

Gestational benzophenone-3 exposure induces litter-dependent ovarian proteomic reprogramming linking reduced fatty acid β -oxidation to impaired ovulatory responsiveness

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Research Article

Keywords: Benzophenone-3, Multiparity, Ovarian programming, Ovary, Proteomics, Ovulation

Posted Date: August 13th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10605849/v1>

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Additional Declarations: No competing interests reported.

Abstract

Maternal exposure to benzophenone-3 (BP3; oxybenzone) during successive pregnancies produces litter-dependent alterations in ovarian function. We previously found that adult female offspring from the second pregnancy exhibited impaired ovulatory responsiveness to gonadotropin stimulation, whereas first-litter offspring retained a normal response. Whether intrinsic differences in the ovarian molecular landscape are already established before gonadotropin stimulation and contribute to this divergent phenotype remains unknown. We therefore investigated the basal ovarian proteome of prepubertal female offspring born from the first and second pregnancies of dams topically exposed to BP3 (50 mg/kg/day) or vehicle during gestational days 0–9. Ovaries were collected on postnatal day 21 and analyzed by label-free liquid chromatography–tandem mass spectrometry, followed by functional enrichment and pathway mapping. BP3 exposure produced distinct, litter-dependent proteomic responses. First-litter offspring showed 25 differentially abundant proteins, mainly involving cytoskeletal organization, desmosomal components, and signaling pathways potentially related to tissue remodeling and functional compensation. In contrast, second-litter offspring displayed 48 differentially abundant proteins, with a predominance of decreased abundance. These changes were enriched in fatty acid β -oxidation, oxidative phosphorylation, mitochondrial function, proteasome regulation, and chromatin remodeling. Key reductions included proteins involved in mitochondrial energy production and lipid catabolism. These findings indicate that ovarian molecular differences are already present before gonadotropin stimulation. The second-litter ovary exhibited a metabolically vulnerable proteomic profile that may contribute to the impaired gonadotropin responsiveness previously observed in adulthood.

1. INTRODUCTION

Among widely used endocrine disrupting chemicals (EDCs), the UV filter benzophenone-3 (BP3; oxybenzone) is of increasing concern. BP3 is detected in human serum, urine, amniotic fluid, placenta, breast milk, and follicular fluid, reflecting continuous exposure throughout reproductive life (Wnuk et al. 2022; Teiri et al. 2023; Beck et al. 2024). Although BP3 is considered a weak estrogenic compound (Dutta et al. 2025), converging evidence indicates that it can interfere with several reproductive endpoints, including follicular assembly, oocyte quality, hormone-dependent signaling, and early embryo development (Santamaría et al. 2019; Mustieles et al. 2023; Abud et al. 2024; Dutta et al. 2025; Gomez 2025). Particularly, maternal exposure to BP3 during early pregnancy affects early embryo stages and pregnancy outcomes (Wnuk et al. 2022; Teiri et al. 2023). We previously reported that dermal exposure to BP3 during the first 10 days of gestation altered fetal growth and sex ratio (Santamaria et al. 2020). More recently, using in vitro models, we demonstrated that BP3 compromises two critical reproductive processes: blastocyst implantation and trophoblast migration at low doses (Abud et al. 2024). Together, these findings indicate that BP3 can contribute to fetal growth restriction through both direct and indirect mechanisms.

The ovary is another key target of BP3. The ovarian follicle is a highly sensitive target for EDCs, particularly during early development when primordial follicle assembly and ovarian organization are

established (Priya et al. 2021). Disruption of these processes may lead to impaired folliculogenesis, accelerated follicular atresia, ovulatory dysfunction, and long-term impairment of female fertility (Tricotteaux-Zarqaoui et al. 2024). Our group has shown that BP3 perturbs ovarian development at multiple stages. Using ex vivo rat whole-ovary cultures, Santamaría et al. (2019) showed that BP3 disrupts early follicular assembly, leading to an altered distribution of follicular types and a reduction in the establishment of the primordial follicle pool. We subsequently reported that perinatal exposure to BP3 induces early subfertility in female offspring due to an impoverished oocyte stockpile (Galliani et al. 2024). These findings support that BP3 interferes with the ovarian reserve, follicular development and steroidogenesis, ultimately compromising long-term reproductive outcomes.

A physiologically relevant scenario rarely considered in endocrine disruption research is multiparity (Bafandeh et al. 2023; Magaye et al. 2025). Repeated gestations may induce persistent changes in maternal endocrine, metabolic, and immune states, potentially influencing offspring susceptibility to EDCs (Rebholz et al. 2012; Bridges 2015; Merrill et al. 2021; Vicente et al. 2022; Moon et al. 2023). We recently addressed this question using a murine multiparity model, demonstrating that gestational exposure to BP3 or their combination with BPA markedly reduces ovarian responsiveness to gonadotropin stimulation in a litter-dependent manner. Specifically, female offspring from the second gestation (F2) exhibited a significant impairment in ovulation, whereas first-litter offspring (F1) retained normal responsiveness (Galliani et al. 2025). However, the molecular mechanisms underlying this litter-dependent susceptibility remain unknown. In particular, it is unclear whether intrinsic differences in the ovarian molecular landscape are already present prior to ovulatory stimulation and may account for the divergent responses observed between F1 and F2 offspring. The present study builds on our previous work demonstrating litter-dependent impairment of ovulatory responsiveness following gestational BP3 exposure in a multiparity model. Using ovarian tissues collected from that experimental model, we performed an untargeted proteomic analysis to identify molecular pathways underlying this phenotype. We hypothesized that second-litter offspring exhibit a distinct ovarian proteomic profile, reflecting altered biological pathways that predispose them to impaired ovulatory responses.

2. Materials and Methods

2.1. Experimental design and sample collection

The ovarian samples analyzed in the present study were obtained from animals included in a previously published experiment investigating the effects of gestational BP3 exposure on ovarian responsiveness to gonadotropin stimulation (Galliani et al. 2025). The present study represents a secondary analysis of those samples, aimed at characterizing the basal ovarian proteomic landscape underlying the previously reported functional phenotype. Briefly, offspring were generated by housing C57BL/6 females with BALB/C males. This allogeneic pairing was selected as it mimics natural mating conditions observed in mammals, including humans, thereby increasing the translational relevance of the model (Müller et al. 2018). Pregnant dams were exposed to vehicle (olive oil) or BP3 via daily topical application at a dose of 50 mg/kg body weight (bw). This dose was selected to reflect realistic levels of estimated human dermal

exposure following the application of topical formulations (Janjua et al. 2008; Wang and Ganley 2019; Santamaria et al. 2020).

