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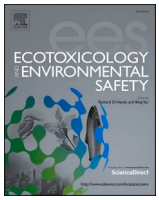

Expression of carboxylesterase and paraoxonase in the placenta and their association with chlorpyrifos exposure during pregnancy

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ABSTRACT

Exposure to environmental chemicals during pregnancy, including organophosphate pesticides, can affect the health of both the mother and the fetus, and have repercussions later in life. The present study aimed to determine whether the A-esterases paraoxonases (PON) and the B-esterases carboxylesterases (CES) are modulated in the placenta of pregnant women residing in an intensive pesticide use scenario. A total of 104 healthy pregnant women were recruited between 2018 and 2022 and were classified according to their residential settings in rural (RG) and urban (UG) groups. Chlorpyrifos (CP) level in the placenta was determined by GC-ECD, and confirmed by GC-MS. To analyze possible impacts in esterases, the CES and PON activity, mRNA transcript and protein expression levels were studied. Significantly higher CP levels were detected in RG vs UG. Also, CES activity determined with 1-naphthyl acetate substrate was significantly lower in RG vs UG. In contrast, PON arylesterase and lactonase activities were up modulated in RG vs UG. Likewise, mRNA transcript levels of CES1, CES2 and PON2 were upregulated in the RG along with increases in CES2 and PON2 protein expressions. Moreover, a positive significant correlation was determined between CP concentration and CES1 and CES2 mRNA levels. Rural samples showed elevated CP concentrations and alterations in esterases, which elucidate the impact of CP exposure in mRNA CES and PON regulation. These findings highlight the need for further investigation into the effects of pesticide exposure during pregnancy and to deepen the knowledge about the function that esterases play in the placenta.

1. Introduction

There is an increasing interest in studying the changes that exposure to environmental chemicals, including pesticides, can induce in human beings. It is known that exposure to xenobiotics may start as early as gestation begins. The exposure to xenobiotics during pregnancy may

Abbreviations: 4-MUB, 4-methylumbelliferon; 4-MUBA, 4-methylumbelliferyl acetate; CES, carboxylesterase; CP, chlorpyrifos; DHC, dihydrocoumarin; ECD, electron capture detector; EI, electron ionization; GC–MS, gas chromatography-tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MFA, Multiple Factor Analysis; O-HPPA, 3-o-hydroxyphenyl propionic acid; OP, organophosphate pesticides; PON, paraoxonase; RG, rural group; TCP, 3,5,6-trichloro-2-pyridinol; UG, urban group; YWHAZ, Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide.

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impact both the mother and the fetus. Exposure to environmental pesticides during gestation can lead to a variety of adverse health consequences for the neonate, both in early childhood and later in life (Treviño et al., 2023). Pesticides may also impact the placenta at molecular, cellular, and physiological levels (Espinoza et al., 2016; Fouyet et al., 2022; Guiñazú et al., 2012; Ridano et al., 2017). The placenta is a transient organ that plays a crucial role in intrauterine development and growth of the fetus. This organ secretes hormones and growth factors and acts as a semi-permeable barrier, facilitating the exchange of gases, nutrients, and metabolic waste products between the mother and fetus (Nelissen et al., 2011). The transport of potentially hazardous chemicals through placentas can result in various adverse effects on the fetus, which can manifest during embryo-fetal development or predispose individuals to diseases later in life (Mathiesen et al., 2021). It has been recognized that a healthy placenta is a requisite for a healthy pregnancy (Huppertz and Schleußner, 2023). The placenta is also a sensitive tissue that can be affected by environmental contaminants. In this sense, the reported placenta alterations induced by pesticides include changes in the placenta transcriptome (Wang et al., 2025), changes in hormone levels and signaling (Padmanabhan and Veiga-Lopez, 2024; Rivero Osimani et al., 2016), DNA damage (Capriati et al., 2023), changes in enzyme activity (Bulgaroni et al., 2013), among others.

Organophosphate pesticides (OP) are commonly used worldwide for pest control, with chlorpyrifos (CP) being the most widely used OP worldwide (Sharma et al., 2019). The acute neurotoxic effect of OP is based on its inhibitory effects on the esterase enzyme acetylcholinesterase (Costa, 2018), other toxic targets and effects may occur at low exposure levels, especially in susceptible subpopulations, such as pregnant women, and children (Treviño et al., 2023; Costa, 2018). Alternative toxicity targets of CP include oxidative stress and endocrine disruption, among others (Chiapella et al., 2014; Lukaszewicz-Hussain, 2010; Ubaid ur Rahman et al., 2021; Ventura et al., 2016) Prenatal exposure to CP impacts the neonate morphometric parameters as well as the neurocognitive development, among other deleterious effects (Eskenazi et al., 2004; Hertz-Picciotto et al., 2018). The use of CP is being revised worldwide, the European Union banned CP in 2020 (EFSA, 2019), later in 2022 the US EPA banned CP for agricultural and food uses (EPA, 2021). Argentina is a country with high pesticide usage for agrarian production (Sharma et al., 2019), with CP being the most consumed insecticide (Alvarez et al., 2019) until it was banned in June 2023 (SENASA, 2021). Moreover, CP was frequently detected in marketed fruits and vegetables (Mac Loughlin et al., 2018) as well as in the environment (Alvarez et al., 2019). Nevertheless, CP is still being used in several countries worldwide, including India and China (Foong et al., 2020).

The esterase enzyme family was classified into A, B, and C esterases as they interact with OP. The paraoxonases (PON-1, 2, and 3) are A-esterases, and share a 60 % similarity at the amino-acid level, though their localization, stress response, and functions differ (Schweikert et al., 2012). The PONs show differences in their paraoxonase, arylesterase, and lactonase activities but share antioxidant properties (Taler-Verčič et al., 2020), and play a role in cellular protection against oxidative stress. PON1 is found to be associated with serum high-density lipoproteins (HDL), PON2 is found to be associated with endoplasmic reticulum and mitochondria, while PON3 is found to be associated with endoplasmic reticulum, mitochondria, and HDL (Levy et al., 2019). PON2 and PON3 have been reported to be expressed in the placenta (Dikbas et al., 2017). It has been demonstrated that low PON1 activity may contribute to the developmental toxicity and neurotoxicity of OP (Costa et al., 2013). Nevertheless, the isoforms PON2 and PON3 have been studied to a lesser extent.

B-esterases, including acetylcholinesterase and carboxylesterases (CES), are inhibited by OP. Hence the B-esterases are considered exposure biomarkers to OP. CES play a role in hydrolyzing carboxylic acid esters, amides, and thioesters. Three human CES have been catalytically characterized: CES1, CES2, and CES3 (Yan, 2014). These enzymes are known to play critical roles in detoxifying organophosphate, carbamate, and pyrethroid pesticides. This study aimed to determine whether there were any changes in the expression of A and B esterases (CES and PON), which are essential for the metabolism and reduction of the toxicity of OP compounds. Furthermore, the objective was to ascertain whether these modifications/alterations were associated with CP exposure among pregnant women residing in rural and urban areas.

2. Materials and methods

2.1. Reagents

CP standard solution and PCB-103 (internal standard) were purchased from Absolute Standards (Connecticut, USA), and Ultra Scientific (California, USA), respectively. The chemicals used were of reagent grade and were obtained from Merck (Buenos Aires, Argentina), antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA) or Merck (Buenos Aires, Argentina). Primers were purchased from Macrogen (Seoul, Korea).

2.2. Participant recruitment

The study included one hundred four (104) healthy pregnant women (18–45 years old). These participants were recruited from the San Lucas prenatal clinic in Neuquén City, located in North Patagonia, Argentina. Samples were collected from 2018 to 2022. Women in the third trimester of pregnancy were invited to participate in this study and were included if they had a moderate income level, identified as Hispanic, and were schedule for a planned cesarean section either due to a previous cesarean section or fetal breech presentation. Women were excluded if they smoked, suffered infectious, chronic disease, were medicated (except those included in Group A according to the U.S. Food and Drug Administration), were involved in agricultural work, or developed a pregnancy complication (i.e., gestational diabetes, hypertension, or preeclampsia). The objective of this work was to study possible associations between the modulation of enzymes relevant to OP metabolism and the exposure to pesticides in the human placenta. Exclusion criteria included maternal characteristics that may interfere with the results such as maternal smoking, chronic diseases (i.e diabetes Mellitus) and medication, that may regulate the expression of enzymes, the oxidative status of the placenta, the placenta weight as well as neonate morphometric characteristics (Walker et al., 2019; Wang et al., 2014; Singh et al., 2024). Infectious diseases were also excluded to meet biosecurity criteria for handling human samples in our laboratory facilities. At the time of recruitment, the physician assisted the participant in completing a guided questionnaire including place of residence, previous childbirth, the maximum education level achieved, and pesticide exposure risk conducts such as indoor fumigation and well water consumption (Table SM1). Information about the neonate morphometric parameters at birth (weight, height, head circumference), and gestational age were collected from medical records. The placentas were weighed immediately after delivery. Written informed consent was obtained from each participant. This study was approved by the ethical committee of the local Advisory Committee of Biomedical Research in Humans (RIS 08.07.08 CAIBSH). For all determinations, several inner parts of the placenta were randomly collected to obtain a representative sample. The samples were washed twice in PBS (phosphate buffer saline, pH=7.4) and frozen at −80 ºC, until analysis.

