Received: 12 July 2021 | Revised: 5 October 2021 | Accepted: 25 October 2021

DOI: 10.1096/fj.202101131R

THE FASEB JOURNAL

RESEARCH ARTICLE

Maternal background alters the penetrance of growth phenotypes and sex-specific placental adaptation of offspring sired by alcohol-exposed males

Kara N. Thomas | **Katherine N. Zimmel** | **Alexis N. Roach** | **Alison Basel** | **Nicole A. Mehta** | **Yudhishtar S. Bedi** | **Michael C. Golding**

Department of Veterinary Physiology & Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas, USA

Correspondence
Michael C. Golding, Department of Veterinary Physiology & Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843-4466, USA
Email: mgolding@cvm.tamu.edu

Funding information
HHS | NIH | National Institute on Alcohol Abuse and Alcoholism (NIAAA), Grant/Award Number: R01AA028219; W. M. Keck Foundation (W.M. Keck Foundation)

Abstract

Epigenetic mechanisms of paternal inheritance are an emerging area of interest in our efforts to understand fetal alcohol spectrum disorders. In rodent models examining maternal alcohol exposures, different maternal genetic backgrounds protect or sensitize offspring to alcohol-induced teratogenesis. However, whether maternal background can mitigate sperm-inherited alterations in developmental programming and modify the penetrance of growth defects induced by preconception paternal alcohol exposures remains unaddressed. In our previous studies examining pure C57Bl/6J crosses, the offspring of alcohol-exposed sires exhibited fetal growth restriction, enlarged placentas, and decreased placental efficiency. Here, we find that in contrast to our previous studies, the F1 offspring of alcohol-exposed C57Bl/6J sires and CD-1 dams do not exhibit fetal growth restriction, with male fetuses developing smaller placentas and increased placental efficiencies. However, in these hybrid offspring, preconception paternal alcohol exposure induces sex-specific changes in placental morphology. Specifically, the female offspring of alcohol-exposed sires displayed structural changes in the junctional and labyrinth zones, along with increased placental glycogen content. These changes in placental organization are accompanied by female-specific alterations in the expression of imprinted genes *Cdkn1c* and *H19*. Although male placentae do not display overt changes in placental histology, using RNA-sequencing, we identified programmed alterations in genes regulating oxidative phosphorylation, mitochondrial function, and Sirtuin signaling. Collectively, our data reveal that preconception paternal

Abbreviations: Ascl2, achaete-scute family BHLH transcription factor 2; Atp5e, ATP synthase F1 subunit epsilon; Atp5l, ATP synthase membrane subunit G; Atp5mf, ATP synthase membrane subunit F; Cdkn1c, cyclin-dependent kinase inhibitor 1C; Cox7b, cytochrome C oxidase subunit 7B; EIF2, eukaryotic translation initiation factor 2A; FASDs, fetal alcohol spectrum disorder; H19, hepatic transcript 19; microCT, microfocus computed, X-ray tomography; Mt-Cytb, mitochondrially encoded cytochrome B; Mt-ND5, mitochondrially encoded NADH:Ubiquinone oxidoreductase core subunit 5; Ndufa7, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7; PEG3, paternally expressed gene 3; Pgk1, phosphoglycerate kinase 1; Ppia, peptidylprolyl isomerase A; Slc22a18, solute carrier family 22 member 18; Slc38a2, solute carrier family 38 member 2; Slc3a2, solute carrier family 3 member 2; Tpbpa, trophoblast specific protein alpha; Untr6, untranscribed region of chromosome 6; Ywaz, monooxygenase/tryptophan 5-monooxygenase activation protein zeta; Zfy1, zinc finger protein Y-linked.

alcohol exposure transmits a stressor to developing offspring, that males and females exhibit distinct patterns of placental adaptation, and that maternal genetic background can modulate the effects of paternal alcohol exposure.

KEYWORDS

developmental programming, fetal alcohol spectrum disorder (FASDs), genetic background, mitochondrial disfunction, oxidative phosphorylation, paternal epigenetic inheritance, placental adaptation, placental dysfunction, preconception exposure, RRID:IMSR_CRL:22, RRID:IMSR_JAX:000664, RRID:SCR_002798

1 | INTRODUCTION

The recent discovery of non-genetic mechanisms of paternal inheritance has exposed a significant gap in our understanding of the developmental origins of disease. Work across various species, including worms, flies, mice, and humans, reveals that sperm harbor epigenetic information that exerts a powerful influence on offspring growth and development.[1,2] These studies now prompt us to reexamine the exclusive role of acute maternal environmental exposures in teratogenesis and more thoroughly consider the influence of preconception paternal lifestyle choices and exposure history.[3]

Despite the exclusive association of fetal alcohol spectrum disorders (FASDs) with maternal drinking, emerging research now indicates that paternal alcohol use is a relevant factor in this debilitating condition. Preclinical animal studies from multiple laboratories demonstrate that male alcohol exposures before conception associate with numerous behavioral, growth, metabolic, and physiologic effects in the offspring.[4] Furthermore, these data suggest that male alcohol use prior to conception is a significant modifier of the teratogenic potential of alcohol and a contributing factor to the enormous variation observed in FASD phenotypes and incidence.[5]

Clinical studies demonstrate that maternal genetic factors substantially modify offspring susceptibility to or resistance against FASD phenotypes. For example, maternal genetic vulnerabilities associated with alcohol metabolism and thyroid function significantly modify the penetrance and severity of FASD phenotypes.[6,7] In further support of the importance of maternal genetic factors in determining offspring susceptibility, reciprocal crosses in mice reveal pregnancies carried in C57BL/6J strain dams are highly susceptible to alcohol-induced teratogenesis, while in contrast, F1 offspring carried in other strains are resistant.[8–11] Collectively, these studies indicate that maternal genetics and the uterine environment have a critical influence on the severities of FASD growth and morphological phenotypes. However, no studies have yet examined the ability of maternal genetic background or the uterine environment to modify the penetrance of paternal epimutations influencing fetal growth and development.

Emerging evidence indicates that altered maternal-placental-fetal exchange and signaling mediate the growth and metabolic phenotypes associated with maternal and paternal preconception alcohol exposures.[12,13] However, these studies utilized inbred strains of mice and rats. As humans are genetically diverse, an important consideration is whether differences in maternal genetics may sensitize or predispose offspring to altered paternal epigenetic programming. Indeed, mouse studies examining the development of numerous genetic mutants reveal that maternal genetic background significantly modifies the penetrance of both growth restriction and embryonic lethal phenotypes.[14–18] Significantly, these differing sensitivities associate with strain-specific differences in the histological organization of the placenta (reviewed in Ref. [19]). Therefore, we sought to determine whether an altered maternal genetic background protects or sensitizes offspring to alcohol-induced alterations in the paternally inherited epigenetic program.

CD-1 mice are an outbred strain exhibiting high fecundity, used to study normal pregnancy and fetal development. In contrast, C57Bl/6 mice are a commonly used inbred strain with mediocre fertility, used as the background strain for transgenic and knockout mouse models.[20,21] Previously, we identified late-term fetal growth restriction in the offspring of alcohol-exposed males, which correlated with placental overgrowth and a significant decline in placental efficiency.[12,22,23] In these studies, we employed C57Bl/6J mice due to their consistent levels of ethanol consumption using the Drinking In The Dark model of exposure and behavioral evidence of intoxication.[24] Compared to the C57Bl/6J strain, CD-1 mice exhibit higher fecundity, enhanced growth rates in late gestation, increased litter sizes but similar placental efficiencies.[20] Therefore, we sought to determine if the more robust CD-1 maternal background would modify

the growth and placental abnormalities observed in pure C57Bl/6J crosses. We hypothesized that similar to studies examining maternal genetic vulnerabilities to intrauterine alcohol exposures, the genetic background of the dam would modify the penetrance of growth defects induced by alcohol-mediated errors in the paternally inherited epigenetic program. Here, we find that the fetuses sired by alcohol-exposed, C57Bl/6J males bred to CD-1 dams do not display the previously described growth restriction phenotypes. Surprisingly, male fetuses exhibit smaller placentas, while the female offspring display significant alterations in the architecture of the placental junctional zone, increased amounts of placental glycogen, and altered imprinted gene expression. These data reveal that preconception paternal alcohol exposure transmits a stressor to their progeny and that maternal genetic background can modulate this effect. However, sex-specific differences in placental adaptation ultimately determine how the phenotype manifests.

2 | MATERIALS AND METHODS

2.1 | Ethics

All experiments were conducted under AUP 2017-0308 and approved by the Texas A&M University IACUC.

2.2 | Animal husbandry and preconception male alcohol exposure

This study utilized two strains of mice. Males were of a C57BL/6J background (RRID:IMSR_JAX:000664), while females were CD-1 (RRID:IMSR_CRL:22). We obtained mice from the Texas A&M Institute for Genomic Medicine, where they were housed using a 12-h light/dark cycle. We maintained mice on a standard diet (catalog# 2019, Teklad Diets, Madison WI, USA). Stress is a confounding variable known to influence the male inherited epigenetic program.[25] Therefore, we implemented additional animal husbandry measures to improve animal welfare and reduce stress through enhanced shelter and environmental enrichment.[26,27] Specifically, we implemented colored tunnels for males and igloos for females (catalog# K3322 and catalog# K3570, Bio-Serv, Flemington NJ, USA).

On postnatal day 83, we began acclimating adult males to individual cages. Beginning on postnatal day 90, one week later, we exposed males to either the control (water) or 10% (w/v) ethanol (catalog# E7023; Millipore-Sigma, St. Louis, MO, USA) preconception treatments using the Drinking in the Dark limited access model of voluntary exposure.[24] We implemented the preconception treatments beginning one hour after the initiation of the dark cycle and ceased treatments after four hours of exposure. To ensure identical handling conditions for control males, we switched between two water bags. At the conclusion of each week, we recorded the weight of each mouse (g) and the amount of fluid consumed (g) and then calculated weekly fluid consumption as grams of fluid consumed per gram body weight.