BP3 or vehicle was applied from gestational day 0 (gd0) to gd9 during two sequential pregnancies of the same dams. This window encompasses critical early developmental events highly sensitive to endocrine disruption, such as embryo implantation, spiral artery remodeling (Charalambous et al. 2012; Rusidzé et al. 2023), and the specification and migration of primordial germ cells (Richardson and Lehmann 2010). Furthermore, this approach allows for a more realistic assessment of cumulative risks, considering how the physiological adaptations inherent to multiparity may modify susceptibility to exposure (Bridges 2015; Vicente et al. 2022).

Female offspring from both the first (F1.P1) and second pregnancy (F1.P2) of the Control and BP3 groups were selected. Females were sacrificed at postnatal day 21 (dpn21), prior to the onset of endogenous estrous cycles or the administration of exogenous gonadotropins and thus at a basal state. Ovaries were surgically excised and immediately processed under a stereoscopic microscope to remove the surrounding adipose tissue and the ovarian bursa. Finally, the organs were stored at -80°C to preserve tissue integrity for subsequent proteomic processing. Three biological replicates were analyzed per experimental group. In both first pregnancy (F1.P1), and second pregnancy (F1.P2), biological replicates consisted of pools of two ovaries prior to protein extraction to increase protein yield

2.2. Total protein extraction and solubilization

Protein extraction from ovaries was performed using TRIzol™ reagent (Thermo Fisher Scientific) according to the manufacturer's protocol, based on phase separation. Following protein pellet recovery, a modified version of the method described by Simões et al. (2013) was employed for solubilization.

Pellets were resuspended in a buffer solution consisting of a 1:1 mixture of 1% SDS and 8M Urea in 1M Tris-HCl (pH 8.0). To ensure complete denaturation and solubilization of protein complexes, samples underwent five sonication cycles (15 seconds of sonication followed by 30 second incubation periods on ice). Sonication was performed using a VC 505 Vibra-Cell™ ultrasonic processor (Sonics & Materials, Inc., Newtown, CT, USA) with the amplitude adjusted to 80% and pulse to 90%. Once resuspended, the samples were centrifuged to remove insoluble debris, and the resulting supernatant was quantified using BCA assay. To minimize interference from high buffer concentrations, samples were diluted 1:10 in distilled water prior to quantification.

2.3. Protein Quantification

Protein concentrations were determined using the bicinchoninic acid (BCA) assay with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). To minimize potential interference from high concentrations of Tris-HCl (1M) and Urea (8M) in the solubilization buffer, all samples were diluted 1:10 in ultra-pure water prior to the assay. A standard calibration curve was generated using known concentrations of bovine serum albumin (BSA) as a standard. Absorbance was measured at 562 nm using a microplate

reader, allowing for the normalization of protein loading to 30 µg per lane for subsequent SDS-PAGE analysis. Following quantification, samples were stored at -20°C until further processing.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein separation was performed under denaturing and reducing conditions using SDS-PAGE. A discontinuous gel system (0.75 mm thickness) was employed, consisting of a 4.5% stacking gel and a 12% resolving gel (Bio-Rad Laboratories).

Samples were diluted in a Laemmli sample buffer containing SDS and β-mercaptoethanol, then incubated at 95°C for 5 minutes to ensure complete denaturation.

A total of 30 µg of protein was loaded into each lane. Electrophoresis was conducted in Tris-glycine-SDS running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% w/v SDS, pH 8.3) at a constant voltage of 10 V/cm. Electrophoresis was terminated once the dye front had migrated approximately 1 cm into the resolving gel. This restricted short-run strategy was designed to concentrate the total proteome into a single, narrow band, ensuring that subsequent gel excision for mass spectrometry analysis did not exceed dimensions of 1 cm x 1 cm, thereby optimizing the in-gel digestion process.

2.4. Colloidal Coomassie Blue gel staining and band excision

For protein visualization, gels were stained with Colloidal Coomassie Blue G-250 according to a protocol optimized for mass spectrometry compatibility (CEQUIBIEM, University of Buenos Aires, <http://cequibiem.qb.fcen.uba.ar/>). Briefly, gels were fixed for a minimum of 3 hours (or overnight) in a solution of 30% ethanol and 2% phosphoric acid. Following three 30-minute washes with distilled water, gels were pre-incubated for 1 hour in a staining buffer (18% methanol, 17% ammonium sulfate, and 2% phosphoric acid). Subsequently, Coomassie Blue G-250 (0.5 g/L) was added to the solution to generate a colloidal suspension. Gels were maintained under gentle agitation for 24–48 hours in hermetically sealed containers to prevent keratin contamination and evaporation. After achieving optimal staining intensity, gels were thoroughly rinsed with ultra-pure water. The protein-containing bands (1 cm x 1 cm) were precisely excised, transferred to individual microcentrifuge tubes, and stored at 4°C until in-gel digestion and subsequent proteomic analysis.

2.5. Proteomic analysis via NanoHPLC-MS/MS

In-gel Tryptic Digestion Proteomic analysis was conducted at the Center for Chemical and Biological Studies by Mass Spectrometry (CEQUIBIEM, UBA-CONICET, Argentina: <http://cequibiem.qb.fcen.uba.ar/>). The 1 cm x 1 cm gel bands were subjected to in-gel tryptic digestion. Proteins were reduced by 20 mM dithiothreitol (DTT) for 45 minutes at 56°C and subsequently alkylated with 50 mM iodoacetamide for 45 minutes in the dark at room temperature. Proteolytic digestion was performed using sequencing-grade modified trypsin (Promega) for 12–16 hours at 37°C. The resulting peptides were extracted from the gel

matrix using acetonitrile, concentrated by centrifugal evaporation (SpeedVac), and resuspended in 30 μ L of 0.1% trifluoroacetic acid (TFA). Samples were desalted using C18 reverse-phase resins (Merck) according to the manufacturer's instructions before chromatographic injection.

