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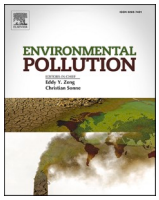

Interactions between blood lead (Pb) concentration, oxidative stress, cellular immune response and reproductive status in livestock from a mining area☆

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ABSTRACT

Chronic exposure to lead (Pb) in livestock grazing in abandoned mining areas affects animal welfare and productivity, as well as represents a significant food safety risk. Here, we evaluate the physiological effects of Pb exposure in goats maintained under extensive farming conditions in a non-remediated mining area. We monitored blood, fecal, and milk Pb levels in two groups of goats, pregnant (n = 17) and lactating (n = 24), kept in different enclosures with high soil Pb concentrations (geometric means of 270 and 143 μg/g, respectively) in Sierra Madrona mining district (Spain). We also studied the influence of Pb exposure on the ability to mount a cellular immune response, and on oxidative stress and biochemical biomarkers measured in blood. Blood Pb concentration was higher in pregnant than in lactating goats, but this difference was not observed in fecal Pb concentration. Pb levels in feces and milk concentrations were correlated with those measured in blood, with 11% of milk samples showing Pb concentrations above the maximum level (ML) for Pb in raw milk established by the EU (0.02 μg/g wet weight). Animals with increased blood Pb levels showed reduced concentrations of retinol in plasma, but these Pb levels did not affect the cellular immune response. The stimulation of the cellular immune response in lactating goats was associated with an increase in blood Pb and calcium levels. The reproductive status and age of goats significantly affected several oxidative stress, antioxidants and plasma biochemistry variables. Goats grazing on soils contaminated by past Pb mining activities may be susceptible to detrimental health effects mediated by retinol deficiency. In view of the detected transfer of Pb through milk, special attention should be paid to the food safety of derived products (i.e. cheese).

1. Introduction

Lead (Pb) mining activity has contributed to the extensive release of Pb into natural environments for centuries around the world (Gutiérrez et al., 2016; Strzebońska et al., 2017). In most cases, the affected areas have never been remediated and remain contaminated after the cessation of mining activity. Currently, many of these abandoned areas are grazed by wildlife species and livestock under extensive or semi-extensive farming conditions (Millán et al., 2008; Rodríguez-Estival et al., 2011a, 2011b;2012, 2014; Khan et al., 2015; Bakowska et al., 2016; Pareja-Carrera et al., 2014). Once dispersed, Pb from mining activities can pollute air, water, sediment and soil, and consequently, enter biota via different food chain pathways (Ma, 2011; Kumar et al., 2020). This pollution represents a risk for ecosystems and both animal and human health. Livestock can become exposed to Pb through several routes, with soil ingestion (involuntary or deliberate; geophagy)

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representing the most important one, along with other routes like the inhalation of wind-blown Pb-laden dust, or the ingestion of contaminated water and plants (Beyer et al., 2007; Smith et al., 2009; Johnsen and Aaneby, 2019). Lead bioavailability in mammals is markedly influenced by the chemical form of Pb (Haque et al., 2021), the interactions with other dietary components such as calcium (Ca) and phosphorus (P) (Ahamed and Siddiqui, 2007; Ettinger et al., 2009; Rodríguez and Mandalunis, 2018; Pareja-Carrera et al., 2020), and the age and physiological status of the animal, including the pregnancy and lactation (National Research Council, 2005; Ettinger et al., 2006). Following absorption, Pb can be distributed through the blood stream to the different body tissues, and excreted very slowly via feces, urine and other biological fluids such as milk (Skerfving and Bergdahl, 2007). During lactation, Pb is transported from maternal blood to the mammary gland and is secreted into milk along with essential elements like copper and zinc (Pilarczyk et al., 2013), which makes milk a significant route of exposure to Pb in suckling animals (Pierezan et al., 2023; Fuchs et al., 2021).

Among the wide range of adverse effects of the absorbed Pb on animal health, reproduction and immunocompetence are especially relevant because of their implications in population dynamics. Adverse effects on male and female reproductive functions have been frequently observed in birds and mammals exposed to Pb (Castellanos et al., 2015; Vallverdú-Coll et al., 2016). Some of these effects include increased abortion, delayed development and reduced fertility in females (Acharya et al., 2006; Kumar, 2018; Verma et al., 2018; Pintus and Ros-Santaella, 2021) and reduced sperm quality in males (Reglero et al., 2009; Giulioni et al., 2023). On the other hand, the reproductive status can also be a risk factor of the adverse effects of Pb in females because, among other reasons, high Ca requirements for fetus development and milk production can be accompanied by a higher Pb absorption or mobilization (Fuchs et al., 2021). Similarly, immunotoxic effects of Pb, affecting both cellular and humoral immune functions, have been identified in birds and mammals (Vallverdú-Coll et al., 2015; Fenga et al., 2017), which could compromise the ability of Pb-exposed populations to deal with pathogenic outbreaks.

In addition to the potential impact on animal health and a reduction in farm animal production, Pb exposure in livestock may pose a risk to human health from ingestion of meat, offal and milk because Pb concentrations in tissues and milk of exposed animals can reach levels above the safety thresholds established for food intended for human consumption (Sharpe and Livesey, 2006; Pareja-Carrera et al., 2014; Bates and Payne, 2017). In general, acute and chronic exposures of livestock to Pb present in the environment may represent a socioeconomic problem in regions of extensive livestock farming where grazing areas have been impacted by old mining activities or other sources of Pb pollution (Waldner et al., 2002; Cowan and Blakley, 2016).

Here, we aimed to evaluate the Pb exposure of two groups of goats with different reproductive status (i.e. pregnant and lactating) maintained under extensive farming conditions in an area contaminated by Pb mining activities in Central Spain. With this purpose, we measured Pb concentrations in blood, feces and milk. We hypothesize that goats in this contaminated site will show blood Pb levels of concern and the transfer of Pb to the milk and other products (i.e. cheese) will be a risk to human consumers. Additionally, we studied the effect of Pb exposure on the immune response, antioxidant system and plasma biochemistry of the goats. We hypothesize that goats with higher blood Pb concentrations will have reduced cellular immune response and disruption of the antioxidant system, possibly showing oxidative stress (i.e. higher lipid peroxidation or lower antioxidant levels). Some of these effects of Pb may be also influenced by interactions with the reproductive status of the individual.

2. Materials and methods

2.1. Study area

Sierra Madrona and Alcudia Valley conform a historical mining district located in the center of the Iberian Peninsula (southwest of the province of Ciudad Real, Spain; Fig. 1). The soils of the area are characterized by siliciclastic rocks with some interlayered volcanics and rare carbonate levels. Galena (PbS) was the most abundant ore mineral, followed by sphalerite (ZnS), chalcopyrite ($CuFeS_2$), pyrite (FeS_2) and marcasite (FeS_2) (Palero-Fernández and Martín-Izard, 2005). Minery in that district began at Roman times, and during the second half of the 19th century the district became the most important Pb-production area in Spain. Because of that long-lasting and intense mining activity, about 500 sites distributed across an area of 2500 km^2 are currently showing high levels of environmental Pb pollution (Palero-Fernández and Martín-Izard, 2005; Higueras et al., 2017). Nowadays, the land of this old mining district contaminated by environmental Pb is used for extensive and semi-extensive livestock farms for meat and milk production, mostly of cattle, sheep, and goat. Previous studies have shown important Pb exposure in both domestic and wild ungulates with consequent adverse effects on reproduction, immune response, bone properties and antioxidant activity of these animals, as well on food safety of their products (see, e.g., Reglero et al., 2009; Taggart et al., 2011; Rodriguez-Estival et al., 2012, 2014; Pareja-Carrera et al., 2014, 2021; Castellanos et al., 2015).

