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Acephate exposure during gestation in rats elicits differing placental transcriptomic responses in male and female fetuses

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ABSTRACT

Acephate, a broadly applied insecticide with endocrine-disrupting properties, has *in utero* effects that are still not fully elucidated. We hypothesized that maternal exposure to a low dose of acephate alters placental function and induces sex-specific offspring changes. Wistar rat dams received water (control) or acephate (4.5 mg/kg/day) by gavage from gestation day (GD) 6.5–18.5, and the placentas and fetuses were collected on GD 18.5. Acephate exposure caused intrauterine growth restriction (IUGR) in both sexes, while placental efficiency (g fetus/g placenta) was reduced in males and increased in females. Placental transcriptomics revealed no significant differential expression in placentas bearing male fetuses. In contrast, 135 downregulated genes and 171 upregulated genes enriched for growth and nutrient transport were identified in placentas bearing female fetuses. A reduction in the profile of proinflammatory cytokines in the amniotic fluid was detected in the fetuses of both sexes, especially in males, whereas the plasma concentration of MCP-1 increased in dams. Taken together, these findings reveal that the response to acephate differs according to fetal sex, suggesting a nontranscriptional mechanism of placental failure in placentas bearing male fetuses, as well as a complex, adaptive transcriptional response in those bearing female fetuses.

1. Introduction

Acephate (O,S-dimethyl acetylphosphoramidothioate) is the active ingredient in insecticides widely used on various crops, such as cotton and beans. As a "moderately hazardous" pesticide, its intermediate compound, methamidophos, is classified as "highly hazardous." Acephate is easily absorbed by plants because of its water-soluble nature. However, this means that this compound can easily contaminate soil and groundwater (Lin et al., 2020). Owing to its molecular characteristics, it is classified as an organophosphate pesticide, exhibiting high affinity for inhibiting the enzyme acetylcholinesterase (AChE). This enzyme is responsible for the degradation of the neurotransmitter acetylcholine (ACh) in nerve endings, ensuring that ACh is removed from the synaptic space after the transmission of nerve impulses. Blocking AChE leads to the accumulation of ACh in synapses, causing the death of insects due to excessive neuronal excitation (Thapa et al., 2017). According to the World Health Organization (WHO, 2003), the no observed adverse effect level (NOAEL) for acephate is 5 mg/kg of body weight (bw) per day for maternal toxicity and 20 mg/kg bw per day for developmental toxicity. In a document published by the U.S. Environmental Protection Agency (EPA, 2023), acephate should only be used on specific crops, with the maternal and fetal NOAEL set at less than 0.5 mg/kg/day. The dermal NOAEL is 150 mg/kg/day, and the oral NOAEL, which is based on cerebral AChE inhibition, is approximately 0.272 mg/kg/day. In contrast, the European Food Safety Authority (EFSA, 2008) has not approved acephate as an active ingredient in the European Union since 2003.

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These parameters are crucial for assessing the safety of using these compounds in agricultural contexts.

Human and animal exposure to this pesticide occurs not only through food intake but also through water intake. Various organisms can suffer from exposure to acephate, from microorganisms to invertebrates and vertebrates. In mammals, cognitive damage, neurodegenerative and respiratory diseases, endocrine disruption, and damage to the reproductive system are already seen as consequences of exposure to this compound (Fernandes Mendonca Mota et al., 2023). Studies in rodents have shown that acephate is capable of inhibiting androgen production in a dose-dependent manner (0.5, 5, and 50 μM) in *in vitro* cultures of immature rat Leydig cells, in addition to promoting a significant reduction in the expression of key genes and proteins involved in androgen synthesis, such as 3 beta-hydroxysteroid dehydrogenase (HSD3B1) and steroidogenic acute regulatory protein (Star) (Wang et al., 2020b).

The administration of a high dose of acephate (140 mg/kg bw) induced reversible hyperglycemia in rats, most likely caused by gluconeogenesis resulting from exposure (Joshi and Rajini, 2009). During pregnancy and lactation, exposure can cause changes in maternal glucose metabolism (Ribeiro et al., 2016). In humans, direct exposure can lead to hyperglycemia, oxidative stress, DNA damage, and cancer. Owing to these factors, many countries have already banned its use, mainly because of the risks to human and animal health and environmental impacts. However, countries such as China, Australia, Canada, Chile, India, Japan, New Zealand, and Brazil still authorize the use of this pesticide (Fernandes Mendonca Mota et al., 2023), reaffirming the need for omics and biochemical studies of organisms to change the current situation.

According to the Developmental Origins of Health and Disease (DOHaD) hypothesis, exposure to environmental factors, including endocrine-disrupting chemicals (EDCs), during early life, especially during critical windows of development such as pregnancy and lactation, can program the body for a predisposition to chronic diseases in adulthood. This occurs because these factors can alter fundamental biological processes, such as gene expression and epigenetics, permanently influencing an individual's physiology. Events that occur during early development, especially during intrauterine development, can be closely related to future health. Intrauterine growth restriction (IUGR), a marker of intrauterine stress, predisposes individuals to obesity, type 2 diabetes, hypertension, cardiovascular disease, and metabolic disorders (Barker et al., 2002; Rinaudo and Lamb, 2008).

Studies indicate the risks of gestational exposure to acephate. The offspring of mice of both sexes exposed to doses of 0.3 and 300 ppm acephate in their drinking water during pregnancy and lactation showed significant effects on the development of the reproductive system, including delayed prepucial and vaginal opening, testicular degeneration, and a decrease in the number of healthy follicles, as well as postnatal growth deficits (Sasaki et al., 2023). Exposure to acephate during the gestational and lactational periods programs offspring for greater susceptibility to type 2 diabetes in adulthood (Ribeiro et al., 2016). In the intrauterine environment, exposure to this pesticide also affects the autonomic nervous system, induces respiratory problems, and alters the behavior of newborns (Eskenazi et al., 1999). A reduction in the number of live fetuses, an increase in resorption, and the presence of skeletal malformations in fetuses were also observed, in addition to a reduction in placental weight (Farag et al., 2000).

During pregnancy, embryo-fetal development depends directly on the formation and functionality of the placenta, a semipermeable organ essential for regulating the exchange of gases, nutrients, and metabolites between the mother and fetus. The effectiveness and selectivity of these exchanges are determined by the placental barrier, which consists of a complex network of systems, including membrane transporters, enzymes, and export pumps, which act in a coordinated manner to control the flow of substances (Prouillac and Lecoeur, 2010). This set of mechanisms ensures an adequate intrauterine environment and healthy pregnancy progression.

When the effects of intrauterine exposure to xenobiotics in animal models are investigated, significant changes in the placental transcriptomic profile are observed. These compounds are capable of modifying both the structure and molecular machinery of the placenta, influencing its morphology, and acting on specific transporters and receptors, such as steroids and nicotinic acetylcholine receptors (Rosenfeld, 2021). Other studies with organophosphate compounds, such as flame retardants, have shown sex-specific effects in males, where exposure disrupted placental tryptophan metabolism and affected fetal forebrain development (Rock et al., 2020).

Given that the placenta is the primary interface between the mother and fetus during pregnancy and given the evidence that EDCs, such as pesticides, can compromise its structure and function, thereby affecting fetuses development in a sex-dependent manner, this study focuses on evaluating the effects of exposure to the pesticide acephate on the placenta for the first time. We hypothesize that exposure to a dose below the NOAEL affects placental and fetal development differently in male and female, resulting in changes in the biometric and transcriptomic profiles of fetuses during intrauterine life.

