2.34)	0.89 (0.35–2.30)	0.92 (0.36–2.40)
	p-trend		0.68	0.61	0.63
	DCCA (ref: <LOD)				
	LOD-P75	62	0.97 (0.44–2.15)	1.04 (0.46–2.33)	1.00 (0.44–2.27)
	>P75	30	0.86 (0.33–2.25)	0.97 (0.36–2.58)	0.92 (0.34–2.49)
	p-trend		0.75	0.93	0.81
	ETU (ref: <LOD)				
	LOD-P75	62	2.47 (1.01–6.02) *	2.37 (0.96–5.84)	2.34 (0.95–5.81)
	>P75	43	1.66 (0.63–4.34)	1.63 (0.62–4.30)	1.66 (0.62–4.40)
	p-trend		0.92	0.91	0.89
	1-NPL: ≥ vs. <LOD	50	0.45 (0.21–0.95) *	0.42 (0.20–0.91) *	0.41 (0.19–0.89) *

LOD: Limit of detection; P75: 75th percentile.
Model 1: adjusted for cohort, age, and urinary creatinine (mg/dL).
Model 2: adjusted for cohort, age, urinary creatinine, and maternal education.
Model 3: adjusted for cohort, age, urinary creatinine, maternal education, and child BMI z-score (continuous).
*p-value<0.05.
[a] N = 147 for MDA and DCCA.
[b] N = 149 for MDA and DCCA.

co-exposure.

Despite increasing restrictions on their utilization, OP insecticides remain widely used in agriculture (Hernández et al., 2019) and were detected in 31–87% of the present urine samples (collected in 2012–2019). MDA was the most prevalent, with concentrations that were higher than those reported for children aged 5–12 years from Valencia, Eastern Spain (detection frequency [DF] = 87% and P95 = 5.42 μg/L in the present study *vs.* DF = 7% and P95 = 1.39 μg/L in Valencia) (Fernández S. et al., 2020b), reflecting generalized exposure to malathion, currently authorized in the EU. Surprisingly, IMPy was detected in the urine of 67% of participants, despite the EU prohibition of its parent compound, diazinon, since 2006 (THE COMMISSION OF THE EUROPEAN COMMUNITIES, 2007), although IMPy concentrations were lower than those found in 2010 in 6–11-year-old children from Valencia (median = 0.24 *vs.* 5.16 μg/g creatinine) (Roca et al., 2014), but slightly higher than those found in 5–12-year-old children from Valencia in 2016 (P95 = 4.28 *vs.* 0.75 μg/L) (Fernández S. et al., 2020b). However, TCPy was detected in only 31% of the boys, with concentrations that were lower than those described by Roca et al. (2014) for Valencian children (P75 = 0.03 μg/g in the present study *vs.* P50 = 3.40 μg/g), although chlorpyrifos was approved in the EU during the urine sample collection period (The European Commission, 2020). The observation of lower TCPy concentrations in the present study compared to other studies remains unclear. The association between exposure to OP insecticides and delayed genital and gonadal development is consistent with their anti-androgenic action and with the finding in

Table 5
Association between urinary pesticide metabolites and pubertal development score (PDS).