The predominant vegetation in the area consists of Mediterranean forests with scrubland and scattered pastures (Rivas-Martínez, 1987). To conduct the experiment, we selected an extensive sheep-cattle-goat farm in a location where evidence of historical mining activities could be observed, including tailings, dumps, and abandoned mines and buildings. Animals in this farm usually feed on pasture, especially after rains when such pasture is abundant, or other available plant materials like fresh buds and shoots, and oak acorns. In addition, animals are supplemented with corn grain during dry periods, when natural vegetation is less abundant, and also during lactation. Previous studies in this mining area have shown Pb concentrations of (mean ± SE) 97.5 ± 37.5 μg/g dry weight (d.w.) in Gramineae, 21.1 ± 14.8 μg/g d.w. in holm oak (*Quercus ilex*) leaves and 13.6 ± 2.65 μg/g d.w. in gum rockrose (*Cistus ladanifer*) leaves (Reglero et al., 2008), which are the most abundant plants in the enclosures. In the same study site, soil Pb concentrations are extremely high in some sites grazed by livestock (mean, range: 8897, 414–65858 μg/g d.w.), and the same was observed for water (26.6, 12.9–43.9 μg/L) and plants (52.5, 2.3–182.7 μg/g d.w.) (Rodríguez-Estival et al., 2014). As a result of this Pb contamination, about 73% of adult sheep from this farm show subclinical Pb poisoning (Pareja-Carrera et al., 2014) and clinical cases have been observed in cattle (Rodríguez-Estival et al., 2014).

2.2. Study design and on-site immune challenge

We carried out a field trial in June of 2017. During the study, animals were managed using standard livestock operation practices on that farm, so the experimental design has some constraints that limit some of the conclusions reached with the obtained results. Animals were kept in the areas used as part of their normal management, fed according to the usual regime established by the farmer (natural pasture and supplemented corn grain), and provided with water *ad libitum*. Therefore, differences in diet and soil Pb concentrations in the grazed enclosures may affect Pb exposure in the animals. In order to know the distribution of soil Pb concentrations in the area where the experimental enclosures were located, surface soil samples (n = 202) were collected on a grid of 200 × 200 m and processed as described by Pareja-Carrera et al. (2021).

A total of forty-one female goats were managed in two groups according to their reproductive status: pregnant group, n = 20 (only 3 being milked close to the end of lactation); lactating group n = 21 (non-

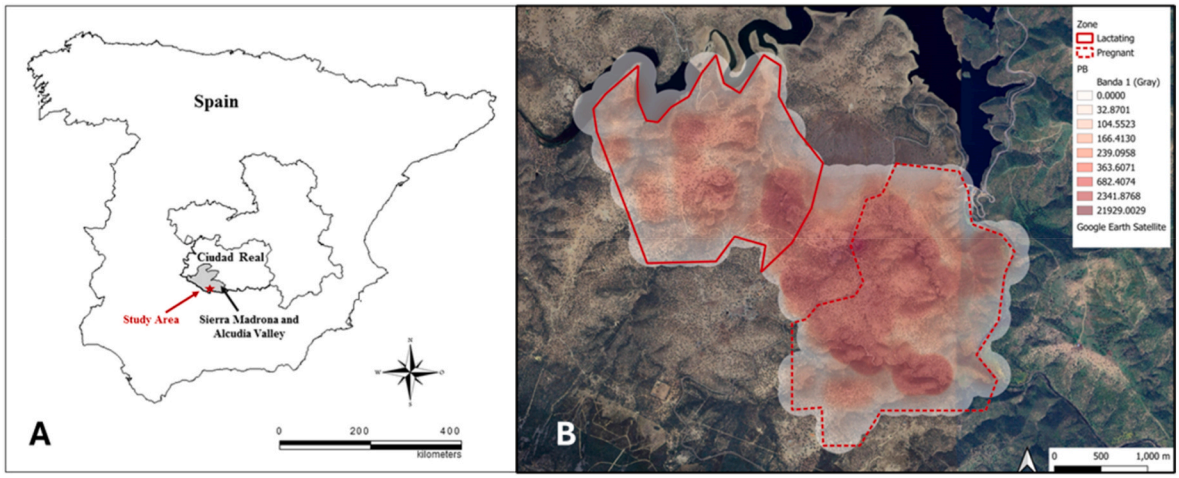

Fig. 1. A. Map of the old mining district of Sierra Madrona – Alcudia Valley study area, located in the province of Ciudad Real (Spain). The shaded area represents the ancient mining area according to Palero-Fernandez and Martín-Izar (2005). The selected farm is marked in red. B. Map of the studied farm, with the soil Pb concentrations (μg/g) in the enclosures of the pregnant and lactating goats. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pregnant and being milked around the lactation peak). Based on standard management for dairy goats, these animals were supplemented in the last 20 days before starting the study with a mineral block that was selected as the most efficient in reducing gastrointestinal Pb bioaccessibility in previous experimental studies (Pareja-Carrera et al., 2020, 2021). This mineral block (IBERBLOCK 10-7-1, 10 kg; Leches Maternizadas, S.A.) contained a mixture of monocalcium phosphate, Ca carbonate, magnesium (Mg) oxide and sodium (Na) chloride, for a total composition of 21% Na, 10% Ca, 7% P and 1% Mg (Pareja-Carrera et al., 2020). Lead concentrations in the mineral block and the supplied fodder were below the limit of detection (<0.15 μg/g d.w.; Pareja-Carrera et al., 2021). Age of goats ranged between 1 and 10 years old (mean ± SE; pregnant: 3.9 ± 0.6 years, non-pregnant: 4.0 ± 0.5 years). Animals were kept in two similar stables and grazing enclosures of about 247 ha. We evaluated the immune function of goats through a functional test conducted in their field enclosures. Specifically, we injected 100 μL of phytohemagglutinin (PHA; Sigma Aldrich) diluted in phosphate buffered saline (PBS; 5 mg/mL) intradermally into the skin on the lateral side of the neck of each goat in both the pregnant and lactating groups. PHA is a mitogen lectin that stimulates a proliferative response of circulating T-lymphocytes that accumulate at the injection site, hence thickening of the skin associated with the PHA injection is used as an indirect indicator of the T-cell mediated immune response of the organism. We used a micrometer (Mitutoyo Absolut 547-401) to measure, to the nearest 0.01 mm the thickness of one patch of skin at the injection site (marked with a non-toxic marker) before challenging with PHA. After 24 h of injection, we measured the skin thickness again to calculate the change and estimate the intensity of cell-mediated response.