2. Experimental procedure

2.1. Experimental design

The experimental design was approved by the Ethics Committee for the Care and Use of Experimental Animals of the Institute of Biology Roberto Alcantara Gomes of UERJ (protocol number 012/2022). All animal procedures were conducted in accordance with the ARRIVE 2.0 guidelines (Percie du Sert et al., 2020) and the NIH Guide for the Care and Use of Laboratory Animals (National Research Council, US). The animals were kept in a room with a controlled temperature (± 22 °C) and light–dark cycle (7:00 a.m.–7:00 p.m.) with free access to water and food. Throughout the experiment, body weight and food intake were regularly monitored. At 3 months of age, nulliparous Wistar rats were mated at a ratio of two females to one male. After 12 h of mating, the vaginal smears were analyzed, and the gestational day (GD) was 0.5 for those cases where the presence of sperm in the smears or a vaginal plug was detected. Pregnant rats were housed in individual cages and subdivided into two different experimental groups (n = 8 dams/group) that received vehicle (filtered water, control) or acephate (4.5 mg/kg bw/day) by daily gavage. Acephate (CAS number: 30560-19-1) was purchased from Sigma–Aldrich® and solubilized in filtered water (solubility in water, g/100 ml at 20 °C: 79, according to the International Chemical Safety Cards (ICSC): 0748). Water was used as the vehicle to provide a non-toxic, inert medium for acephate administration, avoiding potential effects on development or reproduction (OECD, 2018). Exposure began at 6.5, after embryo implantation and early placental morphogenesis had occurred, and continued through GD 18.5, when euthanasia was performed. This covered the entire organogenesis and placental growth period until near term, when the structure and function of the placenta are critical for fetal nutrient and gas exchange (Fonseca et al., 2012). Dams were euthanized by decapitation, and the placentas and fetuses were collected by cesarean section and weighed. All fetuses and placentas were weighed individually and classified by sex. For the statistical analysis, the mean fetal and placental weights were calculated for each pregnant dam; the dam was considered the experimental unit (n = 8 per group). Only one fetus per sex per dam was included for placental, biochemical, and molecular assessments. Therefore, each analyzed placental sample represents a fetus from a different dam. The placental tissue samples consisted of a standardized fragment of the whole placenta collected in a manner that ensured the inclusion of the labyrinth, junctional zone, and decidua regions.

2.2. Sex determination of the fetuses

Fetal liver tissue was used for DNA extraction following the HotSHOT DNA Method (Truett et al., 2000), and PCR targeting the SRY gene was conducted to determine the sex of the fetuses. The PCR mix was prepared using GoTaq® Polymerase Master Mix (Promega Corporation), and the primers for the SRY gene were (5′-TTT ATG GTG TGG TCC CGT GG-3'; forward) and (5′-GTT GAG GCA ACT TCA CGC TG-3'; reverse). Amplification was carried out using the Applied Biosystems™ SimpliAmp™ Thermal Cycler. After that, 2 % agarose gel electrophoresis was performed to analyze the PCR products, and male fetuses were identified by the presence of specific fluorescent bands corresponding to SRY gene amplification.

2.3. Placental efficiency

To assess the ability of the placenta to support fetal growth, placental efficiency was calculated as the ratio of fetal weight (g) to placental weight (g), indicating the amount of fetal mass that is supported per unit of placental weight. As these parameters are inversely proportional, smaller placentas indicate greater efficiency (Fowden et al., 2006).

2.4. Quantification of proinflammatory cytokines in plasma

To measure the inflammatory profile, plasma obtained from dams, n = 8 dams/group, (blood centrifuged at 13,000×*g* for 5 min) and amniotic fluid, n = 8 fetuses per sex per group (one male and one female fetus per dam), were subjected to the measurement of proinflammatory cytokines such as growth-regulated alpha protein/C-X-C motif chemokine ligand 1 (GRO/KC; CXCL1), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1), and tumor necrosis factor alpha (TNF-α), which were quantified using the multiplex immunoassay technique with a Milliplex® MAP Rat Cytokine/Chemokine Magnetic Bead Panel kit (#RECYTMAG-65, Merck Millipore) according to the manufacturer's instructions. The assay's detection limits and sensitivity for each cytokine were as follows: GRO/KC, 19.7 pg/mL; IL-1β, 2.8 pg/mL; IL-6, 30.7 pg/mL; MCP-1, 9.0 pg/mL; MIP-1, 0.8 pg/mL; and TNF-α, 1.9 pg/mL. Some cytokines were not detected in maternal plasma (GRO/KC, IL-1β, IL-6 and MIP-1).

2.5. RNA extraction and sequencing (RNA-Seq)

Total RNA was extracted from placental samples using TRIzol® (n = 6 fetuses per sex per group; one male and one female fetus per dam). After extraction, the samples were treated with the TURBO DNA-free kit to eliminate potential genomic DNA contamination. The quantification of the extracted RNA was carried out using NanoDrop and Qubit (DNA–High Sensitivity kit) equipment. RNA integrity was assessed by electrophoresis on a 1.2 % agarose gel prepared in 1 × TAE buffer and run at 80 V for 1 h and 10 min. Information regarding RNA concentration and integrity is provided in Supplementary Table 1.

For RNA library preparation, the Illumina Stranded mRNA Prep® protocol (Illumina) was used. The initial step of this protocol involves enriching the total RNA extraction for messenger RNAs (mRNAs), followed by fragmentation and denaturation of these mRNAs, cDNA synthesis, polyA tail addition, ligation with specific beads, and PCR amplification. The final library concentration was quantified again using QuBit 4, this time with the DNA High Sensitivity kit. The fragment size was assessed on a Tapestation (Agilent Technologies) using the D1000 High-Sensitivity ScreenTape System.

Library sequencing was performed using the Illumina NextSeq550 System platform at the Institute SENAI for Innovation in Biosynthetics and Fibers, employing a paired-end read protocol with 300 (2 × 150) base pair reads.

2.6. Statistical analysis of biometric and biochemical parameters

The results presented in the graphs are expressed as the mean ± standard error of the mean. Student's t-test with Welch's correction was used, which does not assume homogeneity of variances, with a significance level set at $p < 0.05$. The results were analyzed separately for male and female fetuses. The software used was GraphPad Prism version 8.0 for Windows (GraphPad Software©).

2.7. RNA-Seq analysis and differential expression of placental genes

RNA sequencing was performed using 12 randomly selected samples, resulting in 12 libraries labeled according to the experimental group: control/male (CM), acephate/male (AM), control/female (CF), and acephate/female (AF). Quality control of the RNA-seq data was performed using the FastQC v0.12.0 tool (Andrews, 2010) to verify the quality parameters of the sequenced samples, such as the presence of adapter sequences, base quality along the reads, read length, and guanine–cytosine (GC) content. On the basis of the quality report from the previous step, the TrimGalore v0.6.10 tool (Krueger, 2012) was used to remove adapter sequences, reads shorter than 70 base pairs, and bases with a Phred score or quality lower than 25 (–paired –length 70 –nextera -q 25).

The filtered reads were then aligned to the reference genome using the STAR v2.7.10b tool (Dobin et al., 2013). The reference genome used for creating the mapping index was that of the species *Rattus norvegicus*, assembly version mRatBN7.2 (GCA_015227675.2), available in the Ensembl database version 110 (Harrison et al., 2024), including autosomal and mitochondrial chromosomes. Reads were considered mapped if they had only one mapping in the genome and if the length of the read mapping covered at least 70 % of the read sequence length (–outFilterMultimapNmax 1 –outFilterMatchNminOverLread 0.7).

Gene expression quantification was performed using FeatureCounts v2.0.6 (Liao et al., 2014), and mapped reads were assigned to *R. norvegicus* gene annotations available in Ensembl version 110. Read mapping was attributed to a gene if both paired read ends mapped to the same gene and were not chimeric fragments, indicating that their paired read ends were not aligned to different chromosomes (-B –C). Genes were removed from the dataset if they did not reach a minimum count of 10 in at least the number of samples corresponding to the smallest experimental group included in the comparison.

Differential expression analysis was conducted using the DESeq2 package (Love et al., 2014), allowing for the identification of differentially expressed genes (DEGs) between experimental groups. The selection criteria used were an adjusted p value (FDR or false discovery rate) ≤ 0.01 and a fold change ≥ 2. Venn diagrams and scatter plots were generated using the R package. After we applied principal component analysis (PCA) and generated a heatmap based on Pearson correlation distances to assess sample clustering and variability (Supplementary Fig. 1 and Fig. 2), one library initially assigned to the control/female group and another to the acephate/female group were excluded from further analyses because of their distinct separation from the remaining samples and to ensure the reliability of differential expression analyses.

Enrichment analysis of differentially expressed genes (upregulated and downregulated) was performed using the g:Profiler tool (Reimand et al., 2007) to identify Gene Ontology (GO) terms (Ashburner et al., 2000) for molecular functions, cellular components, and biological processes; pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2017); and Reactome (Gillespie et al., 2022). Additionally, the g:Orth tool was used to identify orthologous genes between humans and rats to enable comparison of the results found in this study with the literature. The results were displayed in pie charts using the ggplot2 v3.5.1 (Wickham, 2016) package in R v4.4.0 (R Core Team, 2024).

Table 1
Effects of maternal acephate exposure on the biometrical and plasma parameters on gestational day 18.5.

Parameter	Control	Acephate	p-value
Body weight (g)	292.1 ± 9.6	261.7 ± 13.9	p = 0.09
Spleen (g/100 of bw)	0.34 ± 0.02	0.37 ± 0.04	p = 0.54
Glycemia (mg/dL)	70.9 ± 0.9	80.2 ± 1.5*	p = 0.04
MCP1 (pg/mL)	220.4 ± 41.7	431.4 ± 81.7*	p = 0.04
TNFα (pg/mL)	11.8 ± 0.8	11.3 ± 0.2	p = 0.60

The values are presented as the mean ± SEM; *p = 0.04 vs. the control group, as determined by Student's *t*-test (n = 8 dams/group).