After six weeks of treatment, we began breeding exposed males to naïve dams and continued the preconception treatments over the four-week period required to generate the offspring. We synchronized female reproductive cycles using the Whitten method[28] and placed a single naturally cycling female into the home cage of the treated male. During breedings, we did not give the males access to the preconception treatments. After six hours, we confirmed matings by the presence of a vaginal plug and returned the female mice to their original cages. We rested males for 72 h, during which we resumed the preconception exposures and then used them again in a subsequent mating. We repeated this procedure until we obtained the requisite number of pregnancies. To validate our preconception treatment, we bred exposed males to C57BL/6J dams to generate pure B6 offspring, which we used to assay alcohol-induced changes in placental efficiency, as described previously.[22] During this time, we also bred exposed males to CD-1 dams to generate F1 hybrid offspring.

We subjected pregnant dams to minimal handling until gestational day 10.5, when we calculated the change in dam bodyweight between gestational day 0 and 10. We used a bodyweight gain of approximately 1.8 g as confirmation of pregnancy.[29] On gestational day 16.5, we euthanized pregnant dams by CO_2 asphyxiation and cervical dislocation. We then dissected the female reproductive tract, excised the gestational sac, and weighed fetal-placental tissues. After assessing physiological measures, we either froze collected tissues on dry ice and stored them at −80°C or fixed them using 10% neutral buffered formalin (catalog# 16004-128, VWR, Radnor, PA, USA).

2.3 | Determination of plasma alcohol levels

We isolated plasma from exposed males after two-weeks of treatment and at the end of the four-hour exposure window. We measured male plasma alcohol concentrations using an Ethanol Assay Kit (catalog# ECET100; BioAssay Systems, Hayward, CA, USA) according to the manufacturer's protocol.

2.4 | Determination of fetal sex

During fetal dissections, we removed the tail and isolated genomic DNA using the HotSHOT protocol.[30] Using the GoTaq Green 2× Mastermix (catalog# M7128, Promega, Madison, WI, USA), we used PCR to amplify the region of the Y-chromosome encoding *zinc finger protein Y-linked* (*Zfy*), or as a positive control, an untranscribed region of chromosome 6 (Untr6), which provides a measure of genomic background in ChIP assays examining transcription factor binding.[31] See Table 1 for a list of primer sequences.

2.5 | RNA isolation and sequencing

We isolated total RNA from placentae using the RNeasy Plus Mini Kit (catalog# 74134, Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. We then randomized samples and used the Illumina mouse TruSeq Stranded Total RNA kit (catalog# RS-122-2202, Illumina, San Diego, CA, USA) to generate sequencing libraries, following the recommended protocol. Finally, we sequenced pooled libraries on an Illumina NextSeq 500 within the Texas A&M Institute for Genome Sciences and Society Experimental Genomics Core.

2.6 | RNA isolation and RT-qPCR analysis of gene expression

We isolated total RNA from gestational day 16.5 placentae using the Qiagen AllPrep Kit (catalog# 80004; Qiagen, Germantown MD, USA) according to the manufacturer's instructions. We assessed RNA purity and concentration using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). We then seeded approximately 1 ug of isolated RNA into a reverse transcription reaction using the High-Capacity cDNA Reverse Transcription Kit (catalog# 4368814; Thermo-Fisher, Waltham, MA, USA). We brought the reaction mixture to 25°C for 10 min, 37°C for 120 min, and then 70°C for 5 min. Next, we determined relative levels of candidate gene transcripts using the Dynamo Flash SYBR qPCR kit (catalog# F-415XL; Thermo-Fisher, Waltham, MA, USA) according to the recommended protocol. Reactions were performed on a Bio-Rad CFX384.

TABLE 1 Sequence of the RT-qPCR primers used in this study

Determination of fetal sex		
Zfy	AAGATAAGCTTACATAATCACATGGA	CCTATGAAATCCTTTGCTGCACATGT
Untr6	CAGGCATGAACCACCATACC	CAACATCCACACGTCCAGTG
RT-qPCR analysis		
Reference genes		
Pgk1	CTGACTTTGGACAAGCTGGACG	GCAGCCTTGATCCTTTGGTTG
Ywhaz	TTGATCCCCAATGCTTCGC	CAGCAACCTCGGCCAAGTAA
Ppia	AGATGCCAGGACCTGTATGCTT	TGTGCCAGGGTGGTGACTTTA
Candidate genes		
Slc22a18	TGATGTCCAGTGTGCTCCAT	AGAGTTCGGGTCAATGGTTG
Slc2a3	GATCGGCTCTTTCCAGTTTG	CAATCATGCCACCAACAGAG
Slc3a2	TGATGAATGCACCCTTGTACTTG	GCTCCCCAGTGAAAGTGGA
Slc38a2	ACTCATACCCCACCAAGCAG	CACAATCGCATTGCTCAGAT
Slc38a4	TGATTGGGATGTTAGTCTGAGG	GGCCTGGGTTAAAATGTGTG
Tpbpa	TGAAGAGCTGAACCACTGGA	CTTGCAGTTCAGCATCCAAC
Cdkn1c	AACGTCTGAGATGAGTTAGTTTAGAGG	AAGCCCAGAGTTCTTCCATCGT
H19 Fwd	TGATGGAGAGGACAGAAGGGC	CTTGATTCAGAACGAGACGGACT
mPeg3 Rev	TTCTCCTTGGTCTCSCGGGC	AAGGCTCTGGTTGACAGTCGTG
Ascl2	TGCCGCACCAGAACTCGTAG	GCCTCGGTTGCTCCAGATC
Mt-ND5	CCTGGCAGACGAACAAGACAT	GGCGAGGCTTCCGATTACTA
Mt-Cytb	CAATCGTTCACCTCCTCTTCCT	GAGCGTAGAATGGCGTATGC
Ndufb10	ATGCCAAGAACCGAACCTACTA	CTCAGCCTCATAGATACACAGAACA
Mt-Co1	CAATAGTAGAAGCAGGAGCAGGAA	GTTTAGGTTGCGGTCTGTTAGTAGT
Mt-Nd1	ATTCTAATCGCCATAGCCTTCCT	TGGGTGTGGTATTGGTAGGGG

Procedures for normalization and data handling are described below; primer sequences are listed in Table 1.

2.7 | Placental histology and glycogen assay

Microfocus computed, X-ray tomography (micro-CT) allows 3D quantification of whole tissue samples with high contrast and resolution, without sample destruction. Multiple laboratories have employed contrast-enhanced micro-CT to characterize the growth and morphology of early embryonic development in mice.[32,33] In these previous studies, the authors could discriminate specific morphological features of the conceptus, including the different placental layers.[32] Using phosphotungstic acid to enhance tissue contrast, we employed the recent refinements described by Lesciotto et al.[34] to identify changes in placental morphology between the control and alcohol preconception treatments.

Briefly, we incubated half placentae in 5% phosphotungstic acid (w/v) dissolved in 90% methanol for four hours and then held samples in 90% methanol overnight. Subsequently, we incubated samples in progressive reductions of methanol (80%, 70%, 50%) each for one day and then moved placentae into PBS with 0.01% sodium azide for long-term storage. Finally, we imaged samples on a SCANCO vivaCT 40 (SCANCO Medical AG Brüttisellen, Switzerland) using a 55 kVp voltage x-ray tube and 29 uA exposure. The resulting microCT image voxel size was 0.0105 mm^3, with a resolution of 95.2381 pixels/mm.

After scanning, we used the open-source medical image analysis software Horos (Version 3.3.6; Nibble Co LLC, Annapolis, Maryland, USA; https://horosproject.org/) to measure placental features. In the 2D-viewer feature, we scrolled through slices to a medial view immediately around the maternal canal. Using sequential digital slices, we then measured area and volume in or across slices to a depth of ~50 microns. After samples had been imaged, we processed placentae for histological sectioning and staining using the periodic acid Schiff stain (catalog# ab150680; Abcam, Cambridge, MA, USA). We captured images using an Olympus VS120 system. To measure placental glycogen, we used the Glycogen Assay Kit (catalog# ab65620; Abcam, Cambridge, MA, USA).

2.8 | Informatic analysis

We performed quality control using MultiQC[35] on the raw paired-end, total RNA sequence files and trimmed Illumina adapters with Trimmomatic[36] using the open-source, web-based Galaxy server[37] (usegalaxy.org). Next, we used RNA STAR[38] to map the reads to the *Mus musculus* reference genome (UCSC version GRCm39/mm39). We determined read abundance for all genes followed by annotation versus M27 GTF (GENCODE, 2020) through the featureCounts function[39] with a minimum mapping quality per read of 10. Next, we used the DEseq2 function[40] with default parameters on the featureCounts files to generate a PCA plot. We then used the Volcano Plot function on DEseq2 results to produce a graphical representation of the log2FC gene expression levels for the top 25 significant genes. We exported differentially expressed genes into the Ingenuity Pathway Analysis software package[41] and conducted gene enrichment analysis. The sequence data can be obtained in the GEO database under accession number GSE179973.