The study was performed under the experimental procedure (PR-2016-02-04) approved by the Committee of Ethics and Animal Experimentation of the University of Castilla-La Mancha and the Regional Government (Project 12–2016).

2.3. Animal sample collection

Blood samples were collected from every goat two days before the PHA injection, and again after the second skin thickness measurement. Feces and milk samples were collected on the day of PHA injection. The protocols for handling and sample collection were supervised by a veterinarian and complied with the animal welfare standards as established by legislation on the use of animals for experimental purposes.

Blood samples (10 mL) were taken with syringes from the jugular vein and collected in heparinized tubes. Fecal samples (≈8 g) were collected directly from the rectum and stored in polyethylene bags. Milk samples (≈50 mL) were collected from the lactating goats in falcon tubes. All samples were maintained refrigerated in a portable cooler, where they were immediately transported to the laboratory. Upon arrival in the laboratory, blood samples were separated in three aliquots; a small volume of whole blood was used to fill a capillary tube that was used to calculate hematocrit (%) after centrifugation at 10,000 rcf for 5 min. A second aliquot was stored at −20 °C as whole blood for Pb analysis, and the third aliquot was centrifuged to separate plasma from the cellular fraction (pellet), both of which were stored at −80 °C for further analysis of biochemical parameters and vitamins in plasma and oxidative stress variables in the pellet (see below). All samples were processed and stored within 4 h of blood collection.

2.4. Elemental analysis

Lead levels were determined in blood, milk and feces from study animals and in soils of the enclosures. Digestions of whole blood aliquots (1 mL) were performed in open quartz tubes that were placed in a heating block with an electronic temperature controller. Each blood sample was digested with 2 ml nitric acid and 2 ml hydrogen peroxide, following Rodríguez-Estival et al. (2011b). Feces were oven-dried at 80 °C for 4 days to a constant mass, then homogenized samples (0.2 g) were acid-digested using the same protocol as for blood but in Pyrex instead of quartz tubes. Milk samples were lyophilized and 0.2 g d.w. were acid-digested as described for blood samples. Digested samples were diluted with Milli-Q grade water to a final volume of 10 mL for blood or 50 mL for feces and milk, and then stored at 4 °C until Pb analysis. Dried soil samples were analyzed as described by Pareja-Carrera et al. (2021).

Lead was determined using graphite furnace atomic absorption spectroscopy (GF-AAS; AAnalyst 800, PerkinElmer). Certified commercial solutions (Panreac) were used to prepare calibration standards. In each digestion batch, four blanks (including digestion reagents only) and several certified reference materials (lobster hepatopancreas, 0.35 ± 0.13 μg Pb/g d.w., TORT-2, National Research Council, Canada, n = 4; spiked skim milk powder, 2.002 ± 0.026 μg Pb/g d.w., BCR151, Community Bureau of Reference, EU, n = 3; and certified soil, 1447 ± 203 μg Pb/g d.w., RTC-CRM025-050, Resource Technology Corporation, USA, n = 2) were included and analyzed together with the samples. Element concentrations were reported in μg/dL for blood and μg/g d.w. for feces and milk, and in wet weight (w.w.) for milk. Limits of detection (LOD) were 0.1 μg/dL for blood and 0.01 μg/g d.w. for feces and milk.

Lead recoveries (mean ± RSD) of the certified reference material were 76.2 ± 2.25% (TORT-2), 108.81 ± 0.36% (RTC-CRM025-050) and 86.68 ± 0.08% (BCR151).

2.5. Determination of oxidative stress biomarkers, plasma biochemistry and vitamins

Blood pellet samples were analyzed for several oxidative stress biomarkers. Concentration of malondialdehyde (MDA), a lipid peroxidation product, was analyzed by high performance liquid chromatography (HPLC) using an Agilent 1100 Series system with a 5-mm ODS-2 C-18 (4.0 mm × 250 mm) column, and coupled with a fluorescence detector (FLD), following the method described by Romero-Haro and Alonso-Alvarez (2014). The activities of the enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD) were recorded spectrophotometrically using commercially available kits (Ransel and Ransod, respectively; Randox Laboratories) with an A25-Autoanalyser automated spectrophotometer (BioSystems). The activities of both enzymes were expressed relative to total protein concentration in blood pellet, which was quantified following Bradford (1976).

A series of biochemical parameters, including Ca, Mg, P, cholesterol, triglycerides, glucose, creatinine, urea, uric acid, total proteins, alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), gamma-glutamyltransferase (g-GT), lactate dehydrogenase (LDH) and creatine kinase (CK) were measured in plasma using commercial kits (BioSystems). All parameters were determined spectrophotometrically with the same automated spectrophotometer used for GPx and SOD following the instructions from the kit manufacturer.

Free retinol (vitamin A) and α-tocopherol (vitamin E) levels in plasma were determined by HPLC coupled to FLD and to a diode array detector (DAD) using an Agilent 1100 Series system and following the methodology described by Rodríguez-Estival et al. (2011a). Calibration curves for vitamin measurements were prepared with standards of free retinol and α-tocopherol (Sigma), using retinyl acetate and tocopheryl acetate as internal standards for retinol and α-tocopherol quantification, respectively. The percentage recovery was >90% for the two analyzed vitamins. Vitamin concentrations are given as micromoles per liter of plasma (μM).

2.6. Statistical analysis

Blood Pb concentration (log-transformed to fit a normal distribution) was tested as a response variable influenced by other physiological factors and as a predictor of effects on functions potentially affected by Pb. In the first case, generalized estimating equations (GEE) with normal distribution and identity link function were used to study the effects on blood Pb concentration (repeated measurements, intrasubject variable: day) of the reproduction status (group: pregnant or lactating), the influence of stimulating the goat cellular immune response by means of the PHA challenge (day: before or after PHA injection) and the age of the goats (continuous variable in years). The interaction between day (pre- or post-PHA challenge) and group (pregnant or lactating) was also included in the model to test the presence of different effects of PHA challenge depending on the reproductive status. In the second case, GEE of repeated measurements were used to study the effect of Pb exposure, as well as of reproductive status and age, on the studied biomarkers potentially affected by Pb (cellular immune response, hematocrit, oxidative stress biomarkers, vitamins and plasma biochemistry). Blood Pb concentration (log-transformed) was included as a covariate, and reproductive group and age as fixed factors. In addition, the models for hematocrit, oxidative stress biomarkers and plasma biochemical parameters also included the day (pre- or post-PHA) as fixed factor to study the effect of the PHA challenge. Finally, generalized linear models (GzLM) were used to study the effects of the reproductive group and age (fixed groups) and blood Pb (covariate) on fecal and milk Pb concentrations (log-transformed). The influence of the immune challenge on fecal and milk Pb levels could not be determined because these samples were collected only before PHA injection. The models were selected by backward elimination of the less significant variables, and in some those cases with several potential explanatory models, the quasi-likelihood information criterion (QIC) was used to select the descriptive variables in the GEE models (Pan, 2001). Soil Pb concentrations (log-transformed) in the two enclosures were compared with *t*-test. The percentage of milk samples above the EU maximum level (ML) of 0.02 μg/g w.w. was calculated (Commission Regulation (EU) No 2023/915). Significance was set at the 0.05 level. Statistical analyses were performed with IBM SPSS statistics version 28.