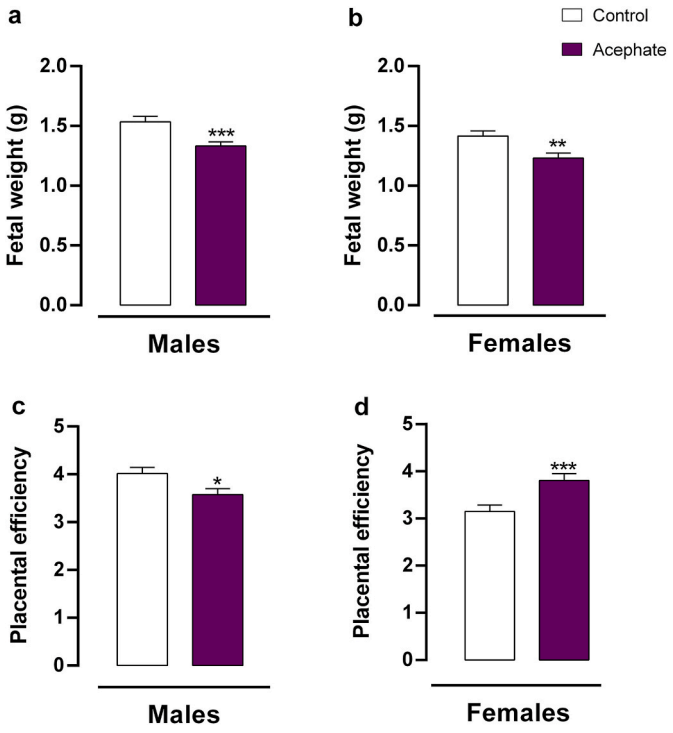

Fig. 1. Effect of acephate exposure on fetal weight and placental efficiency in male and female fetuses on gestational day 18.5. (a) Fetal weight in males and (b) females; (c) placental efficiency (g fetus/g placenta) of male and (d) female fetuses. The data are expressed as the mean ± SEM. Student's t-test with Welch's correction was used for statistical analysis, with significance indicated by p = 0.02 (*), p = 0.003 (**), and p < 0.001 (***); n = 8/dams/group.

2.8. Protein–protein interaction (PPI) network

The STRING database v12.0 (Szklarczyk et al., 2023) was used to investigate potential protein–protein interactions among the proteins encoded by the DEGs. The upregulated and downregulated DEGs were analyzed separately. A medium confidence score (0.40) was applied as the minimum threshold for interaction significance. The resulting network was subjected to clustering analysis using the k-means algorithm to identify functional gene/protein modules.

2.9. Hub gene identification

Hub genes are the genes that have a large number of interactions with other genes in the interaction network and are considered central genes. Hub gene analysis was performed separately for the up- and downregulated genes. For this purpose, we visualized the interaction networks built through the STRING database using Cytoscape software v3.10.3 (Shannon et al., 2003). To identify the central genes, we used the CytoHubba plug-in (Chin et al., 2014) with the maximal clique centrality (MCC) algorithm and visualized the 10 hub genes.

3. Results

3.1. Maternal parameters

No significant differences in maternal body weight, spleen weight, or TNF-α levels were detected between the groups. However, glycemia increased by approximately 13 % and circulating MCP-1 levels by 95 % in acephate-exposed dams compared with controls (Table 1).

3.2. Fetal weight and placental efficiency

There was a 13 % reduction in fetal weight for both males and females compared with that of the control group. Placental efficiency differed between the sexes: in males, a 10 % reduction was observed, whereas in females, placental efficiency increased by 20 % (Fig. 1).

3.3. Proinflammatory cytokines in amniotic fluid

Amniotic fluid from male fetuses showed marked reductions in the proinflammatory cytokines GRO/KC (−25 %, Fig. 2a), IL-1β (−25 %, Fig. 2b), IL-6 (−22 %, Fig. 2c), MCP-1 (−23 %, Fig. 2d), MIP-1 (−44 %, Fig. 2e), and TNF-α (−37 %, Fig. 2f). In females, the decline was restricted to MCP-1 (−23 %, Fig. 2j) and MIP-1 (−36 %, Fig. 2k).

3.4. Placental differential gene expression in response to acephate exposure

The sequenced libraries exhibited approximately 20–39 million reads per sample. After preprocessing, readings that did not meet the base quality and length criteria were removed. Read mapping revealed rates ranging from 90 % to 92 % for uniquely mapped reads. The proportion of mapped reads assigned to genes ranged from 89 % to 90 % (Supplementary Table 1).

After removing genes that had fewer than 10 counts assigned to them in at least the number of samples corresponding to the smallest experimental group included in the comparison, 14,385 genes were detected in the placentas bearing female fetuses. In the placentas bearing male fetuses, a total of 13,164 genes were detected. The majority or totality of genes present in the control and acephate groups may suggest that acephate does not cause an overall change in gene expression but rather modifications in specific pathways or biological functions.

To evaluate when the fold change in gene expression was statistically significant, we performed a differential expression analysis between the control and acephate-exposed groups. We considered genes to be significantly regulated when they had an adjusted p value ≤ 0.01 and a fold change ≥ 2 (upregulated) or ≤ -2 (downregulated). The placental transcriptome of female fetuses revealed 135 genes whose expression was downregulated and 171 genes whose expression was upregulated in the acephate-treated group compared with the control group (Fig. 3a).

However, only one DEG, the procollagen C-endopeptidase enhancer 2 (PCOLCE2) gene, was detected in placentas bearing male fetuses between the control and acephate groups (Fig. 3b). Therefore, the following analyses were only performed on placentas bearing female fetuses.

To identify which biological processes, molecular functions, cellular components and pathways were overrepresented in the set of DEGs, we performed a functional enrichment analysis of the female group (Fig. 4). Functional enrichment analysis of the DEGs in placentas bearing female fetuses revealed that the genes were associated with numerous significant biological themes. The downregulated genes were associated with processes such as the response to BMP and the transforming growth factor beta receptor superfamily signaling pathway, involving genes such as bone morphogenetic protein 6 (*Bmp6*), transforming growth factor, beta 3 (*Tgfb3*), and chordin (*Chrd*). Processes related to matrix structure and remodeling, including collagen fibril organization and cell adhesion, were also enriched for downregulated genes, such as matrix

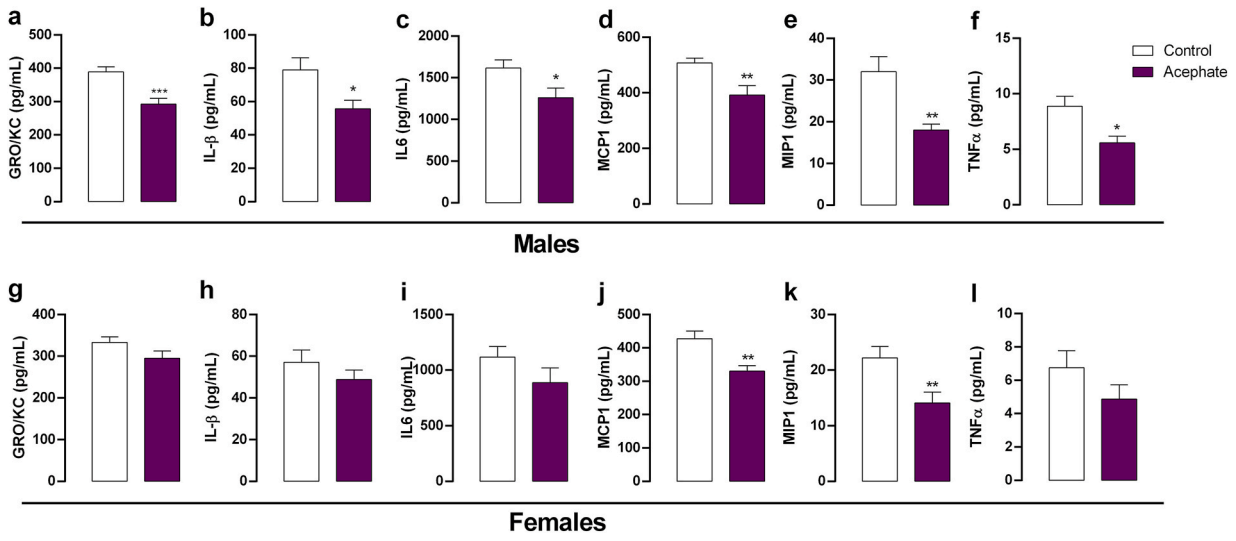

Fig. 2. Effects of acephate exposure on proinflammatory cytokines in the amniotic fluid of male and female fetuses on gestational day 18.5. (a–f) Proinflammatory cytokines in the amniotic fluid of male and (g–l) female fetuses. The data are expressed as the mean ± SEM of n = 8 fetuses per sex per group (one male and one female fetus per dam). Student's t-test with Welch's correction was used for statistical analysis, with significance indicated by $p < 0.01$ (*), $p < 0.001$ (**), and $p = 0.0004$ (***). GRO/KC: growth-regulated alpha protein; IL-1β: interleukin-1 beta; IL-6: interleukin-6; MCP-1: monocyte chemoattractant protein-1; MIP-1: macrophage inflammatory protein-1; and TNF-α: tumor necrosis factor-alpha.