2.6. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Peptide separation and analysis were performed using an EASY-nLC 1000 chromatograph coupled to a Q-Exactive mass spectrometer equipped with an Orbitrap analyzer and a Higher-energy Collisional Dissociation (HCD) cell (Thermo Fisher Scientific). Chromatographic separation was achieved on an EASY-Spray™ Accucore reverse-phase column (C18, 2 μ m, 100 Å, 75 μ m x 250 mm) at a constant temperature of 35°C.

A 120-minute linear gradient was employed at a flow rate of 200 nL/min. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. The elution gradient increased from 5% to 35% in phase B over the first 100 minutes. Ionization was performed via an EASY-Spray™ source with a spray voltage between 1.5 and 3.5 kV in positive mode. The spectrometer operated in data-dependent acquisition (DDA) mode (Top 12). Full MS scans were acquired at a resolution of 70,000 (m/z range 400–2,000) with an Automatic Gain Control (AGC) target of 3e6. The 12 most intense precursor ions were selected for fragmentation (MS/MS) at a resolution of 17,500 (AGC target 1e5). Dynamic exclusion was enabled to optimize the detection of low-abundance peptides.

2.7. Data processing and bioinformatic analysis

Protein Identification and Quantification Raw data processing was performed using Proteome Discoverer™ software (v2.2, Thermo Fisher Scientific). MS/MS spectra were searched against the *Mus musculus* reference database (UniProt UP000000589) using the Sequest HT engine. Search parameters included: trypsin as the proteolytic enzyme with up to two missed cleavages; mass tolerances of 10 ppm for precursors and 0.05 Da for fragments; carbamidomethylation of cysteines as a static modification; and oxidation of methionines as a dynamic modification. Protein quantification and result consolidation were conducted through Multiconsensus analysis (MC/LFQ) using the Feature Mapper node to align retention times (10 min window) and minimize missing values. A False Discovery Rate (FDR) of 0.01 was applied using the Percolator node. Only proteins identified with "High" confidence and a minimum of two unique peptides were retained.

2.8. Statistical analysis

Downstream statistical processing was conducted using Perseus software (v1.6.6.0). To ensure dataset homogeneity, a technical replicability assessment was performed; one treated sample from the G1 cohort was identified as an outlier and subsequently excluded from the analysis. In contrast, no outliers were identified in the G2 cohort, and all samples were retained for comparison. Protein abundance data were log2-transformed, and quality filters were applied to remove potential contaminants and reverse hits. To handle missing values, imputation was performed from a normal distribution. Differentially

expressed proteins (DEPs) were identified using a Student's t-test ($p < 0.05$) and visualized via Volcano plots.

2.9. Functional enrichment and systems biology integration

A comprehensive bioinformatic integration was performed on the lists of DEPs to characterize the molecular alterations in the ovaries. Protein identifiers were mapped to the UniProt database to retrieve detailed functional annotations and categorize proteins according to Gene Ontology (GO) terms (Biological Processes and Molecular Functions). Metabolic pathway enrichment was analyzed using the KEGG Mapper – Color tool (<https://www.genome.jp/kegg/mapper/color.html>), which allowed for the systematic mapping of up-regulated and down-regulated proteins onto KEGG metabolic maps. Functional enrichment analyses were performed using the STRING database v11.5 (<https://string-db.org/>) to identify significantly enriched biological categories among up- and down-regulated proteins. Finally, differential abundance profiles were visualized using heatmaps generated with the Morpheus online tool (<https://software.broadinstitute.org/morpheus/>).

3. Results

3.1. Ovarian proteomic profile in F1.P1 and F1.P2

To evaluate the impact of prenatal BP3 exposure, we compared the ovarian proteome of offspring from the first (F1.P1) and second (F1.P2) gestations with their respective controls. Volcano plots revealed differentially expressed proteins (DEPs) in F1.P1 (Figure 1a) and F1.P2 (Figure 2a), indicating distinct profiles between gestations.

In F1.P1, 25 DEPs ($p < 0.05$) were identified (Table S1 and S2): 17 were up-regulated and 8 were down-regulated (Figure 1a). Hierarchical clustering analysis (heatmap) showed consistency across biological replicates (Figure 1b).

In contrast, F1.P2 exhibited a proteomic profile characterized by 48 DEPs (Table S3 and S4), of which 33 were down-regulated and 15 were up-regulated (Figure 2a). The heatmap also showed consistency among samples (Figure 2b).

The Venn diagram (Figure 3) indicated no overlap in protein identities between F1.P1 and F1.P2, either among up-regulated or down-regulated proteins.

3.2. Functional Enrichment Analysis of DEPs in F1.P1 Ovaries

To evaluate the biological impact of prenatal BP3 exposure on the first pregnancy offspring, functional enrichment analyses were performed on DEPs identified in F1.P1 ovaries.

Functional enrichment of down-regulated proteins revealed a strong impact on ovarian structural organization (Figure 4a). Among the enriched terms, “Intermediate filament organization” was the most significant ($\text{FDR} = 3.0 \times 10^{-7}$) and included the largest number of proteins. Other affected processes included “Keratinocyte differentiation” and “Epithelial cell differentiation” ($\text{FDR} < 0.05$).

The up-regulated proteome in F1.P1 showed enrichment of proteins containing the Armadillo-type fold, representing the most significantly over-represented structural domain in this group (Figure 4b).

3.2.1. KEGG Pathway Mapping in F1.P1 Ovaries

To evaluate the impact of prenatal BP3 exposure, DEPs were mapped into KEGG pathways. From this analysis, representative pathways relevant to ovarian structure and signaling were selected for further characterization.

In the Desmosomal Cadherin pathway (Figure S1a), core structural elements were down-regulated, including Desmoplakin (DSP) and intermediate filament components (IF).

In contrast, mapping of up-regulated proteins identified activation of the Oxytocin signaling pathway (Figure S1b), highlighted by the up-regulation of the Gq protein (Gnaq).