3. Results

3.1. Lead levels in blood, feces, and milk

Blood Pb concentration was higher in the group of pregnant goats than in the lactating goats ($p < 0.001$) and this concentration increased after the PHA challenge ($p < 0.001$), especially in the group of lactating goats as shown by the significant interaction between these two factors ($p < 0.001$; Table 1).

Fecal and milk Pb levels were not significantly different between both groups, but they were, in both cases, positively associated with blood Pb levels (feces: $\beta = 0.356 \pm 0.124$, $p = 0.004$, Fig. 3a; milk: $\beta = 1.191 \pm 0.507$, $p = 0.019$, Fig. 3b). Two out of the 18 analyzed milk samples (11%) exceeded the ML of 0.02 μg/g w.w. established by the EU for Pb in raw milk.

The soil Pb concentrations in the enclosure grazed by pregnant goats were higher (geometric means, min-max: 270, 58–20,727 μg/g d.w.; $n = 103$) than in the enclosure of the lactating goats (143, 50–678 μg/g d.w.; $n = 74$; Fig. 1b; $p < 0.001$).

3.2. Hematocrit and oxidative stress biomarkers and cellular immune response

Hematocrit was positively associated with the blood Pb concentration ($p < 0.001$, Table 2). The reproductive status of the goats showed a significant influence on several biomarkers of oxidative stress. Lactating goats showed higher MDA concentration and GPx activity in red blood cells, and higher retinol and tocopherol concentrations in plasma than pregnant goats (Table 2). On the other hand, the pregnant goats showed higher SOD activity in red blood cells than the lactating ones. The PHA challenge increased the levels of MDA and GPx activity in red blood cells ($p < 0.001$ and $p = 0.006$, respectively), especially GPx activity in pregnant goats as shown by the interaction between reproductive group and sampling day observed in the GPx model ($p = 0.024$; Table 2). The only observed effect of age on oxidative stress biomarkers was reduction of SOD activity in elder goats compared to younger ones ($p = 0.037$; Table 2). No effects of blood Pb concentration were found on the analyzed oxidative stress biomarkers and the cellular immune response.

3.3. Plasma biochemistry and vitamins

Plasma retinol concentration decreased with age ($p = 0.018$; Table 2), and was negatively affected by increased blood Pb concentration ($p = 0.025$; Fig. 3c–Table 2).

Pregnant goats showed higher concentrations of glucose and P ($p < 0.001$ and $p = 0.033$, respectively), whereas lactating goats had higher AST activity and Ca, Mg, creatinine and urea concentrations (all $p < 0.01$; Table 3). The PHA challenge was associated with higher concentrations of Ca (Fig. 2b), glucose and triglycerides (all $p < 0.05$, Table 3). The increase in these two latter variables after PHA injection was especially noticeable in the lactating goats, as revealed by the significant interaction between reproductive group and sampling day observed in the models for glucose and triglycerides (both $p < 0.001$, Table 3). On

Table 1
Concentrations of Pb in blood, feces and milk of goat from a lead mining area and results of Generalized Linear Models (GzLM) with the effects of group (reproduction status), day (phytohemagglutinin challenge), blood Pb concentration (log) and age.

Variable	Day	Group	N	Geometric mean (95% IC)	Range	Generalized Linear Models (effects, β±SE, p values)
Blood Pb (μg/dL)	PrePHA	P	20	5.35 (4.40–6.51)	2.19–12.70	Group (↑ Pregnant, $\beta = 0.143 \pm 0.041$, $p < 0.001$) + Day (↑ PostPHA, $\beta = 0.184 \pm 0.022$, $p < 0.001$) + Day*Group ($p < 0.001$)
		L	21	2.47 (2.18–2.81)	1.43–4.14	
	PostPHA	P	20	5.70 (4.933–6.59)	3.54–11.87	
		L	21	3.91 (3.52–4.35)	2.46–5.80	
Feces Pb (μg/g d.w.)	PrePHA	P	20	26.65 (21.60–32.98)	15.39–67.89	Blood Pb ($\beta = 0.356 \pm 0.128$, $p = 0.009$)
		L	19	20.83 (17.88–24.26)	11.03–34.63	
Milk Pb (μg/g d.w.)	PrePHA	P	3	0.054	0.014–0.115	Blood Pb ($\beta = 1.191 \pm 0.538$, $p = 0.042$)
		L	15	0.023 (0.014–0.038)	0.014–0.163	
Milk Pb (μg/g w.w.)	PrePHA	P	3	0.009	0.003–0.017	
		L	15	0.003 (0.002–0.006)	0.001–0.023	

PrePHA: before phytohemagglutinin challenge. PostPHA: one day after phytohemagglutinin challenge. Group P: pregnant goats without mineral block supplementation. Group L: lactating goats with mineral block supplementation.

the contrary, PHA challenge reduced ALT, CK, g-GT and LDH activities, and creatinine and urea concentrations (all $p < 0.01$). The significant interaction between sampling day and group in the urea model showed that the PHA-related reduction in urea concentration happened in pregnant goats ($p < 0.001$; Table 3). Age was positively associated with total protein concentration ($p < 0.001$) and negatively with P concentration and CK activity (both $p < 0.05$).

4. Discussion

Livestock reared under extensive production in areas with Pb contaminated soils are at risk of accumulating this metal in tissues at levels that can affect their health status and the herd productivity or can be of concern for human consumers. Here we have observed elevated exposure levels in goats grazing around the abandoned mining district of Sierra Madrona and Valle de Alcudia. The milk produced by these animals exceeded the maximum level established by the EU regulations for this food product in 11% of the cases. However, most of the biomarkers of oxidative stress or the cellular immune response were not affected by the blood Pb concentration in the goats. Only plasma retinol levels were negatively correlated with blood Pb concentration. The present study has the constraints of an experiment conducted under the real conditions of a non-experimental farm. This limits the possibility of obtaining conclusions for each individual factor, but some insights were gained by integrating Pb levels in animals as an exposure biomarker with the effect biomarkers as follows.

4.1. Interpreting blood Pb concentration and its determinant factors

Blood Pb is considered a reliable biomarker of exposure to environmental Pb pollution, since it provides a sensitive means for monitoring Pb body burden and the associated health effects (Ma, 2011). The pregnant goats in our study had blood Pb levels within the range of 6–35 μg/dL that corresponds with subclinical intoxication (clinical lead poisoning is observed above 35 μg/dL), whereas the lactating goats showed concentrations below 6 μg/dL that can be considered within the normal range for grazing livestock (Ma, 2011). The higher blood Pb in the group of pregnant goats than in the lactating goats could be explained by several factors: (1) higher soil Pb concentration where the pregnant goats were kept, (2) the supplementation of lactating goats with mineral blocks containing Ca and P at levels that can diminish the exposure/bioavailability of Pb (Ettinger et al., 2009; Pareja-Carrera et al., 2021), or (3) the Pb excretion through the lactation that could benefit the lactating goats (Swarup et al., 2005).

