DECIPHERING VARIATION IN FETAL ALCOHOL SPECTRUM DISORDERS

THE MISSING PIECE: DAD

A Dissertation

by

KARA NICHOLE THOMAS

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Chair of Committee,	Michael Golding
Committee Members,	Gladys Ko
	Lindsay Dawson
	Rajesh Miranda
Head of Department,	Larry Suva

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ABSTRACT

Fetal Alcohol Spectrum Disorder (FASD) is the most prevalent, yet completely preventable birth defect. Over the last half century, we have seen many milestones in fetal alcohol research, from the 1981 US Surgeon General warning to limit alcohol during pregnancy, to the 2005 US Surgeon General warning to avoid alcohol altogether, during pregnancy. Although major advancements, these health messages only provide a partial picture by focusing solely on maternal alcohol consumption. This research questions the maternal centric focus and sheds light on the implications of paternal alcohol use. By building upon recent research linking paternal alcohol consumption to fetal growth defects, this research brings the urgent message of the dangers of paternal alcohol exposure closer to clinical practice.

Using a clinically relevant mouse model, we first investigated the effects of maternal genetics on paternal alcohol exposure. We found that maternal factors have the capacity to modulate the fetal and placental changes induced by paternal alcohol consumption. Next, we tested the capacity of different doses of paternal alcohol to exert effects on fetal development. Interestingly, we found a biphasic dose response, with lower doses resulting in placental and fetal overgrowth and higher doses leading to fetal and placental growth deficiency. Equipped with the knowledge of a maternal-paternal crosstalk and a clinically relevant paternal alcohol dose that resulted in FASD-like growth restriction, we next implemented a multi-factorial model to evaluate the effects of maternal, paternal, and dual parental alcohol exposures on craniofacial development. The most severe form of FASD, Fetal Alcohol Syndrome, results in distinct craniofacial phenotypes and through this groundbreaking research we found that maternal, paternal, and dual parental alcohol exposure resulted in distinct craniofacial growth deficiencies. Finally, as

neurological development is closely associated with craniofacial development, we investigated the lasting impact of maternal, paternal, and dual parental alcohol exposure on offspring behavior during adolescence and adulthood. Importantly, we found that these early life exposures led to long lasting impacts on health and behavior, providing support for the Developmental Origins of Health and Disease hypothesis. Collectively, these studies identify the father as a significant inducer and contributor to FASD.

DEDICATION

To my son, Brayden, in whom I found the inspiration and strength to continue my pursuit of knowledge in hopes of making your world a better place. May you find joy in the search of life's truths, just as I continue to do.

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Contributors

This work was supervised by a dissertation committee consisting of Professor Michael Golding [advisor] and Professor Lindsay Dawson of the Department of Veterinary Physiology and Pharmacology, Professor Gladys Ko of the Department of Veterinary Integrative Biosciences, and Professor Rajesh Miranda of the Department of Neuroscience and Experimental Therapeutics.

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NOMENCLATURE

ANOVA	Analysis of variance
ARBD	Alcohol Related Birth Defect
ARND	Alcohol Related Neurodevelopmental Disorder
ARRIVE	Animal Research: Reporting of In Vivo Experiments
Ascl2	Achaete-scute family basic helix-loop-helix transcription factor
ATP	Adenosine triphosphate
Atp5e	ATP synthase, H+ transporting, mitochondrial F1 complex, subunit epsilon
Atp5l	ATP synthase membrane subunit G
Atp5mf	ATP synthase membrane subunit F
AUP	Animal Use Protocol
cDNA	Complementary deoxyribonucleic acid
Cdkn1c	Cyclin-dependent kinase inhibitor 1C
ChIP	Chromatin Immunoprecipitation
CO_2	Carbon dioxide
Cox7b	Cytochrome C oxidase subunit 7B
DID	Drinking in the Dark
DNA	Deoxyribonucleic acid
EIF2	Eukaryotic translation initiation factor 2A
Esx1	Extraembryonic, Spermatogenesis, Homeobox 1
EtOH	Ethanol
F1	Filial 1

FAS	Fetal Alcohol Syndrome
FASD	Fetal Alcohol Spectrum Disorder
GD	Gestational day
GEO	Gene Expression Omnibus
H19	Hepatic transcript 19
IACUC	Institutional Animal Care and Use Committee
IUGR	Intrauterine growth restriction
Kcnq1	Potassium voltage-gated channel subfamily Q member 1
Kcnq1ot1	Kcnq1 overlapping transcript 1
micro-CT	Microfocus computed, X-ray tomography
mitosRNA	Mitochondrial genome enriched small ribonucleic acid
mt-Co1	Mitochondrially encoded cytochrome c oxidase I
mt-Cytb	Mitochondrially encoded cytochrome B
mt-ND1	Mitochondrially encoded NADH dehydrogenase (ubiquinone) subunit 1
mt-ND5	Mitochondrially encoded NADH dehydrogenase (ubiquinone) subunit 5
NADH	Nicotinamide adenine dinucleotide hydrogen
ncRNA	Non-coding ribonucleic acid
ND-PAE	Neurobehavioral Disorder Associated with Prenatal Alcohol Exposure
Ndufa7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 7
Ndufb10	NADH dehydrogenase (ubiquinone) subunit B10
PBS	Phosphate buffered saline
PC	Principal component
PCA	Principal component analysis

Peg3	Paternally expressed gene 3
pFAS	Partial Fetal Alcohol Syndrome
Pgk1	Phosphoglycerate kinase 1
Phlda2	Pleckstrin homology like domain family A member 2
PND	Postnatal day
Ppia	Peptidylprolyl isomerase A
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
Slc22a18	Solute carrier family 22 member 18
Slc2a3	Solute carrier family 2 member 3
Slc3a2	Solute carrier family 3 member 2
Slc38a2	Solute carrier family 38 member 2
Slc38a4	Solute carrier family 38 member 4
Tpbpa	Trophoblast specific protein alpha
Untr6	Untranscribed region of chromosome 6
w/v	weight to volume
Ywhaz	3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta
Zfy	Zinc finger protein Y-linked

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CHAPTER I

INTRODUCTION

1. Fetal Alcohol Spectrum Disorder

Fetal Alcohol Spectrum Disorder (FASD) is an umbrella term used to encompass a wide range of mental and physical developmental disabilities that leave a lasting impact across the lifespan (Clarke & Gibbard, 2003; Jones & Smith, 1973). According to the American Academy of Pediatrics, the FASD umbrella includes five diagnoses: (1) Fetal Alcohol Syndrome (FAS), (2) Partial Fetal Alcohol Syndrome (pFAS), (3) Alcohol Related Birth Defect (ARBD), (4) Alcohol Related Neurodevelopmental Disorder (ARND), and (5) Neurobehavioral Disorder Associated with Prenatal Alcohol Exposure (ND-PAE). The multitude of diagnoses under the FASD umbrella highlight the broad variation seen in FASD patients. Moreover, individuals within each FASD category also experience a wide range in variation in presenting symptoms, features, and severity.

Recent studies estimate as many as 10% of US children are currently living with FASD (May et al., 2018, p. 2). Additionally, it is estimated that 40,000 babies are born each year with FASD in the United States alone (Eberhart & Parnell, 2016). For individuals with FASD, the additional total lifetime cost is approximately $2 million per affected individual, thus, making FASD both an important public health and economic issue (Mattson et al., 2019; Popova et al., 2011).

For children with FASD, earlier diagnosis is considered a protective factor (O'Connor et al., 2006; Schaefer & Deere, 2011). The most severe form of FASD, Fetal Alcohol Syndrome (FAS), presents with readily visible phenotypes that are more easily detected (Gupta et al., 2016). However, the average age of diagnosis of FAS is approximately two years of age, with the higher

range falling around eight years of age (Moberg et al., 2014). Furthermore, FAS only accounts for 10 to 30% of the FASD population, meaning that the vast majority of FASD individuals are living with disabilities that may evade detection for far longer, or may never be diagnosed (Jonsson, 2019). In fact, a recent study conducted in a population of first grade US children, found that 99% (220 of 222 children) of FASD cases had been undiagnosed (May et al., 2018). Consequently, without the proper FASD diagnosis, it is impossible to get these children the intervention and care required in order to cope with this debilitating disease.

Another hindrance to early detection and diagnosis of FASD is the long-held dogma that FASD is exclusively the mother's fault. As a result of the negative stigma surrounding maternal alcohol use, many women deny consuming alcohol during pregnancy due to perceived judgment (Corrigan et al., 2017; Wozniak et al., 2019). Until recently, knowledge of maternal alcohol use during pregnancy was required for FASD diagnosis. Meaning that even if FASD cases were identified based upon presenting symptoms, diagnosis did not occur due to the lack of documented maternal alcohol consumption during pregnancy (Bandoli et al., 2022).

2. Alcohol and Reproduction

Alcohol is the most socially acceptable, widely abused, and easily attained recreational drug. Within the population reproductive age adults, alcohol accounts for 20% of deaths (Esser et al., 2022). Given its broad use within this demographic, it is not surprising that alcohol is the most common cause of preventable birth defects with an estimated 40,000 babies born each year with FASD in the United States alone (Eberhart & Parnell, 2016).

In the central nervous system (CNS), alcohol is a biphasic drug, specifically, at lower doses alcohol acts a stimulant, but as the dose increases it becomes a sedative (Hendler et al., 2013). Just

like its effects on the CNS, alcohol also has a similar pattern of effect on the male reproductive tract. Specifically, it has been reported that at high levels of alcohol consumption males experience decreased semen volume, sperm concentration, and testosterone level, accompanied with abnormal sperm morphology (Kucheria et al., 1985; Muthusami & Chinnaswamy, 2005). In contrast, males with moderate alcohol consumption, have been reported to have higher testosterone levels and no adverse effects on seminal fluid and sperm (Jensen et al., 2014).

3. Fetal Development and Placentation

Fetal development consists of three phases: germinal, embryonic, and fetal. The germinal phase begins when the sperm fertilizes the egg and lasts until about week three of human pregnancy. The embryonic phase encompasses week three to week eight, while the fetal phase consists of the remainder of gestation. Importantly, a woman typically discovers that she is pregnant around the first missed period, which coincides with week three to seven of gestation, meaning that the pregnancy could almost be in the fetal period at time of discovery. In addition, almost half of the pregnancies in the United States are unintended (Finer & Zolna, 2011). Without the intent to conceive, many pregnancies may be subjected to environmental exposures that would have otherwise been avoided, such as alcohol. Moreover, organogenesis occurs during the embryonic stage and it is within this window that many organ systems have the greatest sensitivity to teratogens (Alwan & Chambers, 2015).

Although it is often disregarded due to being a temporary organ, the placenta is a crucial mediator of pregnancy health. In fact, many adverse pregnancy outcomes are associated with defects in placentation (Brosens et al., 2011). By connecting to the uterine wall, the placenta is the

mediator of nutrient and waste exchange between the mother and fetus. Importantly, alcohol readily crosses the placenta and can exert effects on the developing fetus (Popova et al., 2021).

4. FASD Mouse Models

Studies conducted in rodents provide an excellent way to investigate FASD (Almeida et al., 2020). However, these models vary by route of alcohol administration, dose, frequency, and timing, which in turn, leads to incongruence in models of FASD and skepticism of the results. Previous work by our lab has established a physiologically relevant model of preconception alcohol exposure using a limited access voluntary consumption model (Y. Bedi et al., 2019; Chang et al., 2017; Chang, Wang, et al., 2019). Importantly, our model minimizes stress while still achieving physiologically relevant blood alcohol concentrations that induce FASD-like outcomes.

5. Epigenetics

Recent advances in the field of epigenetics have demonstrated that paternal environmental exposures can induce intergenerational impacts on health and disease (Breton et al., 2021; Lee & Conine, 2022; Van Cauwenbergh et al., 2020). Epigenetic mechanisms result in a change in gene expression without changing the DNA sequence and are highly sensitive to environmental factors (Rompala & Homanics, 2019). Exposure to alcohol has the ability to alter epigenetic factors in both somatic cells and the germline (Chastain & Sarkar, 2017). Collectively, these new insights provide a link between preconception paternal alcohol exposure and offspring outcomes that highlight the need for further investigations into paternal contributions to FASD (Immler, 2018; Le Blévec et al., 2020).

CHAPTER II

MATERNAL BACKGROUND ALTERS THE PENETRANCE OF GROWTH PHENOTYPES AND SEX-SPECIFIC PLACENTAL ADAPTATION OF OFFSPRING SIRED BY ALCOHOL-EXPOSED MALES*

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 (Immler, 2018; Le Blévec et al., 2020). 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 (Fleming et al., 2018).

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 (Rompala & Homanics, 2019). 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

*Reprinted with permission from "Maternal background alters the penetrance of growth phenotypes and sex-specific placental adaptation of offspring sired by alcohol-exposed males" by Thomas et al., 2021. *FASEBJ.* 10.1096/fj.202101131R, Copyright © 2021 Thomas, Zimmel, Roach, Basel, Mehta, Bedi, Golding. The FASEB Journal published by Wiley Periodicals LLC on behalf of Federation of American Societies for Experimental Biology.

FASD phenotypes and incidence (Schaefer & Deere, 2011).

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 (Donald et al., 2018; Eberhart & Parnell, 2016). 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 (Downing et al., 2009; D. M. Gilliam & Irtenkauf, 1990; F. Gilliam et al., 1997; Sittig et al., 2011). 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 (Chang et al., 2017; Kalisch-Smith et al., 2019). 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 (Dackor, Caron, et al., 2009; Dackor, Li, et

al., 2009; Kraut et al., 1998; Tunster et al., 2011, 2014). Significantly, these differing sensitivities associate with strain-specific differences in the histological organization of the placenta (reviewed in Ref. [(Tunster et al., 2012)]). 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 (Albers et al., 2018; Rennie et al., 2012). 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 (Y. Bedi et al., 2019; Chang et al., 2017; Chang, Wang, et al., 2019). 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 (Thiele et al., 2014). 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 (Albers et al., 2018). 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 (Chan et al., 2020). Therefore, we implemented additional animal husbandry measures to improve animal welfare and reduce stress through enhanced shelter and environmental enrichment (Bailoo et al., 2018; van Praag et al., 2000). 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 (Thiele et al., 2014). 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 (Whitten et al., 1968) 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 (Y. Bedi et al., 2019). 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 (Heyne et al., 2015). On gestational day 16.5, we euthanized pregnant dams by CO2 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 (Truett et al., 2000). 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 (Sofronescu et al., 2010). See Table 2.1 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 (cata- log# 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. Procedures for normalization and data handling are described below; primer sequences are listed in Table 2.1.

Table 2.1. Sequence of the RT-qPCR primers used in this study. [Reprinted from Thomas et al., 2021]

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

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 (De Clercq et al., 2019; Hsu et al., 2016). In these previous studies, the authors could discriminate specific morphological features of the conceptus, including the different placental layers (De Clercq et al., 2019). Using phosphotungstic acid to enhance tissue contrast, we employed the recent refinements described by Lesciotto et al. (Lesciotto et al., 2020) 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 mm3, 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://horosproje ct.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 (Ewels et al., 2016) on the raw paired-end, total RNA sequence files and trimmed Illumina adapters with Trimmomatic (Bolger et al., 2014) using the open-source, web-based Galaxy server (Afgan et al., 2018) (usegalaxy.org). Next, we used RNA STAR (Dobin et al., 2013) 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 (Liao et al., 2014) with a minimum mapping quality per read of 10. Next, we used the DEseq2 function (Love et al., 2014) 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 (Jiménez-Marín et al., 2009) 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 (Hayward et al., 2016) 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 (Carnahan et al., 2013). These

included transcripts encoding Phosphoglycerate kinase 1 (Pgk1) and 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (Ywhaz). We then used the -ΔΔCT method (Schmittgen & Livak, 2008). 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 2.1A). 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 2.1B). Using Sidak's multiple comparison test, we did not identify any differences in weekly fluid consumption between the control and alcohol treatment groups (Figure 2.1C). At the end of the four-hour exposure window, alcohol-exposed males exhibited average plasma alcohol concentrations of 50 mg/dl (Figure 2.1D). 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 ap- proximate weight of C57Bl/6J males after ten weeks of treatment. Consistent with our previous studies examining pure C57Bl/6J crosses (Chang, Wang, et al., 2019) we did not observe any differences in CD-1

maternal body weight relative to litter size (Figure 2.1E; gestational day 16.5 shown) or the litter size (Figure 2.1F) between the alcohol and control preconception treatments.

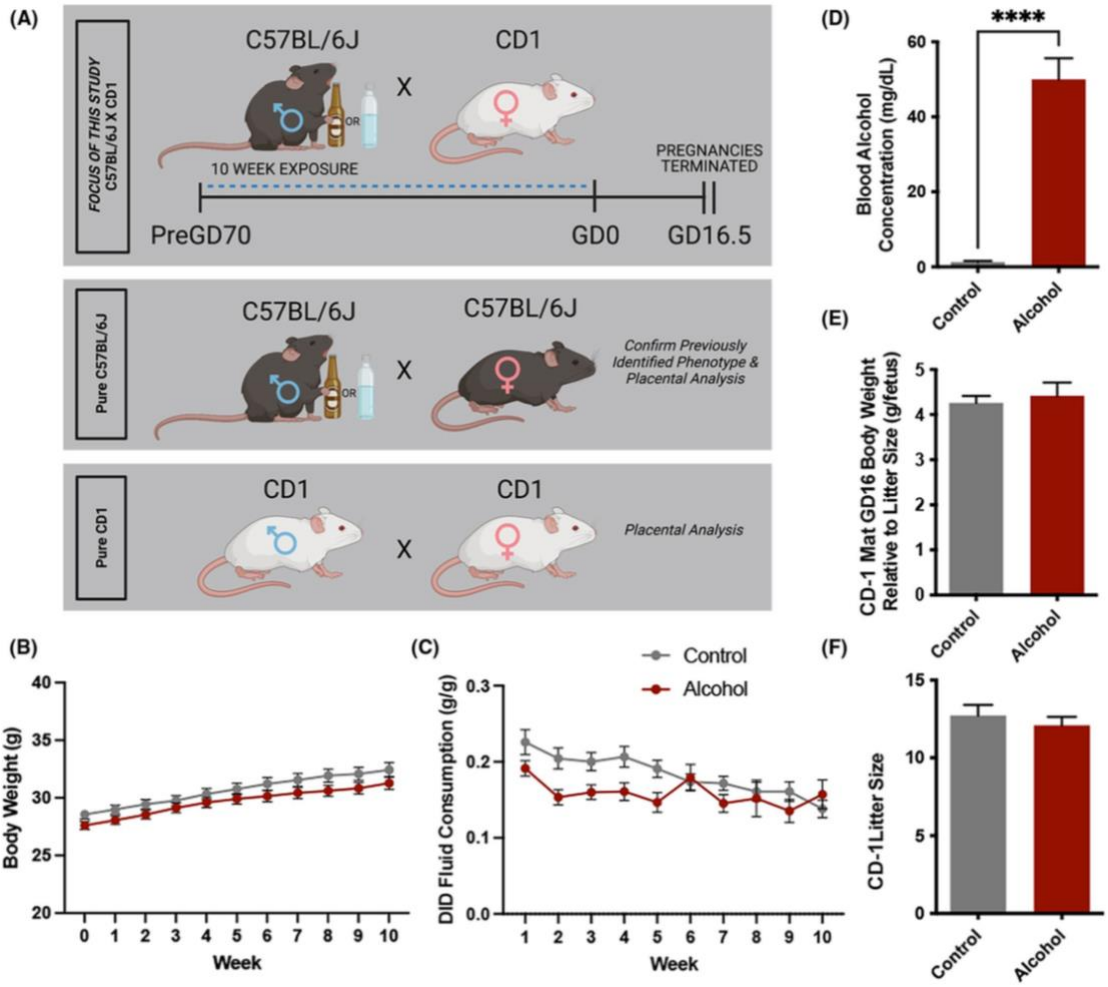

Figure 2.1. Mouse model of preconception paternal alcohol exposure. (A) Graphic representation of the experimental paradigm. Postnatal day 90, C57BL/6J males are exposed to 10% ethanol for 10 weeks (Pre-Gestational Day [PGD] 70), then bred to either naïve CD-1 (top) or naïve C57BL/6J dams (middle). We 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 [Reprinted from Thomas et al., 2021]

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 2.2A–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 2.2D–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 2.2A– C). Consistent with our previous publications, pure C57Bl/6J male offspring sired by alcohol-exposed males exhibited increased placental weights (Figure 2.2D) and decreased placental efficiency (Figure 2.2F). 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 2.2D), reduced placental diameter (−5%, $p < .01$; Figure 2.2E), and a general increase in placental efficiency ($p = .088$; Figure 2.2F). Female fetuses did not exhibit significant changes in placental growth or efficiency (Figure 2.2D–F). Collectively, these data reveal that maternal genetic background can significantly modify phenotypic outcomes arising from sperm-inherited alterations in epigenetic programming.

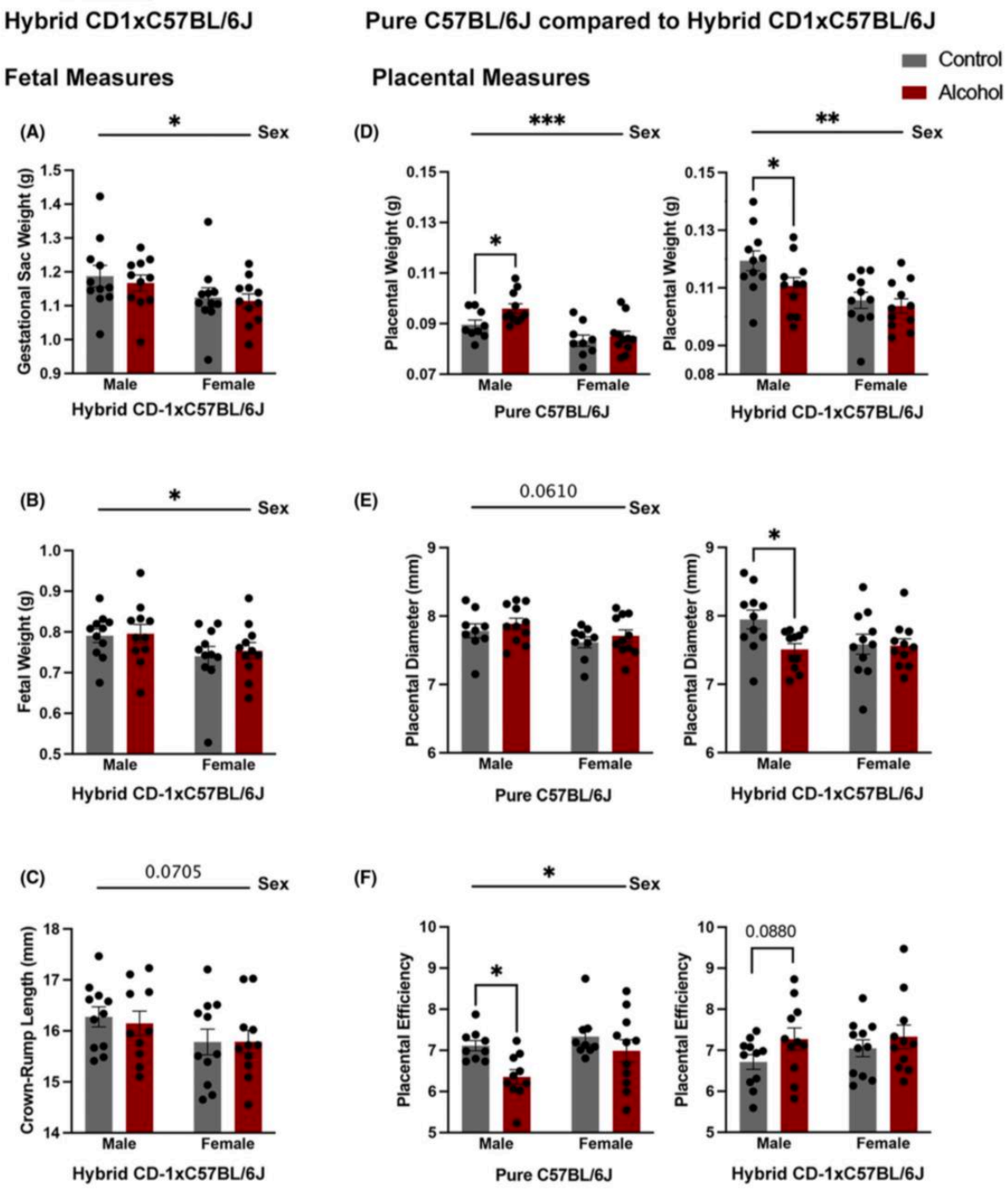

Figure 2.2. Influence of Maternal background on placental growth phenotypes induced by preconception paternal alcohol exposure. Comparison of (A) gestational sac weight, (B) fetal weight, and (C) crown-rump length between hybrid CD-1xC57BL/6J litters sired by alcohol-exposed and control males. Comparison 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 [Reprinted from Thomas et al., 2021]

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 (Rossant & Cross, 2001). Micro-CT allows three-dimensional quantification of whole tissue samples with high contrast and high resolution, including discrimination of the different placental layers (De Clercq et al., 2019). Using phosphotungstic acid to enhance tissue contrast, we employed the methods described by Lesciotto et al (Lesciotto et al., 2020) 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 2.3A). Overall, the placental histology of F1 hybrid CD-1xC57BL/6J offspring appeared more similar to the maternal CD-1 strain (Figure 2.3A). Using these interstrain differences, we confirmed the ability of enhanced tissue contrast micro-CT imaging to identify discrepancies in placental morphology (Figure 2.3B). 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 2.3C) 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 2.3D). 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 2.3D–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 2.3H–I). These 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.

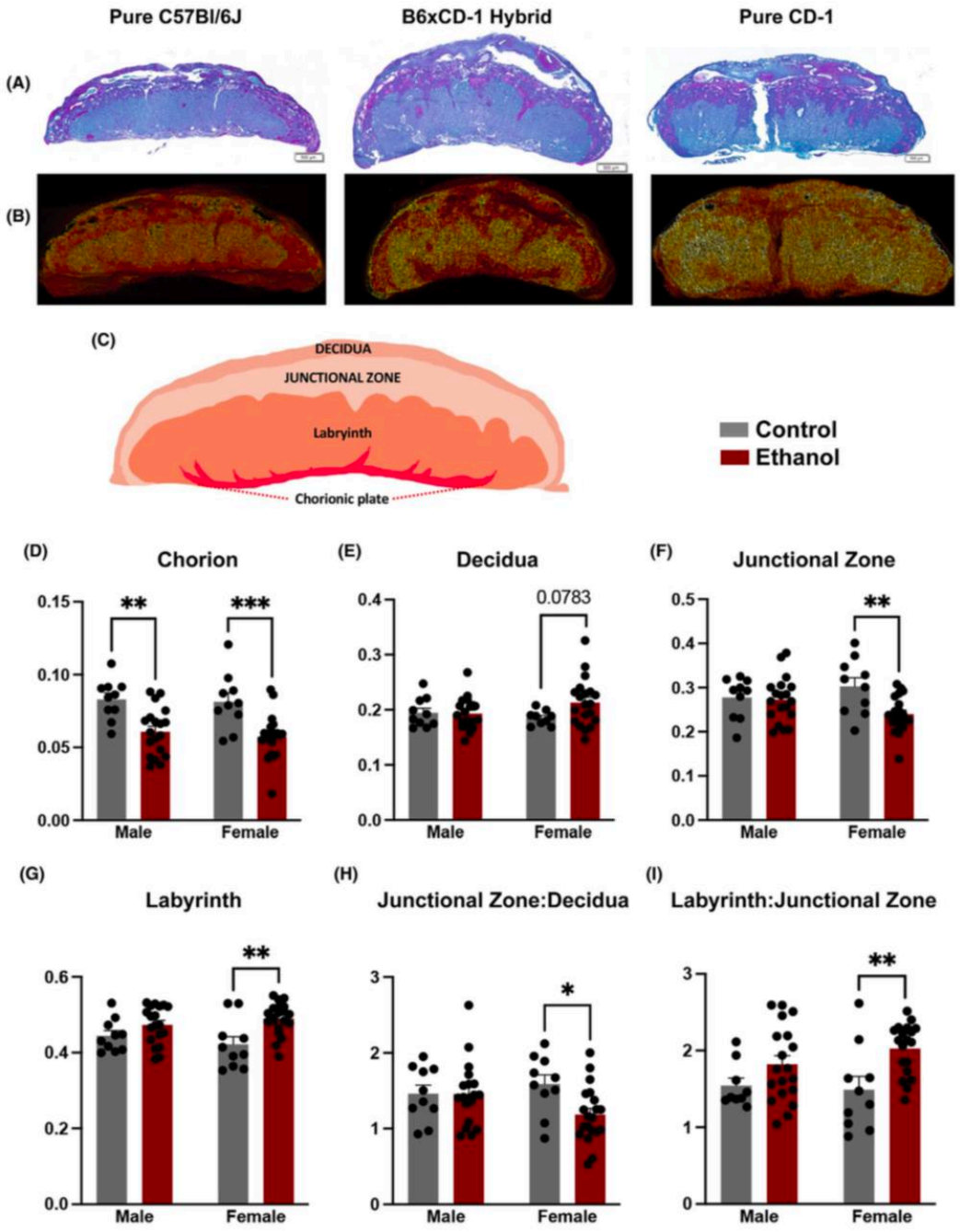

Figure 2.3. Preconception paternal alcohol exposure induces sex-specific changes in placental architecture. (A) Comparison of placental histology between pure C57BL/6J (left), pure CD-1 (right), and hybrid CD-1xC57BL/6J (middle) offspring. (B) MicroCT imaging of placentae derived from pure C57BL/6J, 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 [Reprinted from Thomas et al., 2021]

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 lower nuclei density than surrounding cell types. This lower nuclei density could induce hypoattenuated areas (negative space) in the micro-CT 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 2.4A–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 2.4E). We did not observe any differences in placental glycogen content between males and females.