2.3. Site description

The study included participants from the High Valley of the Rio Negro and Neuquén in the North Patagonia region. The participants were classified into urban and rural groups The urban group (UG, n = 47) lived in Neuquén City and had no history of pesticide exposure, and the rural group (RG, n = 57) lived on farms or in proximity to the

orchards (≤ 5000 m.) (Cecchi et al., 2021; Rodriguez et al., 2023), in places where pears, apples, and peaches are cultivated, for both domestic consumption and export purposes (SENASA, 2022). Additionally, approximately 1200 ha are cultivated with vegetables and fine fruits (SENASA, 2022; INTA, 2020). At the time of participant recruitment, chemical pest control of codling moth, scale insects, and caterpillar, whitefly, among others, primarily involved the use of OP (mainly CP), pyrethroids, and neonicotinoids (Cecchi et al., 2021; Cichón et al., 2017). The applications were performed using air blast sprayers, dispersing pesticides as droplets or particles. CP use was banned in Argentina in June 2023 (SENASA, 2021). All samples were collected before 2023.

2.4. Pesticide determination

CP determination was performed according to Rodriguez et al., (2023). Briefly, 2 g of the placenta was Twisselman extracted with n-hexane:dichloromethane mixture (pesticide grade). Lipid content was determined gravimetrically after gel permeation chromatography using Bio-Beads S-X3 (200–400 mesh, Bio-Rad Laboratory, USA). The pesticide fraction was purified by silica gel chromatography. Extracts were concentrated to 1 ml and kept in sealed vials at −20 °C before gas chromatography analysis.

CP was determined using a gas chromatograph Shimadzu 17-A equipped with a ^{63}Ni electron capture detector (ECD) (Rodriguez et al., 2023) and confirmed by gas chromatography-tandem mass spectrometry (GC–MS) with electron ionization (EI) Shimadzu Q2010. Details of chromatographic analyses are in the supplementary material. CP concentration was expressed as ng/g lipid weight (ng/g lw). Data under the limit of detection (LOD) were considered as LOD/2 for statistical analyses (USEPA, 2022). LOD was 0.19 ng/ml for GC-ECD.

2.5. Enzyme assays

Placental samples (120 mg) were homogenized using 2.5 ml of 0.1 M phosphate buffer, pH 6.5, containing 0.5 % Triton X-100. Then, centrifuged at 12,400 xg for 10 min. at 4 ºC. Supernatants were then collected for enzymatic activity determination. CES activity was measured using 1-naphthyl acetate at 550 nm, in a 96-well microtiter plate by a colorimetric assay previously described (Vera et al., 2012).

CES activity was also determined through the hydrolysis of the substrate 4-methylumbelliferyl acetate (4-MUBA) to the product 4-methylumbelliferone (4-MUB), at 37 °C by fluorometry (Lamego et al., 2015), using an F7000 fluorescence spectrophotometer (Hitachi). Placental samples (100 mg) were homogenized using 1 ml of 20 mM Tris/HCl (pH 8.0) and centrifuged at 10,000 xg for 5 min. at 4 °C. Then the homogenates were incubated with 0.05 mM 4-MUBA (in a mixture of 1:1 DMSO, 90 mM KH_2PO_4, 40 mM KCl, pH 7.4). After 15 minutes of incubation, activity was recorded. A calibration curve of 4-MUB (0.5–25 μM) was included. Hanks balanced salt solution, was used as reaction buffer, with a final volume of 300 μL per well.

Paraoxonase (PON) in placental samples was measured via arylesterase and lactonase activities using the substrates phenylacetate and dihydrocoumarin (DHC), respectively. Placenta arylesterase activity was determined using a working solution of 20 mM Tris/HCl buffer pH 8.0, containing 4 mM phenylacetate and 1 mM $CaCl_2$. Measurements were conducted at 270 nm at 25 °C. Arylesterase activity was calculated with the phenol molar absorbance coefficient 1310 $M^{-1}cm^{-1}$. One unit of arylesterase activity was defined as 1 μmol phenylacetate hydrolyzed/min and expressed as U/mg protein (Schrader et al., 2012).

Placenta lactonase activity was determined kinetically using a working solution of 50 mM Tris/HCl, 1 mM CaCl2, pH 8.0 containing 1 mM DHC (Solmaz Avcıkurt and Korkut, 2018; Draganov, 2010). Measurements were conducted at 270 nm, 25 °C. The molar extinction coefficient of 3-(o-hydroxyphenyl) propionic acid (o-HPPA) was used to calculate the rate of DHC hydrolysis (1295 $M^{-1}cm^{-1}$).

Total protein content was determined by the Bradford method (Bradford, 1976). All measurements were performed in triplicate and a mean value was considered for the calculations.

2.6. Native electrophoresis of carboxylesterase

Native polyacrylamide gel electrophoresis was carried out on a Bio-Rad Tetra Cell Electrophoresis Unit (Bio-Rad Laboratories, California, USA). Five randomly chosen placenta samples per group (100 mg) were homogenized in 0.1 M phosphate buffer, pH 6.5, containing 0.5 % Triton X-100. Equal amounts of protein, 80 μg per sample, were loaded on 7 % polyacrylamide gel. The electrophoresis was conducted with running buffer (25 mM Tris, 192 mM glycine, pH 8.8) at 75 V for 15 min and subsequently at 150 V until the tracking dye, bromophenol blue, reached the bottom of the gel. The CES activity bands were revealed with a staining solution composed of 100 ml of 50 mM sodium phosphate buffer (pH 6.5), 2.5 ml of 2 % 1-naphthyl acetate, and 0.025 g of fast garnet salt. After staining for 30 min. at room temperature, the gels were washed with water. Stained gels were scanned, and band relative intensity was determined by densitometry analysis (Gel Analyzer 19.1 program).

2.7. Western blot analysis

Eight randomly chosen placenta samples per group were homogenized (100 mg) in an ice bath using 1 ml RIPA buffer (1 % Triton X-100, 0.5 % sodium deoxycholate, 9 % SDS, 5 % DTT,1 mM sodium orthovanadate, 10 μg PMSF, 30 μg aprotinin). Equal amounts of protein (100 μg) were diluted in SDS sample buffer, boiled at 100 °C for 5 min., immersed in an ultrasonic bath for 15 s, loaded onto a 10 % SDS-PAGE gel, and run at 150 V for 1 h. After migration, proteins were electrotransferred to a nitrocellulose membrane (Bio-Rad Laboratories, California, USA) at 100 V for 1 h. Then, the membrane was blocked in TRIS buffer (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) containing 0.05 % Tween 20 and 5 % non-fat dry milk, washed, and incubated overnight with the following primary antibodies: anti-CES2 (1:1000), anti-PON2 (1:1000), and anti-Actin (1:5000). After washing, the blots were incubated with peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies (1:5000 and 1:10000 respectively) for 1 h at room temperature. Protein-antibody complexes were visualized by an enhanced chemiluminescence detection system. Actin protein was used as an internal standard.