Geometric means of soil Pb concentration in both grazing areas were below the European soil guideline values (86/278/ CEE Directive; European Commission, 1986) for grazing and agricultural lands (750 mg/kg; Reimann et al., 2014) as well as the soil Pb threshold of 1146 mg/kg suggested by Ford and Beyer (2014) to protect sheep from toxic exposures However, these mean Pb concentrations were within the range of soil Pb levels on which sludge cannot be used in EU (50–300 mg/kg d.w. in soils with pH = 6–7) (86/278/CEE Directive; European Commission, 1986), and some areas of the grazing plots were well above guideline values for grazing and agricultural lands (13.6% with >750 mg/kg; Reimann et al., 2014). With these high Pb soil concentrations (58–20,727 μg/g d.w.), geophagia can be the most important source of Pb exposure in these animals, considering the observed soil ingestion rates of 7.49–36.49% (mass of diet) in sheep of this study area (Pareja-Carrera et al., 2021), which is possibly similar in goats. Plants (2.3–182.7 μg/g d.w.) and water (12.9–43.9 μg/L; Rodríguez-Estival et al., 2014) may also contribute to this exposure, but to a lesser extent.

In this scenario, fecal Pb concentration can be used as a non-invasive biomarker of exposure to this metal (Martinez-Haro et al., 2011), since a significant portion of Pb ingested by ruminants (the non-bioaccessible fraction) is excreted via feces (Suttle, 2010). The positive correlation between Pb concentration in feces and blood observed here and in previous studies with sheep (Pareja-Carrera et al., 2021) supports the utility of Pb in feces as an indicator of Pb-contaminated soil ingestion. Although the soils in the pregnant goat enclosure had higher Pb concentrations than in the lactating goat enclosure (270 vs 143 μg/g d.w.), the more similar fecal Pb concentrations detected in both groups (26.6 vs 20.8 μg/g d.w.) may indicate that differences in soil Pb concentration is not the only factor to explain the differences between groups in blood Pb concentration (5.35 vs 2.47 μg/dL).

Supplementation with mineral blocks rich in Ca and P may have had a significant effect as reported in previous studies on the same farm (Pareja-Carrera et al., 2021). Mineral supplementation can have a dual effect, by reducing ingestion of contaminated soil through decreasing the need of geophagia to meet mineral requirements, and by reducing Pb bioavailability through the formation of non-soluble complexes with Pb and Ca in the gastric tract of the animals (Pareja-Carrera et al., 2020). The similar fecal Pb concentrations despite the differences in soil Pb concentrations between groups may be therefore an effect of mineral supplementation offered to the lactating goats, which reduced Pb bioavailability and increased its excretion through feces.

Finally, the excretion of Pb in the milk can also contribute to lower blood Pb concentration in the lactating goats. Especially considering the dietary Ca supplementation, which helps reduce Pb blood levels (Ettinger et al., 2009). The milk Pb levels in this study could explain an excretion (geometric mean (max.)) of 8 (326) μg/day with a milk production of 2 kg/day, which is a significant percentage (geometric mean: 8.3%, max.: 42%) of the circulating Pb burden of the goats, assuming 3.75 L of circulating blood in 50-kg goats (7.5% of body mass) according to Luethy et al. (2017).

4.2. Milk Pb concentration and food safety

Lead excreted in the milk by lactating animals represents a risk for

Table 2
Oxidative stress and antioxidant biomarkers in blood and cellular immune response of goats from a Pb mining area and results of Generalized Estimating Equations (GEE) with the effects of group (reproduction status), day (phytohemagglutinin challenge), blood Pb concentration (log) and age.

Variable	Day	Group	N	Mean ± SE	Range	Generalized Estimating Equations (effects, β±SE, p values)
Haematocrit (%)	PrePHA	P	20	36.52 ± 0.68	31–43	Blood Pb (↑, $\beta = 6.997 \pm 1.383$, $p < 0.001$)
		L	21	32.76 ± 0.49	30–39	
	PostPHA	P	20	35.95 ± 0.38	33–39	
		L	21	33.19 ± 0.81	27–44	
RBC MDA (umol/g)	PrePHA	P	20	14.20 ± 0.65	9.48–18.36	Group (↑Lactating, $\beta = 0.004 \pm 0.001$, $p < 0.001$) + Day (↑PostPHA, $\beta = 0.004 \pm 0.001$, $p = 0.002$)
		L	21	17.83 ± 1.23	10.05–28.72	
	PostPHA	P	19	17.88 ± 1.86	10.02–30.83	
		L	21	21.67 ± 1.21	14.76–33.88	
RBC GPx (IU/mg protein)	PrePHA	P	20	0.42 ± 0.07	0.15–1.28	Group (↑Lactating, $\beta = 0.041 \pm 0.128$, $p = 0.006$) + Day (↑PostPHA, $\beta = 0.213 \pm 0.129$, $p < 0.001$) + Group*Day ($p = 0.024$)
		L	21	1.01 ± 0.10	0.32–2.22	
	PostPHA	P	19	1.09 ± 0.09	0.50–2.02	
		L	21	1.11 ± 0.09	0.38–2.16	
RBC SOD (IU/mg protein)	PrePHA	P	20	2.42 ± 0.12	1.50–3.45	Group (↑Pregnant, $\beta = 0.391 \pm 0.093$, $p < 0.001$) + Age (↓, $\beta = -0.041 \pm 0.019$, $p = 0.037$)
		L	21	1.85 ± 0.08	0.71–2.41	
	PostPHA	P	19	2.18 ± 0.10	1.52–3.14	
		L	21	1.99 ± 0.10	1.04–2.89	
Plasma Retinol (μM)	PrePHA	P	20	3.22 ± 0.12	2.24–4.22	Group (↑Lactating, $\beta = 0.404 \pm 0.166$, $p = 0.015$) + Age (↓, $\beta = -0.069 \pm 0.032$, $p = 0.029$)
		L	20	3.55 ± 0.17	2.16–4.75	Blood Pb (↓, $\beta = -0.937 \pm 0.418$, $p = 0.025$) + Age (↓, $\beta = -0.079 \pm 0.033$, $p = 0.018$)
	PostPHA	P	20	3.14 ± 0.09	2.10–3.95	
		L	21	3.61 ± 0.16	2.10–4.61	
Plasma Tocopherol (μM)	PrePHA	P	20	8.01 ± 0.57	2.78–14.79	Group (↑Lactating, $\beta = 1.441 \pm 0.742$, $p = 0.052$)
		L	20	9.47 ± 0.45	6.16–13.53	
	PostPHA	P	20	8.16 ± 0.61	3.58–15.15	
		L	21	9.16 ± 0.41	5.18–12.36	
Skin thickness (cm)	PrePHA	P	20	3.57 ± 0.10	2.96–4.38	Day (↑PostPHA, $\beta = 6.259 \pm 0.428$, $p < 0.001$)
		L	21	3.58 ± 0.10	2.54–4.58	
	PostPHA	P	20	9.91 ± 0.36	6.99–15.11	
		L	21	9.73 ± 0.36	6.99–14.88	

PrePHA: before phytohemagglutinin challenge. PostPHA: one day after phytohemagglutinin challenge. Group P: pregnant goats without mineral block supplementation. Group L: lactating goats with mineral block supplementation.