2.9 | Statistical analysis

To analyze offspring physiologic data, we collated the measures for each fetus in Excel and derived the male and female average values for each litter. To calculate placental efficiency,[42] we divided fetal weight by placental weight, then derived the male and female average for each litter. We obtained measures of placental volume using Horos, and placental glycogen levels using a Tecan Infinite 200 Pro M-Plex plate reader, then collated these data in Excel. We then imported data into the statistical analysis program GraphPad Prism 8 (RRID:SCR_002798; GraphPad Software, Inc., La Jolla, CA, USA). We first identified outliers using the ROUT test ($Q = 1\%$) and then verified the normality of the datasets using the Shapiro–Wilk test. If data passed normality (alpha = .05), we then employed either an ANOVA, followed by a Sidak multiple comparisons test, or an unpaired (two-tailed) *t*-test. If the data failed the test for normality, we then used an unpaired, non-parametric Mann–Whitney test. For RT-qPCR analysis of gene expression, we imported the replicate cycle threshold (Ct) values for each transcript into Excel and normalized expression to the geometric mean of two validated reference genes.[43] These included transcripts encoding *Phosphoglycerate kinase 1* (*Pgk1*) and *3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta* (*Ywhaz*). We then used the -ΔΔCT method[44] to calculate the relative fold change for each biological replicate. After collating datasets in Excel, we input the calculated values into the statistical analysis program GraphPad Prism 8 and set the statistical significance set at alpha = .05. Next, we identified and excluded outliers using the ROUT outlier test. We then verified all datasets for normality using the Shapiro–Wilk test. We then conducted a non-parametric unpaired *t*-test or Mann–Whitney test. Data presented are mean +/− standard error of the mean.

3 | RESULTS

3.1 | CD-1 maternal background modifies alcohol-induced errors in paternal programming

Using the Drinking in the Dark model, we exposed postnatal day 90 C57BL/6J males to either water or 10% ethanol for ten weeks. We then utilized these exposed males to generate pure C57Bl/6J and hybrid CD-1 (maternal) × C57Bl/6J (paternal) offspring (Figure 1A). During the preconception exposure period, we did not observe any significant differences in the weekly weight gain between the alcohol and control treatment groups (Figure 1B). Using Sidak's multiple comparison test, we did not identify any differences in weekly fluid consumption between the control and alcohol treatment groups (Figure 1C). At the end of the four-hour exposure window, alcohol-exposed males exhibited average plasma alcohol concentrations of 50 mg/dl (Figure 1D). After six weeks of treatment, we mated exposed males to naive C57Bl/6J or CD-1 females of similar age. We only used CD-1 females smaller than 35 g, the approximate weight of C57Bl/6J males after ten weeks of treatment. Consistent with our previous studies examining pure C57Bl/6J crosses,[23] we did not observe any differences in CD-1 maternal body weight relative to litter size (Figure 1E; gestational day 16.5 shown) or the litter size (Figure 1F) between the alcohol and control preconception treatments.

Using a two-way ANOVA, we first contrasted patterns in fetal-placental growth between male and female conceptuses. For pure C57Bl/6J and hybrid CD-1xC57Bl/6J offspring, we identified sex differences, with male conceptuses exhibiting increased gestational sac weights, fetal weights, and crown-rump lengths compared to females (C57Bl/6J data not shown, hybrid CD-1xC57Bl/6J Figure 2A–C). In contrast, although we identified sex differences in pure C57Bl/6J crosses for all placental measures, hybrid CD-1xC57Bl/6J offspring did not exhibit sex differences for placental diameter or placental efficiencies (Figure 2D–F).

Our previous studies examining pure C57Bl/6J crosses identified growth restriction in both male and female fetuses sired by alcohol-exposed males. In contrast, using an ANOVA, we did not observe any impact of preconception treatment on hybrid CD-1xC57Bl/6J gestational sac weights, fetal weights, or crown-rump lengths (Figure 2A–C). Consistent with our previous publications, pure C57Bl/6J male offspring sired by alcohol-exposed males exhibited increased placental weights (Figure 2D) and decreased placental efficiency (Figure 2F). In contrast, when bred onto a CD-1 maternal background, the male progeny of alcohol-exposed C57Bl/6J sires displayed decreased placental weights (−7%, $p < .05$; Figure 2D), reduced placental diameter (−5%, $p < .01$; Figure 2E), and a general increase in placental efficiency ($p = .088$; Figure 2F). Female fetuses did not exhibit significant changes in placental growth or efficiency (Figure 2D–F). Collectively, these data reveal that maternal genetic background can significantly modify phenotypic outcomes arising from sperm-inherited alterations in epigenetic programming.

3.2 | Paternal alcohol exposure induces sex-specific changes in placental morphology

Given the reductions in placental weight and diameter observed in the CD-1xC57Bl/6J male offspring of alcohol-exposed sires, we focused on characterizing placental histology between the preconception treatment groups. The murine placenta consists of four distinguishable layers: the chorion, labyrinth, junctional zone, and the maternal decidua.[45] Micro-CT allows three-dimensional quantification of whole tissue samples with high contrast and high resolution, including discrimination of the different placental layers.[32] Using phosphotungstic acid to enhance tissue contrast, we employed the methods described by Lesciotto et al.[34] to measure each of the placental layers between the control and alcohol preconception treatments.

We first compared placental histology between pure C57BL/6J, pure CD-1, and hybrid CD-1xC57BL/6J offspring. Pure C57BL/6J placentae exhibit a reduced junctional zone thickness compared to those derived from pure CD-1 fetuses (Figure 3A). Overall, the placental histology of F1 hybrid CD-1xC57BL/6J offspring appeared more similar to the maternal CD-1 strain (Figure 3A). Using these interstrain differences, we confirmed the ability of enhanced tissue contrast micro-CT imaging to identify discrepancies in placental morphology (Figure 3B). Using this technique, we next examined hybrid CD-1xC57BL/6J placentae to identify changes in histological organization induced by preconception alcohol exposure.

Using micro-CT, we determined the median volume for each placental layer (Figure 3C) and derived the proportion of each layer to the total volume. Although placentae derived from male fetuses of alcohol-exposed sires displayed an overall reduction in both weight and diameter, we only observed a proportional reduction in the volume of the chorion (Figure 3D). In contrast, placentae derived from the female offspring of alcohol-exposed sires displayed decreases in the volume of the chorion and junctional zone ($p < .05$), an increase in the volume of the labyrinth zone, and a modest ($p = .07$) increase in the volume of the decidua (Figure 3D–G). We observed a reduced ratio of the junctional zone relative to the decidua and an increased Labyrinth to Junctional zone ratio in female but not male placentae (Figure 3H–I). These

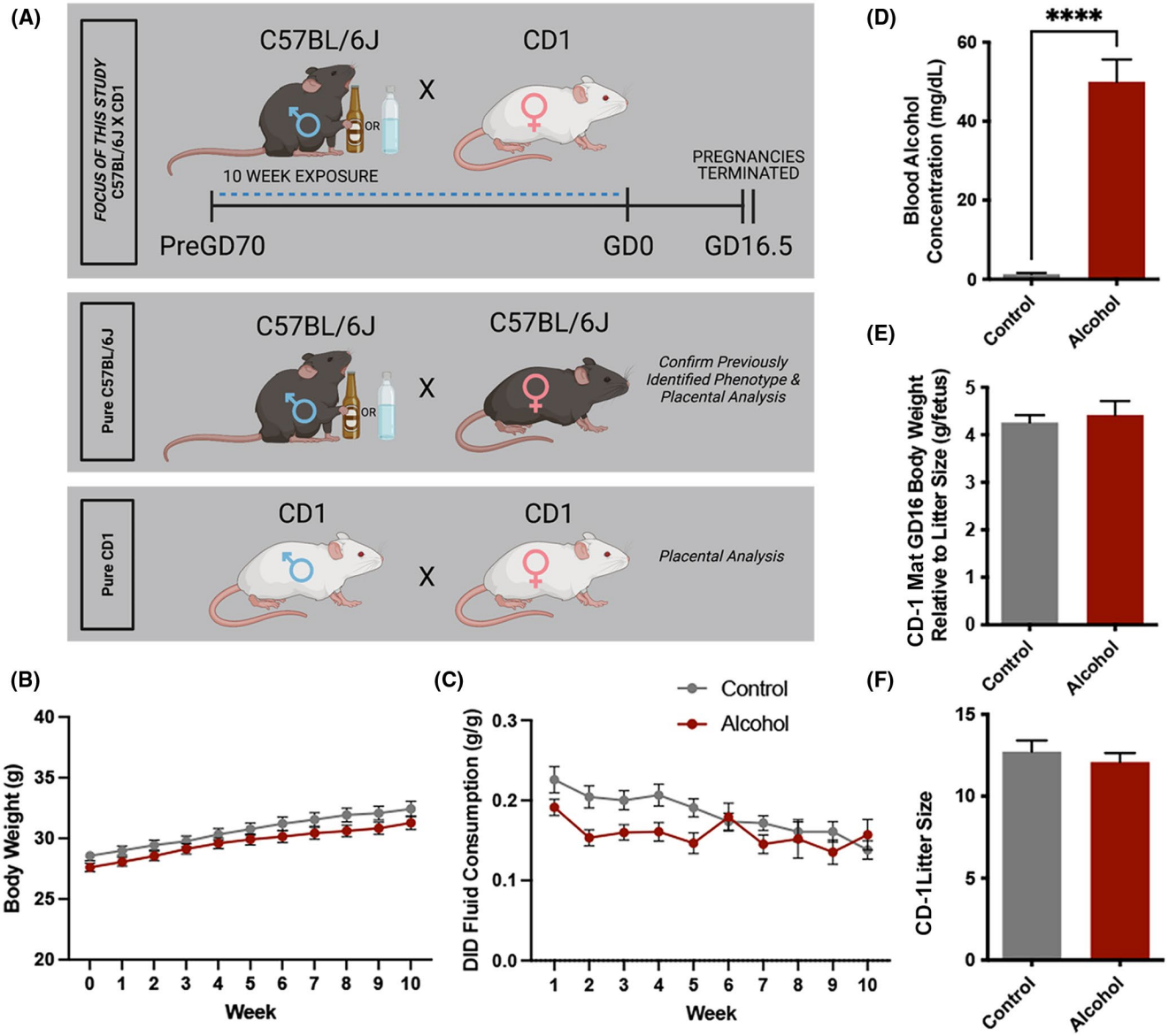


FIGURE 1 Mouse model of preconception paternal alcohol exposure. (A) Graphic representation of the experimental paradigm. Postnatal day 90, C57BL/6J males are exposed to 10% ethanol for 10 weeks (Pre-Gestational Day [PGD] 70), then bred to either naïve CD-1 (top) or naïve C57BL/6J dams (middle). We generated untreated, pure CD-1 offspring for inter-strain comparisons of placental histology (bottom). Image generated using BioRender.com (B) Weekly paternal weights of C57BL/6J males over the 10-week exposure period ($n = 41$ control, 40 alcohol-exposed males). (C) Weekly fluid consumption compared between the 10% ethanol and control (water) preconception treatments. (D) Plasma alcohol levels of alcohol-exposed males measured at the end of the four-hour exposure window ($n = 6$). (E) CD-1 maternal weight normalized to litter size, measured on gestational day 16.5. ($n = 9$ control, 8 alcohol). (F) Litter size of hybrid CD-1 (maternal) × C57BL/6J (paternal) crosses compared between the preconception treatment groups. We used either a two-way ANOVA or a *t*-test to identify differences between treatment groups. Error bars represent the standard error of the mean, ****$p < .0001$