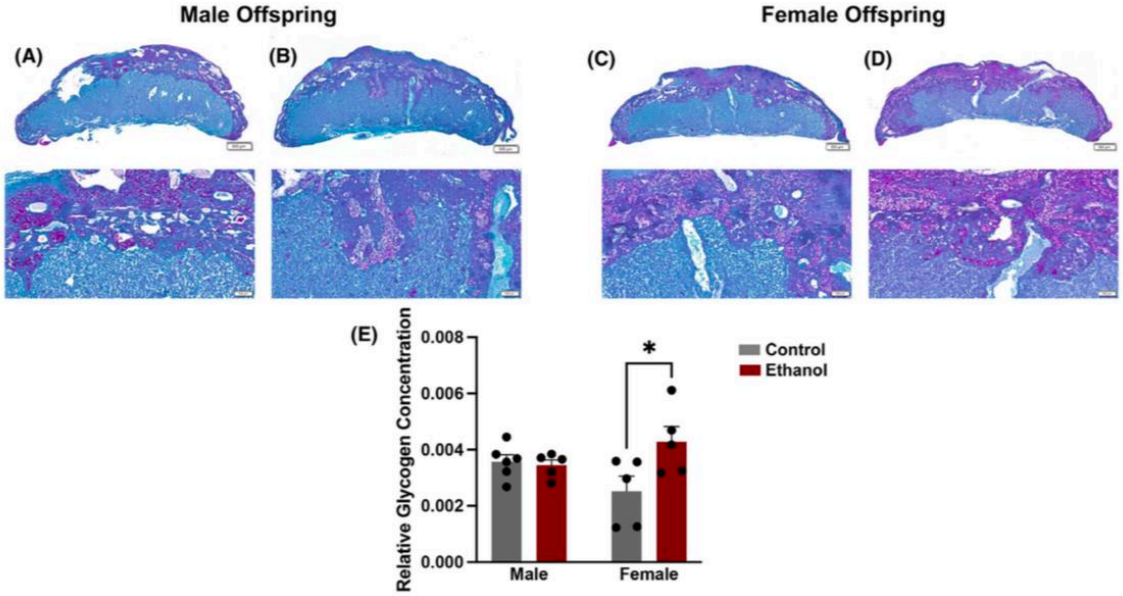

Figure 2.4. Sex-specific increases in placental glycogen in the offspring of alcohol-exposed males. We used the periodic acid Schiff stain to contrast placental histology between the offspring of control (A, C) and alcohol-exposed (B, D) sires. (E) Colorimetric quantification of placental glycogen between 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 [Reprinted from Thomas et al., 2021]

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 (Tunster et al., 2014, 2016). 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 2.5A). In contrast, female placentae exhibited significant reductions in the maternally expressed genes Cdkn1c, H19, and a modest decrease in Slc22a18 (Figure 2.5C). 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 (Coan et al., 2010; Tunster et al., 2016; Watkins et al., 2017), 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 2.5B). In contrast, females exhibited upregulation of Slc3a2 and downregulation of Slc38a2 (Figure 2.5D). We did not observe differences in Tpbpa expression in either male or female placentae.

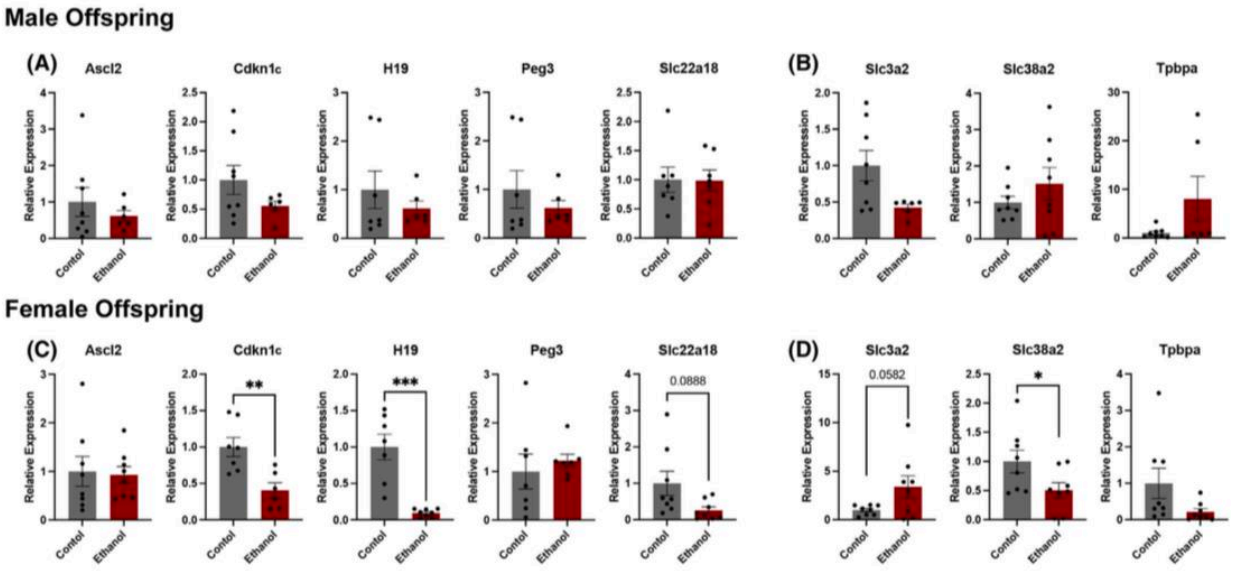

Figure 2.5. Preconception paternal alcohol exposure induces sex-specific changes in imprinted gene expression. Analysis of imprinted gene expression in the (A) male and (C) female offspring of control and alcohol-exposed sires. Expression analysis of key nutrient transporters between (B) male and (D) 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 [Reprinted from Thomas et al., 2021]

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 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 2.6A). In total, we identified 431 differentially expressed transcripts, with 256 upregulated and 175 downregulated genes (Figure 2.6B,C). Gene Enrichment Analysis using IPA identified alterations in the pathways regulating oxidative phosphorylation, mitochondrial function, EIF2, and Sirtuin signaling (Figure 2.6C). We recently described dysfunction of a similar set of pathways in the brains of mice exposed to alcohol in utero (Chang et al., 2021a), 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 2.6D). Interestingly, some of these candidate genes were also differentially expressed in placentae derived from the female offspring of alcohol-exposed sires (Figure 2.6E). These observations suggest the offspring of alcohol-exposed fathers may exhibit programmed alterations in mitochondrial function and oxidative phosphorylation.

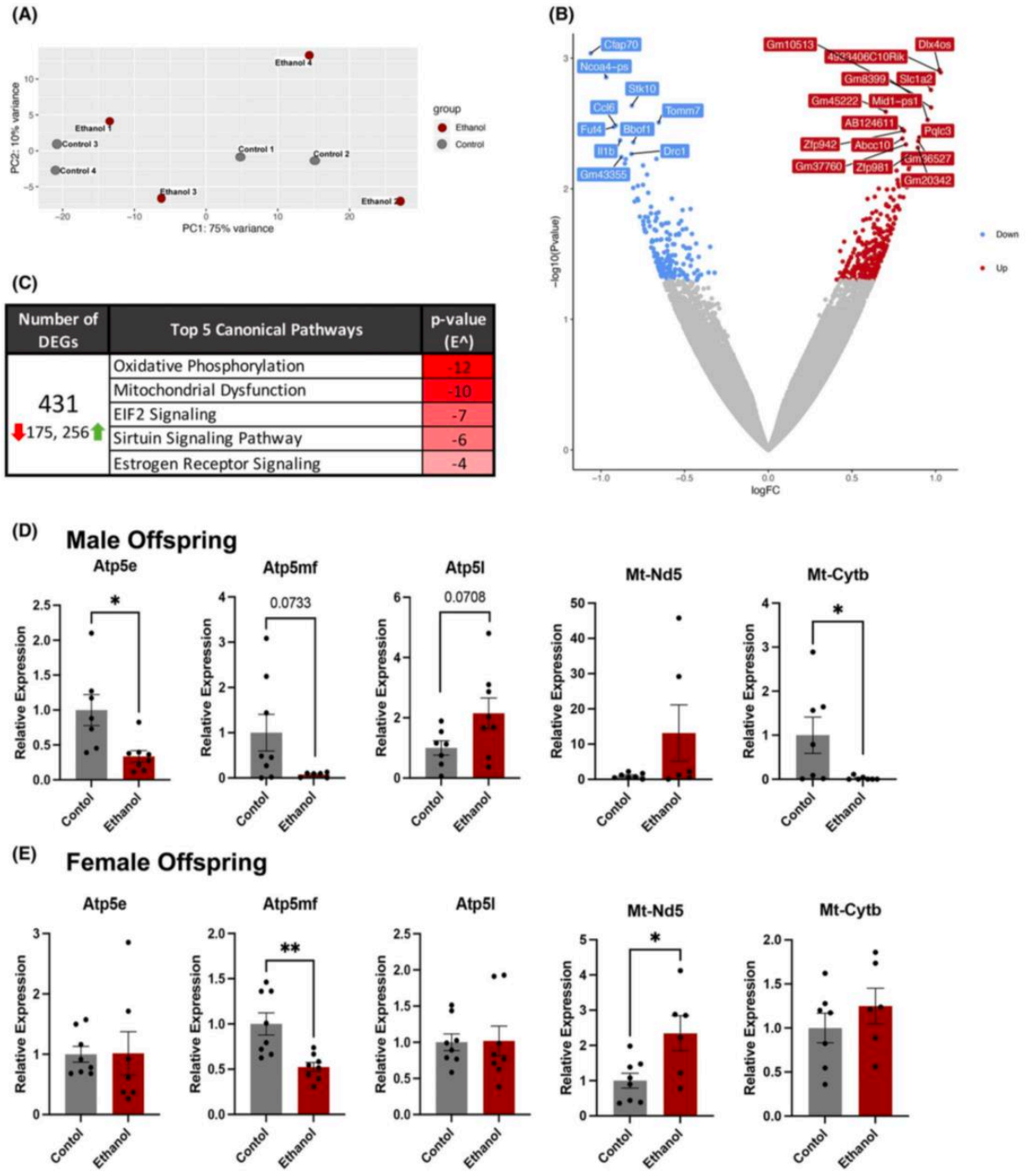

Figure 2.6. RNA-sequencing analysis of male placentae identifies paternally programmed alterations of genes regulating oxidative phosphorylation, mitochondrial function, and Sirtuin signaling. (A) Principle component analysis and (B) Volcano plot comparing the male placental transcriptomic profile between 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 [Reprinted from Thomas et al., 2021]

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 (Champroux et al., 2018). 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 (Downing et al., 2009; D. M. Gilliam & Irtenkauf, 1990; F. Gilliam et al., 1997; Sittig et al., 2011). Although previous studies have examined differing crosses between mouse strains for alcohol-induced, paternally inherited changes in offspring behavior (Rompala et al., 2017), 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 (Y. Bedi et al., 2019; Chang et al., 2017; Chang, Wang, et al., 2019). 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 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 pre-conception alcohol exposures induced an enlarged cross-sectional area of the placental junctional zone and an increased number of glycogen cells (Gårdebjer et al., 2014). 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 (Dackor, Caron, et al., 2009). 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 (Tunster et al., 2011). Similar placental adaptations are also observed in rodent models of nutritional stress (Coan et al., 2010; Pruis et al., 2015), which also exhibit remodeling of the junctional zone, reducing the mass of glycogen cells to adapt to the imposed nutritional deficits (Coan et al., 2010). 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 (Coan et al., 2005; Piedrahita, 2011). 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 (Li & Behringer, 1998; Tunster et al., 2014, 2016). 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 (Rossant & Cross, 2001). Glycogen cell deficiencies and

overabundance both associate with strain-specific patterns of fetal growth restriction (Tunster et al., 2014, 2016). 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 (Tunster et al., 2016). 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 (Gårdebjer et al., 2014). 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 (Kalisch-Smith et al., 2017; Rosenfeld, 2015). 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 (Chang, Wang, et al., 2019). As we and others find that CD-1 males refuse to consume ethanol, even at low percentages (Rhodes et al., 2007), 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 (Chang et al., 2021a). Epigenetic influences on mitochondrial function are an emerging area of interest in addiction and cancer biology (Dong et al., 2020; Sadakierska-Chudy et al., 2014). Importantly, mitochondrial genome enriched small RNAs (mitosRNAs) are present in sperm (Larriba et al., 2018). 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 (Rompala & Homanics, 2019), 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.

CHAPTER III

PATERNAL ALCOHOL EXPOSURES PROGRAM INTERGENERATIONAL HORMETIC EFFECTS ON OFFSPRING FETOPLACENTAL GROWTH*

1. Introduction

Fetal alcohol spectrum disorder (FASD) is a term that describes a broad range of presentations and disabilities attributed to the maternal consumption of alcohol during pregnancy (Cook et al., 2016). In the US, prevalence estimates range from 10 to 50 per 1,000 children, making FASDs the most predominant non-genetic birth defect associated with intellectual disability (Hoyme et al., 2016; May et al., 2018). Unfortunately, despite many years of study, we still lack a firm pathophysiological understanding of this condition and cannot fully explain the enormous variation in FASD phenotypes and prevalence (McCarthy & Eberhart, 2014; Schaefer & Deere, 2011).

While the risks of maternal alcohol use during pregnancy are well-established, emerging clinical studies indicate that paternal alcohol exposures also induce congenital disabilities and impact offspring behavior (Luan et al., 2022; Xia et al., 2018; Zhang et al., 2020). In support of these limited reports, recent preclinical studies reveal that paternal alcohol exposures negatively affect offspring phenotype and long-term health (Rompala & Homanics, 2019). For example, our group has identified short-term impacts of paternal alcohol exposure on fetoplacental growth and long-term influences on inflammation and glucose metabolism (Y. Bedi et al., 2019; Chang et al., 2017; Chang, Wang, et al., 2019; Thomas et al., 2021). Further, research by the Homanics and

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Huffman groups has independently associated preconception paternal alcohol exposures with blunted stress-related responses, altered preference for ethanol, abnormal sensorimotor activities, short-term motor learning impairments, and region-specific changes in gene expression within the brain (Beeler et al., 2019; Conner et al., 2020a; Finegersh et al., 2015; Finegersh & Homanics, 2014; Rathod et al., 2020; Rompala et al., 2017). These later studies have profound implications for the heritability of alcohol use disorders, while collectively, this work demonstrates that chronic male alcohol exposure impacts offspring development and, therefore, must be considered when assessing FASD outcomes.

Our efforts to determine how preconception paternal alcohol exposures affect offspring phenotype have focused on non-genomic, epigenetic mechanisms of inheritance transmitted through sperm, including DNA methylation, histone posttranslational modifications, and small noncoding RNAs. Our studies examining alcohol-exposed sperm identified negligible changes in the genome-wide DNA methylation profile (Chang et al., 2017). However, the Homanics group and our lab have independently identified ethanol-induced changes in sperm noncoding RNAs (Y. Bedi et al., 2019; Rompala et al., 2018, 2020). Although correlative, these data demonstrate that chronic alcohol exposures induce epimutations in sperm, which may negatively influence the developmental program of the offspring. Nevertheless, many questions remain, such as how alcohol influences epigenetic mechanisms in the male reproductive tract, which phases of germline programming represent critical windows, or what levels of ethanol consumption elicit changes in epigenetic programming in sperm.

The question of dose is intriguing as many direct physiological responses to alcohol are J-shaped, meaning lower doses exert distinct outcomes from higher exposures. For example, clinical studies indicate alcohol has a hormetic influence on coronary heart disease and ischemic stroke,

where lower consumption exerts protection beyond those who do not drink, while higher exposures enhance disease onset (Fernández-Solà, 2015). The term hormesis refers to the phenomena where low concentration toxicant exposures stimulate biological systems while higher concentrations inhibit these same processes, resulting in a biphasic response. Importantly, emerging research indicates that epigenetic mechanisms are partially responsible for hormetic programming and the beneficial effects of adaptive conditioning (Leak et al., 2018). Indeed, conditioning an ischemic-tolerant brain requires transcriptional repression by polycomb group (PcG) proteins, and suppressing this complex blocks the programmed gene expression underlying ischemic tolerance (Stapels et al., 2010). Although studies in insects, plants, and coral demonstrate the parental transmission of hormetic responses to their offspring (Brevik et al., 2018; Kishimoto et al., 2017; Putnam et al., 2020), there are no clear examples of intergenerational hormesis described using mammalian systems.

Our previous studies have correlated chronic paternal alcohol exposures with fetal growth restriction, placental overgrowth, and sex-specific changes in placental histology (Y. Bedi et al., 2019; Chang et al., 2017; Thomas et al., 2021). Throughout these studies, we have consistently observed an impact of paternal drinking on the placental weights of male offspring. Therefore, using gestational day 16.5 placental weights within the F1 male offspring as a phenotypic marker, we set out to examine the ability of ethanol to induce dose-dependent effects on the paternally inherited epigenetic program.

We began our studies by defining a clinically relevant range of low and modest exposure levels based on previous works examining the impacts of alcohol on central nervous system function (Eckardt et al., 1998). These previous studies revealed that ethanol concentrations within the range of 0.7–2.0 g/kg reproducibly impacted CNS function in C57BL/6 mice. Therefore, we

employed alcohol concentrations below, as well as at the lower and upper ends of this identified range. Using the previously determined average session fluid consumption of 0.017 g/g (Chang, Wang, et al., 2019), we administered concentrations (w/v) of 3% (0.5 g/kg, a concentration below the identified range), 6% (1.0 g/kg, lower end of the range), and 10% ethanol (1.7 g/kg, upper end of the range). We hypothesized that alcohol exposures falling within the established range would exert an intergenerational impact on placental growth, while the low concentration exposures below this established range would not. Unexpectedly, our studies demonstrate that paternal alcohol use programs nonlinear effects on fetoplacental growth, and significantly, even low dose alcohol exposures influence the sperm-inherited developmental program.

2. Materials and Methods

2.1. Animal husbandry and preconception male alcohol exposures

We used C57BL/6J mice (Strain #:000664 RRID: IMSR_JAX:000664), which we derived from a breeder nucleus housed in the Texas A&M Institute for Genomic Medicine. We housed mice under a reverse 12-h light/dark cycle, with lights out at 8:30 a.m. and lights on at 8:30 p.m. We maintained mice on a standard chow diet (catalog# 2019, Teklad Diets, Madison, WI, United States). We implemented additional animal husbandry measures to minimize stress, including shelter tubes for males and igloos for females (catalog# K3322 and catalog# K3570, Bio-Serv, Flemington, NJ, United States).

Before initiating the EtOH and Control preconception treatments, we acclimated male mice to individual housing conditions for 1week. After this acclimation period, we employed a modified version of the Drinking in the Dark model of voluntary alcohol consumption, initially described by Rhodes et al. (Rhodes et al., 2005). Beginning 3 h into the dark phase, we exposed postnatal day 90 male mice to one of four preconception treatments by replacing the water bottle of their home cage with a bottle containing either: 0% (Control), 3% (Low-concentration), 6% (Medium-concentration), or 10% (High-concentration) w/v ethanol (catalog# E7023; Millipore-Sigma, St. Louis, MO, United States). We simultaneously exchanged the water bottles of Control and ethanol-exposed males to ensure identical handling and stressors. We exposed males to these treatments for 4 h every day and maintained the treatments for 6weeks (preconception period, Figure 3.1A), which encompasses one complete spermatogenic cycle in mice (Adler, 1996). After the 6-week preconception exposure period, we began mating exposed males to naive dams but maintained the preconception treatments during this period. We bred exposed males to naive postnatal day 90 C57BL/6J females by first synchronizing the female reproductive cycle using the Whitten method

(Whitten et al., 1968), then placing the female in the male's home cage immediately after the male's daily exposure window. 6 h later, we confirmed matings by the presence of a vaginal plug and returned females to their original cages. We rested treated males for 72 h, during which the males continued their exposures and then used them again in a subsequent mating. The breeding window lasted 1–12 weeks (7–18 weeks total exposure), depending on the time required to generate the requisite number of litters for each treatment group.

2.2. Sire fluid consumption

We recorded treatment bottle and sire bodyweights weekly. We then quantified weekly fluid consumption by calculating the grams of fluid consumed divided by sire body weight (g/g). Subsequently, we determined the daily ethanol dose (g/kg) by multiplying the weekly fluid consumption (g/g) by the treatment group concentration (0.03, 0.06, or 0.10) and then divided this number by 7 days. Finally, for consistency with clinical studies (Leeman et al., 2010), we converted this number to grams per kilogram (g/kg).

2.3. Plasma alcohol concentration

After 3 weeks of exposure, we isolated plasma from a subset of exposed males at the end of the 4-h exposure window. We then measured plasma alcohol concentrations using the AM1 Alcohol Analyser (Analox Technologies, Toronto, ON, Canada), according to the manufacturer's protocol.

2.4. Fetal dissection and tissue collection

We marked gestational day (GD) 0 by the presence of a copulation plug and recorded female body weight. We diagnosed pregnancies on gestational day ten by an increase in bodyweight of at least

1.8 g. After diagnosing pregnancy, we added additional nesting material (two nestlets) to the female's home cage. We terminated dams on gestational day 16.5 using carbon dioxide asphyxiation followed by cervical dislocation. Subsequently, we dissected the female reproductive tract and recorded fetoplacental measures. We either fixed tissue samples in 10% neutral buffered formalin (catalog# 16004-128, VWR, Radnor, PA, United States) or snap-froze the tissues on dry ice and stored them at −80°C.

2.5. Fetal sex determination

We isolated genomic DNA from the fetal tail using the HotSHOT method (Truett et al., 2000) and determined fetal sex using a PCR-based assay described previously (Thomas et al., 2021).

2.6. Sire tissue collection

We terminated alcohol-exposed males using carbon dioxide asphyxiation and subsequent cervical dislocation. We dissected the testes, seminal vesicle, epididymal tract, liver, pancreas, and spleen, obtained tissue weights, and snap-froze on dry ice before long-term storage at −80°C.

2.7. Placental RNA isolation and RT-qPCR gene expression

We isolated RNA from gestational day 16.5 placentae using the Qiagen RNeasy Plus Mini Kit (cat# 74136, Qiagen, Germantown MD, United States) and seeded approximately one ʋg of isolated RNA into a reverse transcription reaction using the High-Capacity cDNA Reverse Transcription Kit (catalog# 4368814, Thermo-Fisher, Waltham, MA, United States). Using published methods (Thomas et al., 2021), we determined the relative levels of candidate gene transcripts using the Dynamo Flash SYBR qPCR kit (catalog#F-415XL: Thermo-Fisher, Waltham,

MA, United States). We describe the data normalization and handling procedures below; primer sequences are in Table 3.1.

Table 3.1. RT-qPCR primers. [Reprinted from Thomas et al., 2022]

Gene	Forward	Reverse
Ywhaz	TTGATCCCCAATGCTTCGC	CAGCAACCTCGGCCAAGTAA
Pgk1	AGATGCCAGGACCTGTATGCTT	TGTGCCAGGGTGGTGACTTTA
Slc22a18	TGATGTCCAGTGTGCTCCAT	AGAGTTCGGGTCAATGGTTG
Slc3a2	TGATGAATGCACCCTTGTACTTG	GCTCCCCAGTGAAAGTGGA
Slc38a2	ACTCATACCCCACCAAGCAG	CACAATCGCATTGCTCAGAT
Slc38a4	TGATTGGGATGTTAGTCTGAGG	GGCCTGGGTTAAAATGTGTG
Slc2a3	GATCGGCTCTTTCCAGTTTG	CAATCATGCCACCAACAGAG
Tpbpa	TGAAGAGCTGAACCACTGGA	CTTGCAGTTCAGCATCCAAC
Cdkn1c	AACGTCTGAGATGAGTTAGTTTAGAGG	AAGCCCAGAGTTCTTCCATCGT
H19	TGATGGAGAGGACAGAAGGGC	CTTGATTCAGAACGAGACGGACT
mPeg3	TTCTCCTTGGTCTCSCGGGC	AAGGCTCTGGTTGACAGTCGTG
Ascl2	TGCCGCACCAGAACTCGTAG	GCCTCGGTTGCTCCAGATC
Mt-ND5	CCTGGCAGACGAACAAGACAT	GGCGAGGCTTCCGATTACTA
Mt-Cytb	CAATCGTTCACCTCCTCTTCCT	GAGCGTAGAATGGCGTATGC
Mt-Co1	CAATAGTAGAAGCAGGAGCAGGAA	GTTTAGGTTGCGGTCTGTTAGTAGT
Mt-Nd1	ATTCTAATCGCCATAGCCTTCCT	TGGGTGTGGTATTGGTAGGGG
Atp5e	GACAGGCTGGACTCAGCTAC	CCCGAAGTCTTCTCAGCGTT
Atp5mf	CGGACACCAGGACTTCAAGAT	GGGACCCCTCTTCAGTGGA
Atp5l	TACTCGAAGCCTCGATTGGC	AGGGATTTCAGCAGGGGTTG

2.8. Placental histological analysis

To increase tissue contrast, we treated tissue samples with phosphotungstic acid (Lesciotto et al., 2020) and processed them for MicroComputed tomography (microCT) imaging using methods described previously (Thomas et al., 2021). We embedded treated tissues in a 50/50 mixture of polyester and paraffin wax to prevent tissue desiccation during scanning. We imaged treated samples using 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 mm3, with 95.2381 pixels/mm resolution. Finally, we used the open-source medical image analysis software Horos (Version 3.3.6; Nibble Co. LLC, Annapolis, Maryland, United States;

https://horosproject.org/) to quantify layer-specific volumes, as described previously (De Clercq et al., 2019; Thomas et al., 2021).

After tissue scanning, we removed the embedding wax, washed samples in PBS, and processed them for histological analysis using a TP1020 Automatic Benchtop Tissue Processor (Leica, Deer Park, IL, United States). After processing, we paraffin-embedded and sectioned the samples using an RM2255 Rotary Microtome (Leica, Deer Park, IL, United States) and stained slides using a Periodic Acid Schiff (PAS) Stain Kit (Mucin Stain) (catalog #ab150680, Abcam, Boston, MA, United States) following the manufacturer's provided protocol. We imaged samples using the VS120 Virtual Slide Microscope (Olympus, Waltham, MA, United States) and analyzed the images with the included desktop software, OlyVIA (Version 2.8, Olympus Soft Imaging Solutions GmbH, Muenster, Germany). Next, we quantified vascular space using methods described by Neres et al. (Neres et al., 2008). Briefly, using the OlyVia software package, we selected microscopic fields from the labyrinth, one central and one peripheral, each with a magnification of ×20 and a resolution of 19029 × 9029). Using Photoshop (Version 21.0.1, Adobe, San Jose, CA, United States), we standardized the levels, parameters, and saturation of each image and converted the color schemes to black and white, with the cyan color of the blood cells adjusted to black and surrounding tissue showing in gray. Using ImageJ (Version 1.53f51, Wayne Rasband and contributors, National Institutes of Health, United States), we set a threshold to cover the entire tissue area (the gray pixels), excluding open vascular spaces and the area of any blood cells remaining in the tissue. We then used ImageJ to calculate the percent area that was grey, which we subtracted from 100 to derive the percent area of negative (vascular) space (Neres et al., 2008).

2.9. Data handling and statistical analysis

All data generated during this study was subjected to a detailed data management plan that prioritizes safe and efficient data handling and allows long-term storage, retrieval, and preservation. We initially collected physiological measures by hand and then transcribed these data into Google Sheets or Microsoft Excel, where we collated and then transferred them into the statistical analysis program GraphPad Prism 8 (RRID:SCR_002798; GraphPad Software, Inc., La Jolla, CA, United States). For all analyses, we set the statistical significance at alpha = 0.05, used the ROUT test (Q = 1%) to identify outliers, and then verified the normality of the datasets using the Shapiro–Wilk test. If data passed normality (alpha = 0.05), we then employed either an ANOVA 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 measures of fetoplacental growth, we determined the male and female average for each litter and used this value as the individual statistical unit. We calculated placental efficiency (Hayward et al., 2016) by dividing fetal weight by placental weight, then deriving the male and female average for each litter. For offspring organ weights and the analysis of placental histology, we randomly selected male and female offspring from each litter and used measures of these samples as the statistical unit. Graphical depictions of data represent the mean and standard error of the mean. We provide detailed descriptions of each statistical test in Table 3.2.

2.10. RT-qPCR analysis of placental gene expression

We imported the replicate cycle threshold (Ct) values for each transcript into Excel and normalized the expression to the geometric mean of two reference genes. These included transcripts encoding Phosphoglycerate kinase 1 (Pgk1) and 3-monooxygenase/tryptophan 5-monooxygenase activation

protein zeta (Ywhaz). We used the −ΔΔCT Method (Schmittgen & Livak, 2008) to calculate the relative fold change for each replicate. After combining datasets in excel, we input the values into the statistical analysis program GraphPad Prism eight and identified outliers using the ROUT outlier test (Q = 1%). Next, we verified all datasets for normality using the Shapiro-Wilk test. If data passed normality (alpha = 0.05), we employed a One-Way ANOVA or, if the standard deviations were significantly different (Browne-Forsythe), a Welch ANOVA. If normality failed, we performed a non-parametric Kruskal-Wallis test.