Table 3
Plasma biochemistry of goats from a Pb mining area and results of Generalized Estimating Equations (GEE) with the effects of group (reproduction status), day (phytohemagglutinin challenge), blood Pb concentration (log) and age.

Variable	Day	Group	N	Mean ± SE	Range	Generalized Estimating Equations (effects, β±SE, p values)
ALP (U/L)	PrePHA	P	20	778.95 ± 177.81	21.5–1844.2	NS
		L	21	1036.01 ± 265.85	13.7–3864.0	
	PostPHA	P	20	877.04 ± 222.70	22.4–2943.0	
		L	21	850.40 ± 217.59	8.6–2558.4	
ALT (U/L)	PrePHA	P	20	28.37 ± 2.21	7.4–45.4	Day (↑ PrePHA, $\beta = 6.859 \pm 1.183$, $p < 0.001$)
		L	21	24.57 ± 1.75	6.9–39.6	
	PostPHA	P	20	19.25 ± 1.90	4.8–40.6	
		L	21	19.87 ± 1.62	4.8–38.4	
AST (U/L)	PrePHA	P	20	109.47 ± 4.95	78.4–170.6	Group (↑ Lactating, $\beta = 20.207 \pm 7.270$, $p = 0.005$)
		L	21	132.46 ± 6.86	83.3–208.5	
	PostPHA	P	20	114.35 ± 5.02	84.0–167.0	
		L	21	127.76 ± 6.52	87.0–223.0	
Calcium (mg/L)	PrePHA	P	20	8.32 ± 0.59	0.42–10.65	Group (↑ Lactating, $\beta = 2.085 \pm 0.481$, $p < 0.001$) + Day (↑ PostPHA, $\beta = 0.994 \pm 0.392$, $p = 0.011$)
		L	21	10.19 ± 0.35	7.40–15.39	
	PostPHA	P	20	9.03 ± 0.20	7.39–10.33	
		L	21	11.45 ± 0.51	7.13–18.51	
Cholesterol (mg/L)	PrePHA	P	20	98.50 ± 6.17	56.0–171.0	NS
		L	21	94.19 ± 5.21	33.0–122.0	
	PostPHA	P	20	98.50 ± 7.02	55.0–189.0	
		L	21	104.94 ± 4.96	65.0–152.0	
CK (U/L)	PrePHA	P	20	203.40 ± 16.41	99.0–443.0	Day (↑ PrePHA, $\beta = 29.390 \pm 10.799$, $p = 0.006$) + Age (↓, $\beta = -7.797 \pm 3.765$, $p = 0.038$)
		L	21	187.14 ± 16.51	88.0–344.0	
	PostPHA	P	20	177.85 ± 14.60	84.0–364.0	
		L	21	154.12 ± 12.31	61.0–309.0	
Creatinine (mg/L)	PrePHA	P	20	0.88 ± 0.03	0.65–1.19	Group (↑Lactating, $\beta = 0.005 \pm 0.039$, $p = 0.009$) + Day (↑PrePHA, $\beta = 0.171 \pm 0.0290$, $p = 0.001$) + Group*Day ($p < 0.001$)
		L	21	1.11 ± 0.04	0.66–1.40	
	PostPHA	P	20	0.89 ± 0.03	0.66–1.16	
		L	21	0.92 ± 0.03	0.68–1.24	
Glucose (mg/L)	PrePHA	P	20	61.30 ± 4.91	9.0–119.0	Group (↑Pregnant, $\beta = 13.76 \pm 3.645$, $p < 0.001$) + Day (↑PostPHA, $\beta = 25.5 \pm 2.433$, $p < 0.001$) + Group*Day ($p < 0.001$)
		L	21	23.95 ± 2.16	7.0–52.0	
	PostPHA	P	20	65.90 ± 2.78	46.0–91.0	
		L	21	50.72 ± 2.03	31.0–64.0	
g–GT (U/L)	PrePHA	P	19	58.28 ± 3.91	32.0–112.9	Day (↑PrePHA, $\beta = 6.776 \pm 2.395$, $p = 0.005$)
		L	21	64.84 ± 5.46	37.4–139.0	
	PostPHA	P	20	53.33 ± 2.13	35.2–71.8	
		L	21	56.49 ± 4.78	33.0–121.8	
LDH (U/L)	PrePHA	P	20	1007.20 ± 34.06	789.0–1292.0	Day (↑PrePHA, $\beta = 183.1 \pm 44.5$, $p < 0.001$)
		L	21	850.00 ± 39.95	561.0–1304.0	
	PostPHA	P	20	738.85 ± 60.83	313.0–1407.0	
		L	21	748.00 ± 53.01	486.0–1269.0	
Magnesium (mg/L)	PrePHA	P	20	2.06 ± 0.24	0.10–5.34	Group (↑Lactating, $\beta = 3.040 \pm 0.656$, $p < 0.001$)
		L	21	5.24 ± 0.62	1.94–9.10	
	PostPHA	P	20	2.53 ± 0.63	0.09–7.45	
		L	21	6.20 ± 1.00	0.58–12.56	
Phosphorus (mg/L)	PrePHA	P	20	5.61 ± 0.46	2.76–10.30	Group (↑Pregnant, $\beta = 0.908 \pm 0.426$, $p = 0.033$) + Age (↓, $\beta = -0.173 \pm 0.083$, $p = 0.037$)
		L	21	4.84 ± 0.40	1.73–10.24	
	PostPHA	P	20	5.09 ± 0.32	1.52–6.81	
		L	21	4.26 ± 0.31	2.37–8.22	
Total protein	PrePHA	P	20	83.65 ± 1.29	68.90–91.20	Age (↑, $\beta = 1.131 \pm 0.277$, $p < 0.001$)
		L	21	80.41 ± 3.96	5.43–95.00	
	PostPHA	P	20	84.35 ± 1.41	72.0–96.0	
		L	21	85.81 ± 1.86	67.0–99.0	
Triglycerides (mg/L)	PrePHA	P	20	12.66 ± 0.76	8.1–20.0	Day (↑PostPHA, $\beta = 8.446 \pm 3.097$, $p = 0.032$) + Group*Day ($p < 0.001$)
		L	21	5.52 ± 0.95*	0.5–15.4	
	PostPHA	P	20	11.45 ± 1.50	1.0–29.0	
		L	21	15.83 ± 3.21	4.0–71.0	
Urea (mg/L)	PrePHA	P	20	76.98 ± 3.04	61.7–116.5	Group (↑Lactating, $\beta = 5.79 \pm 3.781$, $p = 0.005$) + Day (↑PrePHA, $\beta = 9.207 \pm 4.218$, $p < 0.001$) + Group*Day ($p < 0.001$)
		L	21	45.63 ± 2.70	15.0–60.6	
	PostPHA	P	20	37.75 ± 2.52	24.5–65.5	
		L	21	43.25 ± 3.01	26.4–75.3	
Uric acid (mg/L)	PrePHA	P	20	0.91 ± 0.67	0.01–13.63	NS

(*continued on next page*)

Table 3 (*continued*)

Variable	Day	Group	N	Mean ± SE	Range	Generalized Estimating Equations (effects, β±SE, p values)
		L	21	1.54 ± 1.46	0.01–30.70	
	PostPHA	P	20	0.08 ± 0.01	0.01–0.27	
		L	21	0.07 ± 0.03	0.01–0.77	

PrePHA: before phytohemagglutinin challenge. PostPHA: one day after phytohemagglutinin challenge. Group P: pregnant goats without mineral block supplementation. Group L: lactating goats with mineral block supplementation. NS: Not significant effects.