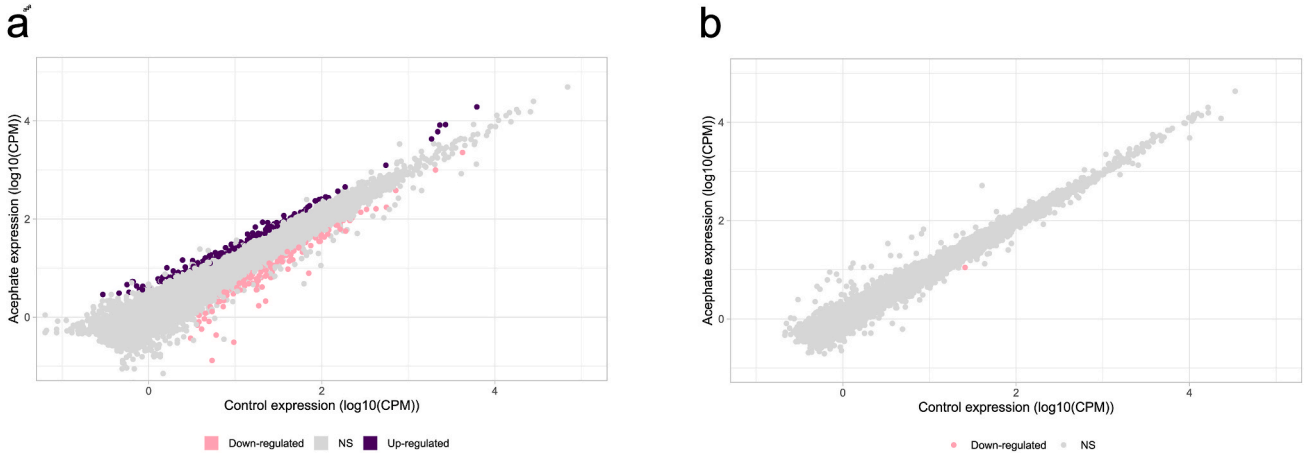

Fig. 3. Scatter plot showing normalized gene expression levels (average $\log_{10}$ CPM) for (a) placentas bearing female and (b) male fetuses. Each point represents a gene, with expression in the acephate-exposed datasets on the X-axis and control datasets on the Y-axis.

metallopeptidase 2 (*Mmp2*) and collagen type V alpha 2 chain (*Col5a2*). Multicellular organismal-level iron ion homeostasis and regulation of angiogenesis, linked to the downregulated heme oxygenase 1 (*Hmox1*) and transmembrane serine protease 6 (*Tmprss6*) genes, were also enriched (Fig. 4a). The upregulated genes were related to processes such as transmembrane transport, with terms enriched such as transport and organic cation transport, involving solute carrier (*Slc*) family genes, such as solute carrier family 4 member 1 (*Slc4a1*) and solute carrier family 40 member 1 (*Slc40a1*). Processes such as cell migration and cell adhesion were also enriched for upregulated genes, including matrix metallopeptidase 14 (*Mmp14*), caveolin 1 (*Cav1*), and KIT ligand *(Kitlg*). The upregulation of genes related to vascular development was also observed, involving processes such as blood vessel development and angiogenesis, involving genes such as Fms-related receptor tyrosine kinase 1 (*Flt1*) and TEK receptor tyrosine kinase (*Tek*), in addition to enrichment in immune system processes, with genes such as complement C1q C chain (*C1qc*) and complement C1q A chain (*C1qa*) as examples (Fig. 4b).

3.5. PPI network analysis of DEGs

Analysis of the protein–protein interaction (PPI) network using the STRING database with an interaction score >0.4 and k-means clustering revealed 9 distinct clusters for the downregulated genes (Fig. 5a). Nodes represent genes/proteins, and edges indicate interactions between them. Additionally, the STRING database provides functional enrichment information in the networks, based on KEGG, Reactome and Gene Ontology analyses. The red cluster, comprising 23 nodes and 37 edges, was enriched in the TGF-β signaling pathway, the Hippo signaling pathway, and protein digestion and absorption. The brown cluster (4 nodes, 3 edges) was associated with arachidonic acid metabolism. The green cluster (2 nodes, 1 edge) shows enrichment for cytokine receptors, chemokine signaling pathways and cytokine–cytokine receptor interactions. The golden cluster (3 nodes, 2 edges) and the remaining clusters (dark green, cyan, dark cyan, blue, and medium blue; each 2 nodes, 1 edge) showed no significant pathway enrichment.

Using the same parameters for the upregulated genes, the PPI network revealed 15 clusters (Fig. 5b). Functional enrichment analysis

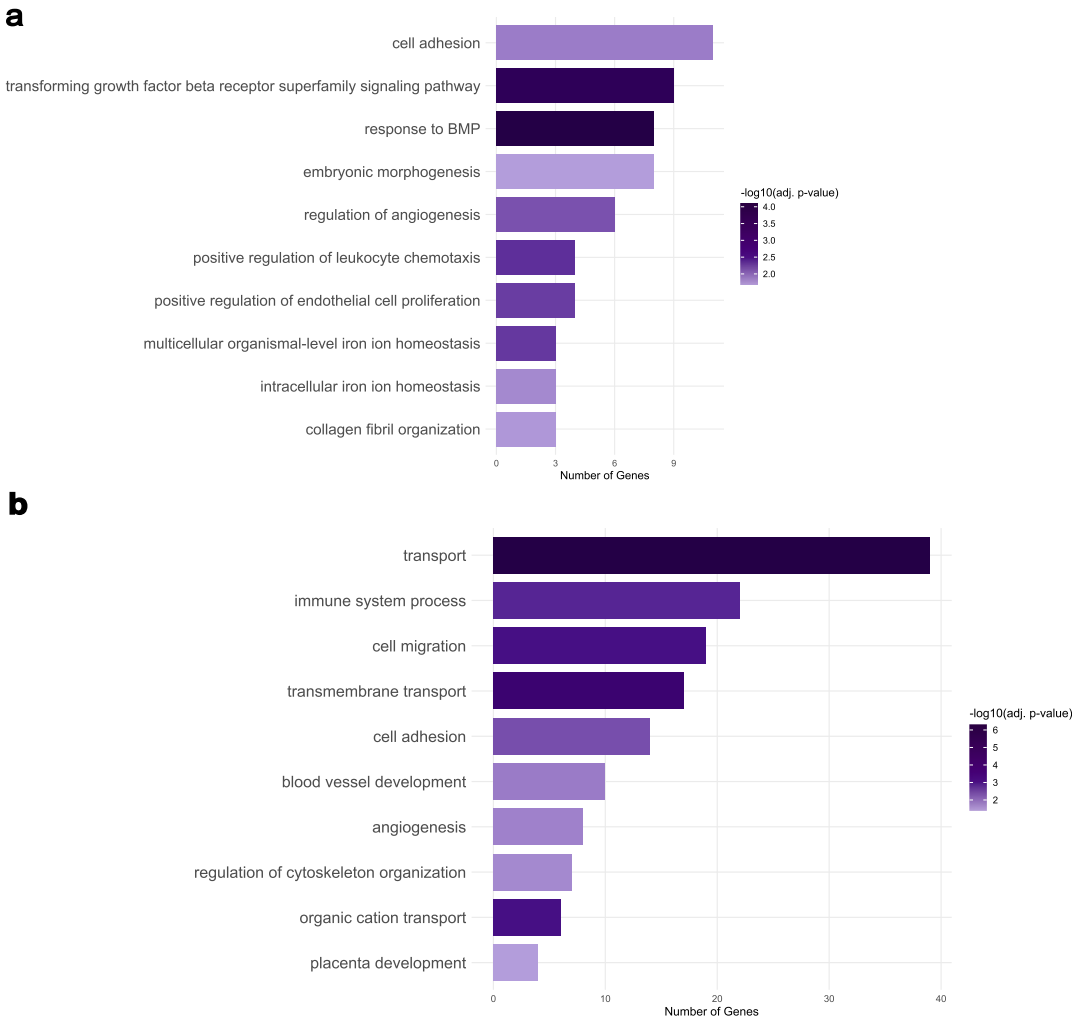

Fig. 4. GO enrichment analysis for DEGs in placentas bearing female fetuses. The bar plots summarize examples of significant and biologically relevant terms identified for (a) downregulated DEGs and (b) upregulated DEGs. The X-axis represents the number of DEGs associated with the biological terms.