2.8. RNA isolation, PCR, and qPCR

Total RNA was isolated from placental tissue using TRIzol® reagent (Sigma Aldrich, St. Louis, MO, USA). cDNA was synthesized using the Strascript reverse transcriptase kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Carboxylesterase 1 (CES1), 2 (CES2) and 3 (CES3) and paraoxonase 1 (PON1), 2 (PON2), and 3 (PON3) transcripts expression in the placenta were first determined by conventional PCR. Briefly, 3 μl cDNA was amplified with the Pegasus DNA polymerase (PBL, Buenos Aires, Argentina) in a 20 μl final volume. The cDNA obtained was subjected to PCR amplification using the following primers; for CES1 forward: 5´AGA GGA GCT CTT GGA GAC GAC AT 3´, reverse: 5´ACT CCT GCT TGT TAA TTC CGA CC 3 (NM_001025195.1), for CES2 forward: 5 AAC CTG TCT GCC TGT GAC CAA GT 3´, reverse: 5´ACA TCA GCA GCG TTA ACA TTT TCT G 3 (NM_003869.6), for CES3 forward: 5 CTG GTC CTT AGC AAG AAG CTG AAA 3´, reverse: 5´CAT TTG GCT TGT GCG TCC GAG TT 3´(NM_024922.6), for PON1 forward: 5´GCT GAG TTG CTG GCT CAT AAG 3´, reverse: 5´TGC AGG AGG ATT CTC TGA GTC 3´(NM_000446.6), for PON2 forward: 5´TCG TGT ATG ACC CGA ACA ATC C 3´, reverse: 5´AAA GTG CCT ATG AGC AGC TTC C 3´(NM_000305.3), for PON3i forward: 5´TGC CAA TGG GAT CAC AGT CTC 3´, reverse: 5´GTT ATC CAC TAA GGT GCC CAA C

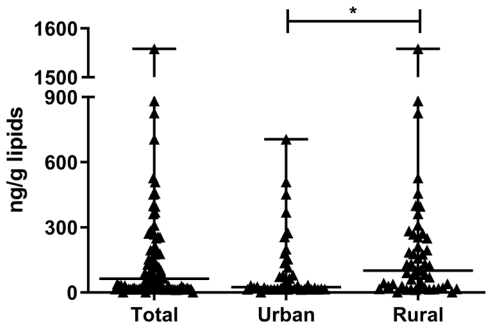

Fig. 1. Levels of chlorpyrifos in the placenta samples. Chlorpyrifos levels (ng/g lw) in placenta samples. Graph shows median and range of chlorpyrifos. *p = 0.017, Mann-Whitney test.

3´(NM_000940.3) and YWHAZ forward: 5´CCT GCA TGA AGT CTG TAA CTG AG 3´, reverse: 5´- TTG AGA CGA CCC TCC AAG ATG −3´(EF094937.1) (Choudhury et al., 2017). The amplification conditions were: 1 cycle at 95 °C for 3 min, 35 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, 1 cycle at 72 °C for 10 min, and 1 cycle at 4 °C for 10 min (Guiñazú et al., 2012). Bands were visualized in a 2 % agarose gel, stained with GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA).

CES1, CES2, PON2, and PON3 transcripts expression levels were measured using a qPCR assay conducted on the Mastercycler® ep Realplex (Eppendorf). In addition, PON3 transcript was measured with 2 different primer pairs (PON3i (see above) and PON3ii forward 5´-TCC TGG CGT TTA GAG AAA GGG-3, reverse 5´-TCA TCT GGC GCA AAG TTT GG-3´). Briefly, 5 µl cDNA of a dilution (1:3) from samples were amplified in 15 µl final volume containing IQTM SYBR® Green Supermix (Bio-Rad Laboratories, California, USA), the forward and reverse primers and DEPC water. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ) gen was used as the internal control (Choudhury et al., 2017). Primer efficiencies reached 98 %. PCR program consisted of an initial denaturation at 95 °C for 10 min; followed by 50 cycles of amplification (95 °C for 15 s and 60 °C for 60 s). Melting curve analysis was also performed. All PCR reactions were run in triplicates and non-template controls were included. PCR products were confirmed by electrophoresis in 2 % agarose gel, stained with GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA). Relative gene expressions were calculated using the $2^{-\Delta Ct}$ method.

2.9. Statistical analysis

Categorical variables (educational level, smoking status, alcohol and groundwater consumption, indoor pesticide use) and the pesticide detection between rural and urban groups were analyzed by Fisher's exact test, since some variables had a small sample size (i.e. groundwater consumption). Statistical significance between medians was determined by the Mann-Whitney test, since some variables were not normally distributed. The associations between analytical variables: CES and PON enzyme activity levels, CES1, CES2, and PON2 mRNA levels, and the neonate and placenta morphometric parameters were estimated by calculating the Spearman correlation coefficient, since some variables were not normally distributed. A Multiple Factor Analysis (MFA) was performed to characterize the quantitative (enzyme activity levels, mRNA levels, placenta weight, placenta weight/neonate weight ratio or Placental Index) and categorical variables (CP detection, urban or rural group, pesticide application period). Possible confounders (age, parity, educational level, body mass index, smoking status, groundwater, indoor pesticide, neonate sex, gestational age) and chlorpyrifos detection were fitted with GLM. None of these possible confounders was statistically significant in the GLM model. The statistical significance was 0.05. Statistical analyses were performed using GraphPad Prism 8.0.2

Table 1
Demographic characteristics of the mothers and anthropometric characteristics of the neonate.

Variable	*Total (n = 104)*	*Urban residence (n = 47)*	*Rural residence (n = 57)*	*Urban vs Rural group p-value*
Age (years)	31.7 ± 5.87	32.0 ± 5.20	31.4 ± 6.37	0.64 (Unpaired t test)
Parity	1.02 ± 0.96	1.02 ± 0.96	1.02 ± 0.97	0.99 (Mann-Whitney test)
Educational level (%)				
Greater	44.1	45.0	43.4	0.99 (Fisher's exact test)
Secondary complete	34.4	42.5	28.3	0.19 (Fisher's exact test)
Primary complete	17.2	12.5	20.8	0.41 (Fisher's exact test)
No education/ primary incomplete	4.3	0	7.5	0.13 (Fisher's exact test)
Body-mass index (%)				
Normal	67.3	72.1	63.6	0.39 (Fisher's exact test)
Smoking status (%)				
Passive smoker	13.3	16.3	10.9	0.55 (Fisher's exact test)
Groundwater consumption (%)	3.1	0	5.5	0.25 (Fisher's exact test)
Alcohol consumption (%)	4.1	4.7	3.6	0.99 (Fisher's exact test)
Indoor pesticides use (%)	9.2	7.0	10.9	0.73 (Fisher's exact test)
Female neonates	62.6	65.1	60.7	0.68
Male neonates	37.4	34.9	39.3	(Fisher's exact test)
Neonate weight (g)	3520.9 ± 428.4	3427.9 ± 391.7	3592.3 ± 444.8	0.06 (Unpaired t test)
Neonate height (cm)	49.2 ± 1.95	49.0 ± 1.62	49.5 ± 2.18	0.20 (Unpaired t test)
Ponderal index	2.94 ± 0.25	2.92 ± 0.28	2.96 ± 0.23	0.68 (Mann-Whitney test)
Head circumference (cm)	35.6 ± 1.11	35.5 ± 1.03	35.6 ± 1.18	0.97 (Mann-Whitney test)
Placenta weight (g)	597.5 ± 128.1	576.9 ± 131.6	613.5 ± 124.1	0.17 (Unpaired t test)
Placenta weight/ neonate weight ratio	0.169 ± 0.029	0.167 ± 0.031	0.170 ± 0.028	0.64 (Unpaired t test)
Gestational age (weeks)	38.5 ± 1.05	38.3 ± 1.13	38.6 ± 0.97	0.36 (Mann-Whitney test)

The results were expressed as mean ± SD or as percentages when indicated. Placenta weight/neonate weight ratio: placenta weight/neonate weight (g/g). Ponderal index: neonate weight/neonate length (g/cm^3). Unpaired *t*-test and Fisher's exact test were used to analyze categorical variables. Mann-Whitney test was used to determine statistical significance between medians.