Outcomes	Pesticide metabolites	n	OR (95% CI)	OR (95% CI)	OR (95% CI)
Overall puberty PDS ≥ 4 (N = 197)*	**TCPy: ≥ *vs.* <LOD**	62	0.64 (0.32–1.29)	0.63 (0.31–1.29)	0.61 (0.29–1.26)
	IMPy (ref: <LOD)				
	LOD-P75	84	1.15 (0.54–2.44)	1.22 (0.57–2.62)	1.36 (0.62–3.02)
	>P75	48	0.61 (0.25–1.53)	0.63 (0.25–1.60)	0.66 (0.26–1.70)
	p-trend		0.71	0.20	0.20
	MDA (ref: <LOD)				
	LOD-P75	95	2.28 (0.68–7.56)	2.12 (0.63–7.12)	1.94 (0.56–6.71)
	>P75	39	2.62 (0.67–10.13)	2.52 (0.65–9.82)	2.60 (0.65–10.38)
	p-trend		0.27	0.27	0.27
	DETP (ref: <LOD)				
	LOD-P75	53	1.20 (0.55–2.63)	1.16 (0.52–2.55)	1.40 (0.61–3.19)
	>P75	49	0.60 (0.25–1.43)	0.58 (0.24–1.40)	0.67 (0.27–1.63)
	p-trend		0.16	0.15	0.19
	DCCA (ref: <LOD)				
	LOD-P75	66	1.15 (0.51–2.61)	1.20 (0.52–2.76)	1.12 (0.51–2.83)
	>P75	40	0.85 (0.32–2.25)	0.91 (0.33–2.46)	0.73 (0.25–2.10)
	p-trend		0.66	0.75	0.43
	ETU (ref: <LOD)				
	LOD-P75	76	1.59 (0.73–3.47)	1.17 (0.56–2.44)	1.71 (0.75–3.91)
	>P75	48	1.56 (0.64–3.78)	0.87 (0.10–7.59)	1.92 (0.76–4.88)
	p-trend		0.53	0.53	0.41
	1-NPL: ≥ vs. <LOD	59	1.84 (0.91–3.71)	1.82 (0.90–3.69)	1.93 (0.94–3.99)
Adrenal development PDS ≥ 4 (N = 197)*	**TCPy: ≥ *vs.* <LOD**	62	0.84 (0.45–1.57)	0.86 (0.46–1.62)	0.81 (0.43–1.54)
	IMPy (ref: <LOD)				
	LOD-P75	84	1.02 (0.39–1.94)	1.02 (0.51–2.06)	1.17 (0.57–2.39)
	>P75	48	0.87 (0.39–1.94)	0.90 (0.40–2.03)	0.96 (0.42–2.18)
	p-trend		0.68	0.75	0.74
	MDA (ref: <LOD)				
	LOD-P75	95	0.35 (0.12–0.99)	0.32 (0.11–0.94)*	0.30 (0.10–0.90)*
	>P75	39	0.41 (0.12–1.40)	0.38 (0.11–1.33)	0.38 (0.11–1.32)
	p-trend		0.42	0.39	0.37
	DETP (ref: <LOD)				
	LOD-P75	53	0.55 (0.26–1.22)	0.51 (0.24–1.09)	0.53 (0.25–1.16)
	>P75	49	0.57 (0.26–1.22)	0.55 (0.26–1.20)	0.57 (0.26–1.24)
	p-trend		0.27	0.28	0.28
	DCCA (ref: <LOD)				
	LOD-P75	66	0.76 (0.35–1.65)	0.74 (0.34–1.63)	0.73 (0.33–1.63)
	>P75	40	0.49 (0.19–1.22)	0.48 (0.19–1.24)	0.49 (0.19–1.28)
	p-trend		0.13	0.13	0.14
	ETU (ref: <LOD)				
	LOD-P75	76	0.94 (0.47–1.91)	0.95 (0.46–1.95)	1.03 (0.50–2.14)
	>P75	48	1.73 (0.78–3.86)	1.79 (0.79–4.03)	2.02 (0.88–4.68)
	p-trend		0.11	0.10	0.09
	1-NPL: ≥ vs. <LOD	59	2.65 (1.33–5.26)*	2.67 (1.33–5.35)*	2.67 (1.32–5.40)*
Gonadal development PDS ≥ 4 (N = 196)*	**TCPy: ≥ *vs.* <LOD**	61	0.54 (0.29–1.02)	0.54 (0.28–1.02)	0.51 (0.27–0.99)*
	IMPy (ref: <LOD)				
	LOD-P75	84	1.20 (0.61–2.38)	1.25 (0.62–2.50)	1.36 (0.67–2.77)
	>P75	47	0.69 (0.31–1.55)	0.72 (0.31–1.62)	0.76 (0.33–1.73)
	p-trend		0.22	0.25	0.27
	MDA (ref: <LOD)				
	LOD-P75	98	1.90 (0.71–5.12)	1.77 (0.65–4.82)	1.69 (0.62–4.62)
	>P75	39	2.13 (0.66–6.87)	2.05 (0.63–6.66)	2.07 (0.64–6.72)
	p-trend		0.31	0.32	0.32
	DETP (ref: <LOD)				
	LOD-P75	53	1.09 (0.53–2.56)	1.03 (0.50–2.16)	1.18 (0.55–2.52)
	>P75	48	0.80 (0.38–1.70)	0.77 (0.36–1.66)	0.85 (0.39–1.85)
	p-trend		0.48	0.46	0.55
	DCCA (ref: <LOD)				
	LOD-P75	66	1.40 (0.66–3.01)	1.49 (0.68–3.24)	1.47 (0.67–3.23)
	>P75	40	1.22 (0.50–2.97)	1.33 (0.53–3.32)	1.20 (0.47–3.08)
	p-trend		0.49	0.46	0.36
	ETU (ref: <LOD)				
	LOD-P75	75	0.88 (0.44–1.79)	0.86 80.42–1.78)	0.90 (0.43–1.86)
	>P75	48	1.22 (0.55–2.71)	1.23 (0.55–2.77)	1.41 (0.61–3.22)
	p-trend		0.78	0.66	0.92
	1-NPL: ≥ vs. <LOD	59	1.08 (0.55–2.11)	1.06 (0.54–2.09)	1.01 (0.54–2.15)