Table 3.2 Statistical analyses associated with each figure. [Reprinted from Thomas et al., 2022]

Graph	Statistical test	Sample size	Outliers
Figure 1: Experimental design for paternal alcohol exposure			
B. Sire fluid consumption by treatment week D. Sire body weight by treatment week	Two-way ordinary ANOVA, multiple comparisons using uncorrected Fisher's LSD, only comparisons to Control were performed	n = 17 Control, 13 low, 12 Medium, 13 High	0
C. Sire plasma alcohol levels	One-way ordinary ANOVA, multiple comparisons using uncorrected Fisher's LSD	n = 3 Low, 4 Medium, 5 High	0
Figure 2: Sire physiological data			
A-F. Testes, seminal vesicle, epididymal tract, liver, pancreas, spleen normalized to body weight G. Litter size	We inserted organ weights into Excel and combined the weights for paired (eg., left right testis) tissues, then divided by total body weight One-way ordinary ANOVA, multiple comparisons using uncorrected Fisher's LSD	A-C. n = 22 Control, 12-25 Low, 15 Medium, 18 High D-F. n = 22 Control, 9 Low, 10-15 Medium, 17 High G. n = 22 Control, 25 Low, 15 Medium, 18 High	0
Figure 3. Fetal and placental physiological data			
A-G. Gestational sac weight, crown-rump length, fetal weight, placental weight, placental diameter, placental efficiency, brain to body weight	Two-way ordinary ANOVA, multiple comparisons using uncorrected Fisher's LSD, only comparisons to Control were performed	A-D/F. Males: n = 20 Control, 24 Low, 15 Medium, 18 High; Females: n = 21 Control, 25 Low, 15 Medium, 18 High [n = litter average] E. n = 15 Control, 13 Low, 11 Medium, 17 High [n = litter average] G. Males: n = 28 Control, 33 Low, 15 Medium, 16 High; Females: n = 29 Control, 51 Low, 19 Medium, 17 High [n = fetus]	A-C & E-G. 0. D. Male: 2 Control, 1 Low, 1 High
Figure 4. Drinking types within the 10% (High) treatment group			
A. Fluid consumption determined drinking types within High group	Split population along average consumption of 0.157 g/g One-way ANOVA, multiple comparisons using uncorrected Fisher's LSD	n = 22 Control, 9 Moderate, 9 Heavy	0
B. Placental weights for male and female fetuses by sire drinker type	Two-way ANOVA, multiple comparisons using uncorrected Fisher's LSD	n = 22 Control, 9 moderate,9 heavy [n = litter average]	Male: 2 Control, 2 Heavy; Female: 1 Control, 2 Moderate
C. Male placental weight vs. Average fluid consumption	Simple linear regression and two-tailed Pearson correlation	n = 18 [n = litter average]	0
Figure 5. Nonlinear models examining the relationship between paternal dose and placental and fetal phenotypes			
A. Male and B. female log transformed relative average placental weight compared to sire daily ethanol dose	We normalized placental weights to the Control average, then log-transformed the average relative placental weights for each litter (dependent variable) and graphed these against the paternal dose of EtOH. Non-linear regression using fourth order polynomial model with least squares regression; Diagnostics: R squared and Runs test	n = 29 [n = averaged individuals at each absolute daily dose]	0
C. Male and female average relative placental weight and D. crown-rump length compared between upper and lower thresholds (inflection points)	Two-way ordinary ANOVA, multiple comparisons using uncorrected Fisher's LSD	n = 10 lower (< 1.0) and 5 upper (> 2.4) [n = averaged individuals at each absolute daily dose]	0
E. Male and F. female average relative crown-rump length compared to sire daily ethanol dose	Non-linear regression using fourth order polynomial model with least squares regression; Diagnostics: R squared and Runs test	n = 29 [n = averaged individuals at each absolute daily dose]	0
Figure 6. Placental histological analysis			
B-E, H-I. Chorion, decidua, junctional zone, labyrinth, junctional zone to decidua, and labyrinth to junctional zone	Two-way ANOVA, multiple comparisons using uncorrected Fisher's LSD	Males: n = 21 Control, 43 Low, 9 Medium, 11 High; Females: n = 18 Control, 42 Low, 9 Medium, 9 High	B, D-E, & I. 0 C. Male: 1 Low; Female: 1 Control H. Male: 1 Low
F/G. Male Dose Response Decidua and Labyrinth	Simple linear regression and two-tailed Pearson correlation	n = 13 [n = averaged individuals at each absolute daily dose]	0
Figure 7. Placenta-Heart Analysis			
J. Male and female central and peripheral labyrinth blood spaces	Three-way (sex, location, treatment) ordinary ANOVA, multiple comparisons using uncorrected Fisher's LSD	Males: 8 Control, 8 Low, 6 High Females: 8 Control, 8 Low, 7 High	0
K. Heart to body weight	Two-way ordinary ANOVA, multiple comparisons using uncorrected Fisher's LSD, only comparisons to Control were performed	Males: n = 13 Control, 16 Low Females: n = 11 Control, 25 Low	0

Figure 8. Placental gene expression analysis

Gene	Statistical Test	Experimental N Outliers		
		Control	Low	High
Ascl2	M: Welch; F: ANOVA	M7; F8 0	M7; F7 0	M8; F8 M3; F0
Cdkn1c	M: Welch; F: ANOVA	M 8; F8 0	M7; F8 0	M8; F7 0
Slc22a18	M & F: Welch	M8; F8 M0; F1	M7; F7 M0; F0	M8; F8 M0; F1
Slc3a2	M: ANOVA; F: Kruskal—Wallis	M7; F7 M1; F0	M8; F7 M0; F0	M8; F8 M3; F0
Tpbpa	M: Welch; F: ANOVA	M7; F6 M1; F0	M7; F7 M0; F1	M8; F8 M0; F0
Mt—Cytb	M: ANOVA; F: Kruskal—Wallis	M7; F8 M0; F1	M7; F7 M2; F2	M8; F8 0
H19	M: ANOVA; F: Welch	M7; F8 0	M7; F7 0	M7; F8 0
mPeg3	F: Kruskal—Wallis	M7; F8 0	M7; F7 0	M7; F8 0
Mt—Nd5	M & F ANOVA	M8; F7 0	M8; F5 0	M8; F8 0

3. Results

3.1. A limited voluntary access model to examine concentration-dependent effects of chronic paternal alcohol exposure

In our previous studies utilizing a prolonged version of the Drinking in the Dark model, male C57BL/6J mice consumed an average of 0.12 g/g/week (Chang, Wang, et al., 2019). These published studies utilized a 10% (w/v) mixture of alcohol and 0.001% Sweet'N Low® to increase palatability. In the current study, we eliminated the Sweet n Low and administered 3, 6, and 10% (w/ v) ethanol (EtOH) solutions. We exposed Control males to water alone and ensured identical handling by concurrently switching between two identical water bottles. In addition, we strictly enforced consumption rates and eliminated males that dropped below a weekly fluid consumption of 0.08 g/g/week, one standard deviation below the previously identified average, for more than three consecutive weeks. In total, we eliminated 0 Low-concentration (3%), 1 Medium-concentration (6%), and 10 High-concentration (10%) males.

We exposed males to the preconception treatments for 6 weeks, encompassing one complete murine spermatogenic cycle (Adler, 1996) (Figure 3.1A). We did not observe any differences in fluid consumption between treatment groups during the preconception treatment period. However, at week ten, during the breeding phase (weeks seven to fifteen), males in the Low-concentration group consumed more fluid than the Control treatment ($p = 0.0334$), while Medium-concentration males consumed more than the High- concentration group ($p = 0.0428$), but not Controls (Figure 3.1B). After 3 weeks of exposure, we collected blood at the end of the 4-h exposure window and measured plasma alcohol levels. We observed an average of ~50 mg/dl in the Low-concentration group, ~150 mg/ dl in the Medium-concentration group, and ~100 mg/dl in the High-concentration group (Figure 3.1C). The plasma alcohol levels we observed in the

Medium- and High-concentration groups were not significantly different and fell within the range previously identified using the Drinking in the Dark model (100–160 mg/dl) (Rhodes et al., 2005; Thiele et al., 2014). Interestingly, plasma alcohol concentrations in the Low-concentration group were significantly lower than those in the Medium-concentration males and below levels observed in previous studies using this same exposure model. We speculate that differences in the palatability of the different concentrations lead to changes in consumption patterns and resulting plasma alcohol concentrations. However, as stress is known to modify the paternally-inherited epigenome (Chan et al., 2018), we subjected males to minimal handling and only measured plasma alcohol concentrations once. During the breeding phase, weeks seven to fifteen, we observed a significant decline in the bodyweights of males within the High-concentration treatment group (Figure 3.1D). We did not observe differences in weekly body weights between any other treatment groups.

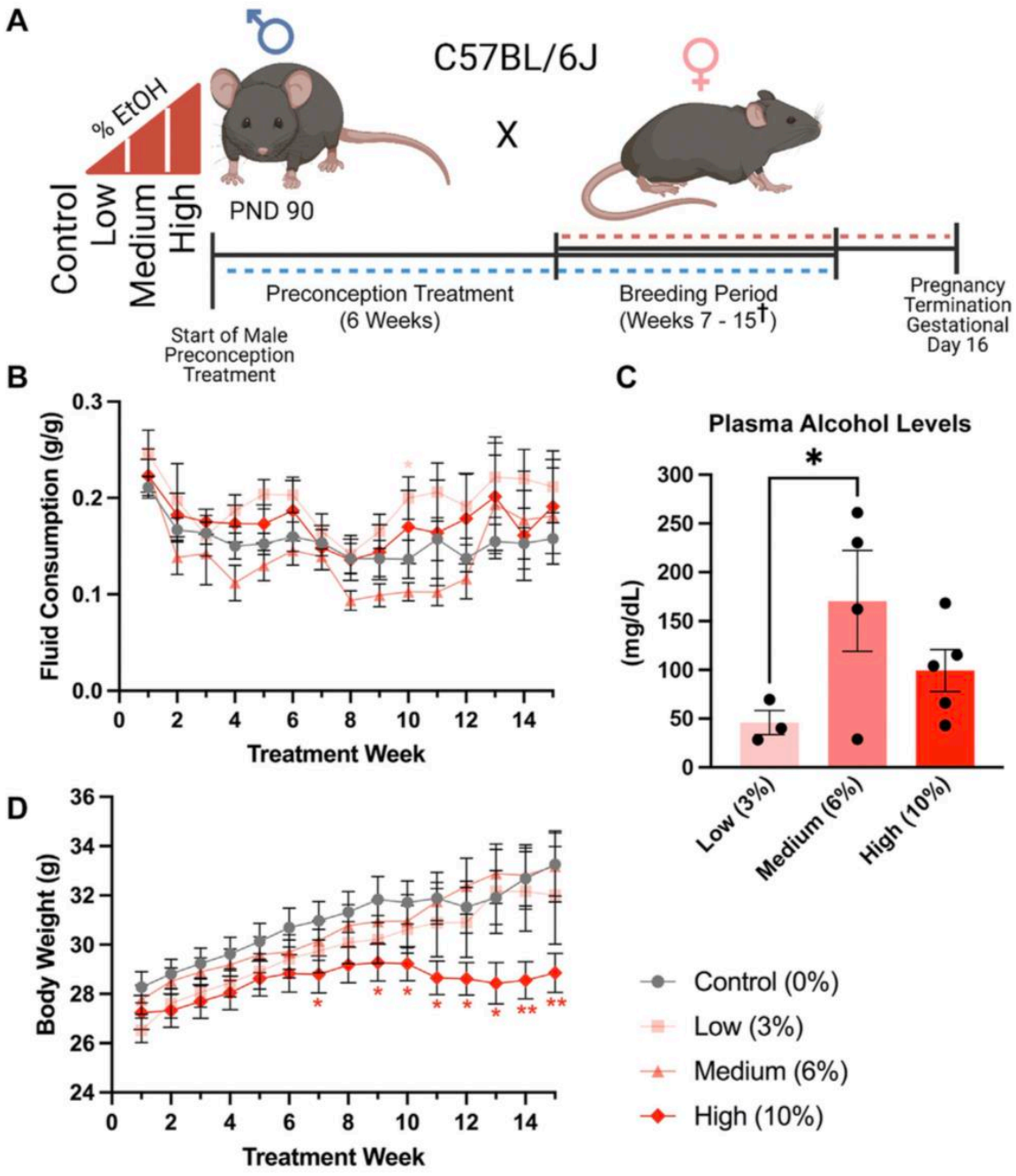

Figure 3.1. A limited access model to study concentration-dependent, ethanol-induced changes in paternal epigenetic programming. (A) Schematic outline of the experimental approach used to model the impacts of increasing ethanol concentrations on offspring fetoplacental growth. The preconception exposure period lasted 6 weeks, with exposures continuing during the breeding phase, which lasted from weeks seven and fifteen († one Control, two Medium- and two High-concentration litters were sired between weeks sixteen and eighteen). (B) Fluid consumption patterns compared between the different treatment groups (n = 17 Control, 13 Low, 12 Medium, 13 High). (C) Comparison of average plasma alcohol levels between treatment groups, measured after 3 weeks of exposure, at the end of the daily treatment window (n = 3 Low, 4 Medium, 5 High). (D) Comparison of sire body weight throughout the 10-week preconception exposure window (n = 17 Control, 13 Low, 12 Medium, 13 High). We used one-way (C) and two-way ANOVAs (B,D) to assay differences between treatment groups. Error bars represent the standard error of the mean, *p < 0.05, **p < 0.01. [Reprinted from Thomas et al., 2022]

The average time to conception was ~4 weeks (10 weeks of total exposure) for the Control and Low-concentration treatments and 5.2 and 5.8 weeks for the Medium- and High-concentration treatments (11.2 and 11.8 weeks of total EtOH exposure). At week 15, we sacrificed the exposure cohort, with the exception of five males (1 Control, 2 Medium- and 2 High-concentration), which required up to three more weeks to sire litters. At sacrifice, we dissected Control and EtOH-exposed sires and examined physiologic measures of their general and reproductive health. We did not observe any differences in the normalized weights of the testes, seminal vesicles, or epididymides, nor any changes in the liver, pancreas, and spleen (Figures 3.2A–F). In addition, consistent with previous studies, none of the examined alcohol treatments impacted litter size (Figure 3.2G). Based on these data, we conclude that none of the examined EtOH treatments negatively impact large-scale measures of male reproductive physiology.

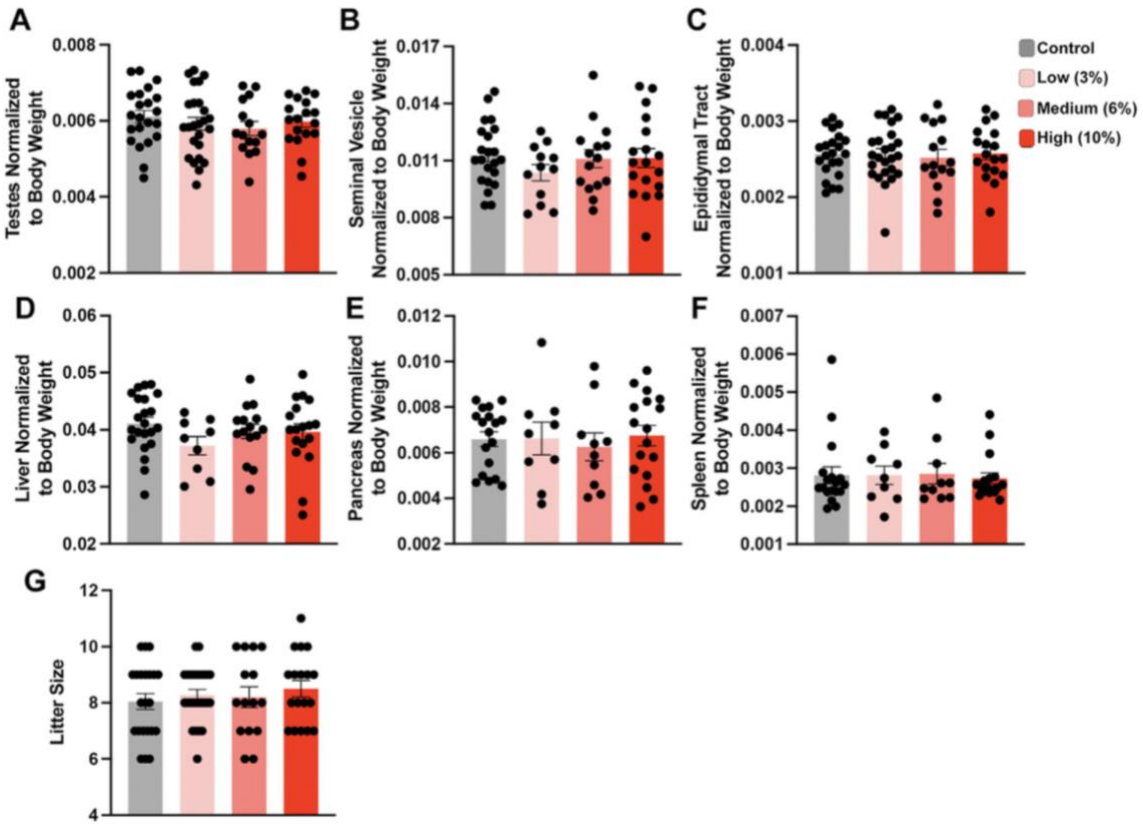

Figure 3.2. Chronic paternal ethanol exposures do not impact macro measures of male reproductive health or fertility. Paternal preconception alcohol exposures do not impact large-scale measures of male reproductive physiology, including normalized (A) testis, (B) seminal vesicle, or (C) epididymal track weights (n = 22 Control, 12-25 Low, 15 Medium, 18 High). Chronic male ethanol exposure does not impact normalized (D) liver, (E) pancreas or (F) spleen weights (n = 22 Control, 9 Low, 10-15 Medium, 17 High). (G) Chronic preconception paternal alcohol exposures do not influence offspring litter size (n = 22 Control, 25 Low, 15 Medium, 18 High litters). To assay changes between treatment groups, we used a one-way ANOVA. Error bars represent the standard error of the mean. [Reprinted from Thomas et al., 2022]

3.2. Chronic low- and medium-concentration preconception ethanol exposures impact offspring fetoplacental growth

We next examined the intergenerational impact preconception paternal exposure to varying concentrations of EtOH has on fetoplacental growth by assessing offspring physiologic measures at gestational day 16.5 (GD16.5). This gestational phase represents a period where placental weights have reached their maximum, and fetal growth is exponential (Mu et al., 2008). Further, we have previously identified EtOH- induced placental overgrowth and fetal growth restriction at this stage (Y. Bedi et al., 2019; Chang et al., 2017; Thomas et al., 2021). Using a one-way ANOVA, we did not find any differences in gestational day 16.5dam weights normalized to litter size (data not shown). Next, using a two-way ANOVA, we contrasted male and female physiologic measures of offspring growth across treatment groups. Unexpectedly, offspring derived from sires in the Low-concentration treatment group exhibited increased average gestational sac weights, crown-rump lengths, and placental weights (Figure 3.3A–F). However, the differences in crown-rump length and placental weights only appeared in the male offspring. In contrast, the offspring of Medium- concentration sires only displayed increased male placental weights, while we did not observe differences in any parameters measured in offspring derived from the High- concentration treatment group. Next, we wondered if an extended exposure duration could drive a progressive increase in litter average placental weights. To address this question, we combined the Low- and Medium-concentration datasets and compared measures before and after 11 weeks, the mean time

to conception for both groups. However, this comparison did not reveal any significant differences in placental growth (data not shown).

Recent studies examining periconceptional maternal exposures suggest that EtOH may impact brain weights in the developing offspring (Steane et al., 2021). Further, increased proportional brain to body weight is a reliable indicator of intrauterine growth restriction (IUGR) (Cortes-Araya et al., 2022). Therefore, we examined the ratio of brain to total bodyweight in the male and female offspring. These analyses revealed that both male and female offspring from sires within the Medium-concentration treatment group exhibited increased normalized brain weights (Figure 3.3G). However, we did not observe any differences in normalized brain weights in offspring derived from sires in the Low and High-concentration treatment groups. These observations collectively indicate that preconception paternal EtOH exposure impacts multiple aspects of fetoplacental growth and suggests that several measures may exhibit concentration-specific outcomes.

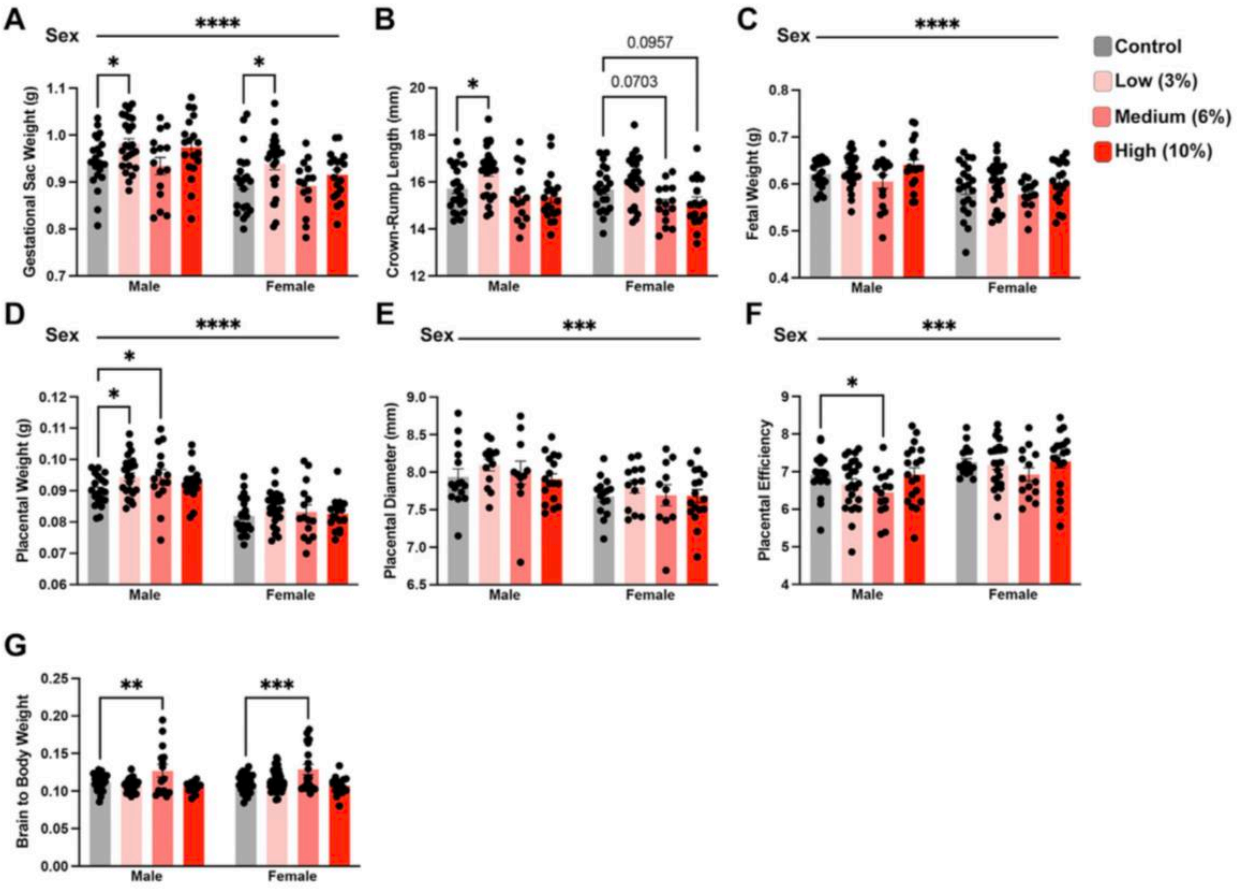

Figure 3.3. Paternal preconception alcohol exposure induces concentration-dependent changes in offspring fetoplacental growth. For all measures of fetoplacental growth, we used the male and female average for each litter as the individual statistical unit. Comparison of litter average (A) gestational sac weight, (B) crown-rump length, (C) fetal weight, (D) placental weight, (E) placental diameter, and (F) placental efficiency between offspring sired by males across treatment groups (A–D/F. n = litter average with males: 20 Control, 24 Low, 15 Medium, 18 High litters; females: 21 Control, 25 Low, 15 Medium, 18 High litters; (E) n = litter average with males and females: 15 Control, 13 Low, 11 Medium, 17 High litters). (G) Comparison of normalized brain weights in the offspring of alcohol-exposed males across treatment groups (n = fetus, randomly selected from each litter, males: 28 Control, 33 Low, 15 Medium, 16 High; females: n = 29 Control, 51 Low, 19 Medium, 17 High). We used either a two-way ANOVA to contrast the impacts of sex and preconception treatments or Brown-Forsythe and Welch's one-way ANOVA. Sex differences are indicated above the figures, while treatment effects are demarcated directly above the bar graphs. Error bars represent the standard error of the mean, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. [Reprinted from Thomas et al., 2022]

3.3. High-concentration paternal alcohol exposures induce dose-dependent effects on placental growth

Given the consistent impact of preconception paternal 10% w/v EtOH exposures on placental weights observed previously (Y. Bedi et al., 2019; Chang et al., 2017; Chang, Wang, et al., 2019; Thomas et al., 2021), we were surprised that we did not detect increased placental growth within offspring sired by males in the High-concentration treatment group. In analyzing sire fluid consumption data, we noted that, although similar to the Controls in the present study, the High-concentration treatment group drank more than males in our previous studies. Further, we also observed larger than anticipated within-group variation. The average fluid consumption level for Control males was 0.157 g/g. Therefore, we split the High-concentration sires into two populations, one below 0.157 g/g (moderate drinkers) and one above (heavy drinkers), and then compared fluid consumption between these males. This comparison revealed significant differences in fluid consumption between heavy and moderate drinkers (Figure 3.4A). Importantly, placental weights of male offspring sired by moderate drinkers were significantly higher than Controls, but those of heavy drinkers were not (Figure 3.4B). To further test the relationship between sire fluid consumption and offspring placental weights, we conducted a Pearson correlation analysis, which revealed increased paternal EtOH exposure correlated with a decline in both male (Figure 3.4C; $r = -0.6041$, p-value = 0.0062) and female ($r = -0.5773$, p-value = 0.0096; data not shown) placental weights. Significantly, these observations reveal that males within the intended treatment groups received different doses of alcohol and that paternal EtOH exposure may induce dose-dependent effects on placental growth.

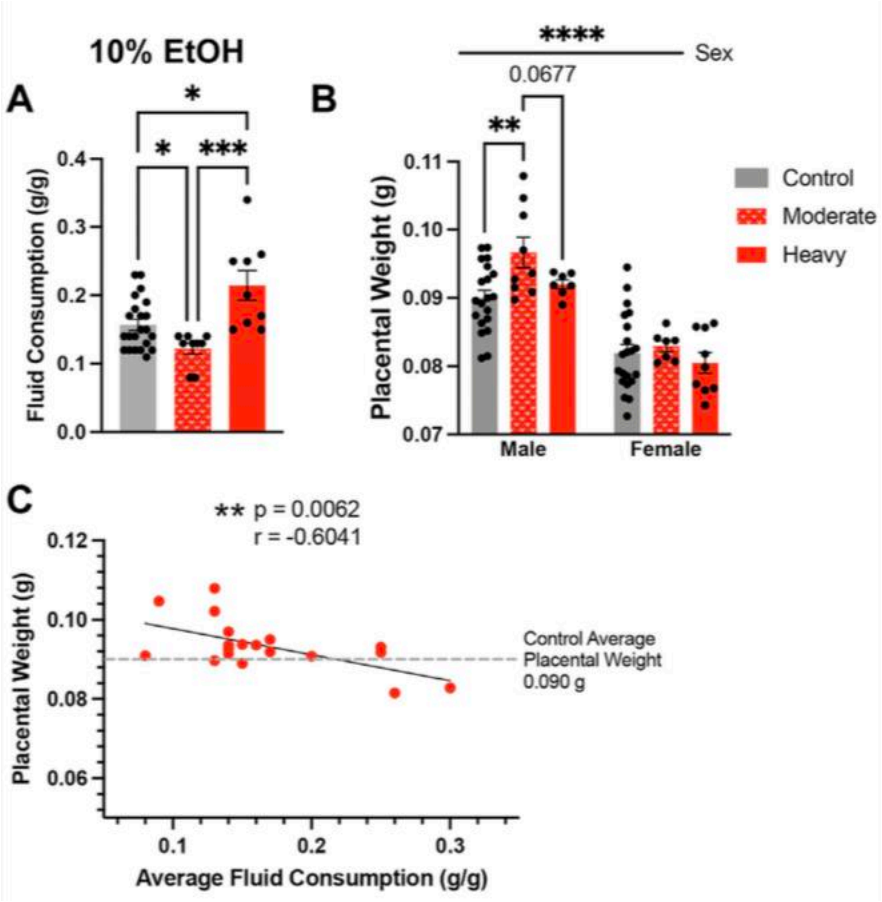

Figure 3.4. High-concentration sires display significant variation in alcohol consumption, which associates with differing effects on placental growth in the male offspring. (A) Comparison of average fluid consumption between the bottom and top consuming males within the High-concentration treatment group (One-way ANOVA, n = 22 Control, 9 Moderate, 9 Heavy). (B) Increased average placental weights in offspring sired by moderate but not heavy drinking males within the High-concentration treatment group (n = litter average with 22 Control, 9 Moderate, and 9 Heavy litters). We used a two-way ANOVA to compare average placental weights between the male and female offspring of Control, Moderate, and Heavy drinking sires. (C) Pearson correlation analysis contrasting male offspring litter average placental weights and average paternal fluid consumption across the High- concentration treatment group (n = 18 litters). Error bars represent the standard error of the mean, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. [Reprinted from Thomas et al., 2022]

3.4. Chronic paternal ethanol exposures induce dose-dependent, hermetic effects on offspring fetoplacental growth

In addition to the variation in weekly fluid consumption observed across all treatment groups, we also observed significant differences in the within-group standard deviations of male and female placental weights ($p < 0.05$). This increased variation suggested a more complex interplay between paternal EtOH exposure and placental growth. As we could correlate increased paternal EtOH consumption with decreasing placental weights in the High-concentration group, we examined this correlation across all EtOH treatment groups. To test this, we first calculated the absolute daily dose of alcohol consumed in grams EtOH per kilogram body weight (see Materials and Methods). However, when examining the offspring of all three original treatment groups, we did not observe a linear relationship between paternal EtOH dose and placental weight ($R2 < 0.05$, Runs test $p < 0.001$). Numerous studies indicate that EtOH exhibits a nonlinear, J-shaped effect on many physiologic systems, with low doses exerting distinct toxic effects, hazard ratios, and neurological outcomes from higher doses (Fernández-Solà, 2015; Kalev-Zylinska & During, 2007; Lundgaard et al., 2018; Sureshchandra et al., 2019; Velázquez-Marrero et al., 2011). Based on these previous studies, we log-transformed the relative average placental weights for each litter (dependent variable) and graphed these against the paternal dose of EtOH. These analyses identified a sine-like relationship, or inverse J-curve, where lower levels of paternal EtOH consumption are associated with increased normalized placental weights. In contrast, relative placental weights decline at higher doses, eventually dropping below the Control group (male $p = 0.0021$, female $p = 0.0317$; Figures 5A,B; data not shown). Using a fourth-order polynomial model, we identified a plateau or upper threshold at ~1 g/kg daily dose and an inflection point at ~2.4 g/kg per day, past which increasing paternal alcohol doses induced a decline in placental weights (Figures 3.5A,B).