observations indicate that the female offspring of alcohol-exposed sires display broad alterations in placental histology. Importantly, when we contrasted volumetric measures to traditional measures examining the area of individual digital slices, we were only able to identify differences in the junctional zone (data not shown). Collectively, these observations reveal that volumetric measures using enhanced tissue contrast micro-CT imaging is more sensitive in identifying differences in placental morphology.

3.3 | Female-specific changes in placental glycogen content between the offspring of control and alcohol-exposed sires

Using micro CT, we observed a decrease in the proportional volume of the junctional zone in the female offspring of alcohol-exposed sires. This reduction may be caused by an increase in glycogen cells, which are much larger and have a

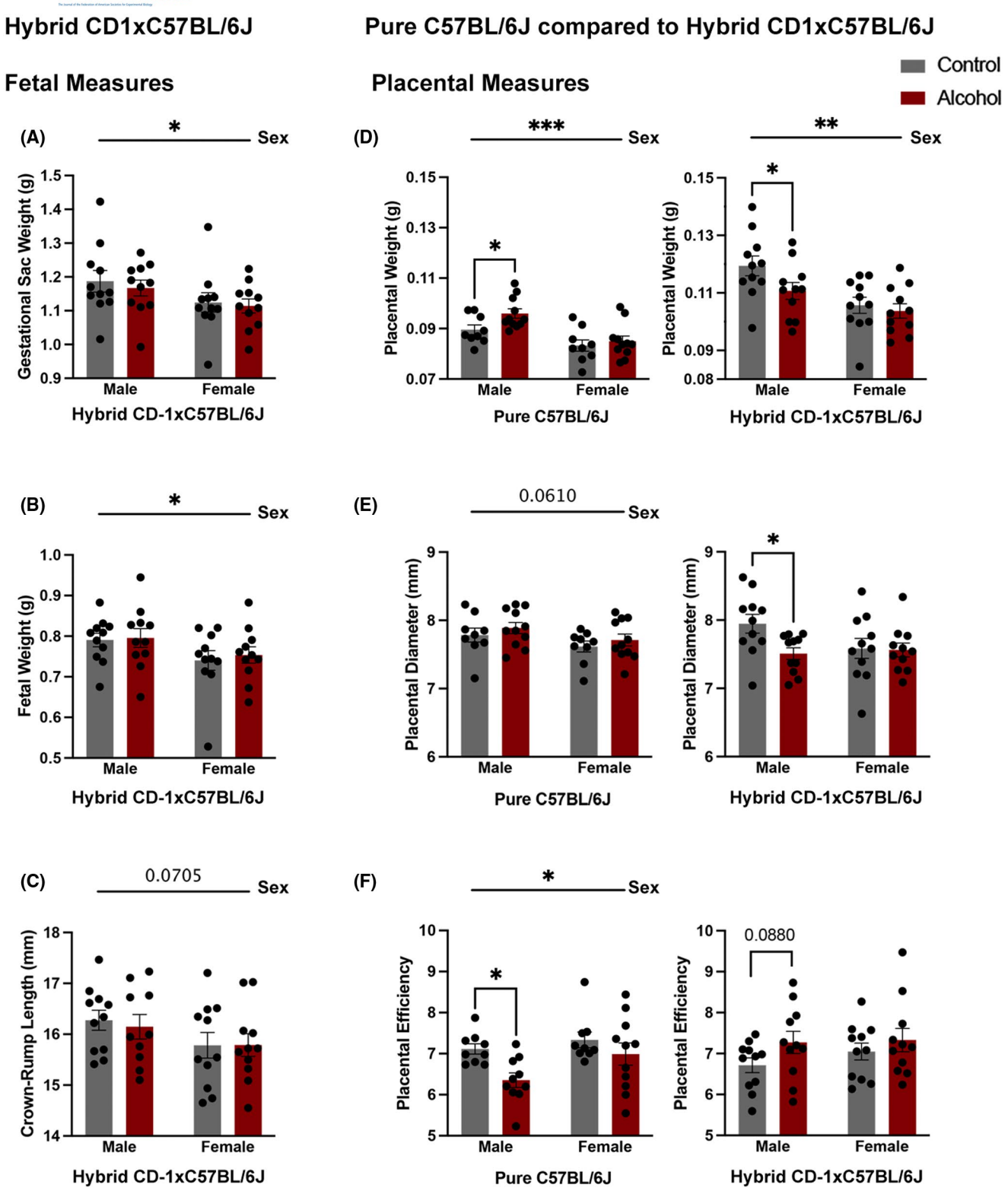


FIGURE 2 Influence of Maternal background on placental growth phenotypes induced by preconception paternal alcohol exposure. Comparison of (A) gestational sac weight, (B) fetal weight, and (C) crown-rump length between hybrid CD-1xC57BL/6J litters sired by alcohol-exposed and control males. Comparison of (D) placental weight, (E) placental diameter, and (F) placental efficiency in pure C57BL/6J and hybrid CD-1xC57BL/6J offspring sired by control and alcohol-exposed males. We used a two-way ANOVA to contrast the impacts of sex and preconception treatments, with sex differences indicated above the figures while treatment effects are demarcated directly above the bar graphs. Pure C57BL/6J offspring were derived from 9 control and 11 alcohol litters, while we derived hybrid CD-1xC57BL/6J offspring from 11 control and 11 alcohol litters. Error bars represent the standard error of the mean, $*p < .05$, $**p < .01$, $***p < .001$