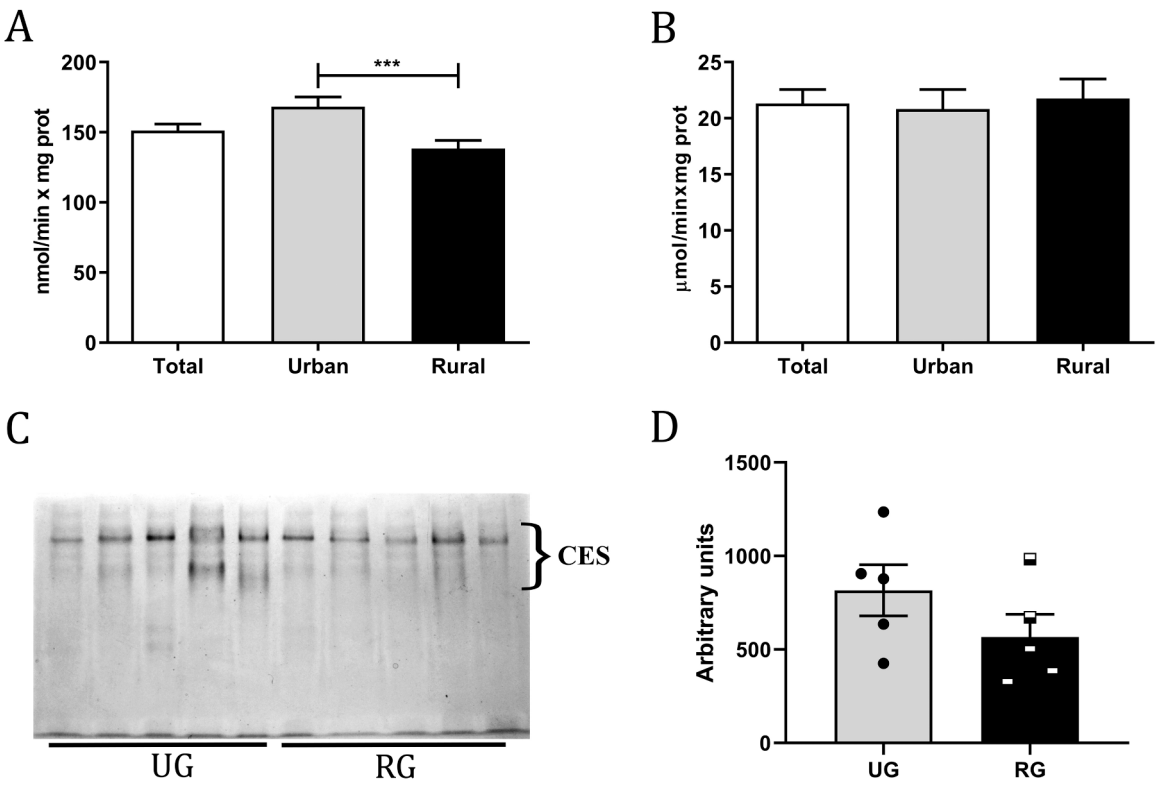

Fig. 2. Activity of carboxylesterase (CES) in placenta samples. (A) CES activity was measured using 1-naphthyl acetate as substrate and expressed as nmol of substrate hydrolyzed/min x mg protein. * **p = 0.0009, Mann Whitney test. (B) CES activity was measured using 4-MUBA as substrate and expressed as µmol of product formed/min x mg protein. (C) Non-denaturing activity gel electrophoresis of placenta samples. 80 µg of placenta lysates were separated on 7 % non-denaturing gel and stained for CES activity with 1-naphthyl acetate as substrate and fast garnet as colorant. Samples shown are urban group (UG) and rural group (RG). (D) CES band relative intensities were estimated by densitometry analysis (Gel Analyzer 19.1 program). The graphs show the mean activity ± SEM.

(GraphPad Software, Inc., USA) and R Statistical Software (R Core Team, 2024).

3. Results

3.1. Chlorpyrifos placenta levels

The mean placental concentration of CP in women (n = 90) residing in North Patagonia was 153.7 ± 234.3 ng/g lw, with a detection frequency of 65.6 %. The level of CP was also analyzed considering the urban or rural setting of the participants. Fig. 1 shows significant differences between the median values (ng/g lw) of UG 25.12 (range <LOD-705.72, n = 40) vs RG 101.1 (range <LOD-1558, n = 50) (p = 0.017, Mann-Whitney). The detection frequency of CP was 55 % in UG and 74 % in RG.

3.2. Population and morphometric characteristics of the neonate and the placenta

The socio-demographic characteristics of the study participants are summarized in Table 1. Women were similar in terms of age (31.7 ± 5.87 years), previous childbirths (1.02 ± 0.96), nutritional state, smoking status, and alcohol consumption. In the UG more women (87.5 %) finished secondary school, in contrast with RG (71.7 %, p = 0.078, Fisher). The risk factors for pesticide exposure, indoor pest control utilization, and well water consumption were also analyzed, but non-significant differences between groups were observed. However, only women residing in rural surroundings reported to consume well water (3 women) (Table 1). The neonate morphometric parameters and the placenta weight were similar between groups (Table 1). Additionally, the placenta weight to neonate weight ratio (an indicator of placenta functional efficiency) (Almog et al., 2011), and the ponderal index (an indicator of fetus nutrition), were evaluated. The results shown in Table 1 exhibit no significant changes between groups.

3.3. Activity and expression levels of placenta carboxylesterase and paraoxonase

As shown in Fig. 2A, placental samples of the UG showed significantly higher CES activity (17.7 %) compared to those in the RG (p = 0.0009, Mann-Whitney), indicating exposure to anticholinesterase compounds in rural surroundings. Since the substrate 1-naphthyl acetate is preferentially metabolized by CES1 isoform (Vera et al., 2012), CES activity was also analyzed using the 4-MUBA substrate, which is preferentially metabolized by the CES2 isoform (Gabriele et al., 2018), to determine the impact on other CES isoforms, As shown in Fig. 2B, CES activity determined by 4-MUBA, showed no significant changes between groups. Changes in CES activity between RG and UG, were also observed by activity determination in native PAGE gels with the substrate 1-naphthyl acetate. As shown in Fig. 2C and D, RG samples show a lower CES activity than UG samples.

Then, we studied CES 1, 2, and 3 transcript expression in the placenta, by conventional PCR we found the expression of CES1 and CES2 isoforms (Fig. 3A), as reported by others (Yan et al., 1999). Next, we studied whether the levels of CES1 and CES2 transcripts were differently modulated in UG vs RG. By qPCR, we determined that both CES1 and CES2 transcripts were significantly upregulated in RG vs UG (Fig. 3B, C). CES1 mRNA increased 11.2 times and CES2 3.08 times in RG compared to UG samples. Finally, we assessed CES 2 protein expression levels by western blot and also demonstrated a tendency of protein induction in RG with respect to UG (Fig. 4 B).

The PON arylesterase and lactonase activities were studied in the

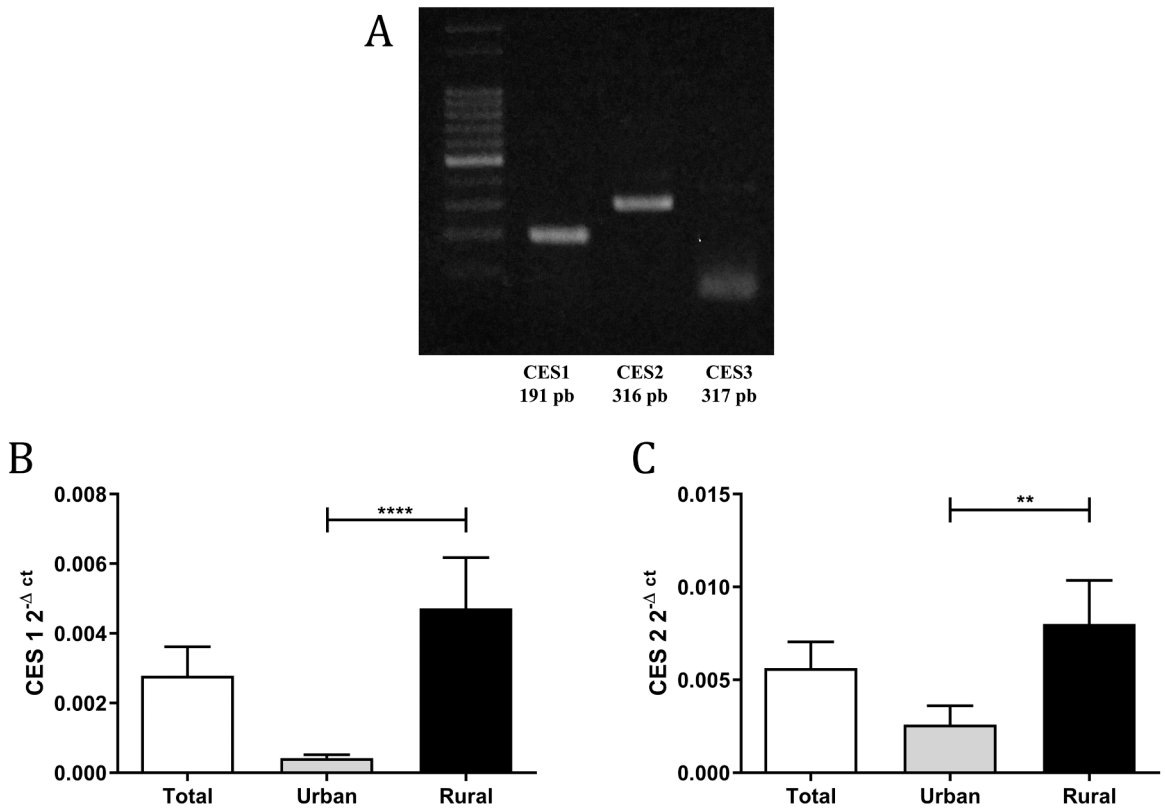

Fig. 3. Carboxylesterase isoforms expression in the placenta. (A) Expression of placenta CES transcripts by conventional PCR visualized in a 2 % agarose gel. (B) CES1 transcript expression levels. ****$p \leq 0.0001$, Mann Whitney test. (C) CES2 transcript expression levels. **$p = 0.004$, Mann Whitney test. The graphs show the mean $2^{-\Delta Ct} \pm$ SEM.