We then separated and compared fetoplacental measures below and above these identified points. These comparisons identified threshold-dependent differences in normalized litter average placental weights for male and female offspring (Figure 3.5C). Fetal crown-rump lengths were significantly different in males and showed a similar trend in female offspring (Figure 3.5D). We did not observe dose- dependent differences in litter average relative fetal weights for either sex (data not shown). Finally, we developed a nonlinear regression model to compare relative crown-rump length and sire ethanol dose for male (Figure 3.5E) and female offspring (Figure 3.5F). These analyses revealed that increases in fetal crown-rump length primarily correlate with increasing paternal EtOH exposures between doses ~0.8–1.3 g/kg/day, past which we do not observe any impacts. Our studies reveal that paternal EtOH exposure induces nonlinear, dose-dependent changes to offspring placental growth and that low-level paternal exposures associate with male-biased increases in crown-rump length.

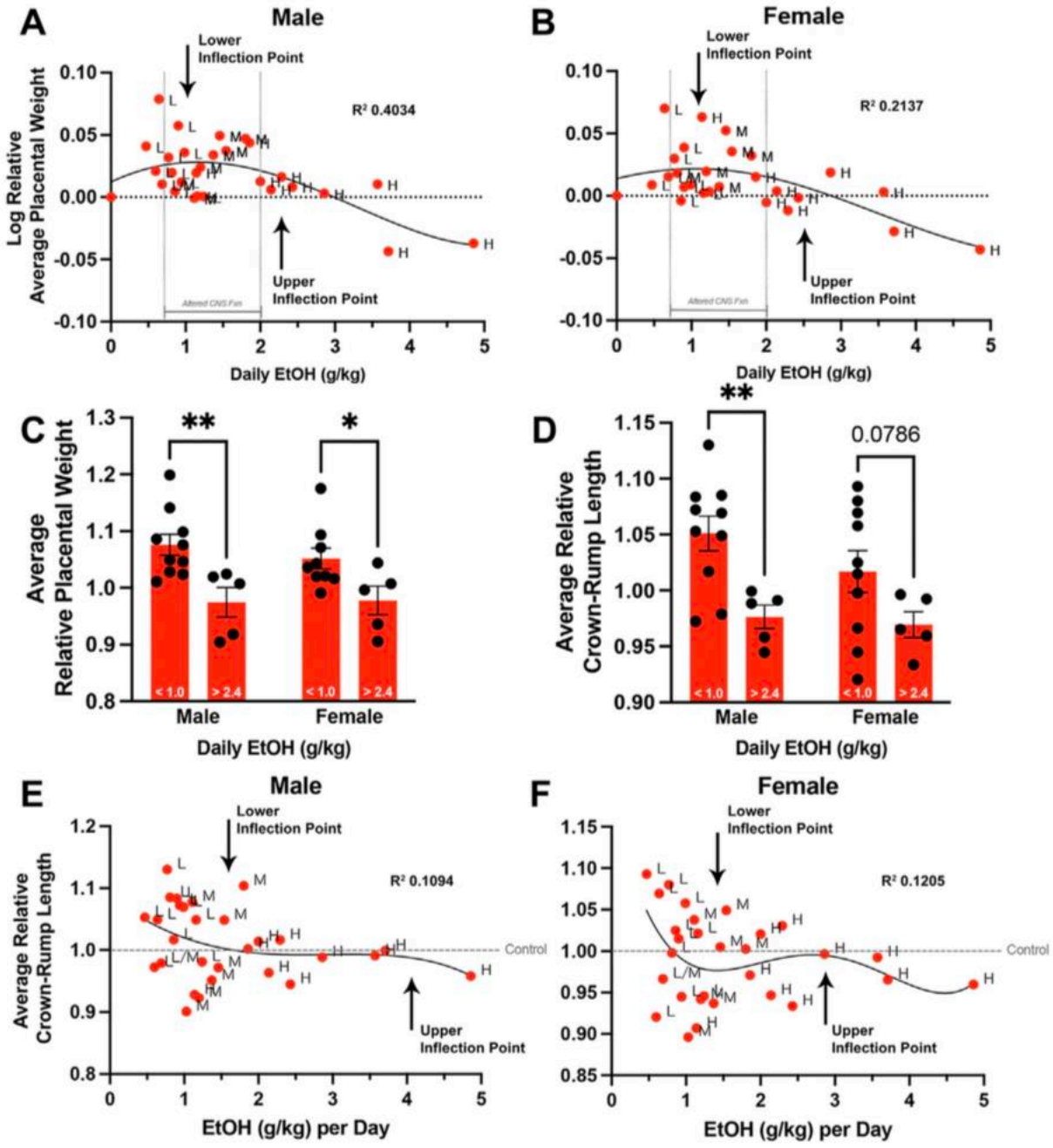

Figure 3.5. Chronic preconception paternal ethanol exposures induce biphasic, dose-dependent effects on offspring fetoplacental growth. We used nonlinear regression to compare the sire's daily ethanol dose to the log-transformed, average relative placental weights of (A) male and (B) female offspring. (n = 29 averages across each absolute daily dose). Comparison of male and female (C) average relative placental weight and (D) average relative crown-rump lengths between offspring sired by males below (< 1.0 g/kg) and above (> 2.4 g/kg) the identified inflection points (two-way ANOVA with n = 10 lower-dose and 5 upper-dose). Nonlinear regression comparing sire daily ethanol dose to (E) male and (F) female average relative crown-rump lengths (n = 29 averages across each absolute daily dose). We used a fourth-order polynomial model with least squares regression to identify inflection points. We eliminated outliers at Q = 1% and verified model fit using R squared analyses combined with Runs testing. Error bars represent the standard error of the mean, *p < 0.05, **p < 0.01. [Reprinted from Thomas et al., 2022]

3.5. Chronic paternal alcohol exposures exert does-dependent changes on the histological organization of the placenta

Using an outbred mouse model, we previously observed that preconception male EtOH exposure induces female-specific changes in the histological organization of the placenta (Thomas et al., 2021). To determine if we could identify concentration-specific changes in placental histology, we used microCT imaging to quantify the different placental layers (De Clercq et al., 2019) (Figure 3.6A). Using phosphotungstic acid to enhance tissue contrast, we determined the proportional volumes of the chorion, labyrinth, junctional zone, and maternal decidua in placentae derived from the offspring of Control and EtOH-exposed sires. These experiments revealed that both the male and female offspring of Low- and Medium-concentration sires exhibited reductions in the chorion, while placentae derived from sires in the High-concentration treatment group did not (Figure 3.6B). Interestingly, we identified progressive, concentration-dependent reductions in the maternal decidua, which were accompanied by increases in the labyrinth layer, but only in the male offspring of EtOH- exposed sires (Figures 3.6C–E). For male placentae, Pearson correlation analysis confirmed linear, dose-dependent decreases in the volume of the decidua ($R2 = 0.4877$, p-value 0.0079), offset by dose-dependent increases in the volume of the labyrinth layer ($R2 = 0.7269$, p-value 0.0183) (Figures 3.6F,G). We did not observe any significant dose-response correlations in the proportional volumes of female placentae (decidua p-value 0.1028, labyrinth p-value 0.2110). Additionally, the male offspring of Low-concentration sires exhibited a modest increase in the junctional zone (Figure 3.6D). When we compared proportional ratios of each layer, we identified differences in the ratio of the junctional zone and decidua but not the proportional ratio of the junctional zone and labyrinth (Figures 3.6H,I), suggesting the endocrine component of

the placenta may remain constant while the region responsible for maternofetal exchange expands with increasing paternal EtOH exposures.

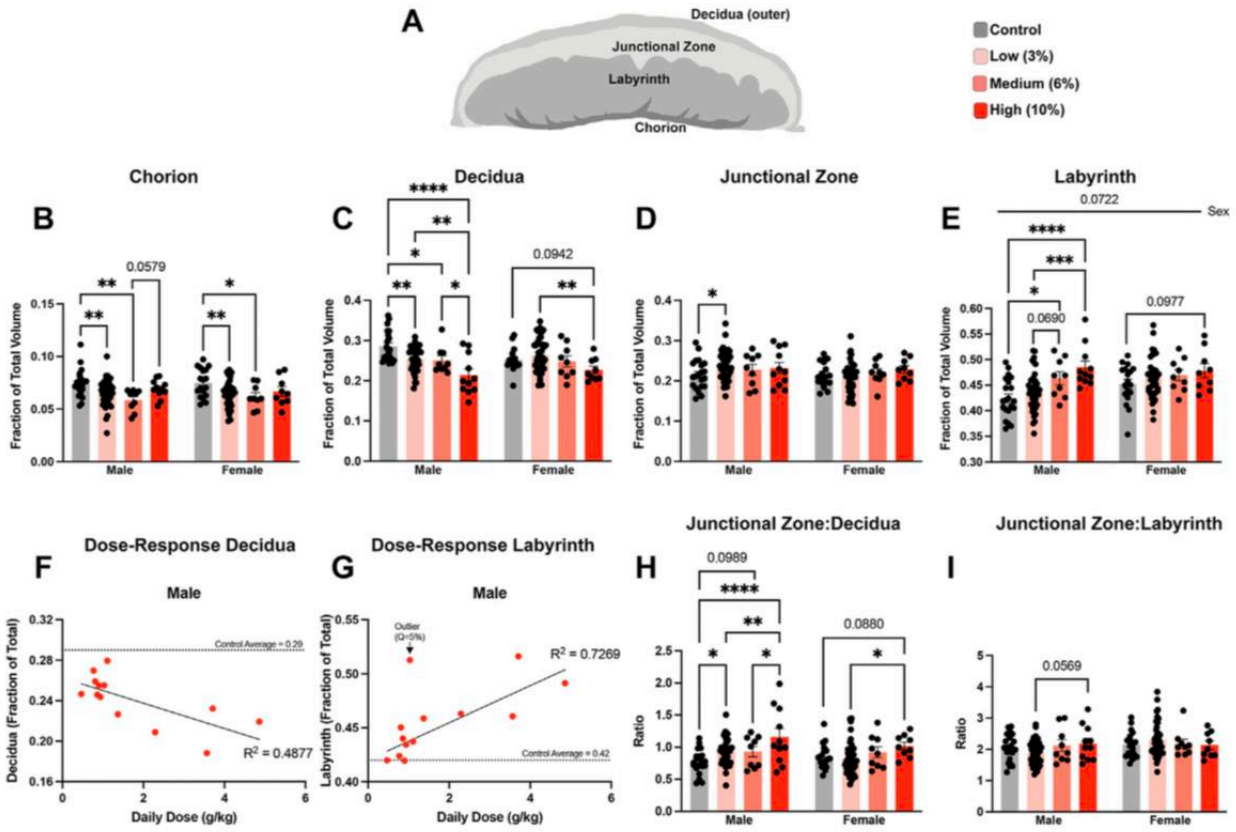

Figure 3.6. Chronic paternal alcohol exposures induce dose- and sex-specific changes in the histological organization of the placenta. (A) Schematic diagram depicting the layers of the murine placenta. Using microCT, we conducted a volumetric analysis of each placental layer and used a two-way ANOVA to compare measures between male and female offspring across treatment groups. Volumes for the (B) chorion, (C) decidua, (D) junctional zone, and (E) labyrinth are expressed as a ratio of the total placental volume (n = fetus, randomly selected from each litter, males: 21 Control, 43 low, 9 Medium, 11 High; females: n = 18 Control, 42 Low, 9 Medium, 9 High). Pearson correlation analysis contrasting proportional volume of the (F) decidua and (G) labyrinth with sire daily ethanol dose (n = 13 averaged individuals at each absolute daily dose). Ratios comparing the proportional volumes of the (H) junctional zone to decidua and (I) labyrinth to junctional zone between male and female offspring across treatment groups. Error bars represent the standard error of the mean, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. [Reprinted from Thomas et al., 2022]

During our histological analysis, we noticed that the labyrinth zone in placentae of offspring sired by Low- concentration males appeared to have increased density compared to placentae derived from the offspring of Control males. For female offspring of EtOH-exposed sires, morphometric analysis of standard histological sections (Neres et al., 2008) revealed a reduction in placental vascular spaces within the central regions of the labyrinth compared to Controls but not in peripheral regions (Figures 3.7A–C). We did not observe changes in vascular space within placentae from male or female offspring derived from High-concentration sires. In contrast, although the density of the labyrinth region of Low- concentration male placentae was not statistically different from the Controls, the peripheral labyrinth of the Low- concentration placentae contained proportionally more vascular space than the central regions (Figure 3.7C). We did not observe proportional differences in central vs. peripheral vascular space in the Controls. These data indicate that while we do not observe alterations in the size of the labyrinth layer in female offspring, we do observe indications of altered vascular structure.

Finally, emerging research suggests a shared developmental axis between the placenta and heart (Woods et al., 2018), and a recent study examining low-level maternal alcohol exposures identified heart defects in the offspring (Nguyen et al., 2014). Therefore, we used a two-way ANOVA to compare normalized heart weights between the Control and Low-concentration treatment groups. This analysis revealed decreased normalized heart weights in the female offspring but not the males (Figure 3.7D). From these observations, we conclude that paternal, Low-concentration alcohol exposures exert sex- specific impacts on placental development, which correlates with sexually dimorphic reductions in offspring normalized heart weights.

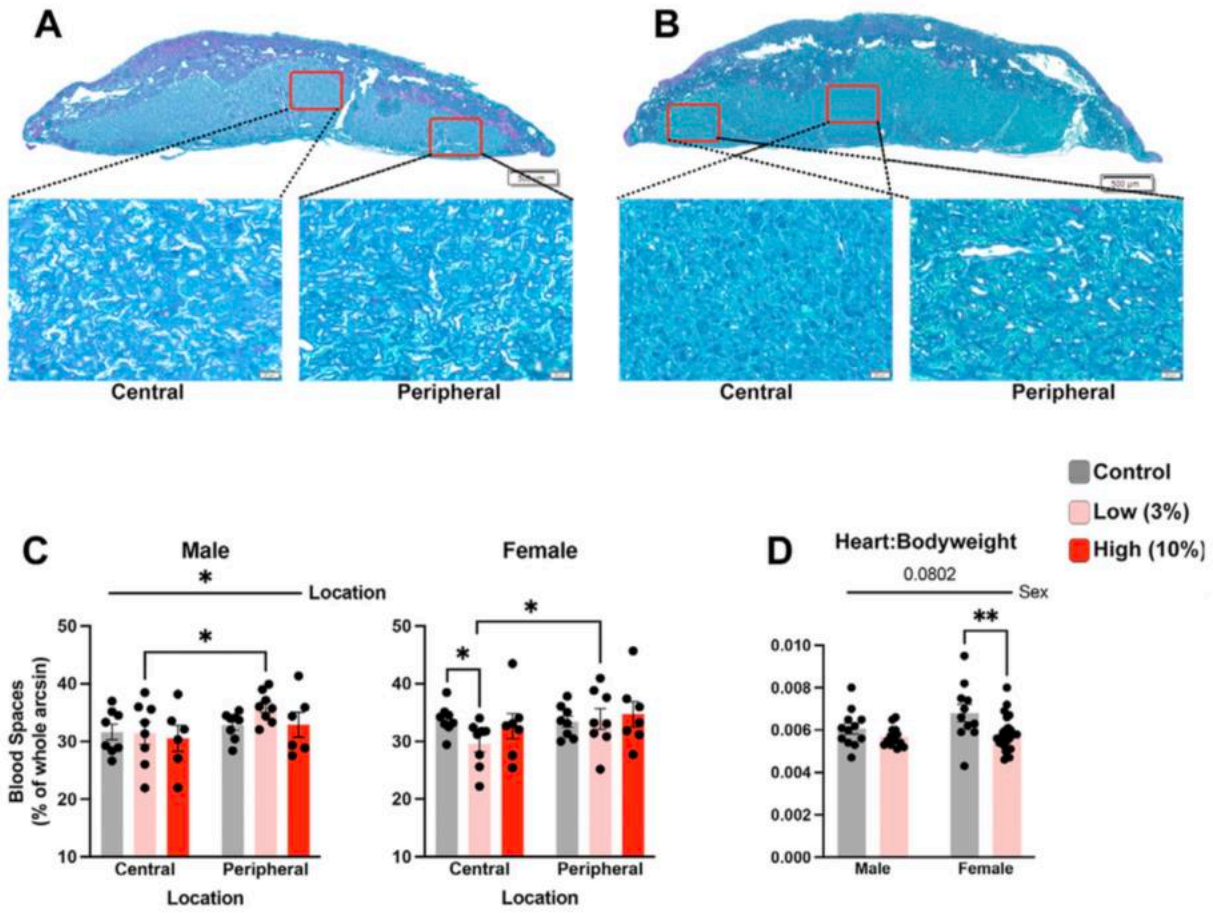

Figure 3.7. Low-concentration paternal alcohol exposures alter placental vascular space. Histological sections comparing central and peripheral vascular spaces between female placentae derived from the offspring of (A) Control and (B) Low-concentration sires. (C) Comparison of placental vascular space between male and female offspring of Control, Low-, and High-concentration sires (n = fetus, randomly selected from each litter, males: 8 Control, 8 low, 6 High; females: n = 8 Control, 8 Low, 7 High). (D) Comparison of relative heart weights between the male and female offspring of Control and Low-concentration sires (n = fetus, randomly selected from each litter, males: 13 Control, 16 low; females: n = 11 Control, 25 Low). 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 < 0.05, **p < 0 0.01. [Reprinted from Thomas et al., 2022]

3.6. Alterations in mitochondrial-encoded and imprinted gene expression accompany paternally programmed changes in placental histology

Epigenetic mechanisms of gene regulation, particularly imprinted genes, play a central role in directing the histological organization and function of the placenta (Piedrahita, 2011; Tunster et al., 2016). Therefore, we assayed the expression of a select cohort of imprinted genes with known roles in organizing placental histoarchitecture. In the male offspring of Low-concentration sires, we identified decreased expression of achaete-scute family BHLH transcription factor 2 (Ascl2) and increased expression of solute carrier family 22 member 18 (Slc22a18), both maternally expressed genes located in the Kcnq1ot1 imprinted domain (Golding et al., 2011) (Figure 3.8A). In contrast, in placentae derived from the male offspring of high-concentration sires, we observed upregulation of cyclin-dependent kinase inhibitor 1C (Cdkn1c), also within the Kcnq1ot1 imprinted cluster but not Ascl2 or Slc22a18. In addition, we observed disruption of maternally expressed H19 but not paternally expressed gene 3 (Peg3). Placentae derived from the female offspring of High- concentration sires also exhibited suppression of H19, while Low-concentration placentae displayed an upregulation of Peg3 (Figure 3.8B).

We next examined the expression of a cohort of genes encoding trophoblast-specific proteins and system A family amino acid transporters known to function downstream of the differentially expressed imprinted genes. Placentae derived from the male offspring of Low-concentration sires exhibited upregulation of solute carrier family 3 member 2 (Slc3a2) and trophoblast specific protein alpha (Tpbpa), while the female offspring displayed upregulation of both amino acid transporters (Slc3a2 and Slc28a2) and Tpbpa (Figure 3.8C). We did not observe any alterations in these same genes in either male or female placenta derived from High-concentration sires. Finally, our previous studies examining alcohol-induced changes in epigenetic

programming have identified altered expression of several mitochondrial-expressed transcripts and nuclear genes regulating oxidative phosphorylation and mammalian target of rapamycin (mTOR) signaling (Chang et al., 2021a; Thomas et al., 2021). Similar to these previous studies, we identify suppression of these same candidate genes in placentae derived from the male and female offspring of Low- concentration sires but not the offspring of High-concentration males (Figure 3.8D). In summary, our studies reveal that Low- concentration EtOH exposures disrupt the regulation of select imprinted genes, which are crucial regulators of placental patterning and function, as well as elements regulating mitochondrial function.

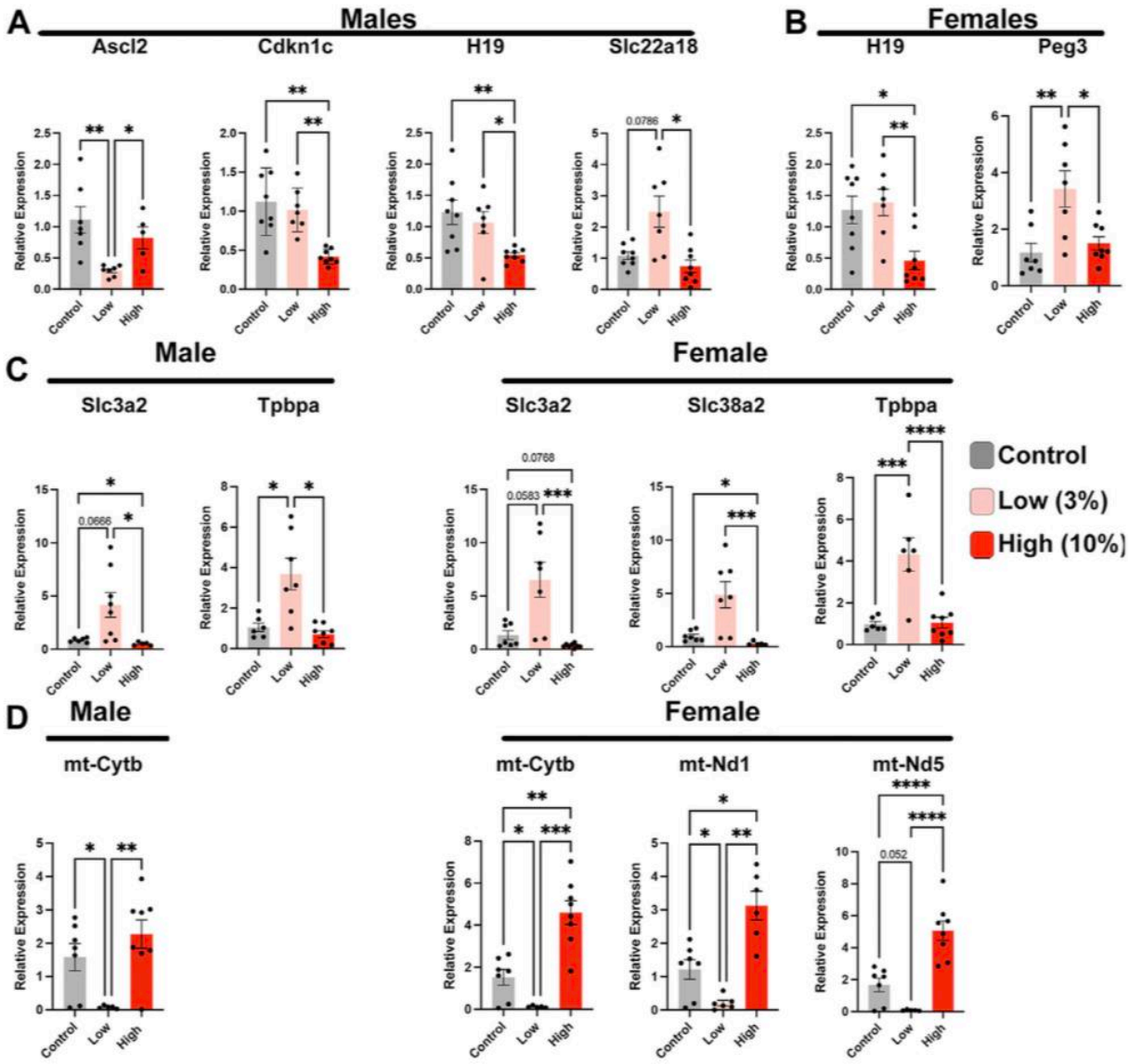

Figure 3.8. Paternal alcohol exposures induce alterations in placental gene expression. Analysis of imprinted gene expression in the placentae of (A) male and (B) female offspring of Control and EtOH-exposed sires in the Low- and High-concentration treatment groups. (C) Expression analysis of critical placental nutrient transporters in male and female offspring sired by Control, Low-, and High-concentration ethanol exposed males. (D) Comparison of mitochondrial-encoded transcripts in placentae derived from the male and female offspring of sires exposed to the Control, Low-, and High- concentration ethanol treatments. We analyzed gene expression using RT-qPCR. Gene expression was normalized to transcripts encoding Pgk1 and Ywhaz; (n = 8). For analysis, we used a one-way ANOVA or a Welch ANOVA. If data were not normally distributed, we used a non-parametric Kruskal- Wallis test. Error bars represent the standard error of the mean, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. [Reprinted from Thomas et al., 2022]

4. Discussion

Dose-response assessments represent a critical element of hazard characterization and typically define the threshold of exposure above which stressors cause adverse effects. Here, we employed a well-defined mouse model of voluntary EtOH exposure to examine the dose-response relationship of paternal drinking on the epigenetic programming of offspring fetoplacental growth. Our studies reveal a nonlinear biphasic or inverted J-shaped dose-response curve, where lower paternal exposures induce placental overgrowth and increased crown-rump length, while higher exposures trend towards placental growth restriction. The progressive, dose-dependent histological changes to the placental labyrinth layer we observe are similar to previous studies examining placental responses during fetal growth restriction (Coan et al., 2008). These observations reinforce the hypothesis that preconception paternal exposures transmit a stressor to the offspring that negatively impacts male placental development and function. Notably, we find that paternal low-dose EtOH exposures also transmit a memory to their offspring, inducing altered imprinted gene regulation and sex-specific impacts on placental histological organization and growth. To the best of our knowledge, this represents the first report describing the paternal epigenetic inheritance of a hormetic, biphasic dose-response in a mammalian system.

Our limited access model does not account for the EtOH preference of individual mice. Even after selecting higher- level drinkers, we still observed wide variation in individual preference, where mice assigned to a particular treatment group based on EtOH concentration achieved higher or lower doses than planned. Only by retrospectively adjusting for fluid consumption could we stratify our data and appropriately define dose-dependent effects, which revealed that lower dose exposures exert a potent influence on fetoplacental growth. Previously, we reported that the offspring of EtOH-exposed sires exhibit sex-specific changes in postnatal

growth and glucose homeostasis (Chang, et al., 2019; Chang, et al., 2019). However, using vapor chambers to chronically expose males to EtOH, Rathod and colleagues did not observe any impacts on offspring growth or glucose metabolism (Rathod et al., 2020). Based on the dose-response data we now report, we suspect these discrepancies are due to the higher dose and longer duration of daily exposures employed in their study. Further, we suspect that sufficiently high doses of EtOH overwhelm and inhibit EtOH processing mechanisms (Pikkarainen, 1971), leading to the epigenetic programming of distinct, high-dose outcomes for various behavioral and growth measures.

In toxicology, the term hormesis describes circumstances where modest exposures to toxicants, hypoxia, or ionizing radiation induce stimulatory effects, while in contrast, sufficiently high exposures cause pathology (Leak et al., 2018). Notably, clinical studies have employed preemptive exposures to low-level stressors to exert beneficial outcomes in advance of high-level stressors or to stimulate recovery. The most notable of these examples is the role brief ischemic episodes have in improving the ability of tissues to recover from enduring ischemic attacks (Pan et al., 2016). Therefore, hormesis is a recognized element of human physiology, enhancing our abilities to recover from injury and adapt to environmental stressors (Leak et al., 2018). Accordingly, transmitting this adaptive information from parent to offspring would be evolutionarily advantageous.

Detoxification of EtOH is a capacity-limited process, where high exposures saturate the underlying enzymatic mechanisms and produce different toxic responses than lower exposures falling within detoxification capacity (Pikkarainen, 1971). For example, work examining the influence of alcohol on memory formation and adaptive responses in the expression of NMDA receptors revealed that low doses of EtOH challenge the brain to induce an adaptive state of

neuroprotection, resulting in improved memory formation. In contrast, higher exposures exert toxic effects that inhibit memory formation (Kalev-Zylinska & During, 2007). Similar biphasic responses arise in the lymphatic and immune systems (Lundgaard et al., 2018; Sureshchandra et al., 2019; Velázquez-Marrero et al., 2011). Therefore, systemic challenges induced by low- dose EtOH exposures may stimulate the germline to transmit nongenomic information that enhances adaptation to this environmental toxicant.