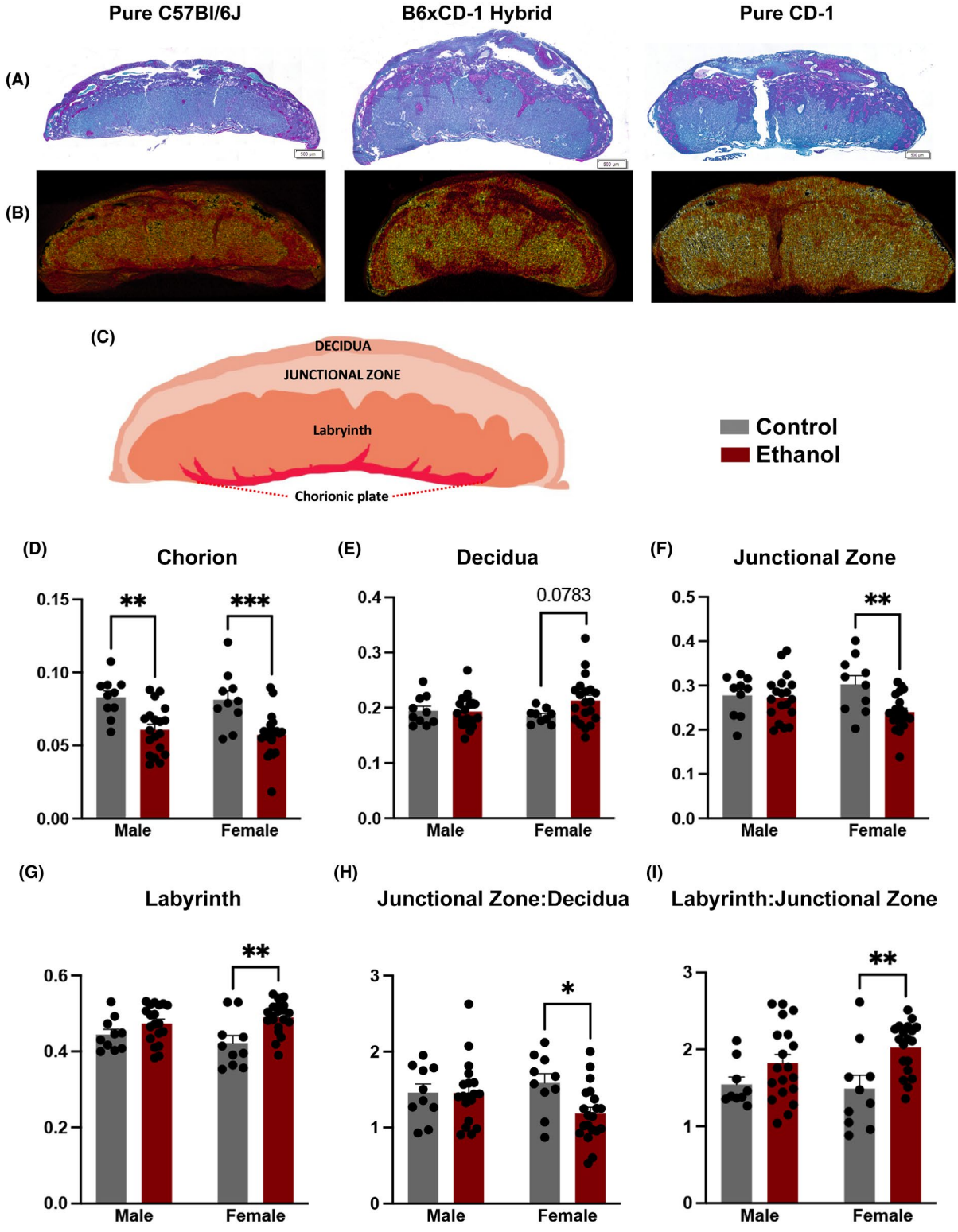


FIGURE 3 Preconception paternal alcohol exposure induces sex-specific changes in placental architecture. (A) Comparison of placental histology between pure C57BL/6J (left), pure CD-1 (right), and hybrid CD-1xC57BL/6J (middle) offspring. (B) MicroCT imaging of placentae derived from pure C57BL/6J, pure CD-1, and hybrid CD-1xC57BL/6J offspring. (C) Schematic diagram depicting the layers of the murine placenta. Volumetric analysis of the placental layers in hybrid CD-1xC57BL/6J offspring sired by control and alcohol-exposed males. Volumes for the (D) chorion, (E) decidua, (F) junctional zone, and (G) labyrinth are expressed as a ratio of the total placental volume (male $n = 10$ control, 19 alcohol; female $n = 10$ control, 20 alcohol, randomly selected across 5 different litters). Ratios comparing the proportional volumes of the (H) junctional zone to decidua and (I) labyrinth to junctional zone between offspring sired by control and ethanol-exposed males. We used a two-way ANOVA to contrast differences between sex and the preconception treatment groups. Error bars represent the standard error of the mean, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

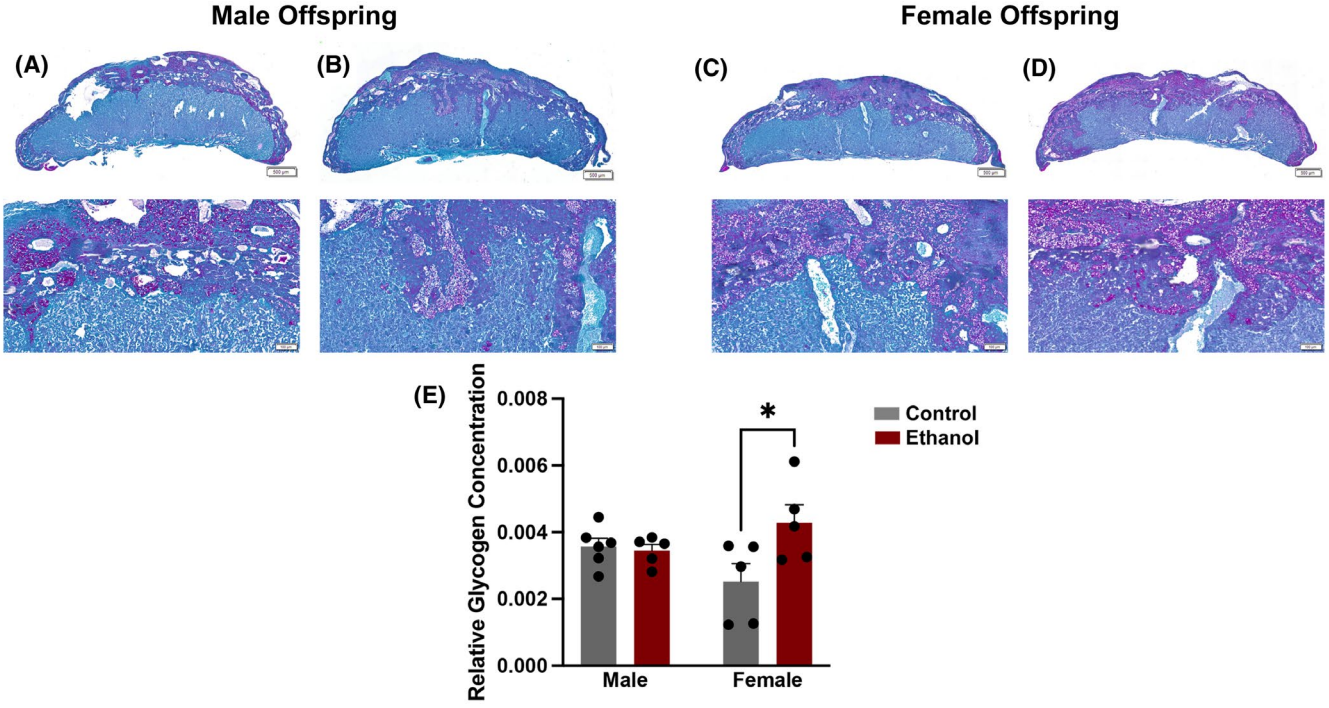


FIGURE 4 Sex-specific increases in placental glycogen in the offspring of alcohol-exposed males. We used the periodic acid Schiff stain to contrast placental histology between the offspring of control (A, C) and alcohol-exposed (B, D) sires. (E) Colorimetric quantification of placental glycogen between male and female offspring of control and ethanol-exposed sires ($n = 5$–6 placenta per treatment). We used a two-way ANOVA to contrast differences between sex and the preconception treatment groups. Error bars represent the standard error of the mean, $^*p < .05$

lower nuclei density than surrounding cell types. This lower nuclei density could induce hypoattenuated areas (negative space) in the microCT image. Therefore, we conducted a standard histological examination, staining sections with the periodic acid Schiff reagent. Here, we observed an increase in glycogen staining in the female offspring of alcohol-exposed sires but not in males (Figure 4A–D). Using an enzyme-based colorimetric assay, and a two-way ANOVA, we confirmed the increased placental glycogen content of only the female offspring of alcohol-exposed sires (Figure 4E). We did not observe any differences in placental glycogen content between males and females.

3.4 | Female-specific alterations in the expression of imprinted genes and placental nutrient transporters

Alterations in imprinted gene expression modulate placental endocrine signaling by controlling the size of the glycogen cell containing junctional zone.[46,47] Therefore, we assayed the expression of select imprinted genes associated with overt changes in placental histoarchitecture. We did not detect any altered imprinted gene expression in male placentae (Figure 5A). In contrast, female placentae exhibited significant reductions in the maternally expressed genes *Cdkn1c*, *H19*, and a modest decrease in *Slc22a18* (Figure 5C). In contrast to these candidates, *Ascl2*, also located within the *Kcnq1ot1* imprinted gene cluster, was not differentially expressed. Expression of the paternally expressed gene *Peg3* was not different between the preconception treatment groups. These data reveal that the expression of these candidate imprinted genes is selectively perturbed in the female offspring of alcohol-exposed sires.

We next assayed the expression of growth and nutrient supply genes known to function downstream of imprinted genes,[46,48,49] including system A family amino acid transporters *Slc3a2* and *Slc38a2*, as well as *Trophoblast specific protein alpha* (*Tpbpa*). Again, male placentae did not display altered expression of any candidate genes (Figure 5B). In contrast, females exhibited upregulation of *Slc3a2* and downregulation of *Slc38a2* (Figure 5D). We did not observe differences in *Tpbpa* expression in either male or female placentae.

3.5 | Changes in placental gene expression induced by paternal preconception alcohol exposure

To better understand the basis of the observed growth defects impacting male placentae, we isolated RNA and

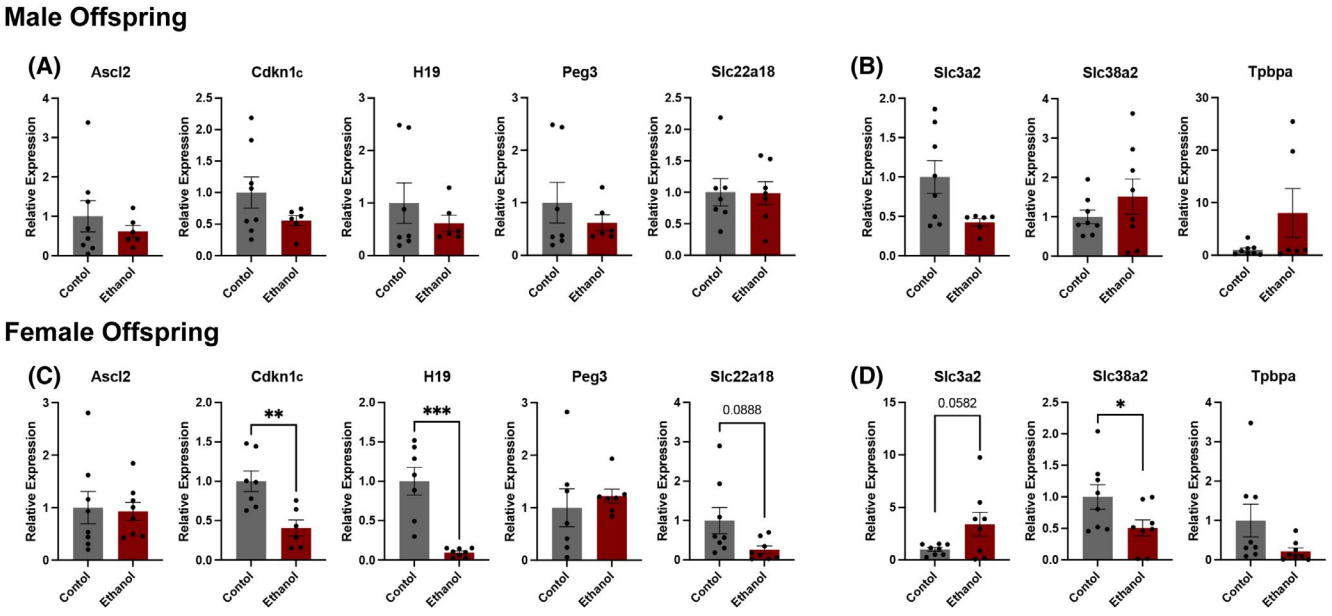


FIGURE 5 Preconception paternal alcohol exposure induces sex-specific changes in imprinted gene expression. Analysis of imprinted gene expression in the (A) male and (C) female offspring of control and alcohol-exposed sires. Expression analysis of key nutrient transporters between (B) male and (D) female offspring sired by control and ethanol-exposed males. Analysis of gene expression carried out using RT-qPCR. Gene expression was normalized to transcripts encoding *Pgk1* and *Ywhaz*; ($n = 8$). Error bars represent the standard error of the mean, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