placentas from UG and RG. Fig. 5A and B, show a significant increase in the PON arylesterase (1.43 times) and lactonase (1.37 times) activities in the RG compared to the UG ($p = 0.032$ and $p = 0.025$ Mann Whitney, for arylesterase and lactonase activities respectively). Conventional RT-PCR was performed to determine the presence of different transcript isoforms of paraoxonase in the placenta (PON1, PON2, and PON3). Fig. 5C, shows the amplification of PON 2 and PON 3. To determine possible changes in PON 2 and 3 transcripts expression qPCR was performed. As shown in Fig. 5D a significantly higher PON2 mRNA transcript level was found in RG vs UG ($p = 0.006$, Mann Whitney). Moreover, the protein expression was also increased in RG vs UG (Fig. 4 C) ($p = 0.030$, *t*-test). The PON3 transcript expression was assessed with 2 different primer pairs and could not be quantified in our experimental conditions.

3.4. Association between variables

Possible correlations between CP concentration, enzyme activities (CES, PON), and enzyme transcript mRNA levels (CES1, CES2, and PON2) were analyzed (Table 2). CP placental levels (ng/g lw) showed a significant but weak positive correlation with CES1 and CES2 mRNA transcript levels. A positive correlation between CP levels and PON2 mRNA was near statistical significance ($p = 0.06$). Interestingly, CES activity with 1-naphthyl acetate substrate showed a significant inverse correlation with PON arylesterase activity, whereas CES activity with the 4-MUBA substrate showed a significant direct correlation with PON arylesterase and lactonase activities. CES1, CES2, and PON2 mRNA showed a significant strong direct correlation with each other. PON2 mRNA transcript levels showed a significant inverse correlation with paraoxonase arylesterase activity.

Additionally, possible correlations between CP concentration, enzyme activities (CES, PON), enzyme transcript mRNA levels (CES1, CES2, and PON2), and the morphometric parameters of the placenta and neonates were also studied (Table 3). Neonate length showed a significant direct correlation with CES2 mRNA transcript levels. Also, placenta weight and Placental Index showed a significant positive correlation with PON lactonase activity.

3.5. Multiple Factor Analysis between the quantitative and categorical variables

The characterization of the relationships between quantitative and qualitative variables was carried out using MFA. MFA is a Principal Component Analysis (PCA) that considers groups of variables, where each group is previously transformed and weighted.

In the present study, two groups of variables are considered. The first group is composed of the quantitative variables enzyme activities (CES 1-naphthyl.acetate, CES 4-MUBA, PON arilesterase activity, PON lactonase activity), enzyme transcript expression (CES1, CES2, and PON2) and the placenta weight and the placental index. The second group is composed of categorical variables: the residence area (rural/urban), the pesticide application period (no application/application), and the detection of Chlorpyrifos (CP>LOD/CP<LOD). The quantitative variables were standardized while the categorical variables were transformed from the complete disjunctive table (Pagès, 2015). Each group was weighted by the inverse of the first eigenvalue of the individual analysis, PCA for quantitative variables ($\lambda = 2.1979$), and Multiple correspondence analysis (MCA) for categorical variables ($\lambda = 0.4157$). After weighting, the groups are concatenated and a PCA is performed. This technique allows the analysis of the existence of associations between variables within and between groups. A vector correlation coefficient RV= 0.10455 was obtained, which is low but highly significant ($p = 0.001223$). This indicated that the information provided by both groups of variables is complementary since the configurations of

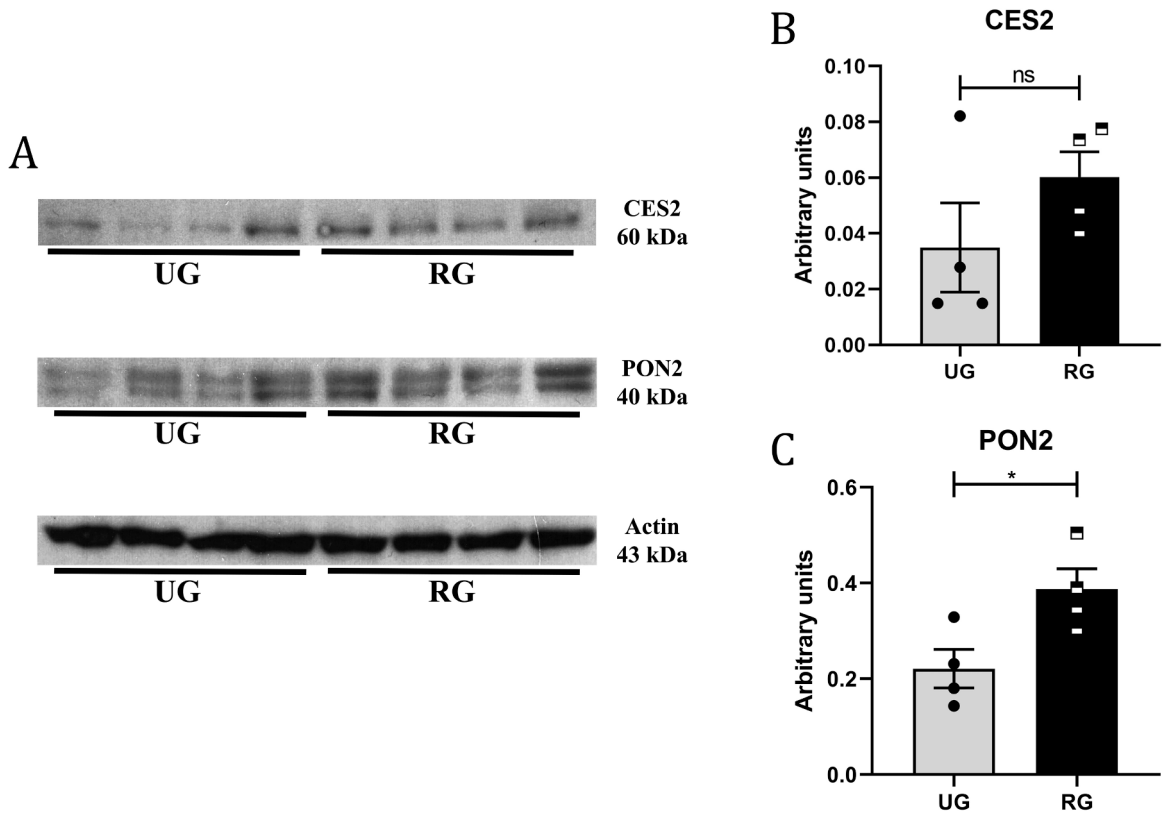

Fig. 4. Protein expression levels of carboxylesterase 2 (CES2) and paraoxonase 2 (PON2). (A) Protein expression in placenta lysates was evaluated by Western blot in 10 % SDS-PAGE gel. Equal amounts (100 μg) of protein obtained from urban group (UG) and rural group (RG) were tested. (B) Band relative intensity of CES2/Actin in placenta. ns $p = 0.219$, *t*-test. (C) Band relative intensity of PON2/Actin in placenta. *$p = 0.030$, *t*-test. Band relative intensity was estimated by standardization with densitometry analysis (Gel Analyzer 19.1 program). Actin was used as a loading control. The graphs show relative intensity as the mean ± SEM.

individuals provided by each group are not homothetic.

As a result of the technique, a plane is obtained in which the variables and individuals are projected. From these graphs, the relationships between quantitative and categorical variables can be analyzed. The main plane formed by the first two dimensions explains 37.16 % of the total variability of the data. Analysis of the vector-variable representation determined that CES1, CES2 and PON2 mRNA transcript levels exhibited the highest correlation with the first dimension (Dim 1), while PON (arylesterase, lactonase) activity, placental weight and placental index were the most strongly correlated with the second dimension (Dim 2) (Table SM2 and SM3).

To analyze the relationships between groups of variables, Fig. 6A and B are displayed together, superimposing them. High CES1, CES2, and PON2 mRNA levels are associated with the presence of CP and have a slight association with pesticide application season. Likewise, high placenta weight and Placental Index, along with low values of CES 1-naphthyl acetate activity are linked to rural areas. Meanwhile, high values of CES 4-MUBA are associated with the non-pesticide application season (Fig. 6A and B).