Researchers suspect that EtOH-induced toxicity arises from reactive oxygen species or other oxidative stress-mediated processes (Herrera et al., 2003). Notably, multiple reactive oxygen species serve as a signaling mechanism for cellular systems to sense and initiate hormetic responses that reprogram gene expression toward a stable adaptive state (Cox et al., 2018; Leak et al., 2018). In worms, these changes alter chromatin structure, specifically histone H3, lysine four trimethylation (H3K4me3) in both the soma and the germline, enabling the transgenerational propagation of an adaptive state (Kishimoto et al., 2017; Tauffenberger & Parker, 2014). We have observed suppression of pathways regulating oxidative phosphorylation in a high-dose model of early gestational EtOH exposure and recently identified this same signature in male placentae sired by EtOH-exposed fathers (Chang et al., 2021b; Thomas et al., 2021). Further, we have identified alcohol-induced increases in H3K4me3 in alcohol- exposed sperm (Bedi et al., 2022). Therefore, we speculate that low levels of oxidative stress program epigenetic changes in sperm, which suppress oxidative stress response pathways in the offspring, including the mTOR, EIf2, and Sirtuin signaling pathways. As hypoxia and oxidative stress stimulate growth and differentiation of the placenta (Pringle et al., 2010), suppressing these protective pathways may explain the enhanced growth we observe. Further, early gestational exposures to transient oxidative stress induce a similar repertoire of metabolic outcomes as those we identified in the male offspring of

alcohol-exposed sires (Dimova et al., 2020). If our mouse studies directly translate to humans, gene-environment differences in oxidative stress responses may play an unappreciated role in the susceptibility to alcohol-induced teratogenesis and contribute to the wide variation in FASD phenotypes and prevalence observed in the clinics (McCarthy & Eberhart, 2014; Schaefer & Deere, 2011). Future studies are required to determine if H3K4me3 or other polycomb-mediated epigenetic changes participate in the intergenerational transmission of these effects.

Although these data are compelling, there are some limitations to our study. First, because we hypothesized that Low-concentration exposures would not impact offspring growth, we have not adequately explored the lower end of the dose-response curve to determine the minimum exposure level eliciting a response in placental growth. Further, we have a limited sample size of males in the higher range of the dose curve. Therefore, additional studies are necessary to model with effects of an occasional or infrequent drinker versus a chronic alcoholic. Second, to avoid the confounding impacts of stress, we subjected the males to limited handling and only measured plasma alcohol concentrations once. Therefore, we do not know if plasma alcohol levels consistently differed between treatments. Further, previous studies modeling binge drinking have revealed that using the Drinking in the Dark model, mice consume most (41%) alcohol during the first 15 min (Linsenbardt & Boehm, 2014). As we did not investigate consumption patterns within the 4-h exposure window, we do not know if differences in palatability between the different concentrations alter drinking behaviors, which could influence the duration and peak magnitude of blood plasma alcohol levels. Most studies modeling binge drinking do not employ low-dose exposures. Therefore, additional studies are required to determine if the Low- concentration treatment alters drinking behaviors and the impacts on plasma alcohol levels, and how this may relate to germline programming in the alcohol-exposed males.

Third, while the preconception period lasted 6 weeks, exposures continued during the breeding phase, with total preconception exposure periods ranging from 7 to 18weeks. Although not the focus of this study, we did not observe any differences between placental phenotypes before or after 11 weeks, the mean exposure time. However, we do not know if longer exposures exert a cumulative impact on the sperm epigenome or if an exposure threshold was reached after the initial 6 weeks. Future studies will address this question, as well as the resilience of the sperm-inherited developmental program to recover after cessation of alcohol exposure. Fourth, we do not know if the observed increases in placental weight or fetal size in the Low-dose group are beneficial or exert long-term consequences on offspring growth and metabolic health. Furthermore, no studies have examined how preconception paternal alcohol exposures may interact with maternal exposures. Therefore, alcohol researchers should interpret these growth data cautiously and not presume these changes as beneficial. Fifth, our study only examines a single developmental time point, and the growth differences we observe may represent transient changes that disappear by birth. Finally, although maternal EtOH exposures also predominantly impact male placental weights (Kwan et al., 2021), we have not identified any further experimental data to explain these sexually dimorphic outcomes. Future experiments will address these questions.

Due to the misconception that sperm only transmit genetic information, reproductive toxicologists have not adequately considered the impacts of paternal lifestyle and exposure history on offspring developmental outcomes. This is especially true for FASDs, where the fault is exclusively attributed to maternal exposures (Cook et al., 2016). Today, we recognize that a wide variety of preconception stressors, including cold, exercise, over and undernutrition, exposure to drugs of abuse, and intense psychological stress, all modify the paternally-inherited epigenetic program, with negative repercussions on offspring health (Champroux et al., 2018; Donkin &

Barrès, 2018; Le Blévec et al., 2020). Our studies demonstrate that a lasting memory of low-dose alcohol exposures transmits to the offspring and provides a dose- response framework to further dissect the molecular mechanisms underlying the mammalian intergenerational inheritance of acquired traits.

CHAPTER IV

PRECONCEPTION PATERNAL ETHANOL EXPOSURES INDUCE ALCOHOL-RELATED CRANIOFACIAL GROWTH DEFICIENCIES IN FETAL OFFSPRING*

1. Introduction

Fetal Alcohol Syndrome (FAS) is characterized by a range of structural birth defects, including facial dysmorphia, central nervous system growth deficits (microcephaly), and prenatal/postnatal growth restriction, which correlate with the magnitude of prenatal alcohol exposure (Hoyme et al., 2016; Suttie et al., 2013). Although exclusively attributed to the maternal consumption of alcohol during pregnancy, multiple clinical studies and case reports have emerged describing instances where infants presenting with alcohol-related birth defects were born to mothers who denied consuming alcohol during pregnancy (Bandoli et al., 2022; Suttie et al., 2013). For example, the Collaboration on FASD Prevalence (CoFASP) research consortium recently conferred a diagnosis of FAS to a cohort of 41 children whose mothers refused to endorse alcohol use during pregnancy (Bandoli et al., 2022). The prevailing rationalization for these reported inconsistencies is that the mothers did not faithfully report their prenatal alcohol use (Jacobson et al., 2002). However, the recent identification of epigenetic mechanisms of paternal inheritance presents an alternative explanation; that the drinking habits of the birth father may contribute to the emergence of alcohol-related phenotypes in their offspring. However, due to the misconception that sperm do not transmit information beyond the genetic code, the influence of paternal drinking on the development of alcohol-related birth defects has not been rigorously examined.

Due to the development of grossly observable alcohol-induced phenotypes consistent with clinical presentations of FAS, mouse models have become a powerful resource for investigating the development of alcohol-induced dysgenesis (Petrelli et al., 2018). Therefore, we developed a multiplex mouse model to test the hypothesis that preconception male alcohol exposures induce alcohol-related craniofacial defects and determine if paternal alcohol use increases the penetrance and severity of alcohol-related phenotypes induced by maternal drinking. Using a 2x2 factorial design, we contrasted the emergence of alcohol-induced developmental defects in offspring generated using maternal (MatExp), paternal (PatExp), and dual parental (DualExp) models of alcohol exposure.

2. Materials and Methods

2.1. Study design

Multiple clinical studies and case reports describe instances where infants diagnosed with Fetal Alcohol Syndrome (FAS) were born to mothers who denied consuming alcohol during pregnancy (Bandoli et al., 2022; Eichler et al., 2016; May et al., 2006, 2008; Suttie et al., 2013). The prevailing explanation offered for these discrepancies is that the mothers lied about their prenatal alcohol use (Jacobson et al., 2002). However, these previous studies did not record the alcohol use of the birth father and, therefore, did not adequately consider paternal epigenetic contributions to this pediatric disorder (Olshan & Faustman, 1993). Three of the four diagnostic criteria for FAS include alcohol-induced structural and growth defects (Hoyme et al., 2016). Therefore, the objective of this study was to investigate the influences of preconception paternal alcohol consumption on the development of alcohol-related birth defects and determine if paternal alcohol exposures could interact with maternal exposures to exacerbate these outcomes.

Previous studies examining alcohol-induced structural birth defects in rodents have primarily employed oral gavage, a potent inducer of the systemic stress response (Brown et al., 2000). Stress hormones alter developmental programming in sperm and oocytes, with demonstrated consequences to offspring neurodevelopmental outcomes (Chan et al., 2018). To avoid this confounder, we utilized a modified version of a voluntary consumption paradigm known as 'Drinking in the Dark' (Rhodes et al., 2005). Using this model, male and female mice consume ethanol (EtOH) according to their individual preference, obtaining physiologically relevant plasma alcohol levels while encountering minimal handling. To maximize the clinical relevance of our model, we continuously exposed male mice to EtOH, while in contrast, we only exposed females during an initial preconception period and the first ten days of gestation. This paradigm models

the behavior of most women, who cease consumption upon pregnancy diagnosis (Pryor et al., 2017). After establishing our exposure model, we employed a 2x2 factorial experimental design to examine alcohol-related growth and structural birth defects in the offspring of unexposed (Control), maternal- (MatExp), paternal- (PatExp), and dual parental-exposed (DualExp) mice. We then assessed established measures of alcohol-induced craniofacial dysgenesis and central nervous system development (Anthony et al., 2010; Boschen et al., 2018; Kaminen-Ahola et al., 2010; C. Klingenberg et al., 2010; Lipinski et al., 2012) to determine the impacts of each treatment on the emergence of growth and structural birth defects.

2.2. Mice

We conducted all experiments under IACUC 2020-0211, approved by the Texas A&M University IACUC. All experiments were performed following IACUC guidelines and regulations, and we report our data per ARRIVE guidelines.

We obtained adult (postnatal day 90) C57BL/6J (Strain #:000664 RRID: IMSR_JAX:000664) mice from the Texas Institute of Genomic Medicine (TIGM) and maintained them in the TIGM facility on a reverse 12-hour light/dark cycle (lights off at 8:30 am) with ad libitum access to a standard chow diet (catalog# 2019, Teklad Diets, Madison, WI, United States) and water. To minimize stress, we implemented additional enrichment measures to the animal's home cage, including shelter tubes for males and igloos for females (catalog# K3322 and catalog# K3570, Bio-Serv, Flemington, NJ, United States).

2.3. Maternal periconceptional exposures and breeding

One week before treatment initiation, we acclimated female mice to individual housing conditions. We then randomly assigned postnatal day 90 females to either the experimental (10% w/v ethanol; catalog# E7023; Millipore-Sigma, St. Louis, MO, USA) or Control (water alone) treatments. Then, beginning four hours after the initiation of the dark cycle, we replaced the water bottle of the animal's home cage with an identical bottle containing the appropriate treatment. We maintained these treatments for four hours, then returned the animal's original water bottle. During all experiments, we simultaneously exchanged the water bottles of Control and EtOH-exposed dams to ensure identical conditions. At the end of each week, during their regular cage change, 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 of body weight.

We initiated maternal exposures ten days (approximately two estrus cycles) before breeding dams to treated males (Pregestational Day Ten; PGD10). After seven to ten days of exposure, we synchronized female reproductive cycles using the Whitten method (Whitten et al., 1968). Then, after the daily Control or EtOH treatment, we placed a single female into the home cage of a treated male. After six hours, we confirmed matings by the presence of a vaginal plug and returned the female mice to their home cage. We ensured males rested for a minimum of 72 hours before the next attempted mating. We subjected dams to minimal handling but maintained the EtOH and Control treatments until gestational day 10.5, when we calculated the change in dam body weight between gestational day Zero and 10.5, then used a body weight gain of approximately 1.8 g as confirmation of pregnancy (Thomas et al., 2022). Upon pregnancy diagnosis, we ceased the Control and EtOH treatments and left females undisturbed until

gestational day 16.5. We repeated this procedure until we obtained the requisite number of pregnancies.

2.4. Paternal preconception ethanol exposures

We exposed male mice to alcohol using a prolonged version of the Drinking in the Dark paradigm described previously (Chang et al., 2017; Chang, Wang, et al., 2019; Thomas et al., 2021, 2022). At the end of each week, we recorded sire weight (g) and the amount of fluid consumed (g) and then calculated weekly fluid consumption as grams of fluid consumed per gram of body weight. Using methods described by our group (Thomas et al., 2022), we maintained males on the preconception treatments for six weeks, then bred exposed males to treated dams as described above.

2.5. Fetal sex determination

We isolated genomic DNA from the fetal tail using the HotSHOT method (Truett et al., 2000) and then determined fetal sex using a PCR-based assay described previously (Thomas et al., 2021).

2.6. Craniofacial analysis: 2D imaging

During dissections, we collected 2D images of fetal heads by excising the fetus from the gestational sac and placing the fetus directly under a stereomicroscope (SZX2-ZB10, Olympus, Shinjuku City, Tokyo, Japan) with an attached digital camera (SC-180, Olympus, Shinjuku City, Tokyo, Japan). We used the cellSens Entry software (cellSens Entry Version 3, Olympus, Shinjuku City, Tokyo, Japan) to acquire and analyze 2D images of the frontal and lateral views of fetal heads for further craniofacial analysis.

2.7. Craniofacial analysis: Geometric morphometrics

FAS is associated with three broad developmental defects: facial dysmorphia, including midline defects and reductions in eye size; central nervous system structural defects, including microcephaly; and prenatal growth restriction (Suttie et al., 2013; Wozniak et al., 2019). Geometric morphometric analysis is a landmark-based analytical technique used to compare the relative positions of facial landmarks and quantify differences in overall biological shape and morphology between populations (Zelditch et al., 2012; Katsube et al., 2022; Klingenberg, 2011). The obtained morphological information includes shape variation, relative shifts in landmark position, differences in feature rotation, and changes in proportional size (Zelditch et al., 2012; Katsube et al., 2022; Klingenberg, 2011). After identifying landmarks, generalized Procrustes analysis (GPA) standardizes all specimens by removing scale from the dataset and minimizing the distance between landmarks using the least squares method (Klingenberg, 2011). This standardization technique then allows the placement of all observed landmark datasets into a common coordinate system. Subsequently, standardized data are examined using canonical variant (CV) analysis to identify the proportional relationships that best distinguish shape differences among groups (Attanasio et al., 2013; Zelditch et al., 2012; Penrice, 2023; Wiseman et al., 2021; Katsube et al., 2022; C. P. Klingenberg, 2011). Accordingly, geometric morphometric analysis is widely used, both clinically and experimentally, to study diverse aspects of craniofacial patterning, including the role of enhancers in driving craniofacial development (Attanasio et al., 2013), the prevalence of craniofacial phenotypes in genetic syndromes (Roussos et al., 2021) and in characterizing fetal alcohol syndrome-associated craniofacial dysmorphology (Kaminen-Ahola et al., 2010; Klingenberg et al., 2010).

We used 12-14 litters per treatment, yielding a sample size of ~48 male and ~48 female offspring per treatment, curated the digital photographs of each fetus within the litter to include their litter ID, sex, and uterine position, then processed images for analysis using the publicly available program MORPHOJ (Klingenberg, 2011). First, we used the publicly available program tpsUtil64 ((Rohlf, 2015); version 1.82) to generate a TPS database. We then imported collected 2D images of fetal heads into the publicly available image analysis software tpsDig ((Rohlf, 2005); version 3.2) and set the reference scale bar included in the picture to 1 mm. Next, we demarcated 16 landmarks on the left/right profile and 18 landmarks on the front profile, following previously established criteria (Zelditch et al., 2012; Perez & King-Heiden, 2018) and morphological landmarks described by Anthony et al. (Anthony et al., 2010). The employed landmarks included:

Left/Right Photograph Landmark Key

1. Tip of nose
2. Nasion (in between eyes)
3. Top of head (highest point of elevation of head)
4. Curve of skull (cusp of where the skull begins to curve downward)
5. Back of skull (Before projection of spinal column, directly behind the ear)
6. Bottom of mandible (lowest part of the jaw)
7. Front of mandible (Closest to mouth opening, furthest point facing out)
8. Upper philtrum (upper lip, closest point below base of nose)
9. Inner mouth (the innermost corner of the mouth)
10. Edge of snout (Light coloration, where the whiskers would end)
11. Central auditory canal (middle of light-colored tissue)
12. 3 O'clock position of eye (closest to the auditory canal, exterior of eye)
13. 12 O'clock position of eye (towards top of head, exterior of eye)
14. 9 O'clock position of eye (closest to the snout, exterior of eye)
15. 6 O'clock position of eye (bottom most part of eye, closest to the jaw)
16. Pupil (center of eye)

Front Photograph Landmark Key

1. Top of head (Central, highest point of head, in line with the nose)
2. Bottom of mandible (Bottom-most point of jaw)
3. Right corner of mouth (Furthest right point where mouth closes)
4. Left corner of mouth (Furthest left point where mouth closes)
5. Top of philtrum (Closest point below the base of nose)
6. Bottom of philtrum (Ventral extent of philtrum, closest junction between two lips)
7. Tip of nose (top, central most part of the nose)
8. Nasion (Central point between the two eyes)
9. 3 O'clock position of left eye (medial-most part of the external eye)
10. 12 O'clock position of left eye (topmost part of the external eye)
11. 9 O'clock position of left eye (medial-most part of the external eye)
12. 6 O'clock position of left eye (bottom most part of external eye)
13. Pupil of left eye (Center of the left eye, lighter color)
14. 3 O'clock position of right eye (medial-most part of the external eye)
15. 12 O'clock position of right eye (topmost part of the external eye)
16. 9 O'clock position of right eye (medial-most part of the external eye)
17. 6 O'clock position of right eye (bottom most part of external eye)
18. Pupil of right eye (Center of the right eye, lighter color)

To ensure consistency, a single individual (N.S.) demarcated the same landmarks in the exact location and order for each image. We then created a linear outline around the head and digitized the landmarks and outlines as a TPS file. tpsDig (Rohlf, 2005) then adds additional landmarks, including the midpoints between features and other aspects of the outline, for a total of 47 landmarks for the front profile and 45 for the left/right. We then imported the TPS files into the MORPHOJ software (Klingenberg, 2011) (software version build 1.07a, Java version 1.8.0_291 (Oracle Corporation)) to conduct geometric morphometric analysis. We added classifiers describing each treatment group, then separately normalized male and female left, right, and frontal datasets for scale, rotation, and translation using the Procrustes fit feature (Klingenberg, 2011). We then generated a covariance matrix, which we used to conduct Principal Component Analysis (PCA). Our PCA analysis revealed that PC1 and PC2 described most (Males Front 68.5%, Left 71.0%, and Right 68.5%; Females Front 69.6%, Left 69.0%, and Right 69.6%) of the variation in our model.

We then used Canonical Variate (CV) analysis to identify differences in facial features between treatments and exported the raw CV scores into the (Attanasio et al., 2013; Zelditch et al., 2012; Penrice, 2023; Wiseman et al., 2021). These included the parametric Multivariate analysis of variance (MANOVA) and non- parametric Analysis of similarities (ANOSIM), and Permutational multivariate analysis of variance (PERMANOVA) tests, followed by Bonferroni correction to identify significant differences in clustering and distance between treatment groups. Finally, we generated the CV lollipop diagrams and scatter plots using the graphing features of MORPHOJ (C. P. Klingenberg, 2011).

2.8. Craniofacial analysis: Linear morphometrics

To validate our morphometric analyses, we conducted linear measurements of fetal craniofacial features using 2D images and the analysis software cellSens Entry (cellSens Entry Version 3, Olympus, Shinjuku City, Tokyo, Japan). We used the length tool on lateral images to measure upper facial depth, midfacial depth, and lower facial depth, following previously described facial landmarks (Anthony et al., 2010). We used the cellSens Entry length tool on frontal images to determine philtrum length, snout-occipital distance, inner canthal distance, and biparietal distance, measuring the latter across the axis of the eyes, as previously described (Anthony et al., 2010).

2.9. Data handling and statistical analysis

We subjected all data generated during this study to a detailed data management plan that prioritizes safe and efficient data handling that allows long-term storage, retrieval, and preservation. We recorded Initial measures by hand, then inserted these into Google Sheets or Microsoft Excel for downstream analysis using GraphPad Prism 8 (RRID:SCR_002798, GraphPad Software Inc., La Jolla, CA, USA). We analyzed all data sets with statistical significance set at alpha = 0.05, then employed the ROUT test (Q = 1%) to identify outliers. Next, we verified the normality of the datasets using the Shapiro– Wilk test and verified equal variance using the Brown-Forsythe test. If data passed normality and variance testing (alpha = 0.05), we employed either a One-way or Two-way ANOVA or an unpaired, parametric (two-tailed) t-test. If the data failed the test for normality or we observed unequal variance, we ran a Kruskal-Wallis test followed by Dunn's multiple comparisons test or a non-parametric Mann–Whitney test.

For measures of fetal weight, we determined the male and female average for each litter and used this value as the individual statistical unit. Subsequently, we identified the tenth

percentile fetal weight for the Control population, determined the proportion of offspring above and below this value, then ran a Chi-square analysis to compare the proportions between treatments. For the analysis of fetal brain weights, we selected the four fetuses closest to the cervix from each litter. We present detailed descriptions of each statistical test, sample size, and resulting p-values in Table 4.1.

Table 4.1. Descriptions of the statistical tests for each figure [Reprinted from Thomas et al., 2023]

GRAPH	STATISTICAL TEST	SAMPLE SIZE
Figure 1. Maternal, Paternal, and Dual Parental alcohol exposures each induce changes in offspring craniofacial patterning.		
b. Male right profile canonical variant analysis	Canonical variant analysis	n = 50 control, 47 maternal, 47 paternal, 48 dual parental
c. Male right profile wire diagram shift in facial features	MANOVA (Wilk's Lambda Statistic), ANOSIM, PERMANOVA	n = 50 control, 47 maternal, 47 paternal, 48 dual parental
d. Males with head size below the 10th percentile of the control population	Chi-square test	n = 61 control, 37 maternal, 66 paternal, 62 dual parental
e. Right ocular size (top: male, bottom: female)	One-way ANOVA, Dunnett's multiple comparison test	Male: n = 52 control, 37 maternal, 57 paternal, 53 dual parental; Female: n = 39 control, 49 maternal, 54 paternal, 48 dual parental
f. Mid facial depth (top: male, bottom: female)	One-way ANOVA, Dunnett's multiple comparison test	Male: n = 103 control, 72 maternal, 111 paternal, 106 dual parental; Female: n = 76 control, 98 maternal, 107 paternal, 96 dual parental
g. Snout-occipital distance (top: male, bottom: female)	One-way ANOVA, Dunnett's multiple comparison test	Male: n = 53 control, 33 maternal, 63 paternal, 52 dual parental; Female: n = 40 control, 45 maternal, 57 paternal, 48 dual parental
h. Snout-occipital distance dose correlation	Pearson correlation, two-tailed	Males: n = 33 maternal, 63 paternal, 52 dual parental; Females: n = 28 maternal, 35 paternal, 30 dual parental

Sex	Treatment	r	R^2	p-value	Summary
Males	Maternal	-0.1926	0.0371	0.2829	ns
	Paternal	-0.3695	0.1365	0.0029	**
	Dual	-0.3447	0.1188	0.0123	*
Females	Maternal	-0.3447	0.1188	0.0219	*
	Paternal	-0.2447	0.0599	0.0655	#
	Dual	-0.3855	0.1486	0.0074	**

i. Normalized brain weights dose correlation	Pearson correlation, two-tailed	Males: n = 20 maternal, 38 paternal, 32 dual parental; Females: n = 28 maternal, 35 paternal, 30 dual parental

Table 4.1. *Continued*

GRAPH	STATISTICAL TEST		SAMPLE SIZE		
Sex	**Treatment**	**r**	**R^2**	**p-value**	**Summary**
Males	Maternal	-0.6114	0.3738	0.0042	**
	Paternal	-0.5466	0.2988	0.0004	***
	Dual	-0.5612	0.3150	0.0008	***
Females	Maternal	-0.6365	0.4051	0.0003	***
	Paternal	0.2870	0.0824	0.0946	#
	Dual	-0.2940	0.0865	0.1148	ns

Supplemental Figure 1. A modified version of the Drinking In The Dark paradigm to study the impacts of maternal, paternal, and dual parental alcohol consumption on offspring health.		
b. Maternal daily food intake **c.** Maternal body weight **d.** Maternal daily treatment fluid consumption	Two-way ANOVA, Šídák's multiple comparisons test	Preconception: n = 27 control, 27 ethanol; Gestation: n = 30 control, 23 ethanol
e. Paternal body weight **f.** Paternal fluid consumption	Two-way ANOVA, Šídák's multiple comparisons test	n = 23 control, 24 ethanol
g. Paternal and maternal daily ethanol dose	Two-way ANOVA, Tukey's multiple comparisons test	n = 38 paternal ethanol, 29 preconception maternal ethanol, 23 gestation maternal ethanol
h. Maternal daily ethanol dose	Two-way ANOVA, Šídák's multiple comparisons test	Preconception: 10 maternal, 14 dual parental; Gestation: 8 maternal, 15 dual parental
i. Paternal and maternal plasma alcohol concentration	Two-way ANOVA, Tukey's multiple comparisons test	n = 11 control, 12 paternal ethanol, 11 maternal ethanol
Supplemental Figure 2. Analysis of pregnancy and fetal offspring physiological measures.		
a. Paternal treatment week at time of conception	One-way ANOVA, Tukey's multiple comparison test	n = 15 control, 13 maternal, 21 paternal, 17 dual parental
b. Conception rate	Chi-square test	n = 136 control, 125 maternal, 135 paternal, 191 dual parental
c. Normalized uterine horn weight **d.** Litter size	One-way ANOVA, Tukey's multiple comparison test	n = 16 control, 13 maternal, 20 paternal, 16 dual parental
e. Sex ratio	Chi-square test	n = control: 64 males, 59 females; maternal: 47 males, 61 females; paternal: 71 males, 75 females; dual parental: 65 males, 61 females
f. Litter average fetal weight	Two-way ANOVA, Tukey's multiple comparison test	Male: n = 16 control, 13 maternal, 19 paternal, 14 dual parental; Female: n = 16 control, 12 maternal, 20 paternal, 16 dual parental
g. Males with body weight below the 10th percentile of the control population **h.** Females with body weight below the 10th percentile of the control population	Chi-square test	Male: n = 63 control, 47 maternal, 71 paternal, 65 dual parental; Female: n = 59 control, 61 maternal, 75 paternal, 61 dual parental
i. Male and female brain to body weight ratio	Two-way ANOVA, Tukey's multiple comparison test	Male: n = 38 control, 18 maternal, 40 paternal, 34 dual parental; Female: n = 27 control, 23 maternal, 32 paternal, 30 dual parental

Table 4.1. *Continued*

GRAPH	STATISTICAL TEST	SAMPLE SIZE
Supplemental Figure 3. Maternal, Paternal, and Dual Parental alcohol exposures program dose-dependent changes in offspring craniofacial patterning.		
a-c. Male front, left, and right profile canonical variant analysis **f-h.** Female front, left, and right profile canonical variant analysis	Canonical variant analysis	Males: n = 50 control, 47 maternal, 47 paternal, 48 dual parental; Female: n = 46 control, 49 maternal, 41 paternal, 54 dual parental

a. Male front profile: MANOVA p = 2.223E-90, ANOSIM p<0.0001, R = 0.6316, PERMANOVA p<0.0001

MANOVA	Control	Maternal	Paternal	Dual
Control		5.29E-22	1.57E-24	2.87E-23
Maternal	5.29E-22		5.12E-26	2.53E-18
Paternal	1.57E-24	5.12E-26		4.26E-30
Dual	2.87E-23	2.53E-18	4.26E-30	

ANOSIM	Control	Maternal	Paternal	Dual
Control		0.0006	0.0006	0.0006
Maternal	0.0006		0.0006	0.0006
Paternal	0.0006	0.0006		0.0006
Dual	0.0006	0.0006	0.0006	

PERMANOVA	Control	Maternal	Paternal	Dual
Control		0.0006	0.0006	0.0006
Maternal	0.0006		0.0006	0.0006
Paternal	0.0006	0.0006		0.0006
Dual	0.0006	0.0006	0.0006	

b. Male left profile: MANOVA p = 4.832E-67, ANOSIM p<0.0001, R = 0.4736, PERMANOVA p<0.0001

MANOVA	Control	Maternal	Paternal	Dual
Control		5.05E-17	2.35E-20	6.92E-24
Maternal	5.05E-17		1.50E-17	6.96E-17
Paternal	2.35E-20	1.50E-17		6.42E-21
Dual	6.92E-24	6.96E-17	6.42E-21	

ANOSIM	Control	Maternal	Paternal	Dual
Control		0.0006	0.0006	0.0006
Maternal	0.0006		0.0006	0.0006
Paternal	0.0006	0.0006		0.0006
Dual	0.0006	0.0006	0.0006	

PERMANOVA	Control	Maternal	Paternal	Dual
Control		0.0006	0.0006	0.0006
Maternal	0.0006		0.0006	0.0006
Paternal	0.0006	0.0006		0.0006
Dual	0.0006	0.0006	0.0006	

c. Male right profile: MANOVA p = 6.737E-87, ANOSIM p<0.0001, R = 0.6178, PERMANOVA p<0.0001

MANOVA	Control	Maternal	Paternal	Dual
Control		2.82E-2	2.07E-21	1.12E-20
Maternal	2.07E-21		6.43E-23	2.92E-24
Paternal	2.82E-23	6.43E-23		3.12E-25
Dual	1.12E-20	2.92E-24	3.12E-25	

ANOSIM	Control	Maternal	Paternal	Dual
Control		0.0006	0.0006	0.0006
Maternal	0.0006		0.0006	0.0006
Paternal	0.0006	0.0006		0.0006
Dual	0.0006	0.0006	0.0006	

PERMANOVA	Control	Maternal	Paternal	Dual
Control		0.0006	0.0006	0.0006
Maternal	0.0006		0.0006	0.0006
Paternal	0.0006	0.0006		0.0006
Dual	0.0006	0.0006	0.0006	

f. Female front profile: MANOVA p = 7.85E-95, ANOSIM p<0.0001, R = 0.6848, PERMANOVA p<0.0001