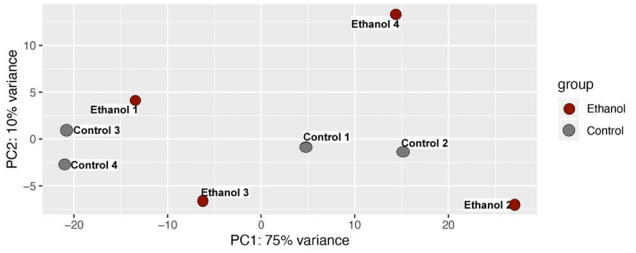
conducted deep sequencing of the placental transcriptome ($n = 4$). Principle component analysis failed to identify a clear separation between male placentae derived from the offspring of control and alcohol-exposed sires (Figure 6A). In total, we identified 431 differentially expressed transcripts, with 256 upregulated and 175 downregulated genes (Figure 6B,C). Gene Enrichment Analysis using IPA identified alterations in the pathways regulating oxidative phosphorylation, mitochondrial function, EIF2, and Sirtuin signaling (Figure 6C). We recently described dysfunction of a similar set of pathways in the brains of mice exposed to alcohol *in utero*,[50] suggesting maternal and paternal alcohol exposures may program similar outcomes. Consistent with our previous study, we identified altered expression of several mitochondrial-expressed transcripts and nuclear genes encoding mitochondrial enzymes (Figure 6D). Interestingly, some of these candidate genes were also differentially expressed in placentae derived from the female offspring of alcohol-exposed sires (Figure 6E). These observations suggest the offspring of alcohol-exposed fathers may exhibit programmed alterations in mitochondrial function and oxidative phosphorylation.

4 | DISCUSSION

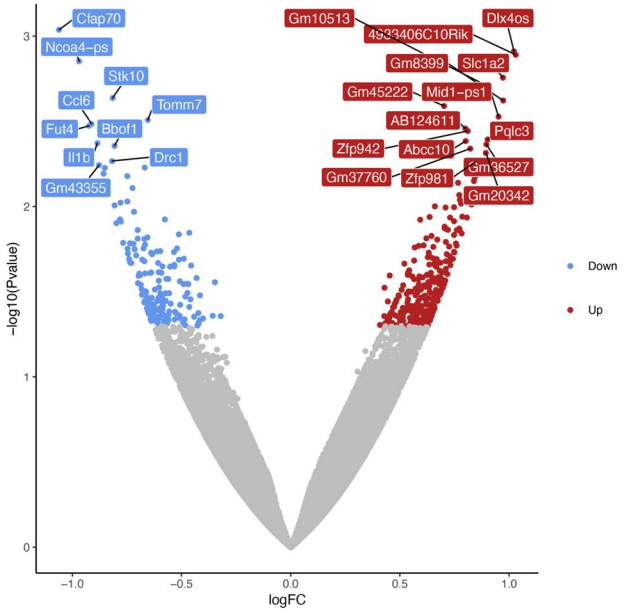
Multiple laboratories have demonstrated that exposing male mice to a high-fat diet, protein restriction, drugs of abuse, or stress before conception exert intergenerational effects on offspring growth, glucose tolerance, addictive behaviors, and stress responsivity.[51] However, these animal studies used inbred strains of mice, eliminating the potential influence of maternal genetic and uterine factors. Maternal factors are significant, particularly in alcohol research, where reciprocal crosses in mice demonstrate that maternal genetic background can protect or sensitize offspring to alcohol-induced growth and structural defects.[8–11] Although previous studies have examined differing crosses between mouse strains for alcohol-induced, paternally inherited changes in offspring behavior,[52] no studies have ever reported changes in fetal-placental growth. Therefore, whether maternal background can modify paternal epimutations and influence the penetrance of sperm-inherited growth defects remains unexplored.

Here, we find that on gestational day 16.5, a CD-1 maternal background protects offspring from the growth restriction phenotypes observed in pure C57Bl/6J crosses. However, alterations in placental physiology persist, with the male offspring of alcohol-exposed sires displaying smaller placentas, in opposition to the previously observed increase.[12,22] Surprisingly, although female offspring did not exhibit overt differences in placental growth, they did display morphological changes in placental architecture, suggesting that female offspring are not refractory to alcohol-induced changes in the paternally inherited developmental program. Thus, establishing how maternal

(A)



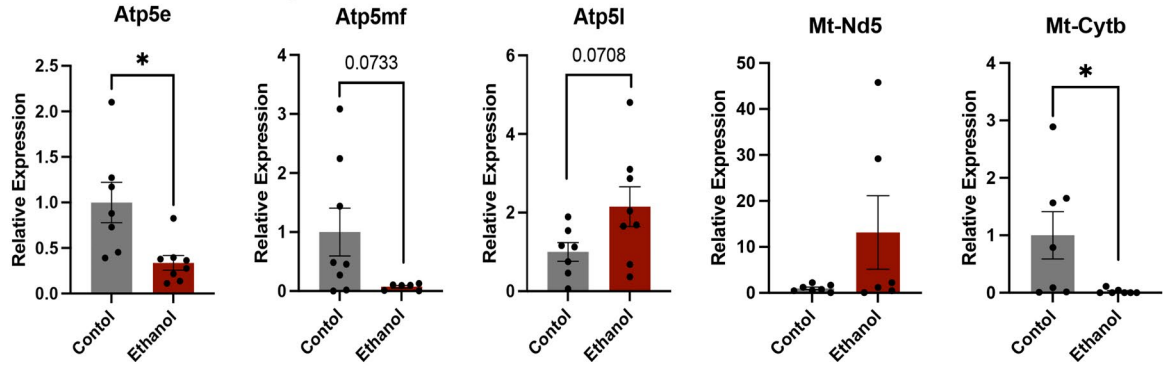
(B)



(C)

Number of DEGs	Top 5 Canonical Pathways	p-value (E^)
431 ⬇175, 256⬆	Oxidative Phosphorylation	-12
	Mitochondrial Dysfunction	-10
	EIF2 Signaling	-7
	Sirtuin Signaling Pathway	-6
	Estrogen Receptor Signaling	-4

(D)



(E)

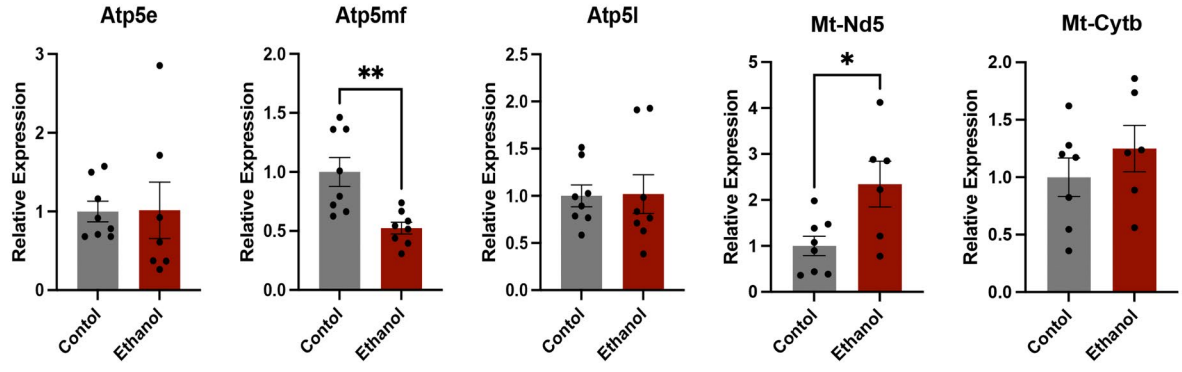


FIGURE 6 RNA-sequencing analysis of male placentae identifies paternally programmed alterations of genes regulating oxidative phosphorylation, mitochondrial function, and Sirtuin signaling. (A) Principle component analysis and (B) Volcano plot comparing the male placental transcriptomic profile between the control and alcohol preconception treatments ($n = 4$). (C) Gene Enrichment Analysis of RNA-seq datasets. Validation of identified candidate genes within the Oxidative Phosphorylation, Mitochondrial Dysfunction, and EIF2 Signaling pathways between (D) male and (E) female offspring sired by control and ethanol-exposed sires. Analysis of gene expression carried out using RT-qPCR. Gene expression was normalized to transcripts encoding *Pgk1* and *Ywhaz* ($n = 8$). Error bars represent the standard error of the mean, $*p < .05$, $**p < .01$

background modifies paternal intergenerational epigenetic inheritance will be an essential next step in determining how the epigenetic memory of paternal experience influences offspring phenotype.