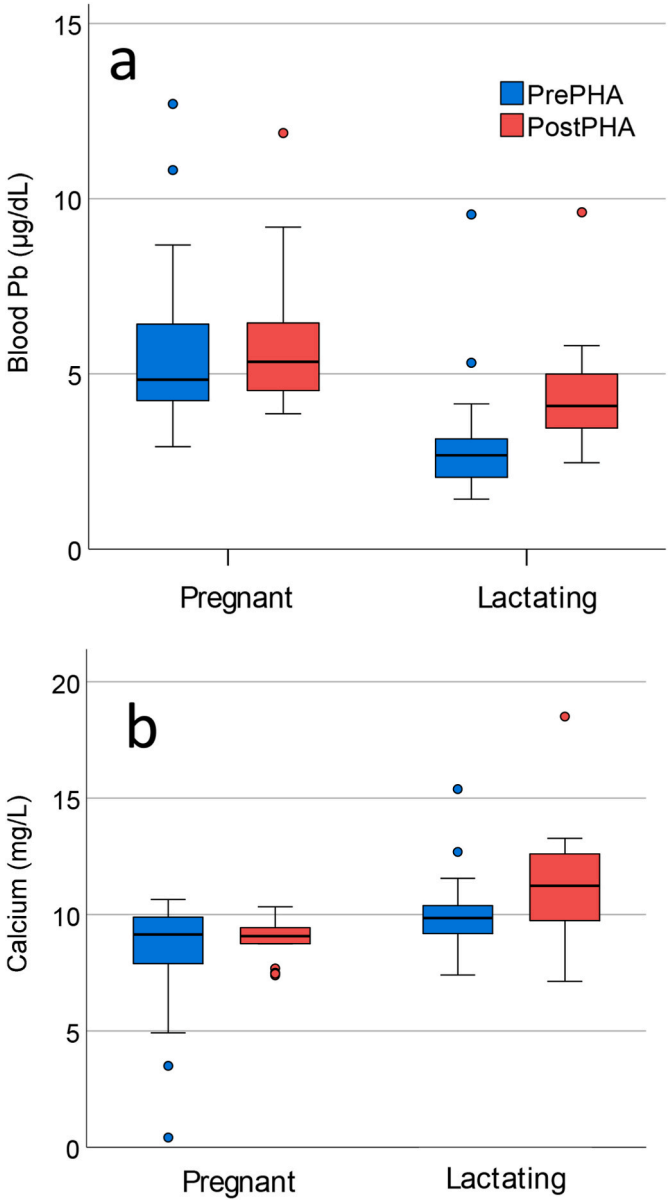

Fig. 2. Blood lead (a) and plasma calcium (b) concentrations in pregnant and lactating goats from a lead mining area before and after the intradermal injection of phytohemagglutinin for the evaluation of the cellular immune response.

human consumers too (Swarup et al., 2005; Bischoff et al., 2014; Pilarczyk et al., 2013). Milk samples in this study had Pb concentrations in the range of 0.001–0.023 µg/g w.w., and 11.1% of the samples were above the maximum permissible limit is 0.02 µg/g w.w. (Commission Regulation, 2023). Other studies have observed Pb concentrations above this threshold, especially in areas or animals affected by Pb contamination (Gonzalez-Montana et al., 2012; Ismail et al., 2017), while cows' milk in non-contaminated sites in Spain has lower Pb concentration (all samples <0.020 µg/g w.w.; Sola-Larrañaga and Navarro-Blasco, 2009; Rey-Crespo et al., 2013). In cheese, average Pb concentrations in 57 types of Spanish cheese ranged from 0.005 to 0.110 µg/g w.w., values not considered of concern for human consumers (Moreno-Rojas et al., 2010). Given the elevated milk Pb concentrations detected here, further studies about the presence of this metal in cheese may be necessary because goat and sheep milk from this area is commonly intended for this dairy product. Previous studies have also shown elevated Pb levels in meat of large game animals of Sierra Madrona-Valle de Alcudia mining area, exceeding EU ML of 0.1 µg/g w.w. (Commission Regulation, 2023) in 84% of wild boar (*Sus scrofa*) and 57% of red deer (*Cervus elaphus*) (Taggart et al., 2011). Similarly, 87.5% of sheep of this study site exceeded in their liver the EU ML of 0.5 µg/g w.w. for offal (Commission Regulation, 2023).

4.3. Interactions between pb, biomarkers and immune and reproductive status

Examining the interaction between the cellular immune challenge and blood Pb levels was one of the main objectives of the present study. Lead can affect the immune response of animals, which predisposes them to infectious diseases or hypersensitivity (Vallverdú-Coll et al., 2019; Dou et al., 2022). We did not see exaggeration or suppression of the cellular immune response in goats with higher blood Pb, so immunotoxicity of T-cell mediated immunity was not evident at the exposure levels of this farm. However, the PHA challenge had a significant effect on increasing blood Pb concentrations in the lactating group. Interestingly, plasma Ca concentration was also higher in the lactating goats after PHA challenge as occurred with Pb. This may be explained by the mobilization of Ca^{2+} for developing the T-cell dependent immune response (Modiano et al., 1999), which could have increased Pb^{2+} levels in blood because of their similarity as divalent cations. T-cell response involves the mobilization of Ca^{2+} from their endothelial reticulum accompanied by an influx of this cation through the plasma membrane into the cytosol to arrange the cytoskeleton for the immune response (Vig and Kinet, 2009). Moreover, the T-cell response can activate bone-resorbing cells (osteoclasts) concentrations (Arron and Choi, 2000), which may increase plasma Pb^{2+} and Ca^{2+}. Further studies may be necessary to elucidate if the increase of blood Pb concentration after PHA challenge is a consequence of Ca^{2+} mobilization for T-cell activation, a result of osteoclast activation by T-cell, or both.

Oxidative stress and plasma biochemistry were not directly affected by blood Pb concentrations in the goats. However, like other studies on animals from Pb-mining areas, plasma retinol concentrations were negatively associated with blood Pb concentration. Wild ungulates from Sierra Madrona and Valle de Alcudia showed lower levels of retinyl esters, but higher levels of free retinol in liver (Rodríguez-Estival et al., 2011a), and lower levels of both vitamin A forms in testes (Rodríguez-Estival et al., 2011b). Wild birds exposed to Pb also show a reduction in plasma retinol levels (Vallverdú-Coll et al., 2015; Sánchez-Virosta et al., 2021). The depletion of storage forms like retinyl esters, and the increase of free retinol in liver, or the decrease of this free form in plasma of animals exposed to Pb contamination may represent an attempt by the organism to cope with tissue damage caused by Pb. As well, it may be due to direct effects of Pb on the expression of genes involved in retinoid homeostasis (Espín and Sánchez-Virosta, 2021).