MANOVA	Control	Maternal	Paternal	Dual
Control		1.55E-2	1.01E-18	3.75E-25
Maternal	9.29E-26		9.29E-26	8.57E-29
Paternal	1.01E-18	9.29E-26		2.04E-2
Dual	3.75E-2	8.57E-29	2.04E-27	

ANOSIM	Control	Maternal	Paternal	Dual
Control		0.0006	0.0006	0.0006
Maternal	0.0006		0.0006	0.0006
Paternal	0.0006	0.0006		0.0006
Dual	0.0006	0.0006	0.0006	

PERMANOVA	Control	Maternal	Paternal	Dual
Control		0.0006	0.0006	0.0006
Maternal	0.0006		0.0006	0.0006
Paternal	0.0006	0.0006		0.0006
Dual	0.0006	0.0006	0.0006	

Table 4.1. *Continued*

GRAPH	STATISTICAL TEST	SAMPLE SIZE

g. Female left profile: MANOVA p = 2.89E-77, ANOSIM p<0.0001, R = 0.5696, PERMANOVA p<0.0001

MANOVA	Control	Maternal	Paternal	Dual
Control		2.64E-21	3.49E-22	2.21E-24
Maternal	5.29E-22		9.38E-21	1.42E-18
Paternal	1.57E-24	9.38E-21		1.23E-18
Dual	2.87E-23	1.42E-18	1.23E-18	

ANOSIM	Control	Maternal	Paternal	Dual
Control		0.0006	0.0006	0.0006
Paternal	0.0006		0.0006	0.0006
Maternal	0.0006	0.0006		0.0006
Dual	0.0006	0.0006	0.0006	

PERMANOVA	Control	Maternal	Paternal	Dual
Control		0.0006	0.0006	0.0006
Paternal	0.0006		0.0006	0.0006
Maternal	0.0006	0.0006		0.0006
Dual	0.0006	0.0006	0.0006	

h. Female right profile: MANOVA p = 1.15E-71, ANOSIM p<0.0001, R = 0.5196, PERMANOVA p<0.0001

MANOVA	Control	Maternal	Paternal	Dual
Control		1.73E-20	5.44E-19	5.69E-20
Maternal	1.73E-20		5.83E-17	5.19E-20
Paternal	5.44E-19	5.83E-17		2.54E-21
Dual	5.69E-20	5.19E-20	2.54E-21	

ANOSIM	Control	Maternal	Paternal	Dual
Control		0.0006	0.0006	0.0006
Maternal	0.0006		0.0006	0.0006
Paternal	0.0006	0.0006		0.0006
Dual	0.0006	0.0006	0.0006	

PERMANOVA	Control	Maternal	Paternal	Dual
Control		0.0006	0.0006	0.0006
Maternal	0.0006		0.0006	0.0006
Paternal	0.0006	0.0006		0.0006
Dual	0.0006	0.0006	0.0006	

d. Male frontal profile lollipop diagram	MANOVA (Wilk's Lambda Statistic), ANOSIM, PERMANOVA	n = 50 control, 47 maternal, 47 paternal, 48 dual parental
i. Female right profile lollipop diagram shift in facial features	MANOVA (Wilk's Lambda Statistic), ANOSIM, PERMANOVA	Female: n = 46 control, 49 maternal, 41 paternal, 54 dual parental
e. Male right profile wire diagram **j.** Female right profile wire diagram	MANOVA (Wilk's Lambda Statistic), ANOSIM, PERMANOVA	Males: n = 50 control, 47 maternal, 47 paternal, 48 dual parental; Female: n = 46 control, 49 maternal, 41 paternal, 54 dual parental
l. Upper facial depth (top: male, bottom: female)	One-way ANOVA, Dunnett's multiple comparison test	Males: n = 103 control, 73 maternal, 111 paternal, 106 dual parental; Female: n = 78 control, 98 maternal, 108 paternal, 96 dual parental
m. Lower facial depth (top: male, bottom: female)	One-way ANOVA, Dunnett's multiple comparison test	Males: n = 103 control, 74 maternal, 111 paternal, 106 dual parental; Female: n = 78 control, 98 maternal, 108 paternal, 96 dual parental
n. Ear length (top: male, bottom: female)	One-way ANOVA, Dunnett's multiple comparison test	Males: n = 104 control, 65 maternal, 124 paternal, 105 dual parental; Female: n = 78 control, 90 maternal, 116 paternal, 94 dual parental
o. Eye ear distance (top: male, bottom: female)	One-way ANOVA, Dunnett's multiple comparison test	Males: n = 104 control, 66 maternal, 124 paternal, 106 dual parental; Female: n = 78 control, 90 maternal, 116 paternal, 95 dual parental
p. Mid facial depth dose correlation (top: male, bottom: female)	Pearson correlation, two-tailed	Males: n = 37 maternal, 55 paternal, 53 dual parental; Females: n = 49 maternal, 53 paternal, 47 dual parental

Table 4.1. *Continued*

GRAPH	STATISTICAL TEST		SAMPLE SIZE		
Sex	**Treatment**	**r**	**R^2**	**p-value**	**Summary**
Males	Maternal	-0.4160	0.1731	0.0104	*
	Paternal	-0.3076	0.0946	0.0223	*
	Dual	-0.2561	0.0656	0.0641	#
Females	Maternal	-0.2535	0.0642	0.0789	#
	Paternal	-0.0249	0.0006	0.8594	ns
	Dual	-0.4414	0.1948	0.0019	**
q. Ocular size (top: male, bottom: female)	One-way ANOVA, Dunnett's multiple comparison test		Males: n = 104 control, 74 maternal, 114 paternal, 106 dual parental; Female: n = 78 control, 99 maternal, 108 paternal, 96 dual parental		
r. Left ocular size (top: male, bottom: female)	One-way ANOVA, Dunnett's multiple comparison test		Male: n = 52 control, 37 maternal, 57 paternal, 53 dual parental; Female: n = 39 control, 50 maternal, 54 paternal, 48 dual parental		
s. Right ocular size (top: male, bottom: female)	One-way ANOVA, Dunnett's multiple comparison test		Male: n = 52 control, 37 maternal, 57 paternal, 53 dual parental; Female: n = 39 control, 49 maternal, 54 paternal, 48 dual parental		
t. Inner canthal distance (top: male, bottom: female)	One-way ANOVA, Dunnett's multiple comparison test		Male: n = 52 control, 37 maternal, 53 paternal, 53 dual parental; Female: n = 39 control, 50 maternal, 53 paternal, 48 dual parental		
u. Biparietal distance (top: male, bottom: female)	One-way ANOVA, Dunnett's multiple comparison test		Male: n = 53 control, 33 maternal, 63 paternal, 52 dual parental; Female: n = 40 control, 49 maternal, 57 paternal, 48 dual parental		
v. Relative normalized inner canthal distance (top: male, bottom: female)	One-way ANOVA, Dunnett's multiple comparison test		Male: n = 52 control, 33 maternal, 53 paternal, 52 dual parental; Female: n = 39 control, 49 maternal, 53 paternal, 48 dual parental		

3. Results

3.1. A modified version of the Drinking in the Dark paradigm to study the impacts of maternal, paternal, and dual parental alcohol consumption on offspring health

During the preconception and pregnancy phases, we did not observe any differences in daily maternal food intake between treatments (Figure 4.1B). Moreover, we did not observe any differences in maternal weight gain between treatments (Figure 4.1C). Pair-feeding is an additional control employed to account for altered maternal nutrition when drug exposures reduce food intake during pregnancy (Spear & Heyser, 1993). However, as maternal alcohol exposure did not measurably impact food consumption or maternal weight gain, we did not implement a pair-fed control. During the exposure window of the preconception phase, we did not observe any differences in dam fluid consumption between treatments (Figure 4.1D). However, during pregnancy, Control dams consistently drank more fluid (g/kg) during the exposure window than EtOH-exposed dams (Figure 4.1D). We did not observe any differences in sire weight gain or fluid consumption between treatments (Figure 4.1E-F).

We determined the daily EtOH dose by multiplying the average weekly fluid consumption (g/g) by 0.10 (10% EtOH), divided this number by 7 (days), and converted the resulting values to grams per kilogram (g/kg), consistent with clinical studies (Leeman et al., 2010). We then compared the daily EtOH doses between the preconception and pregnancy phases. Consistent with publications from other groups (Rhodes et al., 2005), during the preconception phase, females obtained a significantly higher daily EtOH dose than males (Paternal 2.7 g/kg Maternal: 3.9 g/kg, $p<0.01$, Figure 4.1G). However, during the gestational phase (right side of dashed line), pregnant dams obtained a lower daily EtOH dose (2.8 g/kg) and were not significantly different from males (Figure 4.1G). Finally, we did not observe any differences in maternal EtOH average daily dose

between the MatExp and DualExp treatment groups for either the preconception or gestation phases (preconception MatExp: 3.279 g/kg and Dual Exp 3.011 g/kg; gestation MatExp: 2.945 g/kg and DualExp 2.773 g/kg; Figure 4.1H).

Stress during the preconception period modifies the maternally- and paternally-inherited epigenome (Chan et al., 2018). Therefore, we subjected mice to minimal handling and only measured plasma alcohol concentrations once during the preconception phase. We collected blood from a subset of the treated mice (at the end of the four-hour exposure cycle) and measured plasma alcohol levels using an M1 Alcohol Analyzer (Analox Technologies, Toronto, ON, Canada). During the preconception phase, treated dams exhibited average plasma alcohol levels of 84 mg/dL, while males averaged 92 mg/dL. Plasma alcohol levels were not significantly different between EtOH-treated sires and dams (Figure 4.1I). These plasma alcohol levels are entirely consistent with previous studies by our group and others using this model (Chang, et al., 2019; Thiele et al., 2014; Thomas et al., 2021, 2022). Notably, this concentration is equivalent to blood alcohol levels at or slightly above the U.S. legal limit for operating a motor vehicle (0.084 and 0.092) and is representative of the drinking patterns reported for one-third of U.S. adults (Naimi et al., 2003; White et al., 2006).

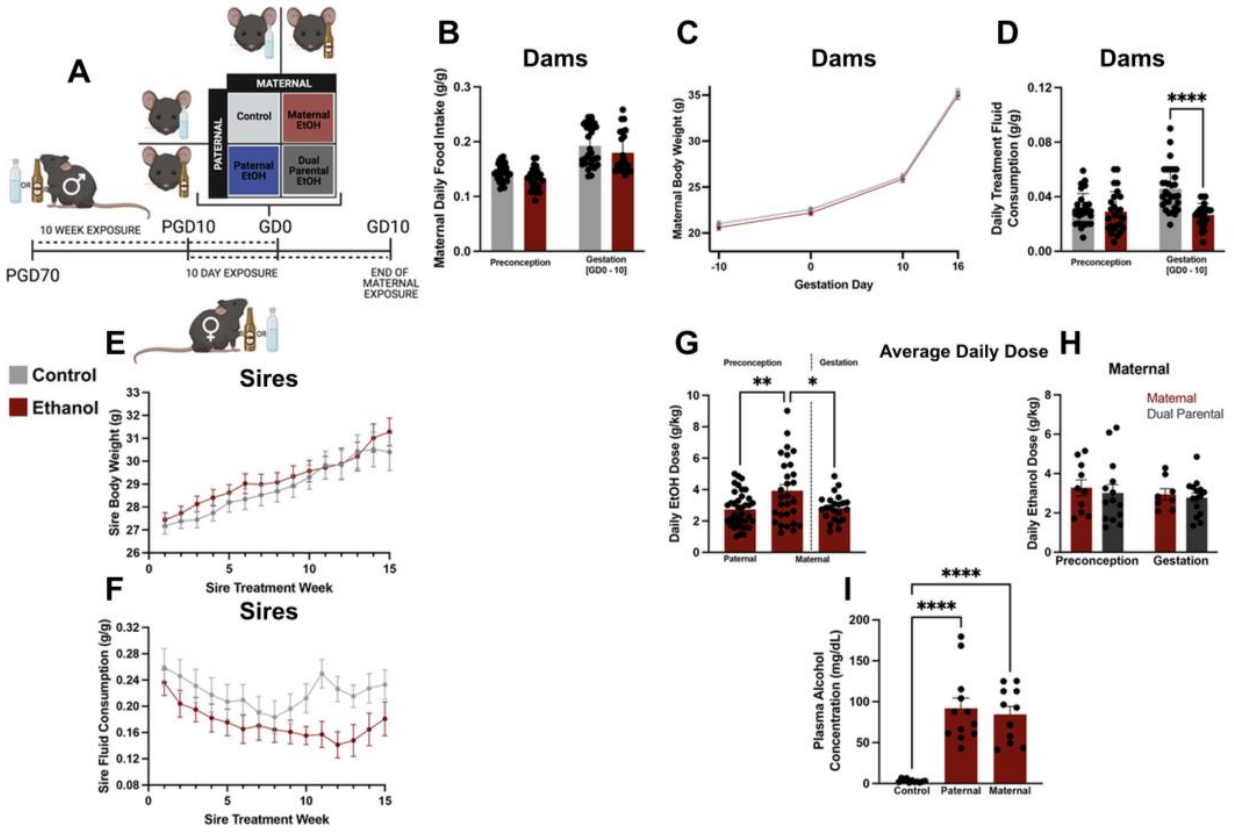

Figure 4.1. A modified version of the Drinking In The Dark paradigm to study the impacts of maternal, paternal, and dual parental alcohol consumption on offspring health. A) Experimental design employed to contrast the impacts of differing patterns of parental alcohol consumption on the emergence of alcohol-related birth defects. B) Comparison of daily maternal food intake between the Control and EtOH treatments, both preconception and during gestation. C) Comparison of dam weight gain measured across the exposure course and into pregnancy. D) Daily maternal fluid consumption between the Control and EtOH treatments during the preconception and gestation phases. Comparisons of sire E) weight gain and F) fluid consumption across the exposure course. G) Average daily dose of EtOH compared between males and females and between the preconception (left of the dashed line) and gestation (right of the dashed line) phases. We calculated the average daily dose by multiplying the average weekly fluid consumption (g/g) by 0.10 (10% EtOH), dividing this number by 7 (days), and converting to g/kg. We used an ANOVA to compare the preconception and pregnancy phases. H) Average daily EtOH dose compared between females within the MatExp and DualExp treatments, between the preconception and gestation phases. I) Comparison of plasma alcohol levels between treated males and females during the preconception phase. Data represent mean ± SEM, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. [Reprinted from Thomas et al., 2023]

3.2. Analysis of pregnancy and fetal offspring physiological measures

On average, males sired offspring after nine to twelve weeks of exposure, and we did not observe any differences between treatment groups (Figure 4.2A). Further, we did not observe any significant differences in pregnancy success rates between treatment groups (Figure 4.2B). After pregnancy diagnosis, we provided dams with additional cage enrichments, including three nestlets, one Manzanita wood gnawing stick (catalog# W0016, Bio-Serv, Flemington, NJ, USA), and one gummy bone (catalog# K3585, Bio-Serv, Flemington, NJ, USA). On gestational day 16.5, we sacrificed pregnant dams using CO2 asphyxiation followed by cervical dislocation, then excised the female reproductive tract. We did not observe differences in normalized uterine horn weights or litter size between treatment groups (Figure 4.2C-D).

We did not observe any differences in the ratio of males and females between treatment groups (Figure 4.2E). In clinical studies, FAS children present as small for their gestational age, exhibiting perinatal weights and head circumference below the 10th percentile (Carter et al., 2016). We did not observe any differences in litter average fetal weights between treatment groups (Figure 4.2F). However, in the DualExp treatment group, we did observe an increase in the proportion of male offspring at or below the smallest 10th percentile of the Control population (Figure 4.2G) but did not observe this change in female offspring (Figure 4.2H). After collecting fetal measures, we imaged offspring heads under a stereomicroscope.

Finally, we selected the four fetuses closest to the cervix from each litter, dissected the fetal brain, and measured brain weights. We observed a significant increase in female brain-to-body weight ratios for offspring in the MatExp and DualExp treatment groups (Figure 4.2I). Due to our inability to definitively identify phenotypic sex at this developmental stage and random sampling, there were fewer male fetuses in the MatExp treatment (Male brains: n = 38 control, 18 maternal,

40 paternal, 34 dual parental), which may have limited our ability to detect changes in male offspring brain weights. After dissections, we either fixed tissue samples in 10% neutral buffered formalin (catalog# 16004-128, VWR, Radnor, PA, USA) or snap-froze the tissues on dry ice and stored them at −80°C.

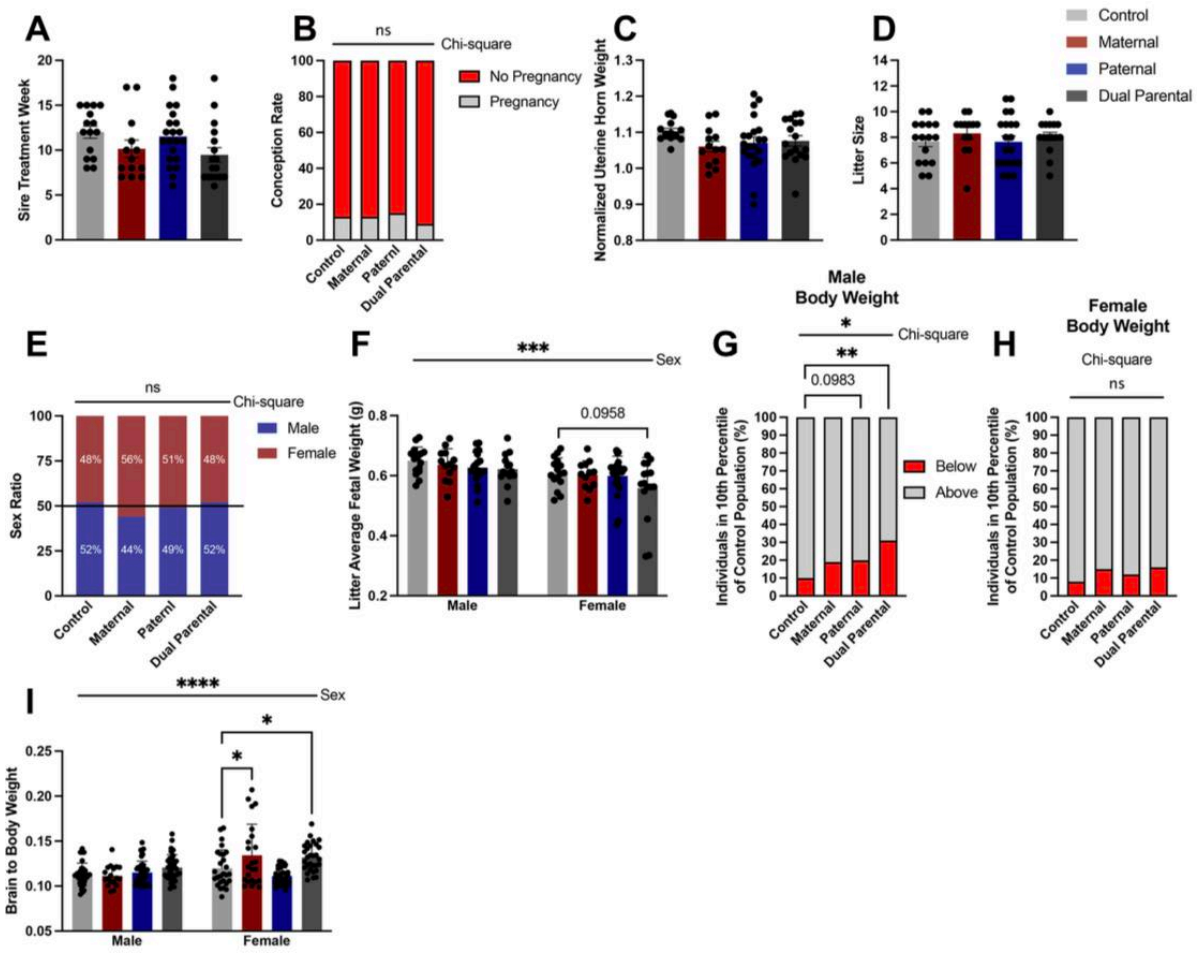

Figure 4.2. Analysis of pregnancy and fetal offspring physiological measures. A) Comparison of sire treatment week at the time of conception between treatment groups. B) Comparison of pregnancy success rates between treatment groups. We did not detect any differences in C) normalized uterine horn weights, D) litter size, E) the ratio of male and female offspring, or F) litter average fetal weights between treatment groups. Differences in the proportion of G) male but not H) female offspring at or below the smallest 10th percentile of the control population. I) Comparison of brain-to-body weight ratios between treatment groups. Data represent mean ± SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. [Reprinted from Thomas et al., 2023]

3.3. Maternal, Paternal, and Dual Parental alcohol exposures each induce changes in offspring craniofacial patterning

We first employed geometric morphometric analyses (Klingenberg et al., 2010) to contrast the geometric relationships between 16-18 craniofacial landmarks in offspring derived from Control, MatExp, PatExp, and DualExp pairings. Canonical variant analysis of geometric facial relationships revealed that MatExp, PatExp, and DualExp treatments each induced distinct signatures of morphometric change, with paternal exposures frequently inducing the most significant shifts in craniofacial patterning (Figure 4.3B). Further, our analyses revealed that alcohol-induced changes predominantly centered on the lower portions of the face, including the mandible (lower jaw), maxilla (upper jaw), and positioning of the ear. As in clinical studies of FAS children (Klingenberg et al., 2010), we identified a shift of midline features to the right.

To validate our observations, we examined offspring for alterations in established measures of alcohol-induced craniofacial defects. Consistent with our geometric morphometric analysis, paternal and dual-parental alcohol exposures induced significant reductions in offspring head size (Figure 4.3D), ocular size (Figure 4.3E), midfacial depth (Figure 4.3F), and snout-occipital distance (Figure 4.3G). Notably, the reductions in ocular size primarily manifested in the right eye (Figure 4.3E).

Finally, we conducted a dose-response analysis, contrasting offspring snout-occipital distance and bodyweight-normalized brain weights with the parental average daily ethanol dose (g/kg). We identified a significant correlation between both measures and parental alcohol dose for male offspring across the PatExp and DuelExp treatments (Figure 4.3H-I). In contrast, we only observed a significant interaction for females in the MatExp and DualExp treatments (Figure 4.3H-I). Aligning with previous studies examining maternal alcohol exposures using the C57BL/6J

mouse model (Petrelli et al., 2018), our dose-response analyses identified a consistent inflection point of 2.9 g/kg, past which paternal alcohol exposures induce significant craniofacial and central nervous system growth deficiencies (Figure 4.3H-I dashed line).

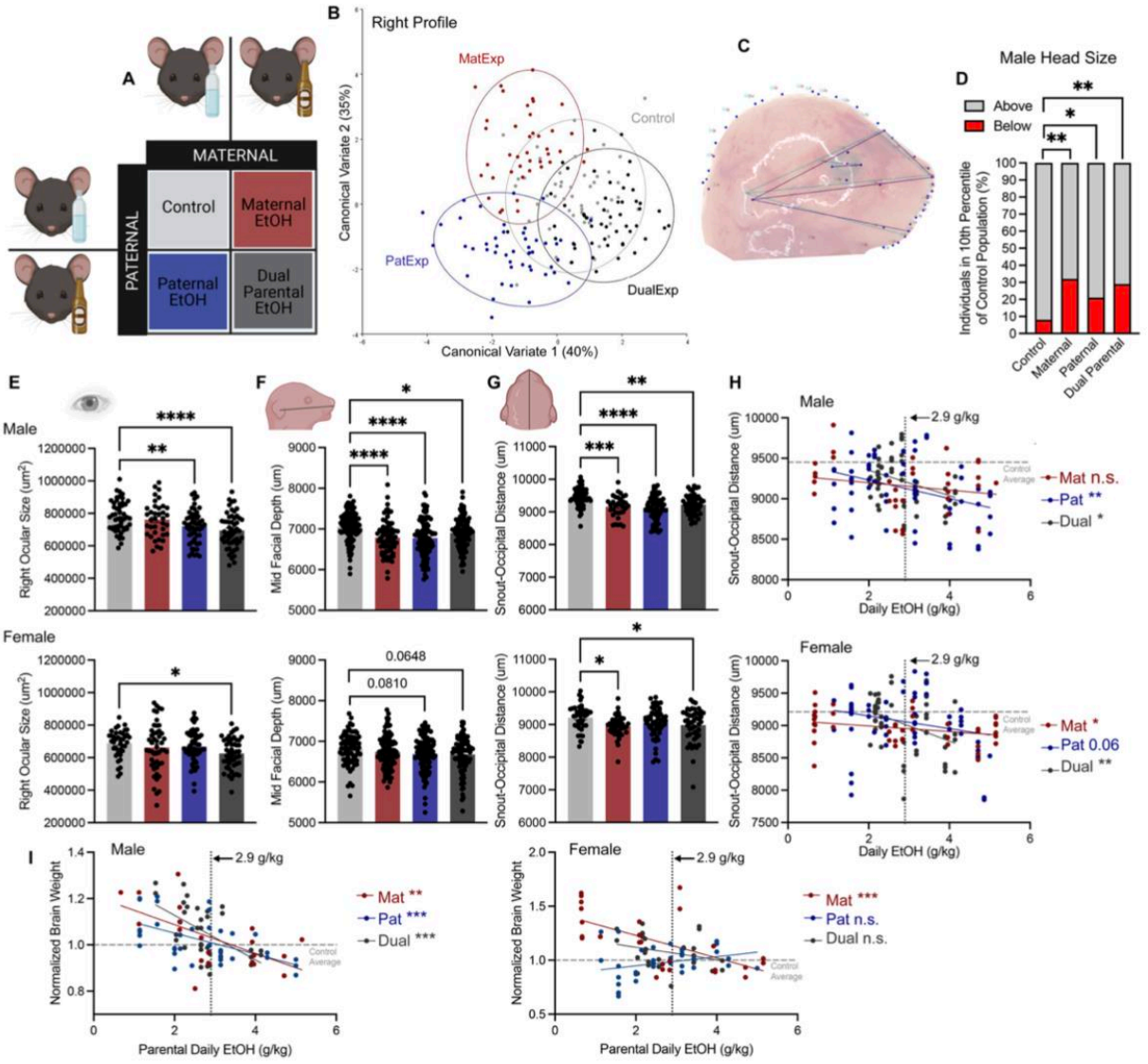

Figure 4.3. Maternal, Paternal, and Dual Parental alcohol exposures each induce changes in offspring craniofacial patterning. A) We used a 2x2 factorial design to contrast craniofacial development between the offspring of maternal, paternal, and dual parental alcohol exposures. Using geometric morphometrics followed by canonical variate analysis and MANOVA, we identified treatment-specific shifts in the profiles of the gestational day 16.5 face (right profile male offspring shown in B, n=~48). C) Representative wire diagram demonstrating the shift in facial landmarks of a PatExp male head along principal component 1. The blue line demarcates the individual sample, while the turquoise line demarcates the average of the entire population. D) Chi-square analysis followed by Holm-Bonferroni correction revealed an increased incidence of microcephaly, with an increased proportion of male offspring exhibiting head sizes at or below the smallest 10th percentile of the Control population. We used ANOVA, followed by Dunnett's multiple

comparison test, to examine established alcohol-related craniofacial phenotypes, including changes in E) ocular size (right eye shown), F) midfacial depth, and G) snout-occipital distance (males top, females bottom; n=30-50). Pearson correlation analysis followed by Holm-Bonferroni correction contrasting offspring H) snout-occipital distance (males top, females bottom) and I) normalized brain weights (males left, females right) with average parental daily EtOH dose (n=30-50, see Supplemental Table 1). Data represent mean ± SEM, * P < 0.05, ** P < 0.01, *** P < 11 0.001, **** P < 0.0001. [Reprinted from Thomas et al., 2023]

3.4. Maternal, Paternal, and Dual Parental alcohol exposures program dose-dependent changes in offspring craniofacial patterning

Our multivariate analysis of male CV scores revealed significant differences between treatments for the front (MANOVA p = 2.223E-90, ANOSIM p<0.0001, R = 0.6316, PERMANOVA p<0.0001; Figure 4.4A), left (MANOVA p = 4.832E-67, ANOSIM p<0.0001, R = 0.4736, PERMANOVA p<0.0001; Figure 4.4B), and right profiles (MANOVA p = 6.737E-87, ANOSIM p<0.0001, R = 0.6178, PERMANOVA p<0.0001; Figure 4.4C), with each treatment exhibiting significant differences during pairwise comparisons (Table 4.1). Notably, Procrustes ANOVA identified significant (p = 0.0103) shifts in overall face shape between treatments. Further analyses of male craniofacial shape revealed that most changes centered around the lower portion of the face, including the mandible (lower jaw), maxilla (upper jaw), and positioning of the ear (Figure 4.4D-E). As in clinical studies of FAS children (16), we identified a shift of midline landmarks to the right (Figure 4.4D).

Our multivariate analysis of female CV scores revealed significant differences between treatments in the front (MANOVA p = 7.85E-95, ANOSIM p<0.0001, R = 0.6848, PERMANOVA p<0.0001; Figure 4.4F) left (MANOVA p = 2.89E-77, ANOSIM p<0.0001, R = 0.5696, PERMANOVA p<0.0001; Figure 4.4G), and right profiles (MANOVA p = 1.15E-71, ANOSIM p<0.0001, R = 0.5196, PERMANOVA p<0.0001; Figure 4.4H), with each treatment exhibiting significant differences during pairwise comparisons (Table 4.1). As with male offspring, most identified changes centered around the lower portion of the face (Figure 4.4I-J). However,

Procrustes ANOVA did not identify any significant shifts in female overall face shape between treatments.