4. Discussion

This work demonstrates the exposure to CP in pregnant women in North Patagonia, insecticide concentrations were significantly correlated with the residing site. Additionally, women residing in rural areas presented significantly higher detection frequencies and concentrations of CP in the placenta compared to women residing in urban settings. These results are consistent with previous reports from this area (Rivero Osimani et al., 2016; Rodriguez et al., 2023), highlighting the exposure of pregnant women to pesticides in the High Valley of Rio Negro and Neuquén. From an economic perspective of farming production, it is noteworthy that 4 % of the global production of apples and pears is derived from this region (Ondarza et al., 2014). Fruit production is characterized by intensive pesticide use and chemical pest control is achieved by ground-based insecticide application of finely dispersed droplets or particles during the spring and summer months (CAFI, 2022). Insecticide contamination in the atmosphere of the Rio Negro watershed associated with chemical pest control of fruit crops was previously reported (Miglioranza et al., 2021) Thus, inhalation is an important exposure pathway that may contribute to CP exposure mainly in rural residents. Moreover, ingestion may be also contributing to CP placental levels, in both rural and urban residents, since CP was widely detected in eatable fruits, vegetables and fishes (Mac Loughlin et al., 2018; Ondarza et al., 2014). Therefore, CP residues were present in both rural and urban placentas, indicating that diverse exposure routes may exist. It has been stated that diet is likely to be a primary pesticide source in persons, in addition, pesticide spray drifting onto nearby houses and the proximity of residence to agricultural fields might be an important source of pesticide exposure in persons living in an agricultural area (Ding et al., 2012; Muñoz-Quezada et al., 2012). In this sense, a study performed on breastfeeding women in Spain reported an 85 % detection frequency of 3,5,6-trichloro-2-pyridinol (TCP) the specific chlorpyrifos and chlorpyrifos-methyl, metabolite in urine. Moreover, participants living near farming activities (<200 m) had higher levels of TCP in urine compared with those living far from these areas (Fernández et al., 2020). Another study determining TCP in pregnant women's urine from Spain reported that samples taken during the summer or fall had the highest metabolite concentrations. However, women who lived in metropolitan, semi-urban or rural zones had a non-significant correlation with TCP levels (Llop et al., 2017). The work by Fernández-Cruz et al (Choudhury et al., 2017)., determined the presence of OP such as CP, methyl parathion, fenthion, diazinon, and dichlorvos in both the placenta and

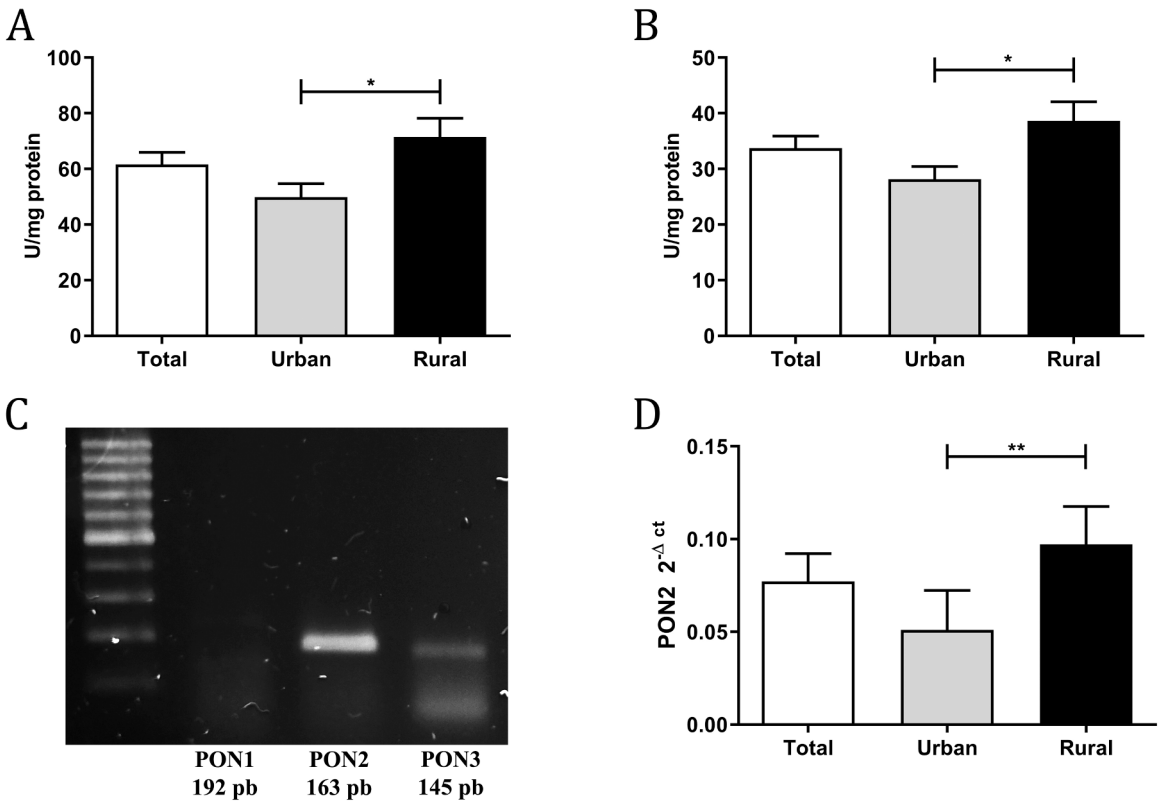

Fig. 5. Activity and mRNA levels of paraoxonases. (A) Arylesterase activity was measured using phenylacetate as substrate. One unit of arylesterase activity was defined as 1 μmol phenylacetate hydrolyzed/min and expressed as U/mg protein. *p = 0.032, Mann Whitney test. (B) Lactonase activity was measured using dihydrocoumarin (DHC) as substrate. One unit of lactonase activity was defined as 1 μmol DHC hydrolyzed/min and expressed as U/mg protein. *p = 0.025, Mann Whitney test. The graphs show the mean activity ± SEM. (C) Expression of placenta paraoxonase transcripts by conventional PCR visualized in a 2 % agarose gel. (D) PON2 transcript expression levels. **p = 0.006, Mann Whitney test. The graph shows the mean $2^{-\Delta Ct}$ ± SEM.

Table 2
Correlation between variables.

	CES 1-naphthyl acetate	CES 4-MUBA	CES 1 mRNA	CES 2 mRNA	PON arylesterase	PON lactonase	PON 2 mRNA
Chlorpyrifos	r −0.125 p 0.280 n 76	r −0.206 p 0.068 n 79	r 0.250 p 0.044 * n 65	r 0.271 p 0.020 * n 74	r −0.037 p 0.765 n 75	r −0.065 p 0.576 n 76	r 0.219 p 0.060 n 74
CES 1-naphthyl acetate		r 0.177 p 0.130 n 74	r 0.080 p 0.510 n 70	r 0.118 p 0.332 n 70	r −0.408 p 0.0005 * ** n 70	r −0.010 p 0.932 n 71	r −0.100 p 0.410 n 70
CES 4-MUBA			r −0.230 p 0.057 n 69	r −0.028 p 0.801 n 82	r 0.255 p 0.041 * n 83	r 0.475 p 4.90E−6 * ** n 84	r −0.021 p 0.851 n 81
CES 1 mRNA				r 0.775 p 5.28E−15 * ** n 69	r −0.411 p 0.0007 * ** n 65	r −0.093 p 0.457 n 66	r 0.732 p 8.52E−13 * ** n 69
CES 2 mRNA					r −0.398 p 0.0003 * ** n 78	r −0.129 p 0.259 n 79	r 0.623 p 4.22E−10 * ** n 82
PON arylesterase						r 0.216 p 0.052 n 81	r −0.327 p 0.004 * * n 77
PON lactonase							r −0.121 p 0.290 n 78

Data are expressed as covariates, Spearman r (r), significance level of p ≤ 0.05 (*), p ≤ 0.01 (**) and p ≤ 0.001 (***), and the number of samples (n).

meconium of women from Spain, and the placenta geometric mean levels of OP were 13 ng/g lw (Fernández-Cruz et al., 2017) and 23 ng/g lw (Fernández-Cruz et al., 2020). In the present study, we reported a higher CP concentration with a geometric mean of 60.92 ng/g lw in the placentas analyzed. The CP detection frequency found was 65.5 % while the detection frequency of OP reported by Fernandez-Cruz, was 89 % (Fernández-Cruz et al., 2020). It has been previously demonstrated that the levels of CP in maternal and cord blood are similar and correlated. Thus, pesticides are readily transferred from the mother to the fetus during pregnancy (Whyatt et al., 2003). Many epidemiological studies have shown that prenatal chlorpyrifos exposure is associated with adverse birth outcomes (Perera et al., 2003; Whyatt et al., 2005), and

Table 3
Correlations between variables and morphometric parameter of the neonate and placenta.