The cellular immune response includes the activation of

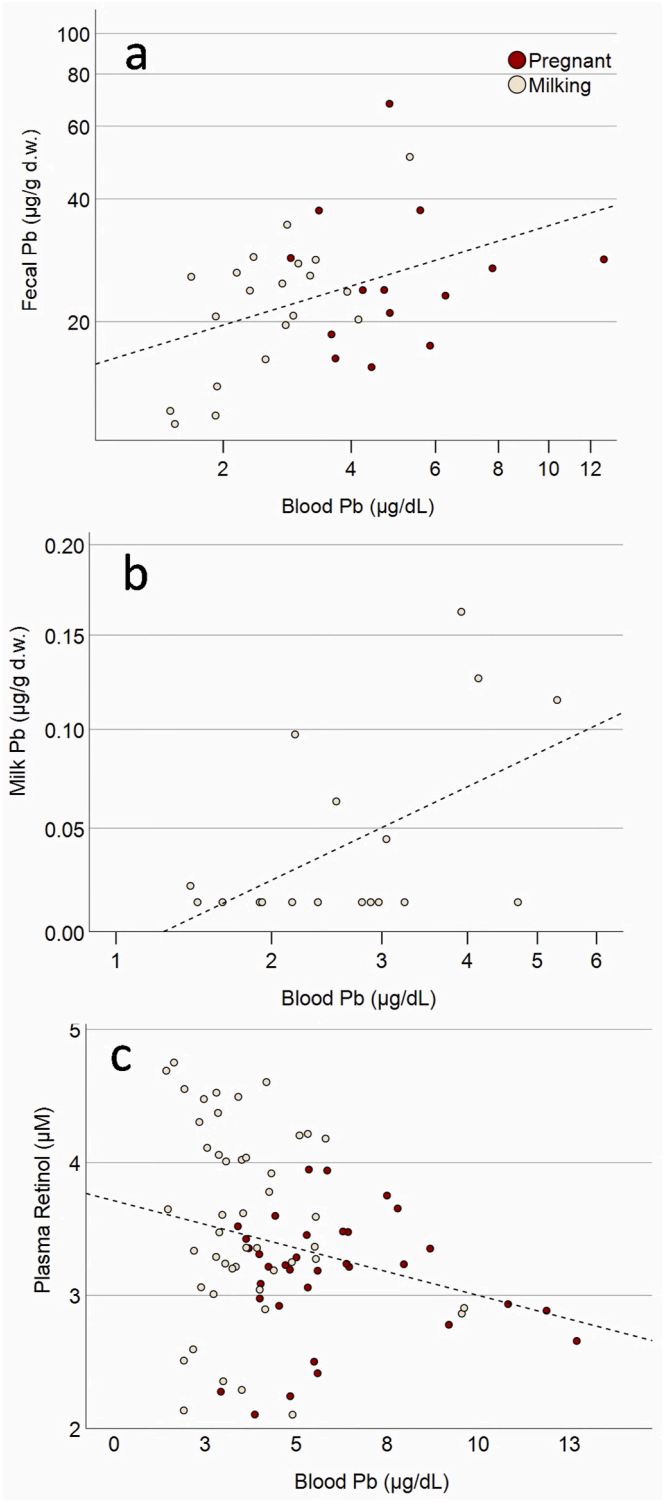

Fig. 3. Relationship between the concentrations of Pb in blood and in feces (a) and milk (b) and with plasma retinol concentration (c) of goats from a Pb mining area.

macrophages that produce reactive oxygen species (ROS) such as nitric oxide (NO) and superoxide ($O_2^{\cdot-}$) to kill pathogens, but when such production overwhelms the antioxidant capacity of the tissue, it can result in oxidative damage (MacMicking et al., 1997; Birben et al., 2012). Here we have observed an increase in lipid peroxidation (MDA) in RBCs after PHA challenge, which could be from ROS production by activated macrophages during the cellular immune response (Costantini and Dell'Omo, 2006), and this was accompanied by an increase in GPx activity in RBC that could be a response to this T-cell response and pro-oxidant production (Matsushita et al., 2015). However, none of these interactions between stimulation of the cellular immune response and activity of the antioxidant system was modulated by blood Pb levels.

The reproductive status of goats can also affect their oxidative status and plasma biochemistry because of the important demands of energy and nutrients during gestation and lactation (Nawito et al., 2016). The lactating goats showed higher MDA levels and GPx activity in red blood cells, as well as higher retinol and tocopherol in plasma compared to the pregnant goats. This may indicate priority allocation of antioxidants in blood to fight the lipid peroxidation produced during milk production (Celi et al., 2010; Nawito et al., 2016). On the other hand, pregnant goats showed higher SOD activity in red blood cells, an important enzyme for the neutralization of superoxide anion overproduction during gestation (Ponnampalam et al., 2022). In fact, SOD activity and retinol levels were negatively associated with the age of the goats, as observed in red deer (Pareja-Carrera et al., 2018), probably as a sign of reproductive senescence (Mingaud et al., 2008). The rest of the plasma biochemistry showed normal allocations for gestation (glucose and P) or lactation (Ca and Mg) (Tharwat et al., 2015).

5. Conclusions

Goats raised in areas contaminated by old mining activity, where soils have never been remediated, showed elevated blood Pb levels, which was reflected in high milk Pb concentrations, sometimes above regulatory levels for human consumption (11.1% of samples). The cellular immune response of goats was not affected by blood Pb levels, but the immune challenge and the reproductive status of the goats were determinants in blood Pb concentrations. In lactating goats, immune challenge increased blood Pb levels in a similar way to that observed for Ca, although compared to the pregnant goats they had lower blood Pb levels, which likely reflects the important elimination route of Pb through the milk (up to 326 µg/day). Plasma retinol was the most sensitive biomarker that was clearly affected by Pb exposure. However, given the significant effect of the immune challenge and the reproductive status on the oxidative balance of animals, exposure to metals like Pb that induce oxidative stress should be considered an additional risk factor in the evaluation of the impact of this contamination. The implications of Pb pollution on animal welfare, food safety and productivity of the farms from the mining area must be considered in the cost-benefit analysis for the restoration of this and other Pb contaminated areas.

CRediT authorship contribution statement

Jennifer Pareja-Carrera: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mónica Martinez-Haro:** Writing – review & editing, Validation, Methodology, Investigation. **Jaime Rodríguez-Estival:** Writing – review & editing, Validation, Methodology, Investigation. **Judit E.G. Smits:** Writing – review & editing, Methodology, Investigation. **Maciej Durkalec:** Writing – review & editing, Investigation. **Araceli Gort-Esteve:** Formal analysis. **Manuel E. Ortiz-Santaliestra:** Writing – review & editing, Methodology, Investigation. **Rafael Mateo:** Writing – review & editing, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.125240.

Data availability

Data is shared in the supplmentary material.

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