Finally, the Estrogen signaling pathway (Figure S1c) showed a mixed pattern, with down-regulation of Krt19 and up-regulation of Gq, indicating differential regulation within this pathway.

3.3. Functional Enrichment Analysis of DEPs in F1.P2 Ovaries

To evaluate the biological impact of prenatal BP3 exposure on the second pregnancy offspring, functional enrichment analyses were performed on DEPs identified in F1.P2 ovaries.

Functional enrichment of the down-regulated proteome in F1.P2 identified significant over-representation of pathways related to lipid metabolism and mitochondrial function (Figure 5a). The most significant terms included “Fatty acid oxidation” and “Fatty acid β -oxidation” ($\text{FDR} < 4.4 \times 10^{-4}$), followed by “Cellular lipid catabolic process” and “Mitochondrial part.”

These findings showed a coordinated reduction in proteins involved in bioenergetics and proteostasis in F1.P2 ovaries.

The up-regulated proteome in F1.P2 showed enrichment in terms associated with protein synthesis (Figure 5b). “Ribosome” was identified as the main over-represented biological process, and the enrichment map indicated activation of ribosomal biogenesis and translational processes, along with terms related to post-translational modification and signaling. These results suggest activation of translational processes together with changes in chromatin-related regulation in F1.P2 ovaries.

3.3.1. Mapping of DEPs into KEGG Pathways in F1.P2 Ovaries

To further characterize the metabolic and structural alterations in the second generation, DEPs were mapped into selected KEGG pathways.

In the Oxidative Phosphorylation pathway (Figure S2a), essential components of the mitochondrial electron transport chain were down-regulated. This bioenergetic suppression was targeted at Complex III (node 7.1.1.8, including the QCR2 subunit) and Complex V (node 7.1.2.2). Within the F-type ATPase, the alpha subunit (Atp5f1a) was identified as a key down-regulated DEP. Consistent with this pattern, the Fatty Acid Degradation pathway (Figure S2b) showed decreased levels of enzymes involved in the final steps of β -oxidation, specifically Acads (1.3.8.1) and Eci1 (5.3.3.8).

Mapping into the Proteasome pathway (Figure S2c) revealed down-regulation of subunits of the 19S regulatory particle, including Psmd13 (RPN9) and Psmd8 (RPN12), as well as Psme3 (PA28 γ).

Finally, the ATP-dependent chromatin remodeling pathway (Figure S2d) exhibited a specific expression imbalance within the SWI/SNF complex. This disruption was characterized by the up-regulation of the catalytic subunit Smarca4 (BRG1, represented by the A2/4 node), alongside the significant down-regulation of the critical coupling subunit Actl6a (BAF53/ACTL6).

Discussion

Previous studies from our group demonstrated that gestational exposure to BP3 does not impair ovulatory responsiveness in first-litter offspring (F1.P1), whereas second-litter offspring (F1.P2) exhibit a marked reduction in the number of ovulated oocytes following gonadotropin stimulation in adulthood (Galliani et al. 2025). Building upon these findings, the present study analyzed the ovarian proteomic profile of prepubertal offspring before the exposure to exogenous gonadotropins in both F1.P1 and F1.P2 litters. Our results demonstrate distinct ovarian molecular states depending on litter order.

Adaptive ovarian phenotype in first-litter offspring (F1.P1)

Functional enrichment analysis in prepubertal F1.P1 ovaries revealed alterations primarily associated with tissue architecture and cytoskeletal organization. In particular, proteins involved in the desmosomal cadherin pathway, including Desmoplakin (Dsp) and several intermediate filament-associated keratins, were significantly down-regulated. Desmosomes and cytoskeletal anchoring structures are essential for maintaining tissue integrity and mechanical stability (Terada et al. 2005; Green and Simpson 2007). Thus, these findings suggest that prenatal BP3 exposure induces an early remodeling of the ovarian structural scaffold.

From a physiological perspective, ovulation requires extensive extracellular matrix remodeling, coordinated follicular wall rupture, and dynamic changes in cell adhesion and tissue mechanics (Robker et al. 2018; Russell and Robker 2019). Therefore, structural weakening of the ovarian tissue could potentially compromise the ability of follicles to adequately respond to periovulatory signals. In addition, cytoskeletal organization is tightly linked to follicular survival and atresia (Tilly et al. 1997; Hussein

2005). Given the central role of extracellular matrix dynamics, cell–cell communication, tissue mechanics, and cytoskeletal organization in follicular development, the down-regulation of desmosomal and intermediate filament proteins observed in F1.P1 ovaries suggests an early alteration of the ovarian structural scaffold (Berkholtz et al. 2006; Woodruff and Shea 2007; Biswas et al. 2022).

Despite these basal alterations, first-litter offspring previously showed preserved ovulatory responsiveness (Galliani et al. 2025). Interestingly, proteomic analysis revealed the activation of pathways potentially associated with adaptive compensation. Specifically, up-regulation of Gnaq, a key mediator of Gq signaling, was detected together with increased expression of Calponin-2 (Cnn2) and Prostaglandin E Synthase 2 (Ptges2). Activation of Gq signaling stimulates phospholipase C activity and intracellular Ca²⁺ mobilization, thereby promoting contractile responses (Leung and Steele 1992; Breen et al. 2013). In the ovary, coordinated contraction of the theca cell layers contributes mechanically to follicular rupture during ovulation (Robker et al. 2018). Similarly, prostaglandin synthesis is indispensable for cumulus expansion, local inflammatory signaling, and extracellular matrix degradation during the periovulatory period (Robker et al. 2018). This concurrent activation of contractile and inflammatory pathways could represent an adaptive response aimed at preserving ovulation despite the structural alterations induced by BP3 exposure.

Nevertheless, proteomic analysis identified reduced expression of Topoisomerase II alpha (Top2a) (Table S2) together with increased abundance of Mre11 (Table S1), a key mediator of DNA double-strand break repair. Developmental exposure to EDCs has previously been associated with meiotic defects, genomic instability, and altered oocyte quality (Hunt et al. 2003; Peretz et al. 2014; Vrooman et al. 2025). Thus, although first-litter offspring preserve ovulatory responsiveness, the presence of early molecular signatures related to DNA repair and meiotic stress suggests that prenatal BP3 exposure may still compromise long-term ovarian quality at the cellular level.