Similar to clinical studies (Basnet et al., 2015), we determined ear size and positioning by measuring the distance from the dorsal to ventral aspects of the external ear and the distance between the central auditory canal and the center of the pupil, respectively (Figure 4.4K). For male offspring, we identified reductions in upper facial depth in the PatExp treatment and a trend towards reduced size in the MatExp treatment ($p = 0.0617$) (Figure 4.4L). We did not observe any differences in the upper facial depth of female offspring across any treatment (Figure 4.4L). Consistent with our geometric morphometric analysis, male offspring across all treatments displayed significant reductions in midfacial and lower facial depth, while female offspring in the PatExp and DualExp treatments displayed similar trends ($p = 0.08$ to 0.06) (Figure 4.3F; Figure 4.4M). Male and female offspring across all treatment groups exhibited significant reductions in ear size (Figure 4.4N). Further, male offspring across all treatments and DualExp female offspring exhibited significant decreases in the distance between the central aspects of the eyes and ears (Figure 4.4O).

In clinical studies, alcohol-induced changes in craniofacial shape exhibit dose-dependent effects (Suttie et al., 2013). Therefore, we used linear regression analysis to compare changes in offspring shape with the parental average daily EtOH dose (g/kg). These analyses revealed dose-dependent effects on male snout-occipital distance, midfacial depth, and body weight normalized brain weights across most treatments (Figure 4.3H-I; Figure 4.4P). Female offspring only displayed dose-dependent effects in a subset of these measures.

We then used the area tool on lateral images to determine ocular size (area um2), then used an ANOVA to compare the impacts of each treatment. Male offspring in the PatExp and DualExp

treatments exhibited significant reductions in ocular size, while only DualExp females were significantly different from the controls (Figure 4.4Q). Previous studies examining EtOH-induced craniofacial dysgenesis using mouse models of maternal exposure demonstrate that reductions in eye size appear right-side dominant (Lipinski et al., 2012; Parnell et al., 2006). Similarly, clinical studies demonstrate that gestational EtOH exposures alter facial symmetry with a right-shift in facial features (Klingenberg et al., 2010). Consistent with these previous studies, we observed significant reductions in ocular size for PatExp and DualExp male and DualExp female offspring in the right but not the left eye (Figure 4.3E, Figure 4.4R-S).

We did not observe any significant differences in philtrum length, although female offspring in the PatExp treatment trended towards a reduction ($p = 0.0706$) (data not shown). Male offspring across all treatment groups exhibited reductions in snout-occipital distance, while only female offspring in the MatExp and DualExp treatment groups exhibited significant reductions (Figure 4.3G). Male offspring in the PatExp and DualExp treatments exhibited reduced inner canthal distances, while female offspring did not exhibit any significant changes in this feature (Figure 4.4T). Male offspring across all treatment groups exhibited reductions in biparietal distance, while only female offspring in the DualExp treatment exhibited reductions (Figure 4.4U). Finally, to determine if changes in inner canthal distance are driven by microcephaly or represent relative changes in eye spacing, we normalized inner canthal distance to biparietal distance and compared offspring between treatment groups. Male and female offspring from all three treatment groups exhibited increases in relative inner canthal distance (Figure 4.4V).

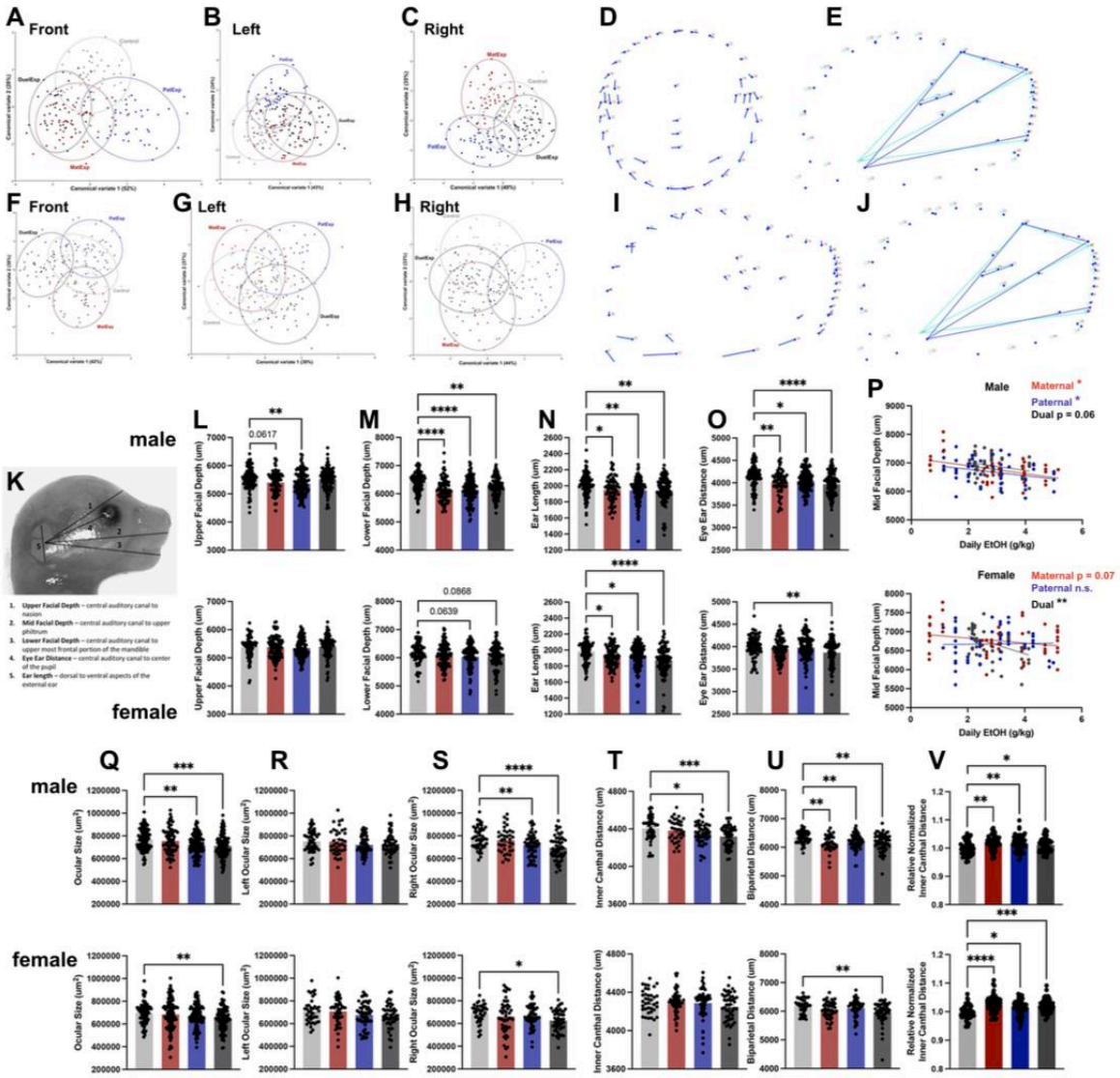

Figure 4.4. Maternal, Paternal, and Dual Parental alcohol exposures program dose-dependent changes in offspring craniofacial patterning. We conducted geometric morphometric analysis on the front, left, and right profile images obtained from ~48 male and ~48 female offspring per treatment, then employed canonical variate analysis to identify significant differences in clustering and distance between treatment groups. We identified significant shifts in the front (A, F), left (B, G), and right (C, H) profiles of male and female offspring. Morphometrics revealed a right-shift in central facial features and a rear shift in the lower jaw. Lollipop diagram describing the shift in central (male offspring D) and lower (female offspring I) facial features along PC1, with the stick of the lollipop signifying the shift further along the PC1 axis. Wire diagrams demonstrating the shift in key facial landmarks along PC1 (male offspring E, female offspring J), with the turquoise line demarcating the average and the blue line demarcating the shift. To validate our morphometric analysis, we assayed established measures of craniofacial morphology (K) disrupted in mouse models of prenatal alcohol exposure (13) and compared the effects of each treatment on male (row top) and female (row bottom) offspring. We measured the effects of parental alcohol exposures on upper facial depth (L), lower facial depth (M), ear length (N), and the distance between the central auditory canal and the center of the pupil (O). We then conducted a Pearson correlation analysis contrasting the midfacial depth of male (top) and female (bottom) offspring with average parental daily EtOH dose (P). We measured the effects of parental alcohol exposures on both eyes (Q), then individually examined the left (R) and right (S) eyes in male (row top) and female (row bottom) offspring. We measured the effects of parental alcohol exposures on inner canthal (T) and biparietal (U) distance in male (row top) and female (row bottom) offspring. We normalized inner canthal distance to biparietal (V) and compared the effects of each treatment on male and female offspring. Data represent mean ± SEM, * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, **** $P = 0.001$. [Reprinted from Thomas et al., 2023]

4. Discussion

In 1981, the U.S. Surgeon General issued a public health advisory warning that alcohol use by women during pregnancy could cause birth defects. Since this time, maternal alcohol use during pregnancy has remained the sole explanation for alcohol-related birth defects. In contrast, paternal drinking and lifestyle choices remain unexamined. Using a physiologically relevant mouse model, our studies are the first to demonstrate that male drinking is a plausible yet completely unexamined factor in the development of alcohol-related craniofacial abnormalities and growth deficiencies. Our study demonstrates the critical need to target both parents in prepregnancy alcohol messaging and to expand epidemiological studies to measure the contributions of paternal alcohol use on children's health.

CHAPTER V

PATERNAL ALCOHOL EXPOSURE LEAVES A LASTING IMPACT ON OFFSPRING HEALTH AND BEHAVIOR ACROSS THE LIFESPAN

1. Introduction

Fetal Alcohol Spectrum Disorder (FASD) is caused by alcohol exposure before birth and can results in a broad range of developmental physical and functional abnormalities. The term "Fetal" in FASD is somewhat misleading as prenatal alcohol exposure is a lifelong disorder and not simply a pediatric condition. However, little is known of later life health outcomes in patients born with FASD (Moore & Riley, 2015).

A clinical study found that after puberty Fetal Alcohol Syndrome (FAS), often referred to as the most severe form of FASD, facial features become less distinctive and body weight deficits diminish, however, maladaptive behavioral issues are highly distinctive (Streissguth et al., 1991). Importantly, although FASD is often described as a continuum in regard to severity, this representation can be misleading when it comes to brain and behavioral outcomes. Patients with "less severe" diagnoses such as Alcohol-Related Neurodevelopmental Disorder (ARND) often experience similar, if not more severe, brain dysfunction (Connor & Streissguth, 1996). Notably, maladaptive behavior due to perinatal alcohol exposure, is one of the most persistent FASD symptoms and can impact individuals across the lifespan.

Although contrary to the long-held dogma that the maternal alcohol consumption is to blame for FASD outcomes, recent work by our lab has shown that preconception paternal alcohol exposure results in craniofacial growth deficiencies during fetal development (Thomas et al., 2023). As craniofacial growth is directly linked to brain anatomical structure and neurological

development, we hypothesized that the offspring born to alcoholic fathers would also exhibit distinct behavioral changes in postnatal life. To test this, we reared offspring from maternal, paternal, and dual parental alcohol exposures (Mat-Exp, Pat-Exp, Dual-Exp, respectively) and analyzed macro measures of offspring health and behavior during multiple phases of the lifespan, including pubescent and sexually mature adolescence and immature and mature adulthood.

2. Materials and Methods

2.1. Ethics

All experiments were conducted under IACUC 2020-0211, approved by the Texas A&M University IACUC. All data is reported per ARRIVE guidelines.

2.2. Animal husbandry

We used C57BL/6J mice (Strain #:000664 RRID: IMSR_JAX:000664), housed under a reverse 12-h light/dark cycle. We maintained mice on a standard chow diet (catalog# 2019, Teklad Diets, Madison, WI, United States) and implemented additional animal husbandry measures to minimize stress, including shelter tubes for males and igloos for females (catalog# K3322 and catalog# K3570, Bio-Serv, Flemington, NJ, United States).

2.3. Maternal and Paternal periconceptional alcohol exposure

We performed periconceptional treatment according to our published protocol (Thomas et al., 2023). Wherein, we exposed postnatal day 90, adult C57BL/6J mice to either 10% w/v ethanol or water as our control using a voluntary limited-access exposure model. Specifically, we exposed mice to their corresponding treatments for four hours during the dark cycle. We exposed males to

chronic treatment described previously (Bedi et al., 2019; Chang et al., 2017; Chang, et al., 2019; Thomas et al., 2021, 2022, 2023). We maintained males on treatment for six weeks (approximately one spermatogenic cycle) and females for ten days (approximately two estrus cycles) before mating. After the preconception exposure window, we synchronized female reproductive cycles using the Whitten method (Whitten et al., 1968) and initiated matings directly following the four-hour exposure window by placing the female in the male's cage. We implemented a multifactorial mating paradigm to generate offspring from maternal, paternal, and dual parental alcohol exposed offspring (Mat-Exp, Pat-Exp, Dual-Exp, respectively). Copulation was confirmed by the presence of a vaginal plug and females were returned to their home cage and continued daily respective treatments. 10 days following copulation, we diagnosed pregnancy by determining weight gain from plug date, with a 1.8-gram body weight increase resulting in a positive pregnancy diagnosis. Following pregnancy diagnosis, we removed pregnant females from treatment and left undisturbed.

2.4. Postnatal offspring

Beginning on postnatal day seven, we began recording weekly offspring body weight and on postnatal day 21, we weaned the offspring and group housed litters in same sex cages.

2.5. Behavioral testing

At six weeks of age, we began behavioral testing according to the behavioral testing procedure depicted in Figure 5.1. We randomly selected 2 offspring of each sex (2 males and 2 females) and conducted behavioral assays during multiple stages of development, including pubescent adolescence, sexually mature adolescence, immature adulthood, and mature adulthood. Behavioral

testing occurred in a behavioral testing procedure room adjacent to the housing room. On testing days, mice were acclimated to the procedure room for a minimum of 30 minutes prior to the onset of behavioral testing and individually caged in a testing cage for a 30-minute acclimation period.

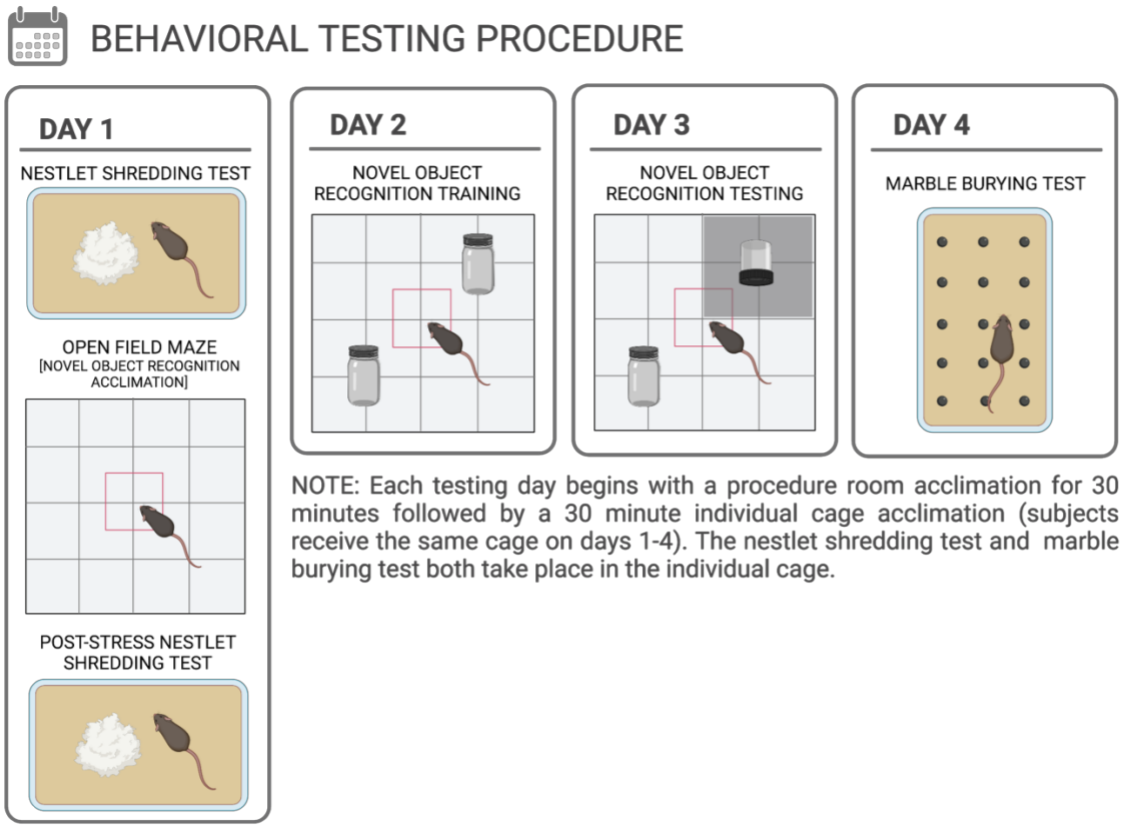

Figure 5.1. Behavioral testing procedure

2.5.1. Nestlet shredding test. We conducted the nestlet shredding test according to a published protocol (Angoa-Pérez et al., 2013). Mice were first acclimated to the testing cage for 30 minutes. After acclimation, we placed a pre-weighed nestlet in the center of the cage and left the subject undisturbed for 30 minutes. At the conclusion of the testing window, we weighed the un-shredded nestlet and then subtracted the value from the initial weight to calculate the amount of nestlet shredded in grams.

2.5.2. Open field maze. We implemented an open field maze testing protocol that was adapted from a published protocol (Seibenhener & Wooten, 2015). We first allowed subjects to acclimate to the testing room for a minimum of 30 minutes, after which we placed the mouse into the center of a smart frame open field station with 16x16 beams (catalog #: SFOF, Kinder Scientific) inside an open field sound attenuation chamber (catalog #: OFSAC, Kinder Scientific). The maze was connected to the behavioral system core 2-station (catalog #: BSC Jump, Kinder Scientific) that allowed testing data to be collected and analyzed with MotorMonitor II application software (catalog #: 2050-0024, Kinder Scientific). We programmed the software for a sixteen-minute data acquisition frame and then excluded the first minute upon analysis in order to allow the mouse to acclimate upon entrance to the testing chamber. After each use the mouse was returned to the appropriate cage and we removed any physical debris (i.e. fecal matter and urine) from the arena and discarded it in the biohazard waste. We then sprayed the arena and a Kimwipe with 100% ethanol. The ethanol saturated Kimwipe was first used to wipe down the walls and floor, making sure to lift the walls and wipe underneath. We allowed the arena to air dry (approximately 5 minutes), until no liquid ethanol was visible.

2.5.3. Post-stress nestlet shredding test. We conducted a second nestlet shredding test as described above, immediately following the mouse's removal from the open field maze. The amount shredded was calculated and a stress index was implemented in order to assess the stress endured during the open field maze, which can induce stress due to the mouse's natural aversion to open spaces and bright lights. We calculated the stress index as follows: (amount shredded during post-stress nestlet shredding test)/[(amount shredded during pre-stress nestlet shredding test)+(amount shredded during post-stress nestlet shredding test)*100.

2.5.4. Novel object recognition. The novel object recognition test is a multiphase behavioral testing procedure consisting of an acclimation, training, and testing phase (Antunes & Biala, 2012). Since the open field maze test is essentially the novel object recognition test acclimation phase, we simultaneously conducted both tests (see Open Field Maze section for details). Mice were then rested for 24 hours, after which we implemented the training phase, wherein two like objects (plastic specimen cup with purple lid) were placed in opposite quadrants of the open field maze testing apparatus and the mouse was placed in the center of the open field chamber. We programmed the software to collect data for an 11-minute window, and upon analysis, we excluded the first minute to allow the mouse to acclimate to the arena. Following the session, we removed the mouse and placed back in the respective cage. In addition to the cleaning procedure described under Open Field Maze, we sprayed the objects with 100% ethanol and wiped them down with an ethanol saturated Kimwipe and allowed them to air dry. Finally, 24 hours after the training phase, we began the testing phase, in which we exchanged one of the like objects for a novel object (glass jar with purple lid, note: both the jar and specimen cup were of approximately equal size). We placed the mouse in the center of the box and began the 11-minute testing window. Again, the first minute was excluded during analysis to allow for acclimation to the testing chamber. Following the test, the mouse was removed, and objects and open field were cleaned as described previously. The novel object discrimination index was calculated by dividing the time spent with the novel object by the total object exploration time and multiplied by 100 (Hattiangady et al., 2014).

2.5.5. Marble burying test. The marble burying test was conducted according to a published protocol (Angoa-Pérez et al., 2013). Specifically, we placed 15 marbles on level bedding in a 5 x 3 pattern. Mice were placed in the center of the cage and tested for 30 minutes. After the elapsed

testing time, we removed the mouse and counted how many marbles were not buried. A marble was considered “not buried” if more than half of the sphere was above the bedding. After use, we soaked the marbles in 10% bleach overnight, and rinsed them the next day with water. Finally, we sprayed the marbles with 100% ethanol and allowed to air dry.

2.5.6. Voluntary physical activity. In mature adulthood, 16 weeks of age, we implemented voluntary physical activity for one female and one male offspring per litter. Specifically, we individually caged mice and placed a low-profile wireless running wheel (catalog #: ENV-047, Med Associates Inc) into the subject’s home cage. We acclimated the subject to the running wheel for one week and then conducted a one-week testing phase (2 weeks of total voluntary physical activity exposure). Data was wirelessly sent to the USB hub device (catalog #: DIG-807, Med Associates Inc) and analyzed using wheel manager and analysis software (catalog #: SOF-860 and SOF-861, Med Associates Inc).

2.6. Dissection and tissue collection

We terminated adult offspring at postnatal week 20 using carbon dioxide asphyxiation and subsequent cervical dislocation. The head was then severed using surgical decapitation scissors. We dissected the heart, liver, kidneys, adrenal glands, thymus, and spleen. We weighed the tissues and snap froze on dry ice before long-term storage at −80°C.

2.7. DXA scan

We conducted dual-energy X-ray absorptiometry (DXA) scans immediately preceding dissection and tissue collection at postnatal week 20. Mice were positioned on a sample tray in a prone

position for analysis. Total body bone mineral density and fat and lean mass were determined using the Faxitron Pro x-ray cabinet (Hologic Inc., MA, USA) and Vision DXA version 2.4.2.Rev3U BETA (64-Bit) software (Hologic Inc., MA, USA).

2.8. Statistical analysis

Statistical analysis was conducted in GraphPad Prism 9. For all analyses statistical significance was set at p=0.05 (95% confidence interval). For analyses consisting of two factors (i.e., treatment and age, or sex and age) we conducted two-way ANOVAs followed by Dunnett's multiple comparison test. If the two factors resulted in a statistically significant interaction, then uncorrected Fisher's LSD was used, which allows each comparison to stand alone and thus mitigating the interacting effect. Specific statistical information is provided in **Table 5.1**.

Table 5.1. Description of the statistical tests for each figure

Figure	Test	Sample size
Figure 5.2. The effects of parental alcohol on gross metrics of offspring health.		
A. Gestation length	One-way ANOVA, Dunnett's multiple comparison test	n = 19 Control, 21 Mat-Exp, 14 Pat-Exp, 10 Dual-Exp
B. Litter size	One-way ANOVA, Dunnett's multiple comparison test	n = 19 Control, 20 Mat-Exp, 15 Pat-Exp, 11 Dual-Exp
C. Neonatal mortality	One-way ANOVA, Dunnett's multiple comparison test	n = 19 Control, 20 Mat-Exp, 15 Pat-Exp, 11 Dual-Exp
D. Early postnatal offspring body weight	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 19 Control, 20 Mat-Exp, 16 Pat-Exp, 11 Dual-Exp
E. Sex ratio	Contingency, Chi-square test	n = Control: 75 males, 60 females, Mat-Exp: 54 males, 63 females, Pat-Exp: 64 males, 54 females, Dual-Exp: 32 males, 33 females
F. Post-weaning body weight gain	Two-way (treatment and sex) ANOVA, Dunnett's multiple comparison test	Male: n = 39 Control, 35 Mat-Exp, 29 Pat-Exp, 13 Dual-Exp Female: n = 32 Control, 32 Mat-Exp, 25 Pat-Exp, 9 Dual-Exp

Table 5.1. *Continued*

Figure	Test	Sample size
G. Adolescent offspring body weight	Two-way (treatment and age) ANOVA, Uncorrected Fisher's LSD *male and female are separate analyses with converged graph	Male: n = 35 Control, 36 Mat-Exp, 29 Pat-Exp, 23 Dual-Exp Female: n = 57 Control, 43 Mat-Exp, 36 Pat-Exp, 26 Dual-Exp
H. Adult male offspring body weight	Two-way (treatment and age) ANOVA, Uncorrected Fisher's LSD	n = 31 Control, 37 Mat-Exp, 28 Pat-Exp, 23 Dual-Exp
I. Adult female offspring body weight	Two-way (treatment and age) ANOVA, Uncorrected Fisher's LSD	n = 52 Control, 33 Mat-Exp, 36 Pat-Exp, 26 Dual-Exp
Figure 5.3. Parental alcohol exposure induces sex-specific changes in activity and anxiety.		
B. Male total distance	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 16 Control, 15 Mat-Exp, 14 Pat-Exp, 7 Dual-Exp (immature adult: 8 Mat-Exp, 5 Dual-Exp)
C. Female total distance	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 12 Control, 17 Mat-Exp, 14 Pat-Exp, 8 Dual-Exp (immature adult: 8 Mat-Exp, 6 Dual-Exp)
D. Male total rest time	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 16 Control, 15 Mat-Exp, 14 Pat-Exp, 7 Dual-Exp (immature adult: 8 Mat-Exp, 5 Dual-Exp)
E. Female total rest time	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 12 Control, 17 Mat-Exp, 14 Pat-Exp, 8 Dual-Exp (immature adult: 8 Mat-Exp, 6 Dual-Exp)
F. Male center entries	Two-way (treatment and age) ANOVA, Uncorrected Fisher's LSD	n = 16 Control, 15 Mat-Exp, 14 Pat-Exp, 7 Dual-Exp (immature adult: 8 Mat-Exp, 5 Dual-Exp)
G. Female center entries	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 12 Control, 17 Mat-Exp, 14 Pat-Exp, 8 Dual-Exp (immature adult: 8 Mat-Exp, 6 Dual-Exp)
H. Male center distance	Two-way (treatment and age) ANOVA, Uncorrected Fisher's LSD	n = 16 Control, 15 Mat-Exp, 14 Pat-Exp, 7 Dual-Exp (immature adult: 8 Mat-Exp, 5 Dual-Exp)
I. Female center distance	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 12 Control, 17 Mat-Exp, 14 Pat-Exp, 8 Dual-Exp (immature adult: 8 Mat-Exp, 6 Dual-Exp)
J. Male time in center	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 16 Control, 15 Mat-Exp, 14 Pat-Exp, 7 Dual-Exp (immature adult: 8 Mat-Exp, 5 Dual-Exp)
K. Female time in center	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 12 Control, 17 Mat-Exp, 14 Pat-Exp, 8 Dual-Exp (immature adult: 8 Mat-Exp, 6 Dual-Exp)

Table 5.1. *Continued*

Figure	Test	Sample size
Figure 5.4. Sex-specific and developmental stage-dependent changes in cognition in offspring exposed to maternal, paternal, and dual parental alcohol.		
B. Male total distance	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 16 Control, 14 Mat-Exp, 17 Pat-Exp, 7 Dual-Exp (immature adult: 5 Dual-Exp)
C. Female total distance	Two-way (treatment and age) ANOVA, Uncorrected Fisher's LSD	n = 12 Control, 15 Mat-Exp, 18 Pat-Exp, 8 Dual-Exp
D. Male total rest time	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 16 Control, 14 Mat-Exp, 17 Pat-Exp, 7 Dual-Exp (immature adult: 5 Dual-Exp)
E. Female total rest time	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 12 Control, 15 Mat-Exp, 18 Pat-Exp, 8 Dual-Exp
F. Male total object exploration time	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 16 Control, 14 Mat-Exp, 17 Pat-Exp, 7 Dual-Exp (immature adult: 5 Dual-Exp)
G. Female total object exploration time	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 12 Control, 15 Mat-Exp, 18 Pat-Exp, 8 Dual-Exp
H. Male time spent in novel object quadrant	Two-way (treatment and age) ANOVA, Uncorrected Fisher's LSD	n = 16 Control, 14 Mat-Exp, 17 Pat-Exp, 7 Dual-Exp (immature adult: 5 Dual-Exp)
I. Female time spent in novel object quadrant	Two-way (treatment and age) ANOVA, Uncorrected Fisher's LSD	n = 12 Control, 15 Mat-Exp, 18 Pat-Exp, 8 Dual-Exp
J. Male discrimination index	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 16 Control, 14 Mat-Exp, 17 Pat-Exp, 7 Dual-Exp (immature adult: 5 Dual-Exp)
K. Female discrimination index	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 12 Control, 15 Mat-Exp, 18 Pat-Exp, 8 Dual-Exp
Figure 5.5. The effects of parental alcohol on compulsive-like behaviors and stress in offspring.		
B. Male marbles buried	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 16 Control, 14 Mat-Exp, 17 Pat-Exp, 8 Dual-Exp (pubescent adolescent: 11 Mat-Exp, 5 Dual-Exp)
C. Female marbles buried	Two-way (treatment and age) ANOVA, Uncorrected Fisher's LSD	n = 12 Control, 17 Mat-Exp, 18 Pat-Exp, 8 Dual-Exp (pubescent adolescent: 12 Mat-Exp, 6 Dual-Exp)
D. Male nestlet shredded	Two-way (treatment and age) ANOVA, Uncorrected Fisher's LSD	n = 14 Control, 13 Mat-Exp, 15 Pat-Exp, 8 Dual-Exp (pubescent adolescent: 5 Dual-Exp; sexually mature adolescent: 6 Dual-Exp)
E. Female nestlet shredded	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 12 Control, 17 Mat-Exp, 18 Pat-Exp, 8 Dual-Exp (pubescent adolescent: 13 Mat-Exp, 6 Dual-Exp)