LOD: Limit of detection; P75: 75th percentile.
Model 1: adjusted for age, cohort, and urinary creatinine (mg/dL).
Model 2: adjusted for age, cohort, urinary creatinine, and maternal education.
Model 3: adjusted for age, cohort, urinary creatinine, maternal education, and child BMI z-score (continuous).
[a]N = 157 for MDA and DCCA.
*p-value<0.05.

Belgian boys aged 14–15 years of an association between urinary concentrations of DAPs and delayed genital development (Croes et al., 2015). Additionally, a Danish cohort study observed that the sons of mothers occupationally exposed during pregnancy to multiple pesticides, including OPs, had smaller testicular size and shorter penis length at the age of 6–11 years compared with sons of non-exposed mothers (Wohlfahrt-Veje et al., 2012). These observations are supported by experimental findings that exposure to chlorpyrifos, diazinon, and malathion induces smaller testicular size and structural abnormalities in adolescent (Jayachandra and D'Souza, 2014; Slimen et al., 2014) and adult (Farag et al., 2010; Joshi et al., 2007) mice. This is biologically plausible because various OP insecticides exert anti-androgen activity by binding to ERα, ERβ, or AR and by interfering with the expression of genes involved in the metabolism of steroid hormones (Kojima et al., 2004; Manabe et al., 2006). In the present study, urinary MDA seems to be associated with smaller TV and delayed adrenarche, consistent with an animal study in which malathion exposure was found to downregulate gonadal development by targeting the expression pattern of transcription factors, activin A, orphan nuclear or sex steroid receptors, and steroidogenic enzymes (Prathibha et al., 2014). Interestingly, in our study in children from the INMA cohorts at the age of 7–11 years, urinary DETP was associated with delayed puberty onset in boys with overweight or obesity, which is in line with the present results, whereas TCPy was associated with earlier genital growth onset (Castiello et al., 2023), suggesting that the effect of pesticide exposure on pubertal development may depend on the age-time window.