Altered ovarian metabolic state in second-litter offspring (F1.P2)

On the other hand, second-litter offspring exhibited a markedly different molecular profile characterized by alterations in energy metabolism, mitochondrial activity, proteostasis, and chromatin remodeling.

Among the most prominent findings was the down-regulation of proteins involved in mitochondrial oxidative phosphorylation and fatty acid β -oxidation, including Uqcrc2, Atp5f1a, Acads, and Eci1. Ovarian follicles are highly energy-demanding structures, requiring continuous ATP production to sustain granulosa cell proliferation, steroidogenesis, cytoskeletal remodeling, and ovulatory competence (Dunning et al. 2010, 2014). In particular, β -oxidation plays a critical role in generating acetyl-CoA to fuel the tricarboxylic acid cycle and support mitochondrial ATP synthesis. In fact, meiotic resumption in the oocyte is accompanied by a significant increase in β -oxidation in the cumulus-oocyte complex (Valsangkar and Downs 2013). Conversely, pharmacological blockade of this pathway with inhibitors such as etomoxir prevents FSH-induced ovulation and subsequent embryonic development to the blastocyst stage (Dunning et al. 2010; Valsangkar and Downs 2013).

Importantly, ovulation itself constitutes an energetically demanding inflammatory-like process that requires rapid transcriptional activation, extracellular matrix remodeling, cytoskeletal reorganization, and steroidogenic activity (Russell and Robker 2019). Thus, the metabolic deficiencies identified in F1.P2 ovaries likely compromise the tissue's ability to adequately respond to the gonadotropin surge. In this context, the proteomic profile identified in second-litter offspring is consistent with a metabolically pre-exhausted ovarian state.

Consistent with the existence of energetic stress, F1.P2 ovaries also exhibited increased expression of *Prkaa1* (Table S3), the catalytic α -subunit of AMP-activated protein kinase (AMPK). AMPK functions as a central cellular energy sensor activated in response to elevated AMP/ATP ratios (Hardie 2008). Once activated, AMPK suppresses anabolic and energy-consuming pathways while promoting metabolic adaptation and stress resistance. In ovarian physiology, AMPK signaling modulates granulosa cell metabolism, steroidogenesis, and follicular dynamics (Tosca et al. 2005; Dupont and Scaramuzzi 2016). Therefore, increased *Prkaa1* expression likely reflects an attempt to restore cellular homeostasis in response to mitochondrial insufficiency. However, the inability of the ovary to respond with an adequate number of ovulated oocytes indicates that this potential adaptive response is insufficient to fully compensate for the underlying energetic dysfunction.

A second major feature of F1.P2 ovaries was the coordinated disruption of proteostasis-related pathways. Down-regulation of multiple proteasome regulatory subunits (*Psmd13*, *Psmd8*, *Psmd5*, *Psme3*) was observed together with reduced expression of CCT chaperonin complex components (*Cct2* and *Cct7*). Proteostasis is essential for maintaining protein quality control through the coordinated regulation of protein folding, trafficking, and degradation (Collins and Goldberg 2017). The inability to adequately fold new proteins and eliminate defective ones favors the accumulation of protein aggregates in the cytoplasm, which is associated with chronic cellular stress and functional decline (Labbadia and Morimoto 2015; Hipp et al. 2019).

Interestingly, some of the molecular alterations identified in F1.P2 ovaries resemble processes commonly associated with ovarian aging, such as mitochondrial dysfunction, (Miao et al. 2009). Although the present study does not directly demonstrate premature ovarian aging, the observed proteomic profile suggests the emergence of an aging-like molecular signature in second-litter offspring exposed to BP3 during gestation.

In parallel with metabolic and proteostatic alterations, F1.P2 ovaries also exhibited dysregulation of ATP-dependent chromatin remodeling machinery. Specifically, the SWI/SNF complex showed increased expression of the catalytic ATPase *Smarca4* (BRG1) accompanied by reduced abundance of the essential coupling subunit *Actl6a*. The SWI/SNF complex is indispensable for nucleosome sliding and regulating chromatin accessibility (Sahu et al. 2020). The SWI/SNF complex is indispensable for nucleosome sliding and regulating chromatin accessibility (Sahu et al. 2020). Since *Actl6a* is crucial for properly coupling the catalytic ATPase to the nucleosome, its down-regulation indicates a functional impairment of the chromatin remodeling machinery. In this context, *Smarca4* up-regulation may

represent an attempt to maintain transcriptional adaptability under conditions of cellular stress. Importantly, appropriate chromatin remodeling is required for granulosa cell responsiveness to gonadotropins and for the acquisition of oocyte developmental competence (Abedini et al. 2022). Therefore, these alterations may contribute to the deficient ovulatory response observed in second-litter offspring.

Unlike first-litter offspring, second-litter offspring exhibit disruption involving energy metabolism, protein quality control, and epigenetic regulation. This molecular state likely reduces the ovary's capacity to respond to gonadotrophin stimulation. Given that F1.P1 and F1.P2 offspring originate from the same mother exposed to BP3, these functional differences may reflect a persistent effect of the toxicant on the maternal ovarian reserve (Bromfield and Piersanti 2019). Importantly, these changes were already present at the prepubertal stage, indicating that the ovary enters adult life in a metabolically compromised state before exposure to gonadotropin stimulation.

Some limitations of the present study should be acknowledged. First, proteomic analyses were performed at a basal prepubertal stage and therefore do not directly capture the molecular dynamics occurring during gonadotropin-induced ovulation. Second, although the data strongly suggest impaired mitochondrial metabolism and β -oxidation, direct functional metabolic assays were not performed. Future studies integrating mitochondrial functional analyses, and targeted manipulation of β -oxidation pathways will be necessary to establish causal links between metabolic dysfunction and impaired ovulatory competence.