	Neonate weight (g)	Neonate length (cm)	Neonate head circumference (cm)	Ponderal index	Placenta weight (g)	Placental index
Chlorpyrifos (ng/g lipid)	r 0.137	r 0.087	r 0.122	r 0.120	r 0.125	r 0.012
	p 0.212	p 0.437	p 0.274	p 0.285	p 0.257	p 0.916
	n 85	n 82	n 82	n 82	n 84	n 84
CES 1-naphthyl acetate	r −0.140	r −0.196	r −0.096	r 0.077	r −0.085	r −0.011
	p 0.204	p 0.088	p 0.408	p 0.507	p 0.441	p 0.923
	n 84	n 77	n 77	n 77	n 84	n 84
CES4-MUBA	r 0.065	r 0.068	r 0.039	r 0.080	r −0.012	r 0.022
	p 0.545	p 0.530	p 0.722	p 0.461	p 0.916	p 0.839
	n 88	n 87	n 87	n 87	n 85	n 85
CES1 mRNA	r −0.008	r 0.013	r −0.002	r 0.030	r 0.124	r 0.054
	p 0.945	p 0.916	p 0.990	p 0.806	p 0.307	p 0.654
	n 70	n 69	n 69	n 69	n 70	n 70
CES2 mRNA	r 0.178	r 0.246	0.139	r 0.045	r 0.159	r 0.046
	p 0.107	p 0.026 *	p 0.213	p 0.685	p 0.160	p 0.688
	n 83	n 82	82	n 82	n 80	n 80
PON arylesterase	r 0.057	r 0.134	r 0.063	r −0.124	r −0.065	r −0.091
	p 0.606	p 0.229	p 0.576	p 0.266	p 0.567	p 0.422
	n 83	n 82	n 82	n 82	n 80	p80
PON lactonase	r 0.077	r 0.098	r 0.060	r 0.044	r 0.226	r 0.221
	p 0.487	p 0.379	p 0.593	p 0.692	p 0.042 *	p 0.048 *
	n 84	n 83	n 83	n 83	n 81	n 81
PON2 mRNA	r 0.046	r 0.042	r 0.041	r 0.034	r 0.143	r 0.099
	p 0.684	p 0.709	p 0.720	p 0.760	p 0.209	p 0.383
	n 82	n 81	n 81	n 81	n 79	n 79

Data are expressed as covariates, Spearman r (r), significance level of $p \leq 0.05$ (*), and the number of samples (n). Ponderal index expressed as neonate weight (g)/neonate length^3 (cm^3). Placental index expressed as placenta weight (g)/neonate weight (g).

impairments in neurodevelopment (González-Alzaga et al., 2014; Burke et al., 2017; Chiu et al., 2021). In this sense, prenatal exposure to OP has been related to neurodevelopmental problems, increased child blood pressure (Harari et al., 2010), short gestation time (Eskenazi et al., 2004), respiratory difficulties (Reardon et al., 2009; Raanan et al., 2016; Ye et al., 2017), obesity, and diabetes (Debost-Legrand et al., 2016; Slotkin, 2011).

Organophosphate pesticides are potent B-esterase inhibitors. B-esterases include the enzymes acetylcholinesterase, butyrylcholinesterase, and CES. The CES are responsible for the metabolism and detoxification of a wide range of ester- and amide-containing compounds, as well as insecticides such as OP and pyrethroids (Xu et al., 2016). Also, the inhibition of CES activity represents a biomarker of anticholinesterase pesticide exposure since CES is a secondary target of oxon toxicity (Casida and Quistad, 2004) and the inhibition persists for days to weeks in animal tissues (Chanda et al., 1997). In this work, as demonstrated previously by Rivero Osimani et al. (2016) and Vera et al. (2012), a lower CES activity in the placenta of women living in proximity to crops was found. CES activity was determined using two different substrates, 1-naphthyl acetate and 4-MUBA. It has been reported that CES1 and CES2 enzymes share 48 % amino acid sequence identity but exhibit distinct substrate and inhibitor specificity (Satoh and Hosokawa, 2006; Parker et al., 2015). In this sense, the catalytic efficiency of CES2 for 4-MUBA hydrolysis is about 30 times that of CES1 (Pindel et al., 1997). We observed an impact in CES1 activity in rural residents compared to urban. In contrast, CES2 activity showed no changes between groups. Previously, it was reported that CES1 activity is a more suitable indicator of OP exposure than CES2 activity in the placenta (Vera et al., 2012). The mRNA transcript levels indicated that in rural residents CES1 and CES2 mRNA are induced. Moreover, the increase in CES2 mRNA was also observed at protein expression levels in rural residents. The increase in CES2 protein expression likely dampens the inhibitory effect of exposure to CP, since non-significant activity changes are observed between groups. On the other hand, CES1 also shows an increase in the expression of mRNA, though protein level by western blot was not assessed. It is probable that the increase in CES1 mRNA expression does not reflect a sufficient increase in protein levels to balance OP enzyme activity inhibition. Also, results shown here indicate that CES2 transcript is more abundant than CES1 transcript in the placenta. Probably, both, differences in the susceptibility to OP inhibition as well as the abundance of both CES in the placenta renders CES1 more susceptible to OP inhibition. In this sense, it has been demonstrated that CES1 and CES2 display different susceptibility to oxon inhibition, recombinant human CES1 display lower IC50 for chlorpyrifos oxon, paraoxon, and methyl paraoxon than recombinant CES2 (Crow et al., 2012).

CES are enzymes widely distributed in the human body and display critical roles in organs important as xenobiotic barriers or metabolism, such as the intestine and liver. In this sense, CES mediate at least 20 % of hydrolysis reactions for marketed drugs and for about 50 % of marketed pro-drugs (Di, 2019). CES metabolize several clinically important classes of drugs such as anticoagulants, angiotensin-converting enzyme inhibitors, antihyperlipidemic agents, antivirals, chemotherapeutics, immunosuppressants, and psychoactive drugs. The CES detoxification system protects against organophosphate, and carbamate pesticide poisoning, and is involved in pyrethroid detoxification (Anand et al., 2006; Nishi et al., 2006). Dysfunction of CES would lead to physiological and pathological changes and might be important in the cumulative toxicity of xenobiotic exposure. Hence, CES1 inhibition could profoundly impact the pharmacologic effect of drugs and other xenobiotics (Phillips and Stapleton, 2019) as well as potentially alter hepatic lipid metabolism (Morris et al., 2014; Di Consiglio et al., 2021).

The PON gene family in humans has three members, *PON1*, *PON2*, and *PON3*. PON1 participates in the protection against organophosphate pesticide poisoning (Shih et al., 1998). The three isoenzymes have been linked to antioxidant defenses (Furlong et al., 2016). This work studied the presence of PON1, PON2, and PON3 mRNA transcripts in the placenta, and detected the expression of PON2 and PON3 mRNA, in accord with previous reports (Dikbas et al., 2017; Mackness et al., 2010). Although PON3 mRNA was determined by conventional RT-PCR, by qPCR a very low transcript abundance was determined and couldn't be accurately quantified. Thus, further studies are necessary to investigate the possible modulation of PON3 transcript in the placenta. On the other hand, a significant increase in both PON2 mRNA and protein expression was observed in rural placentas compared to the urban ones. In line with these results, in the experimental model of apolipoprotein E3 female mice, 15 days after chlorpyrifos treatment, a delayed increase the in