The frequency of urinary 3-PBA detection was substantially lower in these boys than recorded in other biomonitoring studies. In addition, urinary 3-PBA concentrations were much lower (P95 = 0.16 μg/g) than those from larger samples of children from Japan (P50 = 1.40 μg/g and P95 = 13.09 μg/g) (Osaka et al., 2016), the United States (P50 = 2.50 μg/g and P95 = 13.09 μg/g) (Naeher et al., 2010), and China (P50 = 1.42 μg/g and P95 = 12.53 μg/g) (Ye et al., 2017a; 2017b). In Valencia, Spain, 3-PBA was detected in 23% and 79% of urine samples from children aged 6–11 years and 5–12 years, respectively, and concentrations were higher than in the present study (P95 = 12.33 μg/g and P95 = 11.57 μg/L, respectively), while detection of DCCA was higher (68% *vs.* 23%) but its concentrations were lower (6.74 μg/L *vs.* 46.65 μg/L) than those reported for children from Valencia (Fernández et al., 2020a, 2020b). Concentrations of DCCA (metabolite of pyrethroid insecticides permethrin, cypermethrin, and cyfluthrin) were not associated with sexual maturity status in this study, while this metabolite was not analyzed in children from the INMA cohorts at peripubertal age (Castiello et al., 2023). Various *in vitro* bioassays have documented the interaction of certain pyrethroids with estrogen and androgen receptors (Du et al., 2010; Kojima et al., 2004; Tange et al., 2014; Zhang et al., 2008). However, animal studies have reported contrasting results for the impact on sexual maturation according to the compound in question. For instance, exposure to cypermethrin accelerated puberty in mice by inducing the expression of StAr and Cyp11A1 genes related to testosterone production in Leydig cells and by inducing the synthesis of gonadotrophins in pituitary cells (Ye et al., 2017), whereas prenatal and postnatal exposure to bifenthrin was found to reduce testosterone production by downregulating gene expression in Leydig cells (Jin et al., 2013). To our knowledge, no previous human study has examined the association between urinary DCCA and pubertal development.

To our best knowledge, this is the first study to assess the association between 1-NPL and pubertal development in children. 1-NPL is the hydrolysis product not only of the insecticide carbaryl but also of naphthalene, a polycyclic aromatic hydrocarbon. Thereby, exposure misclassification can be caused by differences in exposure source (Meeker et al., 2007). No published study could be traced on the association of 1-NPL with sexual maturity in adolescents. A study of adult males by Meeker et al. (2006) found an association between urinary 1-NPL and lower testosterone levels, in agreement with the association of 1-NPL with delayed genital development in the present study and with lower testosterone and FSH levels in a previous investigation (Freire et al., 2021). A study in adult male rats found that exposure to carbaryl was associated with reduced testicular size, decreased testosterone levels, and increased gonadotrophin levels (Fattahi et al., 2012). Although the mechanisms underlying these relationships are poorly understood, they may involve estrogenic and anti-androgenic effects (Andersen et al., 2002; Klotz et al., 1997; Tange et al., 2016) and GnRH neuronal damage (Smulders et al., 2003) demonstrated in experimental studies. Conversely, the present observation of an association between 1-NPL and accelerated adrenal development is in agreement with our previous finding of an association between urinary 1-NPL and higher serum DHEAS concentrations (Freire et al., 2021), a marker of adrenal development, but it could also be a chance finding.

Dithiocarbamates are the most widely used fungicides in the EU, where Spain is one of the largest consumers of agricultural fungicides (Eurostat, n.d.). Exposure to mancozeb has been associated with adverse reproductive effects in males (Runkle et al., 2017), although no published study could be found that addressed the impact of childhood ETU exposure on sexual maturation. ETU was detected in 63% of the present urine samples, with concentrations similar to those in French children aged 3–10 years living near vineyards frequently treated with dithiocarbamates (Raherison et al., 2019) but higher than those observed in occupationally exposed pregnant women in California (Castorina et al., 2010) and male farmers in North Carolina (Arcury et al., 2017). In this study, ETU was slightly associated with a larger TV but with no evidence of a linear increasing trend. EBDC fungicides have demonstrated anti-androgenic effects *in vitro* (Manabe et al., 2006; Yu et al., 2015) and a toxic effect on testicles and sperm quality in animal models (Kackar et al., 1997; Khan and Sinha, 1996), and they might therefore be expected to delay sexual maturation in adolescents. However, childhood exposure to ETU was associated with earlier puberty development in boys and girls from the INMA cohorts at peripubertal age, particularly earlier breast development in girls and earlier genital development in boys (Castiello et al., 2023). In this line, prenatal exposure of rats to mancozeb was found to increase circulating levels of kisspeptin, a hypothalamic mediator of puberty onset (Overgaard et al., 2013). ETU is a known antithyroid compound (Hurley et al., 1998; Marinovich et al., 1997) that reduces thyroxine levels and increases the production of thyroid-stimulating hormone (TSH) in animal studies (Axelstad et al., 2011; Kackar et al., 1997; Medda et al., 2017; Panganiban et al., 2004; Piccoli et al., 2016). This is of interest due to the known interaction between the thyroid and gonadal axis (Ren and Zhu, 2022), and elevated TSH levels have been proposed as a contributing factor in central precocious puberty in girls (Jung et al., 2019). Nevertheless, the association observed in the present study was weak and could be a chance finding.