In previous studies using a rat model, maternal preconception alcohol exposures induced an enlarged cross-sectional area of the placental junctional zone and an increased number of glycogen cells.[53] Interestingly, in studies examining gene loss-of-function mutants, strain-specific differences in placental physiology modify the penetrance and severity of growth restriction and embryonic lethal phenotypes. For example, a hypomorphic allele of the *epidermal growth factor receptor* that results in gene loss of function presents with severe growth restriction on a 129 background, while C57Bl/6 mice are resistant.[14] Strain-specific differences in the abundance of trophoblast giant and glycogen cells within the junctional zone appear to confer protection or sensitivity to this genetic lesion.[19] Similar placental adaptations are also observed in rodent models of nutritional stress,[48,54] which also exhibit remodeling of the junctional zone, reducing the mass of glycogen cells to adapt to the imposed nutritional deficits.[48] Therefore, dynamic changes in the junctional zone represent a crucial component of placental physiology, facilitating both adaptations to nutritional stressors and resistance to embryonic growth defects.

A substantial body of evidence supports a role for imprinted genes in controlling junctional zone differentiation and function.[55,56] For example, loss of *Ascl2* induces a loss of glycogen cells within the junctional zone, whereas deletion of *Phlda2* and *Esx1* associate with an expansion of the junctional zone and an increase in total placental glycogen content.[18,46,57] The glycogen cells appear in the junctional zone at mid-gestation and provide a store of easily mobilizable energy, supporting the final stages of fetal growth.[45] Glycogen cell deficiencies and overabundance both associate with strain-specific patterns of fetal growth restriction.[18,46] Notably, the impacts of glycogen cell dysfunction appear to spread from *Phlda2* mutant mice to neighboring wild-type fetuses, impacting placental growth and function within the entire litter.[47] Therefore, the glycogen cells of the junctional zone and upstream regulatory pathways involving imprinted genes play a critical role in the adaptability of the placenta. Our identification of altered imprinted gene expression in female placentae supports this assertion and implies that preconception alcohol exposures exert a lasting stress on offspring development, one that necessitates placental adaptation.

Interestingly, preconception paternal and preconception maternal alcohol exposures both increase the glycogen content of the junctional zone but only in female offspring.[53] From our perspective, the changes in imprinted gene expression we observe are likely symptoms of altered developmental programming and not the primary memory of alcohol exposure *per se*. Additional mechanisms beyond the locus control region regulate imprinted genes and the fact that only the female placentae exhibit histological changes and altered imprinted gene expression support this assertion. Although sporadically described, placental adaptability appears different between male and female offspring.[58,59] Therefore, we posit that females are more adaptable, altering their placental physiology to compensate for the paternally inherited stressor. One limitation to our study and the work by the Moritz group is that we each look at a single snapshot in gestational time. Therefore, we acknowledge that this female-specific signature is potentially also present in males, albeit delayed. However, a lack of adaptability in the male offspring during early life may explain the more significant deficits we see in later life.[23] As we and others find that CD-1 males refuse to consume ethanol, even at low percentages,[60] a second limitation to our study is that we were unable to conduct reciprocal crosses using this voluntary model of exposure. Finally, a third limitation is that we do not know if the modest changes in gene expression we identified using RNA-sequencing impact protein expression and placental function or if these changes associate with long-term alterations in offspring health.

How the memory of preconception alcohol exposures transmit through sperm and into early life remains an open question. Identifying alterations in the expression of genes regulating mitochondrial function and oxidative phosphorylation is intriguing, especially given that we recently identified this same signature in a maternal model of alcohol exposure.[50] Epigenetic influences on mitochondrial function are an emerging area of interest in addiction and cancer biology.[61,62] Importantly, mitochondrial genome enriched small RNAs (mitosRNAs) are present in sperm.[63] Future studies will address whether this class of ncRNA plays a role in transmitting the memory of alcohol exposure to the offspring and if therapeutic interventions targeting oxidative pathways influence the emergence of the observed placental phenotypes.

Finally, our studies reveal that preconception paternal alcohol exposures transmit a stressor that negatively influences offspring development but that maternal genetics influence the manifestation and penetrance of these phenotypes. These observations support the notion that sperm-inherited epigenetic information strongly influences placental function and that paternal lifestyle can impact embryonic development. Our work, combined with other published studies,[4] indicates that we need to expand health messaging around prepregnancy planning to include the father and redress the stigma that FASDs are exclusively the mother's fault.

ACKNOWLEDGMENTS

This work was supported by a Medical Research Grant from the W.M. Keck Foundation (M.C.G) and NIH Grant R01AA028219 (M.C.G). The authors thank the Muneoka and Suva laboratories for their assistance with microCT imaging.

DISCLOSURES

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Kara N. Thomas and Michael C. Golding designed the research. Kara N. Thomas, Katherine N. Zimmel, Alexis N. Roach, Alison Basel, Nicole A. Mehta, and Yudhishtar S. Bedi performed the research. Kara N. Thomas, Alexis N. Roach, and Michael C. Golding analyzed the data. Kara N. Thomas and Michael C. Golding wrote the paper.

ORCID

Michael C. Golding https://orcid.org/0000-0003-1631-2175

REFERENCES

1. Immler S. The sperm factor: paternal impact beyond genes. *Heredity*. 2018;121:239-247. doi:10.1038/s41437-018-0111-0
2. Le Blévec E, Muroňová J, Ray PF, Arnoult C. Paternal epigenetics: mammalian sperm provide much more than DNA at fertilization. *Mol Cell Endocrinol*. 2020;518:110964. doi:10.1016/j.mce.2020.110964
3. Fleming TP, Watkins AJ, Velazquez MA, et al. Origins of lifetime health around the time of conception: causes and consequences. *Lancet*. 2018;391:1842-1852. doi:10.1016/S0140-6736(18)30312-X
4. Rompala GR, Homanics GE. Intergenerational effects of alcohol: a review of paternal preconception ethanol exposure studies and epigenetic mechanisms in the male germline. *Alcohol Clin Exp Res*. 2019;43:1032-1045. doi:10.1111/acer.14029
5. Schaefer GB, Deere D. Recognition, diagnosis and treatment of fetal alcohol syndrome. *J Ark Med Soc*. 2011;108:38-40.
6. Donald KA, Wedderburn CJ, Barnett W, et al. Thyroid function in pregnant women with moderate to severe alcohol consumption is related to infant developmental outcomes. *Front Endocrinol*. 2018;9:294. doi:10.3389/fendo.2018.00294
7. Eberhart JK, Parnell SE. The genetics of fetal alcohol spectrum disorders (FASD). *Alcohol Clin Exp Res*. 2016;40:1154-1165. doi:10.1111/acer.13066
8. Downing C, Balderrama-Durbin C, Broncucia H, Gilliam D, Johnson TE. Ethanol teratogenesis in five inbred strains of mice. *Alcohol Clin Exp Res*. 2009;33:1238-1245. doi:10.1111/j.1530-0277.2009.00949.x
9. Gilliam DM, Irtenkauf KT. Maternal genetic effects on ethanol teratogenesis and dominance of relative embryonic resistance to malformations. *Alcohol Clin Exp Res*. 1990;14:539-545. doi:10.1111/j.1530-0277.1990.tb01196.x
10. Gilliam DM, Mantle MA, Barkhausen DA, Tweden DR. Effects of acute prenatal ethanol administration in a reciprocal cross of C57BL/6J and short-sleep mice: maternal effects and nonmaternal factors. *Alcohol Clin Exp Res*. 1997;21:28-34. doi:10.1111/j.1530-0277.1997.tb03724.x
11. Sittig LJ, Shukla PK, Herzing LBK, Redei EE. Strain-specific vulnerability to alcohol exposure in utero via hippocampal parent-of-origin expression of deiodinase-III. *FASEB J*. 2011;25:2313-2324. doi:10.1096/fj.10-179234
12. Chang RC, Skiles WM, Chronister SS, et al. DNA methylation-independent growth restriction and altered developmental programming in a mouse model of preconception male alcohol exposure. *Epigenetics*. 2017;12(10):841-853. doi:10.1080/15592294.2017.1363952
13. Kalisch-Smith JI, Steane SE, Simmons DG, et al. Periconceptional alcohol exposure causes female-specific perturbations to trophoblast differentiation and placental formation in the rat. *Development*. 2019;146. doi:10.1242/dev.172205
14. Dackor J, Caron KM, Threadgill DW. Placental and embryonic growth restriction in mice with reduced function epidermal growth factor receptor alleles. *Genetics*. 2009;183:207-218. doi:10.1534/genetics.109.104372
15. Dackor J, Li M, Threadgill DW. Placental overgrowth and fertility defects in mice with a hypermorphic allele of epidermal growth factor receptor. *Mamm Genome*. 2009;20:339-349. doi:10.1007/s00335-009-9189-2
16. Kraut N, Snider L, Chen CMA, Tapscott SJ, Groudine M. Requirement of the mouse I-mfa gene for placental development and skeletal patterning. *EMBO J*. 1998;17:6276-6288. doi:10.1093/emboj/17.21.6276
17. Tunster SJ, Van De Pette M, John RM. Fetal overgrowth in the Cdkn1c mouse model of Beckwith-Wiedemann syndrome. *DMM Dis Models Mech*. 2011;4:814-821. doi:10.1242/dmm.007328
18. Tunster SJ, Van De Pette M, John RM. Isolating the role of elevated Phlda2 in asymmetric late fetal growth restriction in mice. *DMM Dis Models Mech*. 2014;7:1185-1191. doi:10.1242/dmm.017079
19. Tunster SJ, Van De Pette M, John RM. Impact of genetic background on placental glycogen storage in mice. *Placenta*. 2012;33:124-127. doi:10.1016/j.placenta.2011.11.011
20. Albers RE, Waker CA, Keoni C, et al. Gestational differences in murine placenta: glycolytic metabolism and pregnancy parameters. *Theriogenology*. 2018;107:115-126. doi:10.1016/j.theriogenology.2017.10.049
21. Rennie MY, Detmar J, Whiteley KJ, Jurisicova A, Adamson SL, Sled JG. Expansion of the fetoplacental vasculature in late gestation is strain dependent in mice. *Am J Physiol Heart Circ Physiol*. 2012;302:H1261-H1273. doi:10.1152/ajpheart.00776.2011
22. Bedi Y, Chang RC, Gibbs R, Clement TM, Golding MC. Alterations in sperm-inherited noncoding RNAs associate with late-term fetal growth restriction induced by preconception paternal alcohol use. *Reprod Toxicol*. 2019;87:11-20. doi:10.1016/j.reprotox.2019.04.006
23. Chang RC, Wang H, Bedi Y, Golding MC. Preconception paternal alcohol exposure exerts sex-specific effects on offspring growth and long-term metabolic programming. *Epigenetics and Chromatin*. 2019;12. doi:10.1186/s13072-019-0254-0
24. Thiele TE, Crabbe JC, Boehm SL. "Drinking in the dark" (DID): a simple mouse model of binge-like alcohol intake. *Curr Protoc Neurosci*. 2014;68(1):1–12. doi:10.1002/0471142301.ns0949s68