using the online STRING database revealed that these clusters were associated mainly with transmembrane molecule transport, oxygen transport and angiogenesis (red cluster; 45 nodes, 67 edges), metabolic pathways, purine metabolism (brown cluster; 8 nodes, 7 edges), phospholipase activity (dark golden cluster; 6 nodes, 6 edges), prolactin receptor binding (yellow cluster; 4 nodes, 4 edges; Prolactin-7B1 (*Prl7b1*) and Glial cells missing transcription factor 1 (*Gcm1*), expressed in trophoblast and trophoblast giant cells), channel and ion transport (olive cluster; 3 nodes, 2 edges), and thyroid hormone synthesis (green cluster; 3 nodes, 2 edges; Dual oxidase 2 (*Duox2*), Paired box 8 (*Pax8*), and Protein kinase C beta (*Prkcb*)). Smaller clusters were linked to PPAR signaling (light purple; 2 nodes, 1 edge), organic acid transport (purple; 2 nodes, 1 edge, including solute carrier genes), positive regulation of dendrite development (pink cluster; 2 nodes, 1 edge, including Cordon-bleu WH2 repeat protein (*Cobl*) and Protein kinase C and casein kinase substrate in neurons 1 (*Pacsin1*)), and sphingolipid metabolism (pink; *Sgms1* and *Sgms2*; Sphingomyelin synthase 1 and 2). The remaining clusters did not show significant pathway enrichment.

3.6. Hub genes from the PPI analysis of DEGs

The identification of hub genes, i.e., genes with the highest connectivity, from the protein–protein interaction (PPI) network of differentially expressed genes (DEGs) highlights the most critical interactions. Using Cytoscape software with the Cytohubba plug-in, we identified the top 10 downregulated hub genes: cellular communication network factor 2 (*Ccn2*), *Mmp2,* Cd68 molecule (*Cd68*), *Col5a2, Bmp6, Hmox1, Tgfb3*, lysyl oxidase-like 1 (*Loxl1*), *Chrd*, and integrin subunit beta 2 (*Itgb2*) (Fig. 6a). The top 10 upregulated hub genes (Fig. 6b) were *Slc4a1*, ankyrin 1 (*Ank1*), *Cav1*, alpha hemoglobin stabilizing protein (*Ahsp*), Rh blood group, D antigen (*Rhd*), *Flt1*, solute carrier family 1 member 5 (*Slc1a5*), Kell metallo-endopeptidase (*Kel*), *Tek*, and hemoglobin, beta adult major chain (*Hbb-b1*).

4. Discussion

This is the first study to investigate the influence of gestational exposure to the pesticide acephate on fetal and placental development. Above all, we demonstrate that this influence is distinct according to fetal sex by analyzing placental gene expression with RNA-Seq and biometric and inflammatory parameters.

4.1. Influence of acephate on maternal parameters, fetal weight, placental efficiency and proinflammatory cytokines

As we expected, and consistent with the literature, which has already demonstrated the hyperglycemic potential of acephate, even in acute (Acker and Nogueira, 2014) and chronic (Deotare and Chakrabarti, 1981) exposure models, our study revealed that maternal gestational exposure also caused an increase in maternal glycemia. Maternal

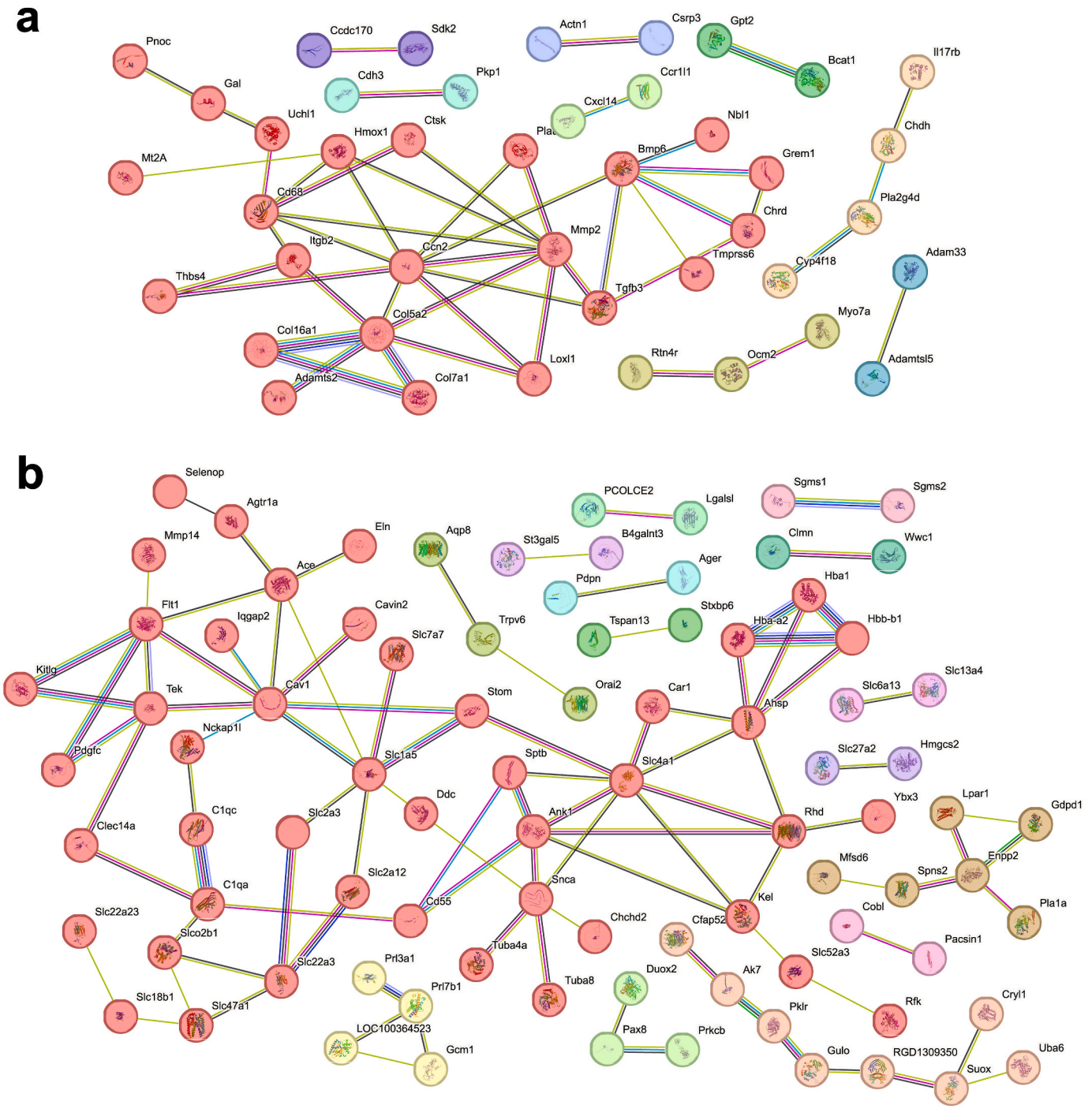

Fig. 5. PPI networks of DEGs in placentas bearing female fetuses generated using the STRING database v12.0 for differentially expressed genes, displaying interactions with a medium confidence score (>0.4). (a) Network of downregulated DEGs and (b) network of upregulated DEGs. Nodes represent proteins encoded by the DEGs, and edges (lines) represent the evidence for the interaction. Node colors represent functional clusters based on k-means clustering. Edge colors indicate the type of evidence supporting the interaction: evidence type is indicated as from curated databases (cyan), experimentally determined (magenta), gene neighborhood (green), gene fusions (red), gene co-occurrence (blue), text mining (yellow), coexpression (black), and protein homology (light purple). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

glucose availability influences placental nutrient sensing, angiogenesis, and transporter expression, thereby affecting fetal growth trajectories (Gaccioli et al., 2013; Eckmann et al., 2021). Therefore, we did not rule out the possibility that acephate-induced hyperglycemia is an important mediator that modulates placental signaling pathways and contributes to the structural and molecular changes observed in this study. However, this metabolic disruption was not accompanied by significant alterations in maternal body weight gain or spleen weight at the dose used. Similarly, a study reported nonsignificant effects on the spleen/-body weight ratio in BALB/c mice at doses of 8.78 mg/kg and 11.7 mg/kg (Sankhala et al., 2012). The lack of biometric change did not extend to the inflammatory profile, as we observed an elevation in circulating maternal MCP-1 and a suppressed inflammatory profile in the amniotic fluid, particularly in males. Different studies have associated exposure to organophosphorus pesticides with the induction of systemic inflammatory processes (Costa et al., 2020; Lopes-Ferreira