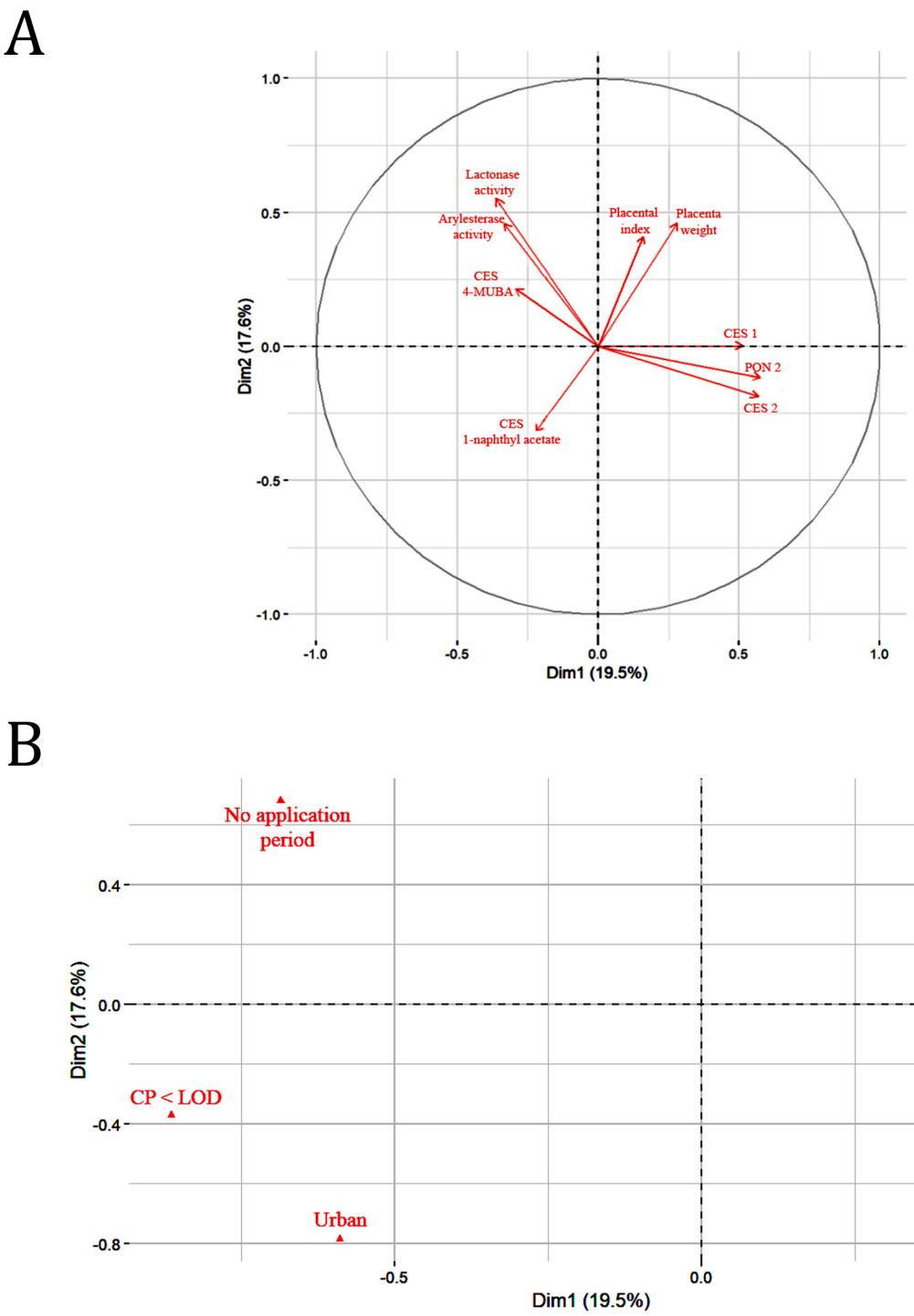

Fig. 6. Multiple Factor Analysis of quantitative and categorical variables. (A) Projection of quantitative variables on the correlation circle. (B) Representation of categorical variables on the principal plane. Dim 1 and Dim 2 correspond to the first two factors, respectively, and together explain 37.16 % of the total variability in the data.

expression of brain PON2 was determined (Basaure et al., 2018). Also, in the retinal pigment epithelium cell line ARPE19 the exposure to CP modulated paraoxonase, arylesterase and lactonase activity as well as increased PON2 mRNA expression (Jasna et al., 2014). Furthermore, in various models, including atherosclerosis, neurodegenerative disorders, ovarian cancer, and cardiovascular disease, PON2 exhibits a protective role (Khalaf et al., 2023). This study highlights increased PON arylesterase and lactonase activities in the placenta under conditions of significant pesticide exposure. However, the specific functions of PON2 in the placenta, whether in physiological or pathological states, remain poorly explored. These findings suggest that PON2 could serve as an important molecule, potentially fulfilling critical roles pertinent to placental function. PON2 is a type II transmembrane protein, that display both arylesterase and lactonase activity (Draganov et al., 2005), and which is dynamically translocated to the plasma membrane in response to oxidative stress to counteract lipid peroxidation (Hagmann et al., 2014). PON2 mRNA expression and lactonase activity toward dihydrocoumarin significantly increased in murine macrophages

incubated under oxidizing conditions *in vitro* (Aviram and Rosenblat, 2004). Moreover, PON2 overexpression in HeLa cells is capable of lowering the oxidative state of cells induced by hydrogen peroxide or Ox-PAPC treatment (Hagmann et al., 2014). Since one possible toxicity mechanism of OP is the induction of oxidative stress in the placenta (Rivero Osimani et al., 2016; Chiapella et al., 2014), PON2 probably increases as an oxidative stress defense mechanism.

The weak positive significant correlation between CP levels and CES1 and CES2 mRNA transcript levels, but not with CES1 and CES2 activity, would indicate that CES mRNA expressions are more sensitive to CP than CES activity. This could be due to the complex exposure scenario, where multiple anticholinesterase pesticides such as CP, phosmet, monocrotophos, and carbamates are used in the area (Cichón et al., 2017; Sánchez et al., 2019). It has been postulated that CES2 participates in cell viability and apoptosis regulation in cancer cells, and its increased mRNA expression is associated with a bad prognosis (Zhang et al., 2020; Chen et al., 2022). Thus, changes in CES expression may have consequences other than changes in drug metabolism. Also, the MFA indicated that the mRNA expression of PON2, CES1 and CES2 are the variables that have a high direct correlation with the first dimension of the analysis. This first dimension is the one that explains most of the variability present in the data set. Additionally, the MFA showed a clear association between residing in rural areas, with a higher CP concentration in the placentas, as well as with the pesticide application period, when insecticides are intensively used. In contrast, residing in urban areas is associated with lower CP levels in the placenta and weakly associated with the non-application period since this latter variable is distant from the other variables on the plane. Thus, reinforcing the hypothesis of the coexistence of multiple exposure pathways. Additionally, the MFA showed an association between high placenta weight and Placental Index with rural areas. This agrees with previous reports performed in this area, where a higher placental weight and a higher Placental Index were found in samples from rural settings (Quintana et al., 2017). Moreover, this study indicates the possible contribution of PON lactonase activity to these parameters, since a positive association between PON lactonase activity and placental index was found.

Limitations of the study should be noted. The collection of samples was performed between 2018 and 2022. It should be noted that during this period, access to human samples was not granted due to the global impact of the SARS-CoV-2 pandemic, and considering the number of participants, the study results should be interpreted with caution. The data collection allowed us to determine pesticide levels at the end of pregnancy, with the potential to underestimate fluctuations in exposure levels during the initial and intermediate periods of pregnancy. Also, other pesticides (historic or current use) may be present in the placenta, and with the experiments performed, we can`t exclude the possible effects of other pollutants. This work emphasizes the study of the esterases CES and PON and other enzymes involved in pesticide metabolism, such as the cytochrome P450 family, were not analyzed. Although we demonstrate CES and PON modulation, the causal relationship between exposure to CP and CES and PON, should be confirmed in an *in vitro* placenta model, which is the current focus of research in our laboratory.

5. Conclusion

The findings of this study demonstrate that pregnant women residing in both rural and urban contexts are exposed to CP at concentrations that vary according to the geographical setting. The highest levels and detection frequencies of CP were observed in rural areas. Among rural women, alterations in the B-esterase CES were observed, characterized mainly by the inhibition of CES1 activity and an increase in protein and mRNA levels of CES1 and CES2. Additionally, the A-esterase PON exhibited induced lactonase and arylesterase activities, along with PON2 induction evidenced by increases in mRNA and protein expression levels.

The bioaccumulation of chlorpyrifos and its potential effects on pregnant women from Argentina and other parts of Latin America are not yet fully understood. The present study addresses this knowledge gap in the region, thereby facilitating the assessment of the health of this vulnerable population group concerning chemical risk, a matter of concern for public health. Taking this into account, it is necessary to promote biological monitoring studies among pregnant women to track the temporal trends in pesticide concentrations. Furthermore, it is essential to provide comprehensive guidance on appropriate actions to safeguard public health.

CRediT authorship contribution statement

Lavalle Andrea: Formal analysis. **Ondarza Paola M.:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Guiñazú Natalia L.:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Rodriguez Piuque M.:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Vera Berta:** Methodology, Investigation. **Burgos Carolina:** Investigation. **Gimenez Gustavo:** Formal analysis. **Miglioranza Karina S.B.:** Writing – review & editing. **Ramirez Cristina L.:** Writing – review & editing, Methodology.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.118285.

Data availability

The data that has been used is confidential.

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