25. Chan JC, Morgan CP, Adrian Leu N, et al. Reproductive tract extracellular vesicles are sufficient to transmit intergenerational stress and program neurodevelopment. *Nat Commun*. 2020;11. doi:10.1038/s41467-020-15305-w
26. Bailoo JD, Murphy E, Boada-Saña M, et al. Effects of cage enrichment on behavior, welfare and outcome variability in female mice. *Front Behav Neurosci*. 2018;12. doi:10.3389/fnbeh.2018.00232
27. van Praag H, Kempermann G, Gage FH. Neural consequences of enviromental enrichment. *Nat Rev Neurosci*. 2000;1:191-198. doi:10.1038/35044558
28. Whitten WK, Bronson FH, Greenstein JA. Estrus-inducing pheromone of male mice: transport by movement of air. *Science*. 1968;161:584-585. doi:10.1126/science.161.3841.584.
29. Heyne GW, Plisch EH, Melberg CG, Sandgren EP, Peter JA, Lipinski RJ. A simple and reliable method for early pregnancy detection in inbred mice. *J Am Assoc Lab Anim Sci*. 2015;54:368-371.
30. Truett GE, Heeger P, Mynatt RL, Truett AA, Walker JA, Warman ML. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques*. 2000;29(1):52-54. doi:10.2144/00291bm09
31. Sofronescu AG, Detillieux KA, Cattini PA. FGF-16 is a target for adrenergic stimulation through NF-kappaB activation in postnatal cardiac cells and adult mouse heart. *Cardiovasc Res*. 2010;87:102-110. doi:10.1093/cvr/cvq025
32. Clercq KD, Persoons E, Napso T, et al. High-resolution contrast-enhanced microCT reveals the true three-dimensional morphology of the murine placenta. *PNAS*. 2019;116:13927-13936. doi:10.1073/pnas.1902688116
33. Hsu C-W, Wong L, Rasmussen TL, et al. Three-dimensional microCT imaging of mouse development from early post-implantation to early postnatal stages. *Dev Biol*. 2016;419:229-236. doi:10.1016/j.ydbio.2016.09.011
34. Lesciotto KM, Perrine SMM, Kawasaki M, et al. Phosphotungstic acid enhanced microCT: optimized protocols for embryonic and early postnatal mice. *Dev Dyn*. 2020;249:573-585. doi:10.1002/dvdy.136
35. Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*. 2016;32:3047-3048. doi:10.1093/bioinformatics/btw354
36. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 2014;30:2114-2120. doi:10.1093/bioinformatics/btu170
37. Afgan E, Baker D, Batut B, et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res*. 2018;46(2018):W537-W544. doi:10.1093/nar/gky379
38. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29:15-21. doi:10.1093/bioinformatics/bts635
39. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30:923-930. doi:10.1093/bioinformatics/btt656
40. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15:550. doi:10.1186/s13059-014-0550-8
41. Jiménez-Marín A, Collado-Romero M, Ramirez-Boo M, Arce C, Garrido JJ. Biological pathway analysis by ArrayUnlock and Ingenuity Pathway Analysis. *BMC Proc*. 2009;3(suppl 4):S6. doi:10.1186/1753-6561-3-S4-S6
42. Hayward CE, Lean S, Sibley CP, et al. Placental adaptation: what can we learn from birthweight: placental weight ratio? *Front Physiol*. 2016;7:28. doi:10.3389/fphys.2016.00028
43. Carnahan MN, Veazey KJ, Muller D, Tingling JD, Miranda RC, Golding MC. Identification of cell-specific patterns of reference gene stability in quantitative reverse-transcriptase polymerase chain reaction studies of embryonic, placental and neural stem models of prenatal ethanol exposure. *Alcohol*. 2013;47(2):109-120. doi:10.1016/j.alcohol.2012.12.003
44. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*. 2008;3:1101-1108.
45. Rossant J, Cross JC. Placental development: lessons from mouse mutants. *Nat Rev Genet*. 2001;2:538-548. doi:10.1038/35080570
46. Tunster SJ, McNamara GI, Creeth HDJ, John RM. Increased dosage of the imprinted Ascl2 gene restrains two key endocrine lineages of the mouse Placenta. *Dev Biol*. 2016;418:55-65. doi:10.1016/j.ydbio.2016.08.014
47. Tunster SJ, Creeth HDJ, John RM. The imprinted Phlda2 gene modulates a major endocrine compartment of the placenta to regulate placental demands for maternal resources. *Dev Biol*. 2016;409:251-260. doi:10.1016/j.ydbio.2015.10.015
48. Coan PM, Vaughan OR, Sekita Y, et al. Adaptations in placental phenotype support fetal growth during undernutrition of pregnant mice. *J Physiol*. 2010;588:527-538. doi:10.1113/jphysiol.2009.181214
49. Watkins AJ, Sirovica S, Stokes B, Isaacs M, Addison O, Martin RA. Paternal low protein diet programs preimplantation embryo gene expression, fetal growth and skeletal development in mice. *Biochim Biophys Acta Mol Basis Dis*. 1863;2017:1371-1381. doi:10.1016/j.bbadis.2017.02.009
50. Chang RC, Thomas KN, Mehta NA, Veazey KJ, Parnell SE, Golding MC. Programmed suppression of oxidative phosphorylation and mitochondrial function by gestational alcohol exposure correlate with widespread increases in H3K9me2 that do not suppress transcription. *Epigenetics Chromatin*. 2021;14:27. doi:10.1186/s13072-021-00403-w
51. Champroux A, Cocquet J, Henry-Berger J, Drevet JR, Kocer A. A decade of exploring the mammalian sperm epigenome: paternal epigenetic and transgenerational inheritance. *Front Cell Dev Biol*. 2018;6:50. doi:10.3389/fcell.2018.00050
52. Rompala GR, Finegersh A, Slater M, Homanics GE. Paternal preconception alcohol exposure imparts intergenerational alcohol-related behaviors to male offspring on a pure C57BL/6J background. *Alcohol*. 2017;60:169-177. doi:10.1016/j.alcohol.2016.11.001
53. Gårdebjer EM, Cuffe JSM, Pantaleon M, Wlodek ME, Moritz KM. Periconceptional alcohol consumption causes fetal growth restriction and increases glycogen accumulation in the late gestation rat placenta. *Placenta*. 2014;35:50-57. doi:10.1016/j.placenta.2013.10.008
54. Pruis MGM, Gellhaus A, Kühnel E, et al. Sex-specific placental differences as a contributor to sex-specific metabolic programming? *Acta Physiol*. 2015;215:127-129. doi:10.1111/apha.12562
55. Coan PM, Burton GJ, Ferguson-Smith AC. Imprinted genes in the placenta – a review. *Placenta*. 2005;26:S10-S20. doi:10.1016/j.placenta.2004.12.009

56. Piedrahita JA. The role of imprinted genes in fetal growth abnormalities. *Birth Defects Res A Clin Mol Teratol*. 2011;91:682-692. doi:10.1002/bdra.20795
57. Li Y, Behringer RR. Esx1 is an X-chromosome-imprinted regulator of placental development and fetal growth. *Nat Genet*. 1998;20:309-311. doi:10.1038/3129
58. Kalisch-Smith JI, Simmons DG, Dickinson H, Moritz KM. Review: sexual dimorphism in the formation, function and adaptation of the placenta. *Placenta*. 2017;54:10-16. doi:10.1016/j.placenta.2016.12.008
59. Rosenfeld CS. Sex-specific placental responses in fetal development. *Endocrinology*. 2015;156:3422-3434. doi:10.1210/en.2015-1227
60. Rhodes JS, Ford MM, Yu C-H, et al. Mouse inbred strain differences in ethanol drinking to intoxication. *Genes Brain Behav*. 2007;6:1-18. doi:10.1111/j.1601-183X.2006.00210.x
61. Dong Z, Pu L, Cui H. Mitoepigenetics and its emerging roles in cancer. *Front Cell Dev Biol*. 2020;8:4. doi:10.3389/fcell.2020.00004
62. Sadakierska-Chudy A, Frankowska M, Filip M. Mitoepigenetics and drug addiction. *Pharmacol Ther*. 2014;144:226-233. doi:10.1016/j.pharmthera.2014.06.002
63. Larriba E, Rial E, del Mazo J. The landscape of mitochondrial small non-coding RNAs in the PGCs of male mice, spermatogonia, gametes and in zygotes. *BMC Genom*. 2018;19:634. doi:10.1186/s12864-018-5020-3

How to cite this article: Thomas KN, Zimmel KN, Roach AN, et al. Maternal background alters the penetrance of growth phenotypes and sex-specific placental adaptation of offspring sired by alcohol-exposed males. *FASEB J*. 2021;35:e22035. doi:10.1096/fj.202101131R