In conclusion, the present study demonstrates that repeated gestational exposure to BP3 induces parity-dependent ovarian prepuberal states. First-litter offspring appear to develop a compensated ovarian phenotype capable of preserving ovulatory responsiveness despite structural alterations, whereas second-litter offspring exhibit an exhausted metabolic profile associated with reduced gonadotropin responsiveness. These findings identify ovarian metabolic vulnerability as a potential mechanistic basis linking repeated developmental exposure to EDCs such as BP3 with long-term reproductive dysfunction.

Declarations

Competing Interests

The authors declare that they have no competing financial or non-financial interests directly or indirectly related to the work reported in this manuscript.

Author Contribution

V.G.: investigation, methodology, formal analysis, data curation, writing – original draft, writing – review & editing. N.C.: investigation and formal analysis. D.A. formal analysis. J.E.A.: supervision. H.A.R.:

conceptualization, funding acquisition, project administration, supervision, writing – review & editing. All authors read and approved of the final manuscript.

Acknowledgement

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Argentina, through grants PICT 2020-08917 and PICT-2021-I-A-00593. The authors gratefully acknowledge Mr. Agustín García for his excellent technical assistance with animal care and handling. The authors also thank Dr. Paula Cardona for her valuable assistance with the omics data analysis.

Data Availability

The data supporting the findings of this study are included within the article and its Supplementary Information files. Supplementary datasets provide the complete lists of differentially expressed proteins and the data underlying the enrichment analyses. Raw mass spectrometry data and additional processed datasets are available from the corresponding author upon reasonable request.

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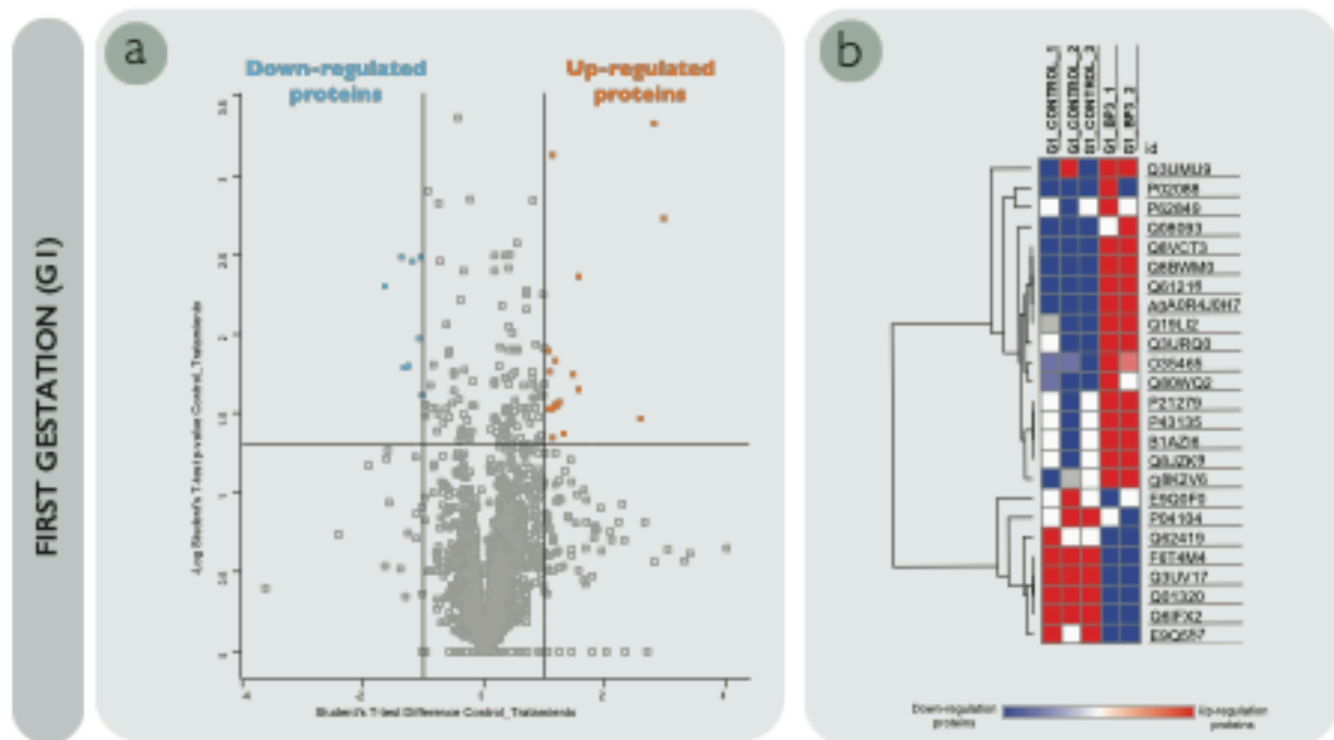
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Global proteomic profiling of F1.P1 ovaries following BP3 exposure. (a) Volcano plot illustrating the distribution of differentially expressed proteins (DEPs) in F1.P1 offspring. Orange squares indicate up-

regulated proteins, blue squares represent down-regulated proteins, and gray squares indicate proteins with no significant differential expression. (b) Hierarchical clustering heatmap of DEPs in F1.P1 ovaries showing the relative abundance of proteins across individual biological replicates. The color scale indicates high (red) and low (blue) protein expression levels.