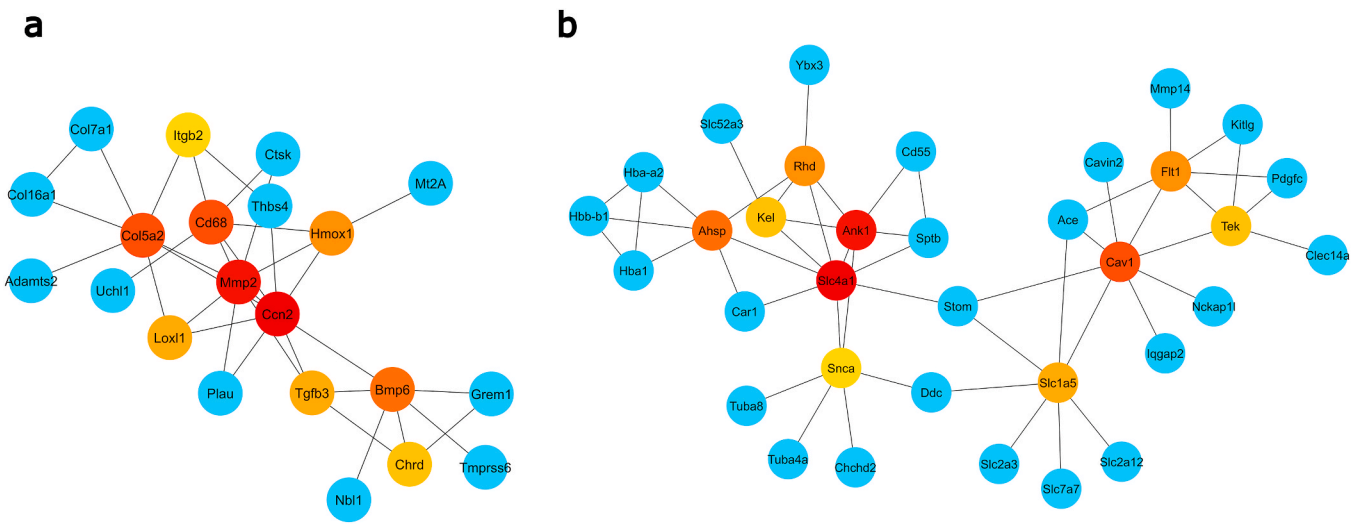

Fig. 6. The networks display the top 10 hub genes identified from (a) downregulated and (b) upregulated genes (DEGs) in placentas bearing female fetuses. Network interactions were retrieved from the STRING database and visualized using Cytoscape. Hub genes were identified and ranked using the MCC algorithm within the Cytohubba plug-in. Nodes are color-coded according to their rank (red, highest rank; orange, intermediate rank; yellow, lowest rank). Light blue nodes represent first-shell interactors connected to the hubs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

et al., 2023). However, a study that evaluated the placenta following exposure to organophosphorus pesticides revealed that this tissue was impacted not through inflammation but rather by an increase in the cytokine IL-13 (Bulgaroni et al., 2013).

The contrast between the maternal and fetal inflammatory profiles requires our attention. The amniotic fluid, considered a reflection of the fetal profile (Chow et al., 2008), presented an immunosuppressed profile in a more pronounced way in males. This immunosuppressed profile may reflect adaptive mechanisms in the fetus or placenta that aim to protect the developing fetus from the pro-inflammatory effects of maternal exposure to acephate. These adaptations may involve cytokine modulation to support placental growth, maintain fetal homeostasis, and prevent excessive inflammation and preterm delivery (Bowen et al., 2002). In addition to inflammation, which is normally associated with pathologies (Hebisch, 1997), an anti-inflammatory state is essential for the maintenance of pregnancy in a normal way (Chow et al., 2008). Therefore, the suppression of cytokines observed in the amniotic fluid of males may demonstrate a protective or repair response induced by acephate in the feto-placental compartment.

Although the female amniotic fluid showed more restricted inflammatory suppression, with only two cytokines reduced, we observed a probable major adaptive response during the gestation of female fetuses, with increased placental efficiency after exposure to acephate, suggesting a protective mechanism. In contrast, male fetuses showed a decrease in both fetal weight and placental efficiency, corroborating the greater sensitivity of these fetuses to organophosphorus compounds, even those from different applications, such as the organophosphate ester flame retardants tributyl phosphate (TBP) and tri-m-cresyl phosphate (TMCP) (Wang et al., 2022a). The protection observed in placentas bearing female fetuses may be associated with the differential expression of X-linked genes, including those that escape inactivation, which confer greater immune and metabolic robustness. Under stressful conditions, compared with males, female fetuses are prioritized because of their greater likelihood of reproductive success in adverse environments (Braun et al., 2022).

IUGR, a dysfunction that predisposes individuals to numerous health problems in adult life (Barker et al., 2002; Rinaudo and Lamb, 2008), can be directly associated with different types of stressors, ranging from environmental contaminants such as PM 2.5 (Tao et al., 2022) and nanoplastics (Chen et al., 2023), for example, to even maternal metabolic alterations (Kretschmer et al., 2020). This association may occur because of the effects of these stressors on processes necessary for placental maintenance, such as spiral artery remodeling, trophoblast differentiation, and metabolism. Consequently, placental efficiency and IUGR, which are parameters related to these structural and vascular factors (Fowden et al., 2009), can be better understood through molecular profiling, as evidenced by our transcriptomic analysis. Although both sexes exhibited IUGR, a sexually dimorphic pattern was observed in placental efficiency, with increased efficiency in females and reduced efficiency in males. This divergence is consistent with growing evidence that male and female fetuses adopt distinct adaptive strategies to adverse intrauterine environments (Kalisch-Smith et al., 2017a). According to Saoi et al. (2020), placentas bearing female fetuses generally exhibit greater adaptive plasticity. They adjust growth and function in order to partially compensate for environmental stress, even if it means sacrificing fetal growth. In contrast, placentas bearing male fetuses demonstrate limited adaptive remodeling, resulting in reduced efficiency and increased vulnerability to environmental or metabolic insults. These differences may be attributed to variations in placental morphology, nutrient transport capacity, and sensitivity to environmental stimuli. These differences could explain why male offspring are at a higher risk of exposure during later development than female offspring.

In this study, we analyzed the placental transcriptome in response to gestational exposure to acephate using RNA-Seq technology, seeking to understand possible changes in gene expression that contribute to impairments in fetal development and in the functionality of the main tissue responsible for maintaining pregnancy. The placenta, which is formed largely by trophoblast cells, which differentiate into cytotrophoblasts and syncytiotrophoblasts (Ander et al., 2019), requires highly coordinated cellular processes. Although most studies on the placenta do not consider the influence of the sex of the fetuses or baby, this variable can influence gene expression and, consequently, the interpretation of results. Under normal conditions, fetal sex influences placental development and function, modifying everything from morphology to gene regulation and responses to environmental stressors (Kalisch-Smith et al., 2017b; Olney et al., 2022). These factors may be related to our study, as we observed differences in gene expression only in placentas bearing female fetuses, rather than in those bearing male fetuses, after exposure to the pesticide.