The sample size was relatively small, offering relatively low statistical power, and the cross-sectional design of the study prevents confirmation of a causal relationship between pesticide exposure and sexual maturation. Study participants from Granada were more likely to reside in an urban area and mothers of boys from both Granada and Menorca were less likely to have a stable partner than those in the original cohorts, and therefore potential selection bias may be considered. Additionally, the quantification of non-persistent pesticide metabolites in spot urine samples indicates recent exposure because their biological half-life is short (4–48 h) (Egeghy et al., 2011), and several studies have shown moderate temporal reliability for urinary pesticide metabolites. For instance, the intra-class correlation coefficient (ICC) for urinary DETP in 7-year-old European children was 0.37 for between-day variability and 0.35 for between-season variability (Casas et al., 2018). The ICC for IMPy ranged between 0.40 and 0.50 in pregnant Spanish women (Bravo et al., 2020), while urinary ETU and TCPy (ICC = 0.67 and 0.52, respectively) showed fair reliability in Costa Rican children (6–9 years) residing in an agricultural area (van Wendel de Joode et al., 2016). Nevertheless, given that the diet is the main source of pesticide exposure in the general population, the exposure may be more or less continuous, with children receiving low daily doses of pesticides with

their food; hence, metabolites of the pesticides can be expected to maintain stable levels in serum and target tissues (Côté et al., 2014; Wielgomas, 2013). Thus, a recent study in non-occupationally exposed adults found good repeatability (ICC>0.75) for urinary TCPy and pyrethroid metabolites 3-PBA and DCCA over a one-year period, concluding that one 24-h urine sample may be considered sufficient to characterize long-term exposure to non-specific pyrethroid metabolites (Klimowska et al., 2020). Future studies should be developed to maximize reproducibility and achieve good characterization of the temporal variability. The detection frequency of some of the analyzed metabolites was low, reducing the sensitivity to detect effects and hampering assessment of the combined effect of mixtures of pesticides (Keil et al., 2020). In this regard, it is very likely that urinary 3-PBA concentrations were substantively underestimated, given that pyrethroid metabolites such as 3-PBA are largely present as phase II conjugates (glucuronide and/or sulphate) in urine (up to 85%) (Baker et al., 2004), and this deconjugation step was omitted. In addition, information on urinary concentrations of methyl phosphate metabolites would have provided a broader picture of the effect of OP insecticide exposure. It is also not possible to rule out the potential confounding effect of other EDCs. The strength of this study is that it is the first to examine the association between biomarkers of exposure to non-persistent pesticides and human male sexual maturation, contributing to scant knowledge on this relationship in this age window (14–17 years). The inclusion of boys from two different geographical areas is an additional strength. However, the study involved multiple comparisons, and it cannot be ruled out that some of the significant results may be due to chance.

5. Conclusions

These findings contribute to increasing evidence that exposure to non-persistent pesticides may be related to sexual maturation, suggesting a delaying effect for OP and carbamate insecticides and an accelerating effect for EBDC fungicides. Prospective studies with larger sample sizes and improved exposure assessment are warranted to verify these results and assess the impact of exposure to mixtures of pesticides.

Author contributions statement

Francesca Castiello: Formal analysis, writing original draft, resources. Beatriz Suárez: Methodology, investigation. José Gómez-Vida: Methodology, investigation. Maties Torrent: Investigation, resources. Mariana F. Fernández: Supervision, resources. Nicolás Olea: Conceptualization, reviewing and editing. Carmen Freire: Conceptualization, review and editing, supervision, project administration, funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.138350.

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