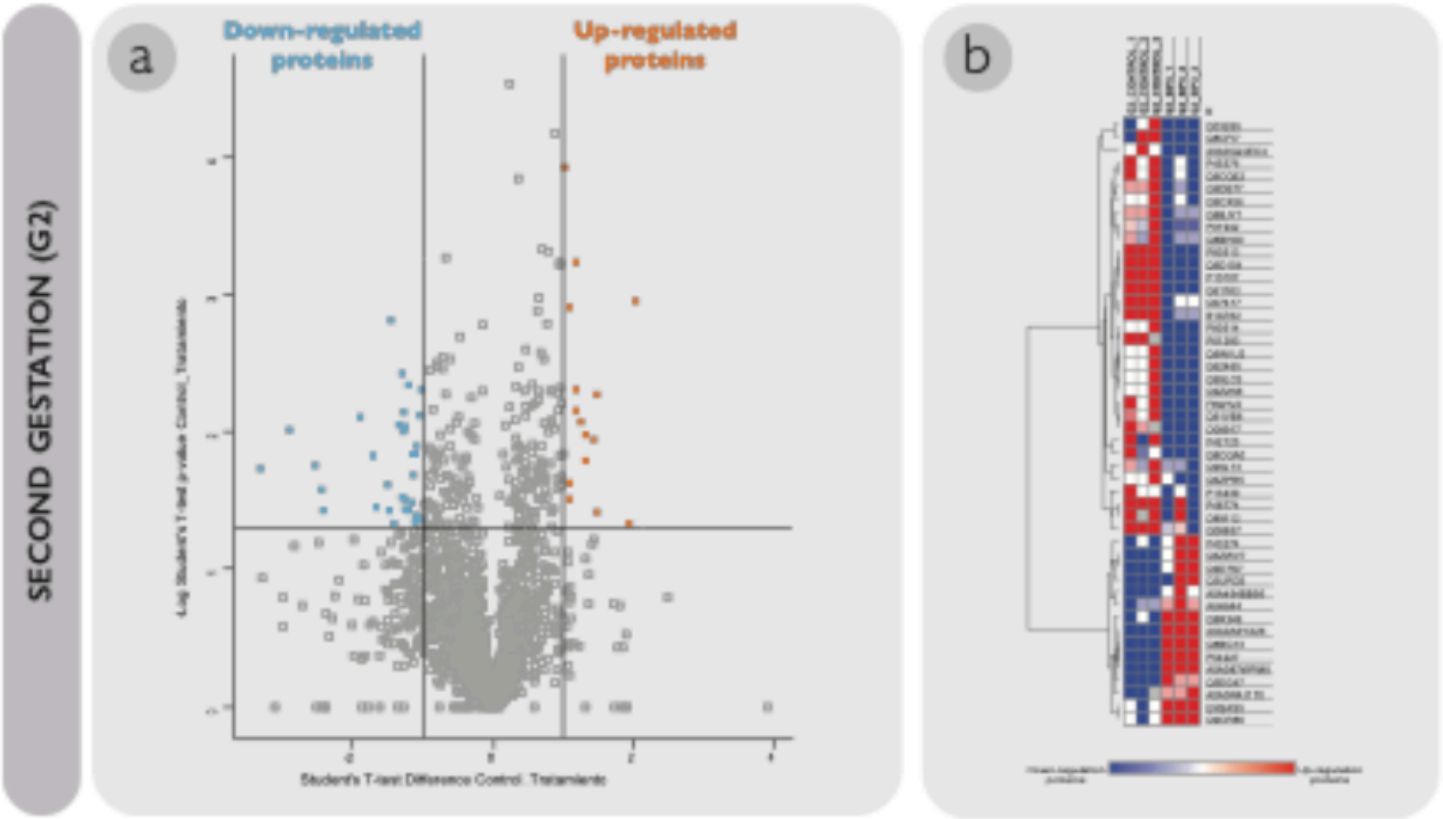


Figure 2

Global proteomic profiling of F1.P2 ovaries following BP3 exposure. (a) Volcano plot illustrating the distribution of differentially expressed proteins (DEPs) in F1.P2 offspring. Orange squares indicate up-regulated proteins, blue squares represent down-regulated proteins, and gray squares indicate proteins with no significant differential expression. (b) Hierarchical clustering heatmap of DEPs in F1.P2 ovaries showing the relative abundance of proteins across individual biological replicates. The color scale indicates high (red) and low (blue) protein expression levels.

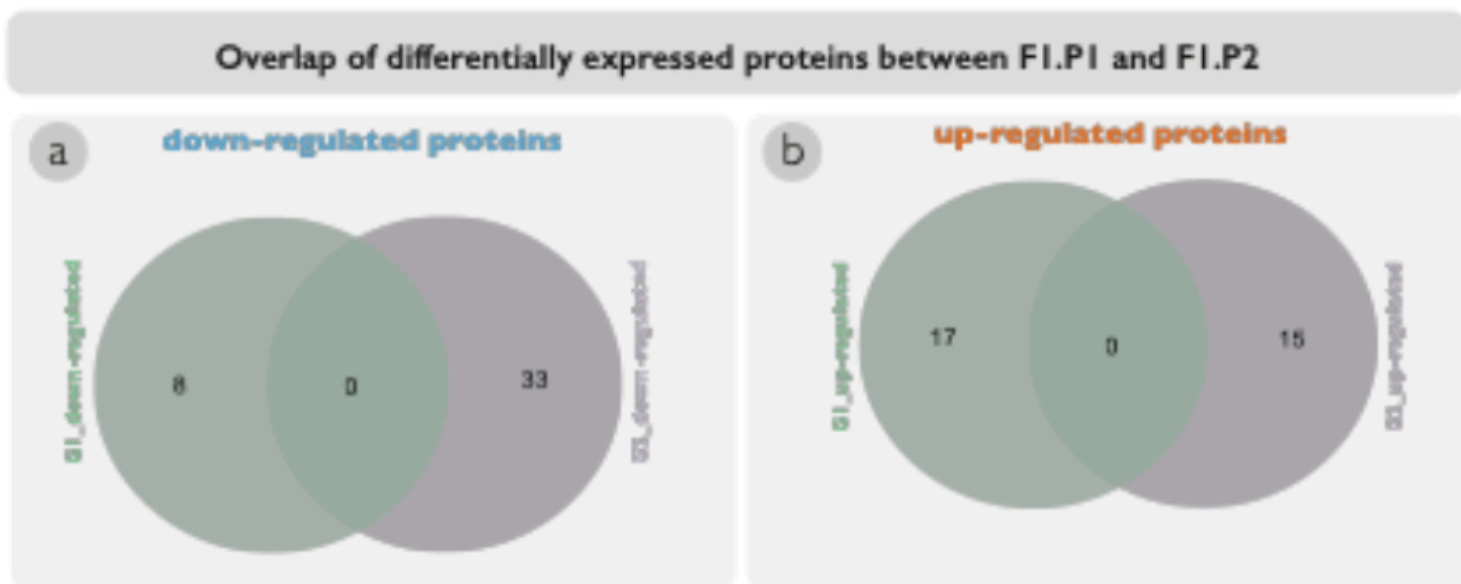


Figure 3

Venn Analysis of Up- and Down-Regulated Proteins in F1.P1 and F1.P2. Venn diagrams comparing the identity of down-regulated (top) and up-regulated (bottom) proteins between F1.P1 and F1.P2, showing the overlap of the molecular response between generations. Venn diagrams were generated using the Venny 2.1 online tool (<https://bioinfogp.cnb.csic.es/tools/venny/>).