4.2. Structural and skeletal effects of placental gene downregulation

The decrease in the expression of genes related to "cell adhesion molecule binding," "regulation of cell communication," and "cell differentiation" may indicate impairments in the formation and maintenance of the placental structure, compromising its functionality and, consequently, affecting the maternal–fetal exchange essential for fetal development. In this context, one study associated exposure to organophosphates with abnormal placentation (Varshavsky et al., 2021). Genes such as *Col5a2* and collagen type VII alpha 1 chain (*Col7a1*), which are involved in the regulation of collagen in the extracellular matrix (ECM), when expressed at low levels, can compromise the maintenance of a robust ECM capable of meeting the structural needs for fetal development. In addition, genes such as *Bmp6*, *Mmp2*, and Cathepsin K (*Ctsk*) are involved in the development of the skeletal system, highlighting another critical area, particularly considering the role of the placenta in supplying minerals and hormones essential for fetal bone development (Kovacs, 2015). Dysfunction in this process can interfere with the adequate supply of calcium and other components necessary for bone formation, resulting in abnormalities in the fetal skeleton. In addition, the placenta also regulates the production of growth factors that influence bone development, and changes in this process can lead to structural defects in the fetal skeleton (Nitta et al., 2016), affecting its development, which is consistent with the reduction in fetal weight.

4.3. Angiogenesis-related transcriptomic changes

The change in angiogenesis regulation is particularly curious, since genes related to this process showed both patterns of regulation. In particular, the gremlin 1, DAN family BMP antagonist (*Grem1*) and thrombospondin 4 (*Thbs4*) genes, flagged in the enrichment analysis as related to angiogenesis, play important roles in modulating this pathway. *Grem1*, an antagonist of the TGF-β signaling pathway, has been implicated in the regulation of trophoblastic invasion and extracellular matrix remodeling, as evidenced by studies demonstrating its ability to reduce decidual resistance to trophoblast invasion (Suhail et al., 2025). *Thbs4*, which is also involved in TGF-β regulation, acts on the invasive function of trophoblast cells and placental vascular development, with inhibition of this gene resulting in reduced cell proliferation and migration, processes essential for placental angiogenesis. Decreased expression of this gene is also observed in preeclampsia, a condition characterized by failures in placental vascular remodeling and inadequate uteroplacental perfusion (Shi et al., 2025). Similarly, other genes associated with angiogenesis and blood vessel development were differentially expressed in our model. The *Cav1* gene has been linked to the regulation of trophoblastic proliferation, migration, and invasion (Dai et al., 2019), whereas C-type lectin domain containing 14A (*Clec14a*), expressed in endothelial cells, has been identified as a mediator of cell–cell adhesion and is necessary for the formation of vascular structures during development (Rho et al., 2011). Dysregulation of placental angiogenesis has been linked to a predisposition to preeclampsia (Putra et al., 2018).

4.4. Growth factor imbalance and preeclampsia

The response to growth factors, an enriched function of positively regulated genes, is essential for trophoblasts to invade the endometrium, develop the placenta, and promote the formation of new tissues during pregnancy (Knofler, 2010). The increase in the expression of the *Flt1* gene, enriched for the term "placental growth factor receptor activity," can be seen as an attempt to promote placental development. However, the *Flt1* gene is upregulated in preeclamptic placentas (Sitras et al., 2009). Moreover, the reduction in *Hmox1* gene expression, which was also observed in our study, is closely linked to this pathology. Preeclampsia is characterized by high levels of the soluble protein sFLT-1, which is known to be suppressed by the Hmox1 pathway, which encodes the HO-1 enzyme (Ahmed and Ramma, 2015). Therefore, the dysregulation of both of these genes in our model suggests a significant angiogenic imbalance, similar to that observed in placental pathologies. HO-1 transcript levels are reduced in the first trimester of human pregnancy in preeclamptic placentas (Ramma and Ahmed, 2014).

Preeclampsia is also characterized by immune hyperactivity, which can lead to fetal death (Deer et al., 2023). GO terms related to the immune response, adaptive and humoral immunity, and leukocyte-mediated immunity were enriched for positively regulated genes. The *C1qc* gene may act by inducing preeclampsia through inflammation and the complement pathway (Wang et al., 2023). Bioinformatic analyses revealed that the upregulation of the *Mmp14* gene may be related to IUGR (Xiao et al., 2022), corroborating the findings of our study.

4.5. Placental transport adaptations under acephate exposure

On the other hand, acephate also induced the upregulation of processes related to transmembrane transport, such as the transport of oxygen, ions, amino acids, vitamins, and neurotransmitters. Among the 135 genes whose expression increased, 15 belong to the solute carrier (Slc) family. A study investigating the effects of organophosphates on Slc transporters revealed that organophosphate pesticides (OPs) can interfere with the activities of these transporters in humans, either by blocking or stimulating them (Chedik et al., 2019). Genes such as solute carrier family 2-member 3 (*Slc2a3*), which encodes the Glucose transporter 3 (GLUT3) protein, and solute carrier family 2 member 12 (*Slc2a12*), which encodes Glucose transporter 12 (GLUT12), are reported to be important for glucose transport in the placenta. During late pregnancy in rodents, another important transporter, the GLUT1 protein, is reportedly replaced by GLUT3 (Gude et al., 2005; Langdown and Sugden, 2001). However, the link between *Slc* transporter expression and pregnancy pathologies such as IUGR is complex, with conflicting reports in the literature. One study (Aiko et al., 2014) revealed that in IUGR placentas, the protein levels of L-type amino acid transporter 1 (LAT1) (encoded by solute carrier family 7 member 7 (*Slc7a7*)) increased. The same study also revealed that while the total protein levels of Alanine, serine, cysteine transporter 2 (ASCT2) (encoded by solute carrier family 1 member 5 (*Slc1a5*)) were unchanged, its functional membrane localization was significantly lower. In contrast, another study (Huang et al., 2018) reported a decrease in *Slc7a7* mRNA and protein expression in IUGR but an increase in *Slc7a7* expression in preeclampsia. Similarly, increased expression of solute carrier family 27 member 2 (*Slc27a2*), which encodes Fatty acid transport protein 2 (FATP2), an essential fatty acid transporter, has been observed in conditions of hypoxia, a key feature of preeclampsia (Jadoon et al., 2015).

The riboflavin transporter RFVT3, encoded by *Slc52a3*, acts to maintain placental homeostasis and embryonic development. *Slc52a3* knockout mice presented cerebral hypoplasia and neonatal lethality, which were reversed by riboflavin supplementation (Jin et al., 2020; Yoshimatsu et al., 2016). In addition, changes in *Slc52a3* expression may compromise placental formation, impact fetal development and cause early embryonic lethality (Intoh et al., 2016). Although not yet described in the literature as associated with placental functions, the riboflavin kinase (*Rfk*) gene, which encodes the Riboflavin kinase enzyme, which is important for riboflavin metabolism in microglial cells (Zhang et al., 2023), is also highly regulated.

The increase in the expression of genes encoding transporter proteins such as solute carrier organic anion transporter family, member 2b1 (*Slco2b1*), which encodes the Organic anion transporting polypeptide 2B1 (OATP2B1) protein, and solute carrier family 47-member 1 (*Slc47a1*), which encodes the Multidrug and toxin extrusion 1 (MATE1) protein, may reflect a fetal defense mechanism against exposure to acephate. OATP2B1, a protein expressed in the placenta, facilitates the uptake of organic anions, such as steroid sulfates and xenobiotics, from