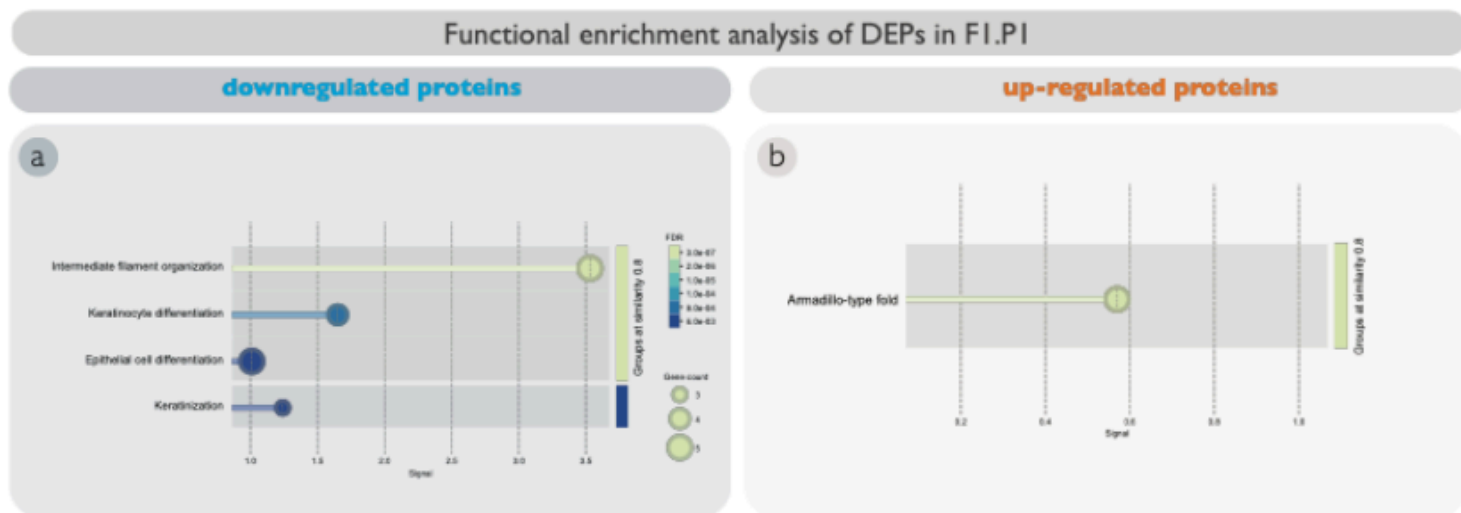


Figure 4

Functional enrichment analysis of DEPs in F1.P1 ovaries following BP3 exposure. a) Enriched functional categories identified among down-regulated DEPs. Bubble size represents the number of proteins mapped to each term, whereas color indicates the false discovery rate (FDR). b) Enriched functional categories identified among up-regulated DEPs. Bubble size represents the number of proteins mapped to each term. Functional enrichment analysis was performed using the STRING database (<https://string-db.org/>).

Functional enrichment analysis of DEPs in F1.P2

downregulated proteins

up-regulated proteins

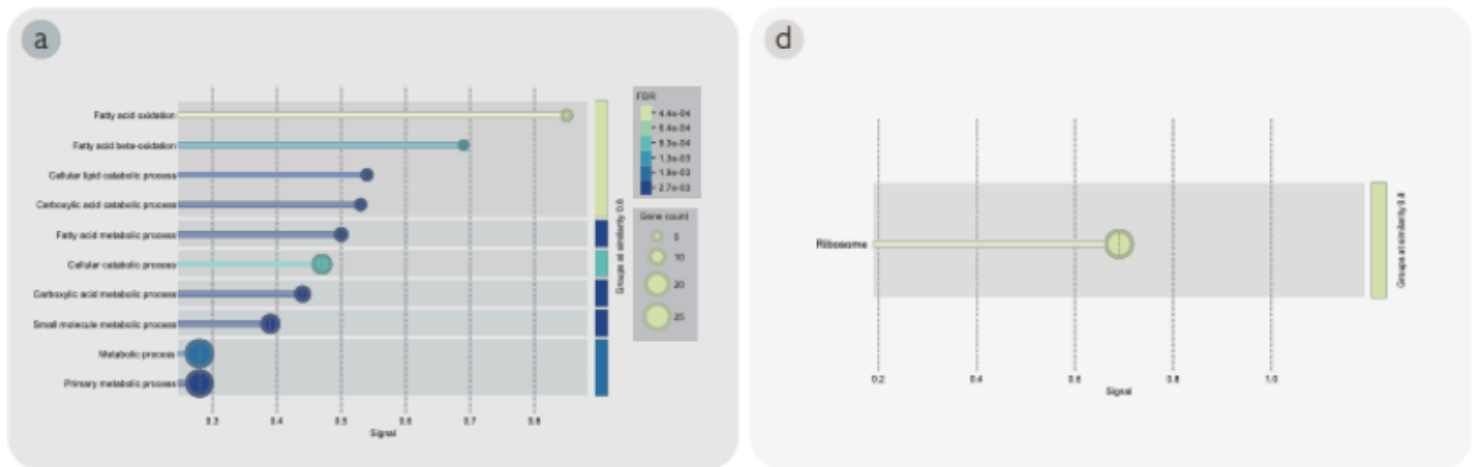


Figure 5

Functional enrichment analysis of differentially expressed proteins in F1.P2 ovaries following BP3 exposure. a) Enriched functional categories identified among down-regulated DEPs. Bubble size represents the number of proteins mapped to each term, whereas color indicates the false discovery rate (FDR). b) Enriched functional categories identified among up-regulated DEPs. Bubble size represents the number of proteins mapped to each term. Functional enrichment analysis was performed using the STRING database (<https://string-db.org/>).

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