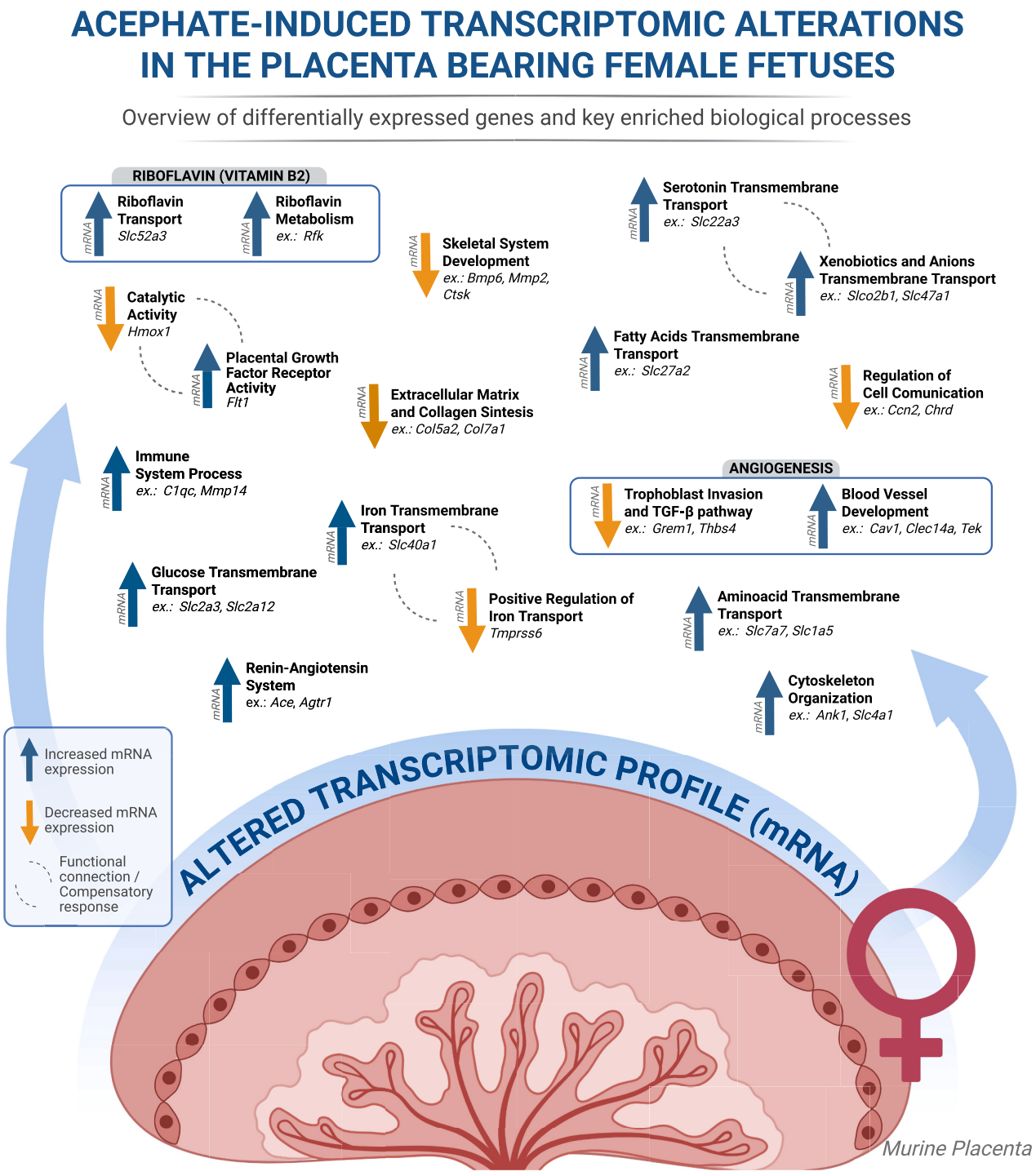

Fig. 7. Schematic representation of differentially expressed genes (DEGs) and key biological themes in placentas bearing female fetuses exposed to acephate. This figure provides a summarized schematic of the transcriptional alterations in placentas bearing female fetuses following maternal acephate exposure, which are discussed in the discussion section of our study. An upward blue arrow indicates an increase in gene expression for that category, whereas a downward orange arrow indicates a decrease. The categories accompanying the arrows represent enriched Gene Ontology (GO) terms or specific biological processes, on the basis of the scientific literature, related to the example gene(s) listed below the category. Rectangles with a blue border encompass categories that are part of a more comprehensive biological process, which may have been regulated in different directions (such as the rectangle titled "ANGIOGENESIS"), or that perform distinct functions but act with a common purpose (such as in "RIBOFLAVIN (VITAMIN B2)"). Finally, the dotted lines indicate connections between the expression of the genes involved in those specific categories (such as the relationship between Hmox1 and Flt1, which encode proteins that regulate each other), possible compensatory responses (such as the Tmprss6 and Slc40a1 relationship), and complementary roles (such as the roles of the proteins encoded by the Slc22a3, Slco2b1, and Slc47a1 genes), as discussed in the text. Created in BioRender: https://BioRender.com/84eapcd. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the fetus into the maternal circulation (Lofthouse et al., 2015; Ugele et al., 2008). Similarly, MATE1 is involved in the efflux of organic cations from the placenta to the maternal circulation, where it participates in fetal protection against toxins (Ahmadimoghaddam et al., 2012, 2013). Solute carrier family 22 member 3 (*Slc22a3*), which encodes the Organic cation transporter 3 (OCT3) protein, is crucial for fetal development because it removes serotonin from the fetal circulation (Karahoda et al., 2020) and plays a complementary role to MATE1, capturing molecules from the fetal circulation to the placenta, which are eliminated to the maternal circulation through MATE1 (Ahmadimoghaddam et al., 2012). The upregulation of these genes may be an adaptive response to mitigate exposure to harmful substances such as acephate, although there is no clear evidence on the ability of the pesticide or its metabolite to cross the placental barrier.

Ferroportin-1 (FPN1) protein is a key component of iron transport across the placental barrier, is responsible for moving iron from the placenta into the fetal circulation (Bastin et al., 2006), and is encoded by the *Slc40a1* gene. In our study, we observed a decrease in the expression of the *Tmprss6* gene, which encodes Matriptase-2. According to the literature, Matriptase-2 deficiency induces an increase in hepcidin, leading to a decrease in iron levels (Willemetz et al., 2014). Hepcidin does this by interacting with the FPN1 protein, causing its degradation (Rauf et al., 2020). Notably, one study (Willemetz et al., 2014) revealed that in *Tmprss6* knockout mice, FPN1 protein expression decreased in the placenta, whereas the mRNA level of the *Slc40a1* gene remained unchanged.

4.6. Upregulation of the RAS pathway in late gestation

A study involving Brown Norway rats, which are considered natural models of placental insufficiency due to inefficient trophoblast remodeling, revealed that their placentas overexpress genes involved in the renin–angiotensin system (RAS), such as *Agtr1* and *Ace* (Goyal et al., 2010). These findings are similar to those placentas bearing female fetuses observed in our study. The placental RAS is involved in different processes, the main of which is the regulation of blood flow for maternal–fetal exchanges and uteroplacental circulation (Vaswani et al., 2015). On the other hand, the expression of these genes increased in the placentas of rats later in gestation, on the 20th gestational day (Vaswani et al., 2015), a gestational stage similar to the day of euthanasia and collection of placentas in our study.

Some DEGs in our studies are also associated in the literature with placental dysfunction; however, these genes are regulated in the opposite manner. The expression of the *CD68* gene is upregulated in the placentas of mothers with gestational diabetes mellitus (GDM) (Mrizak et al., 2014) and in placentas bearing male fetuses exposed to phthalate (Wang et al., 2020a). *ITGB2* was upregulated in human amniotic epithelial cells derived from preeclamptic pregnancies (Kim et al., 2016), as was observed in placental transcriptomic analyses of pregnancies with the same dysfunction (Trifonova et al., 2014). Similarly, reduced expression of the *TEK* gene in humans, which was upregulated in our study, was consistently associated with problems in trophoblast invasion and the establishment of maternal–fetal exchanges, leading to the development of preeclampsia (Wang et al., 2022b). The *SLC4A1* and *ANK1* genes are strongly correlated; both encode proteins associated with cytoskeletal formation, ensuring cellular stability. In the placentas of mothers with GDM, these proteins are negatively regulated (Ge et al., 2023).

A summary of the key functional categories and gene interactions discussed in this section is presented in Fig. 7.

5. Conclusion

For the first time, our study demonstrated the effects of maternal exposure to the insecticide acephate during the gestational period on the development of the fetus and the placenta. Above all, we demonstrated that these impacts are distinct according to fetal sex and are linked to alterations in the expression of placental genes.

On the gestational day near term, acephate exposure led to an increased glycemic profile and an alteration in the maternal inflammatory profile, marked by an increase in MCP-1. In contrast, the inflammatory profile in the amniotic fluid was reduced and more pronounced in the male fetuses. IUGR, a common gestational complication, was observed in both sexes, although only the female fetuses presented an increase in placental efficiency and differential gene expression, suggesting a possible compensatory response for the maintenance of their development. The outcomes observed in the male fetuses may be related to posttranslational alterations, given the absence of placental DEGs and the decrease in the amniotic inflammatory profile.

Although our study is limited to a single gestational time point and to analysis at the RNA level, we provide opportunities for new studies that address exposure to this widely used pesticide, mainly in terms of the placental profile, a tissue that is still rarely discussed in the literature, and that consider the sex of the fetuses. In addition, as a future perspective, region-specific transcriptomic analyses of the placenta may help to elucidate the molecular mechanisms by which acephate differentially affects distinct placental zones.

CRediT authorship contribution statement

Pedro Vinicius Gonçalves Martins: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Vitor Lima Coelho:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Mariana de Souza Pomacena:** Investigation. **Yasmim Petronilho de Souza:** Investigation. **Beatriz Souza da Silva:** Investigation. **Luana Lopes de Souza:** Investigation. **Iala Milene Bertasso:** Investigation. **Egberto Gaspar de Moura:** Resources. **Patricia Cristina Lisboa:** Writing – review & editing, Resources. **Rosiane Aparecida Miranda:** Writing – review & editing, Resources, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2026.115980.

Data availability

Data will be made available on request.

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