Table 5.1. *Continued*

Figure	Test	Sample size
F. Male post-stress nestlet shredded	Two-way (treatment and age) ANOVA, Uncorrected Fisher's LSD	n = 14 Control, 13 Mat-Exp, 15 Pat-Exp, 8 Dual-Exp (pubescent adolescent: 5 Dual-Exp; sexually mature adolescent: 6 Dual-Exp)
G. Female post-stress nestlet shredded	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 12 Control, 17 Mat-Exp, 18 Pat-Exp, 8 Dual-Exp (pubescent adolescent: 10 control, 13 Mat-Exp, 4 Dual-Exp; immature adult: 6 Dual-Exp)
H. Male stress index	Two-way (treatment and age) ANOVA, Uncorrected Fisher's LSD	n = 15 Control, 12 Mat-Exp, 15 Pat-Exp, 8 Dual-Exp (pubescent adolescent: 5 Dual-Exp; sexually mature adolescent: 6 Dual-Exp)
I. Female stress index	Two-way (treatment and age) ANOVA, Dunnett's multiple comparison test	n = 12 Control, 17 Mat-Exp, 18 Pat-Exp, 8 Dual-Exp (pubescent adolescent: 10 control, 13 Mat-Exp, 4 Dual-Exp; immature adult: 6 Dual-Exp)
Figure 5.6. Adult voluntary physical activity due to parental alcohol exposure.		
B. Average active phase revolutions	Two-way (treatment and sex) ANOVA, Uncorrected Fisher's LSD	Male: n = 14 Control, 10 Mat-Exp, 8 Pat-Exp, 6 Dual-Exp Female: n = 12 Control, 11 Mat-Exp, 10 Pat-Exp, 7 Dual-Exp
C. Male average revolutions	Two-way (treatment and time) ANOVA, Uncorrected Fisher's LSD	Male: n = 14 Control, 10 Mat-Exp, 8 Pat-Exp, 6 Dual-Exp
D. Female average revolutions	Two-way (treatment and time) ANOVA, Uncorrected Fisher's LSD	Female: n = 12 Control, 11 Mat-Exp, 10 Pat-Exp, 7 Dual-Exp
Figure 5.7. Voluntary physical activity as a behavioral intervention for parental alcohol exposed offspring activity and anxiety.		
C. Male total distance	Two-way (treatment and VPA) ANOVA, Šídák's multiple comparisons test	NPA: n = 10 Control, 10 Mat-Exp, 6 Pat-Exp, 5 Dual-Exp VPA: n = 13 Control, 11 Mat-Exp, 7 Pat-Exp, 6 Dual-Exp
D. Female total distance	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 10 Control, 8 Mat-Exp, 8 Pat-Exp, 7 Dual-Exp VPA: n = 12 Control, 10 Mat-Exp, 7 Pat-Exp, 7 Dual-Exp
E. Male total rest time	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 10 Control, 10 Mat-Exp, 6 Pat-Exp, 5 Dual-Exp VPA: n = 13 Control, 11 Mat-Exp, 7 Pat-Exp, 6 Dual-Exp
F. Female total rest time	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 10 Control, 8 Mat-Exp, 8 Pat-Exp, 7 Dual-Exp VPA: n = 12 Control, 10 Mat-Exp, 7 Pat-Exp, 7 Dual-Exp

Table 5.1. *Continued*

Figure	Test	Sample size
G. Male center entries	Two-way (treatment and VPA) ANOVA, Šídák's multiple comparisons test	NPA: n = 10 Control, 10 Mat-Exp, 6 Pat-Exp, 5 Dual-Exp VPA: n = 13 Control, 11 Mat-Exp, 7 Pat-Exp, 6 Dual-Exp
H. Female center entries	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 10 Control, 8 Mat-Exp, 8 Pat-Exp, 7 Dual-Exp VPA: n = 12 Control, 10 Mat-Exp, 7 Pat-Exp, 7 Dual-Exp
I. Male center distance	Two-way (treatment and VPA) ANOVA, Šídák's multiple comparisons test	NPA: n = 10 Control, 10 Mat-Exp, 6 Pat-Exp, 5 Dual-Exp VPA: n = 13 Control, 11 Mat-Exp, 7 Pat-Exp, 6 Dual-Exp
J. Female center distance	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 10 Control, 8 Mat-Exp, 8 Pat-Exp, 7 Dual-Exp VPA: n = 12 Control, 10 Mat-Exp, 7 Pat-Exp, 7 Dual-Exp
K. Male time in center	Two-way (treatment and VPA) ANOVA, Šídák's multiple comparisons test	NPA: n = 10 Control, 10 Mat-Exp, 6 Pat-Exp, 5 Dual-Exp VPA: n = 13 Control, 11 Mat-Exp, 7 Pat-Exp, 6 Dual-Exp
L. Female time in center	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 10 Control, 8 Mat-Exp, 8 Pat-Exp, 7 Dual-Exp VPA: n = 12 Control, 10 Mat-Exp, 7 Pat-Exp, 7 Dual-Exp
Figure 5.8. Voluntary physical activity as a behavioral intervention for parental alcohol exposed offspring cognition and memory.		
C. Male total distance	Two-way (treatment and VPA) ANOVA, Šídák's multiple comparisons test	NPA: n = 8 Control, 10 Mat-Exp, 6 Pat-Exp, 5 Dual-Exp VPA: n = 8 Control, 11 Mat-Exp, 7 Pat-Exp, 6 Dual-Exp
D. Female total distance	Two-way (treatment and VPA) ANOVA, Šídák's multiple comparisons test	NPA: n = 5 Control, 9 Mat-Exp, 7 Pat-Exp, 8 Dual-Exp VPA: n = 7 Control, 9 Mat-Exp, 7 Pat-Exp, 7 Dual-Exp
E. Male total rest time	Two-way (treatment and VPA) ANOVA, Šídák's multiple comparisons test	NPA: n = 8 Control, 10 Mat-Exp, 6 Pat-Exp, 5 Dual-Exp VPA: n = 8 Control, 11 Mat-Exp, 7 Pat-Exp, 6 Dual-Exp
F. Female total rest time	Two-way (treatment and VPA) ANOVA, Šídák's multiple comparisons test	NPA: n = 5 Control, 9 Mat-Exp, 7 Pat-Exp, 8 Dual-Exp VPA: n = 7 Control, 9 Mat-Exp, 7 Pat-Exp, 7 Dual-Exp

Table 5.1. *Continued*

Figure	Test	Sample size
G. Male total object exploration time	Two-way (treatment and VPA) ANOVA, Šídák's multiple comparisons test	NPA: n = 8 Control, 10 Mat-Exp, 6 Pat-Exp, 5 Dual-Exp VPA: n = 8 Control, 11 Mat-Exp, 7 Pat-Exp, 6 Dual-Exp
H. Female total object exploration time	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 5 Control, 9 Mat-Exp, 7 Pat-Exp, 8 Dual-Exp VPA: n = 7 Control, 9 Mat-Exp, 7 Pat-Exp, 7 Dual-Exp
I. Male time spent in novel object quadrant	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 8 Control, 10 Mat-Exp, 6 Pat-Exp, 5 Dual-Exp VPA: n = 8 Control, 11 Mat-Exp, 7 Pat-Exp, 6 Dual-Exp
J. Female time spent in novel object quadrant	Two-way (treatment and VPA) ANOVA, Šídák's multiple comparisons test	NPA: n = 5 Control, 9 Mat-Exp, 7 Pat-Exp, 8 Dual-Exp VPA: n = 7 Control, 9 Mat-Exp, 7 Pat-Exp, 7 Dual-Exp
K. Male discrimination index	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 8 Control, 10 Mat-Exp, 6 Pat-Exp, 5 Dual-Exp VPA: n = 8 Control, 11 Mat-Exp, 7 Pat-Exp, 6 Dual-Exp
L. Female discrimination index	Two-way (treatment and VPA) ANOVA, Šídák's multiple comparisons test	NPA: n = 5 Control, 9 Mat-Exp, 7 Pat-Exp, 8 Dual-Exp VPA: n = 7 Control, 9 Mat-Exp, 7 Pat-Exp, 7 Dual-Exp
Figure 5.9. Voluntary physical activity as a behavioral intervention for parental alcohol exposed offspring repetitive-like behaviors and stress.		
C. Male marbles buried	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 8 Control, 6 Mat-Exp, 7 Pat-Exp, 4 Dual-Exp VPA: n = 8 Control, 8 Mat-Exp, 8 Pat-Exp, 5 Dual-Exp
D. Female marbles buried	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 10 Control, 8 Mat-Exp, 8 Pat-Exp, 6 Dual-Exp VPA: n = 11 Control, 10 Mat-Exp, 8 Pat-Exp, 6 Dual-Exp
E. Male nestlet shredded	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 8 Control, 6 Mat-Exp, 7 Pat-Exp, 4 Dual-Exp VPA: n = 8 Control, 8 Mat-Exp, 8 Pat-Exp, 5 Dual-Exp
F. Female nestlet shredded	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 10 Control, 8 Mat-Exp, 8 Pat-Exp, 6 Dual-Exp VPA: n = 11 Control, 10 Mat-Exp, 8 Pat-Exp, 6 Dual-Exp

Table 5.1. *Continued*

Figure	Test	Sample size
G. Male post-stress nestlet shredded	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 8 Control, 6 Mat-Exp, 7 Pat-Exp, 4 Dual-Exp VPA: n = 8 Control, 8 Mat-Exp, 8 Pat-Exp, 5 Dual-Exp
H. Female post-stress nestlet shredded	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 10 Control, 8 Mat-Exp, 8 Pat-Exp, 6 Dual-Exp VPA: n = 11 Control, 10 Mat-Exp, 8 Pat-Exp, 6 Dual-Exp
I. Male stress index	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 8 Control, 6 Mat-Exp, 7 Pat-Exp, 4 Dual-Exp VPA: n = 8 Control, 8 Mat-Exp, 8 Pat-Exp, 5 Dual-Exp
J. Female stress index	Two-way (treatment and VPA) ANOVA, Uncorrected Fisher's LSD	NPA: n = 10 Control, 8 Mat-Exp, 8 Pat-Exp, 6 Dual-Exp VPA: n = 11 Control, 10 Mat-Exp, 8 Pat-Exp, 6 Dual-Exp
Figure 5.10. Changes in mature adult offspring physiology due to parental alcohol exposure.		
A. Fat to lean weight	Two-way (treatment and sex) ANOVA, Dunnett's multiple comparison test	Male: n = 9 Control, 9 Mat-Exp, 9 Pat-Exp, 4 Dual-Exp Female: n = 11 Control, 7 Mat-Exp, 10 Pat-Exp, 6 Dual-Exp
B. Bone area	Two-way (treatment and sex) ANOVA, Dunnett's multiple comparison test	Male: n = 9 Control, 9 Mat-Exp, 9 Pat-Exp, 4 Dual-Exp Female: n = 11 Control, 7 Mat-Exp, 10 Pat-Exp, 6 Dual-Exp
C. Bone mineral density	Two-way (treatment and sex) ANOVA, Dunnett's multiple comparison test	Male: n = 9 Control, 9 Mat-Exp, 9 Pat-Exp, 4 Dual-Exp Female: n = 11 Control, 7 Mat-Exp, 10 Pat-Exp, 6 Dual-Exp
D. Heart to body weight	Two-way (treatment and sex) ANOVA, Dunnett's multiple comparison test	Male: n = 19 Control, 17 Mat-Exp, 19 Pat-Exp, 9 Dual-Exp Female: n = 20 Control, 16 Mat-Exp, 18 Pat-Exp, 16 Dual-Exp
E. Kidney to body weight	Two-way (treatment and sex) ANOVA, Dunnett's multiple comparison test	Male: n = 19 Control, 17 Mat-Exp, 19 Pat-Exp, 9 Dual-Exp Female: n = 20 Control, 16 Mat-Exp, 18 Pat-Exp, 16 Dual-Exp
F. Thymus to body weight	Two-way (treatment and sex) ANOVA, Uncorrected Fisher's LSD	Male: n = 19 Control, 17 Mat-Exp, 19 Pat-Exp, 9 Dual-Exp Female: n = 20 Control, 16 Mat-Exp, 18 Pat-Exp, 16 Dual-Exp
G. Adrenal gland to body weight	Two-way (treatment and sex) ANOVA, Dunnett's multiple comparison test	Male: n = 19 Control, 17 Mat-Exp, 19 Pat-Exp, 9 Dual-Exp Female: n = 20 Control, 16 Mat-Exp, 18 Pat-Exp, 16 Dual-Exp

Table 5.1. *Continued*

Figure	Test	Sample size
H. Spleen to body weight	Two-way (treatment and sex) ANOVA, Dunnett's multiple comparison test	Male: n = 19 Control, 17 Mat-Exp, 19 Pat-Exp, 9 Dual-Exp Female: n = 20 Control, 16 Mat-Exp, 18 Pat-Exp, 16 Dual-Exp
I. Liver to body weight	Two-way (treatment and sex) ANOVA, Uncorrected Fisher's LSD	Male: n = 19 Control, 17 Mat-Exp, 19 Pat-Exp, 9 Dual-Exp Female: n = 20 Control, 16 Mat-Exp, 18 Pat-Exp, 16 Dual-Exp

3. Results

3.1. The effects of parental alcohol on gross metrics of offspring health

Previous studies have reported decreased birth weights, malformations, and mortality in offspring from paternal alcohol exposure (Finegersh et al., 2015). Notably, we recently revealed that paternal alcohol exposure results in distinct craniofacial growth deficiencies and malformations in fetal mice (Thomas et al., 2023). In the current study, we sought to investigate whether preconception paternal alcohol exposure would have a lasting impact on offspring in postnatal life. Using our published multifactorial design, we generated postnatal offspring from maternal, paternal, and dual parental alcohol exposures (Mat-Exp, Pat-Exp, Dual-Exp) (Thomas et al., 2023). We first examined the impact of these parental alcohol exposures on early postnatal life and found that alcohol did not significantly alter gestation length, with all treatments having an average gestation length of 19 days (Figure 5.2A). Average litter size was also not significantly different amongst treatment groups (7 Control, 7 Mat-exp, 8 Pat-exp, 6 Dual-exp) (Figure 5.2B). Furthermore, neonatal mortality, as defined by pup loss prior to weaning on postnatal day 21, was not significantly different with a 96, 96, 99, and 93% pup survival rate in the Control, Mat-Exp, Pat-Exp, and Dual-Exp groups respectively (Figure 5.2C). In order to limit the confounding variable of stress, offspring were not handled until postnatal day 7, at which time weekly weight collection began. Our analysis of early postnatal life offspring body weight did not reveal any significant

differences across treatments (Figure 5.2D). However, it should be noted that other research groups have reported decreased birth weights, but since we did not handle mice during this stage, we are unable present supporting data (Finegersh et al., 2015). We also observed an approximately equal proportion of males and females born to each treatment group (Figure 5.2E). From these results, we report no changes in gross metrics of dysfunction during the early postnatal period.

Husbandry measures include weaning offspring from the dam at postnatal day 21 as adolescence begins in week three of life, thus preventing interbreeding. Specifically, pubertal onset occurs at approximately postnatal day 25 for females and 27-28 for males (Cross et al., 2021). The adolescence period is marked by changes in social relationships, independence, and reproductive competency that are accompanied by drastic changes in hormonal, neural, and behavior (Miguel-Hidalgo, 2013). Adolescence can be divided into three phases: prepubescent from week 3 to 5, pubescent from week 5 to 8, and sexually mature from week 8 to 9 (Brust et al., 2015). Previous paternal alcohol exposure models have reported an increased weight after weaning in males and decreased growth at postnatal day 35 which is the start of the pubescent adolescent stage (Finegersh et al., 2015; Finegersh & Homanics, 2014). Similarly, in the current study, we observed a significant increase in post-weaning (week 3 to 4) body weight gain in the male offspring from the Pat-Exp group only (Figure 5.2F). The increase in post-weaning weight gain was accompanied by an increase in male body weight at four weeks of age in the Pat-Exp group. During pubescent adolescence we observed a decrease in male body weight at six weeks of age, which correlates to previously reported findings by Finegersh and Homanics (Finegersh & Homanics, 2014). Furthermore, upon sexual maturity (9 weeks of age) male offspring from the Pat-Exp and Dual-Exp were significantly heavier than their control counterparts (Figure 5.2G). We did not observe any differences in female post-weaning weight gain (Figure 5.2F), however females from the dual

parental exposed group displayed increased body weight relative to controls one week after weaning (postnatal week 4) (Figure 5.2G). Suggesting that parental alcohol plays a role in offspring growth kinetics in the adolescent period.

Finally, we evaluated whether these changes in growth kinetics had the potential to lead to long lasting effects into adulthood. According to the Jackson Laboratory, mice are considered a mature adult at postnatal day 90, or roughly 13 weeks of age. We observed a nearly consistent significant increase in body weight in males born from paternal and dual parental alcohol exposures (Figure 5.2H). However, we did not any significant differences in female offspring across treatments (Figure 5.2I). Our findings suggest that males are more susceptible to parental alcohol exposures that lead to lifelong changes in growth, while females are less affected, and the effects seem to be minimal and transient.

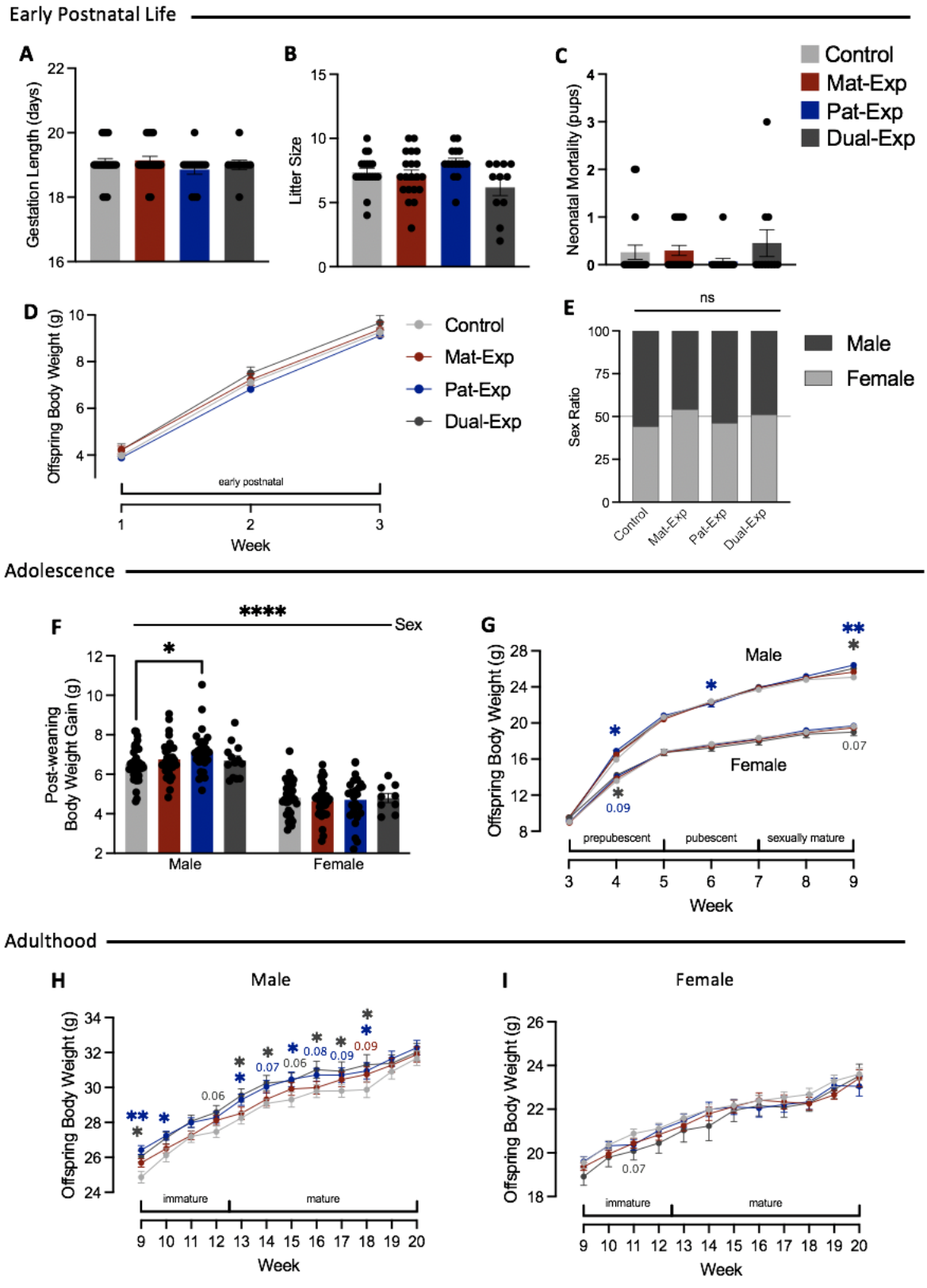
Early Postnatal Life
A
Gestation Length (days)
22
20
18
16
B
Litter Size
15
10
5
0
C
Neonatal Mortality (pups)
4
3
2
1
0
Control
Mat-Exp
Pat-Exp
Dual-Exp
D
Offspring Body Weight (g)
10
8
6
4
early postnatal
1
2
3
Week
Control
Mat-Exp
Pat-Exp
Dual-Exp
E
ns
Sex Ratio
100
75
50
25
0
Control
Mat-Exp
Pat-Exp
Dual-Exp
Male
Female
Adolescence
F
****
Sex
*
Post-weaning Body Weight Gain (g)
12
10
8
6
4
2
Male
Female
G
Offspring Body Weight (g)
28
24
20
16
12
8
Male
Female
**
*
0.07
0.09
prepubescent
pubescent
sexually mature
3
4
5
6
7
8
9
Week
Adulthood
H
Male
Offspring Body Weight (g)
34
32
30
28
26
24
0.06
0.07
0.06
0.08
0.09
0.09
immature
mature
9
10
11
12
13
14
15
16
17
18
19
20
Week
I
Female
Offspring Body Weight (g)
26
24
22
20
18
0.07
immature
mature
9
10
11
12
13
14
15
16
17
18
19
20
Week

Figure 5.2. The effects of parental alcohol on gross metrics of offspring health. One-way ANOVA examining the effect of alcohol exposure on gestation length (days) (A), litter size (B), and neonatal mortality (pups lost per litter) (C). Two-way ANOVA assessing offspring body weight during the early postnatal period (D). Chi-square analysis of the ratio of males to females across treatment groups (E). Two-way ANOVA of post-weaning offspring body weight across treatments by sex (F). Two-way ANOVA of adolescent body weight with males and females in separate comparisons and graphed together (G). Two-way ANOVA of male (H) and female (I) offspring adult body weights. ✱p<0.05, ✱✱p<0.01, ✱✱✱✱p<0.0001. See statistics table for sample size.

3.2. Parental alcohol exposure and behavioral changes across the lifespan

Fetal alcohol syndrome is often referred to as the most severe form of FASD and is associated with distinct craniofacial phenotypes that are accompanied by alterations in neurological development and behavior. In our most recent publication, we found that maternal, paternal, and dual parental alcohol exposure program distinct craniofacial growth deficiencies and malformations (Thomas et al., 2023). Building upon our earlier work, we hypothesized that these changes would also result in behavioral abnormalities.

Behavioral measures are a sensitive indicator of central nervous dysfunction and are an important tool in models of FASD (Abel & Tan, 1988). In the current study, we set out to evaluate the effects of parental alcohol exposures on offspring behavior in pubescent and sexually mature adolescence, as well as immature and mature adulthood. Specifically, we began behavioral testing at week 6, which corresponds to the pubescent stage of adolescence. We used a multi-faceted behavioral testing series (Figure 5.1) to examine different parameters of behaviors across development, with two weeks of rest between repeated testing.

Previous paternal alcohol research has reported dose-dependent decreased activity in prepubertal adolescent mice (Abel & Lee, 1988), while others report sex-specific increases in activity at about the same developmental stage (Conner et al., 2020b). These differences are most likely due to experimental conditions and are not necessarily direct contradictions. The open field test is a measure of hyperactivity and anxiety, in which subjects are placed into an open arena and

allowed to freely explore for an allotted time (Seibenhener & Wooten, 2015). Thigmotaxis is the tendency to remain near the periphery of the open field maze and is directly related to anxiogenic behaviors, meaning, less anxious subjects will venture into the center or open area of the maze (Simon et al., 1994). Through the use of an open field maze apparatus (see materials and methods), we tested activity in male and female offspring exposed to parental alcohol. We did not observe any differences in activity in males (Figure 5.3B,D), while females from maternal and dual parental alcohol exposure groups were more active as mature adults (Figure 5.3C,E). Interestingly, males from the paternal alcohol exposure group were less anxious in pubescent adolescence and then became more anxious in mature adulthood, revealing an inverse in behavior during development (Figure 5.3F,H,J). For female offspring, we did not observe any significant differences until the mature adult stage, when they became less anxious than their control counterparts (Figure 5.3I,K). Importantly, these results signify that early life parental alcohol exposures can induce changes in activity and behavior during mature adulthood.

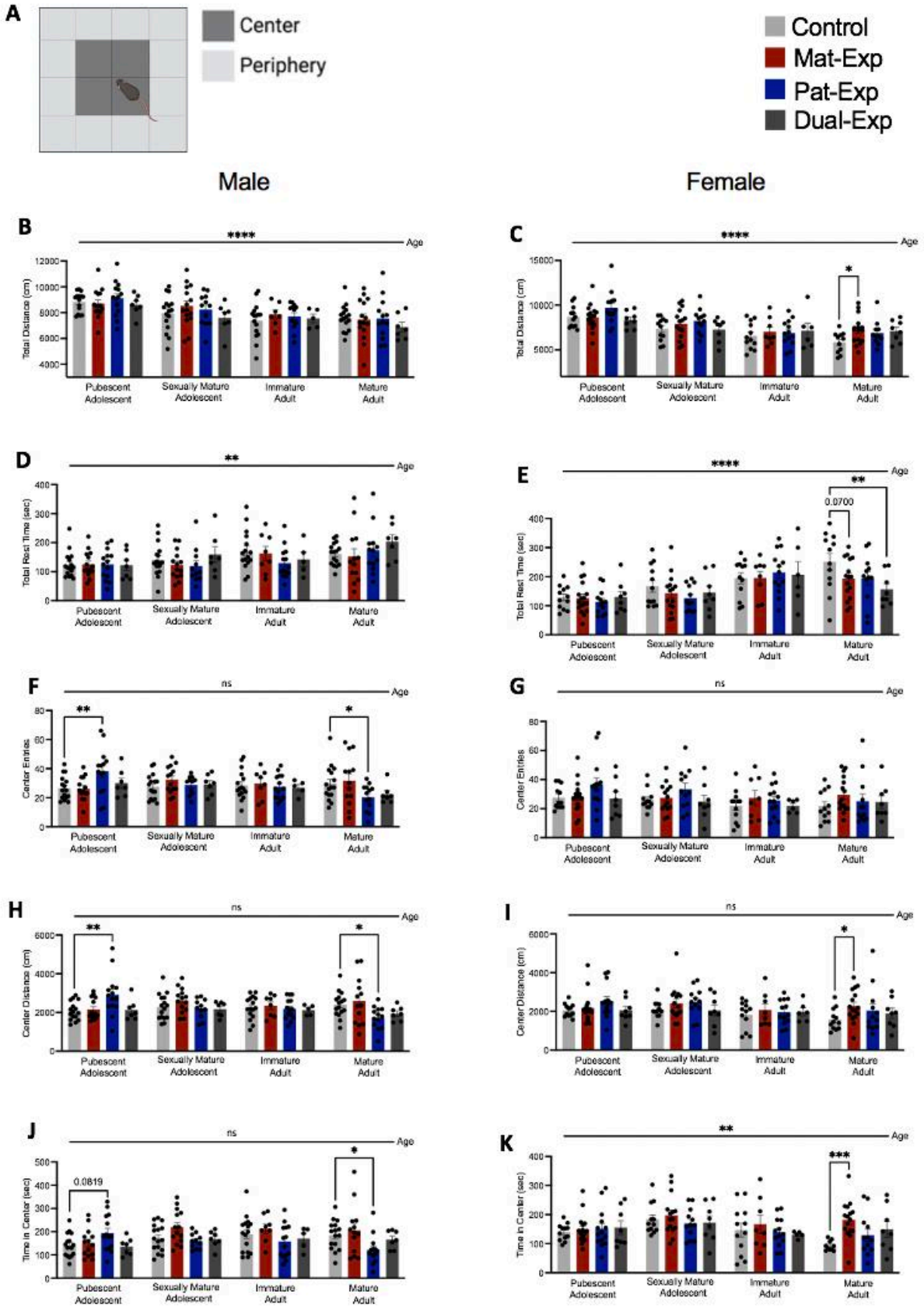
A
Center
Periphery
Control
Mat-Exp
Pat-Exp
Dual-Exp
Male
Female
B
C
D
E
F
G
H
I
J
K
Age
ns
Total Distance (cm)
Total Rest Time (sec)
Center Entries
Center Distance (cm)
Time in Center (sec)
0.0700
0.0819
Pubescent Adolescent
Sexually Mature Adolescent
Immature Adult
Mature Adult

Figure 5.3. Parental alcohol exposure induces sex-specific changes in activity and anxiety. Open field maze paradigm (A). Two-way (treatment and developmental stage) ANOVA of total distance traveled for male (B) and female (C) offspring. Two-way ANOVA of total rest time for male (D) and female (E) offspring. Two-way ANOVA of center entries for male (F) and female (G) offspring. Two-way ANOVA of center distance for male (H) and female (I) offspring. Two-way ANOVA of time spent in the center for male (J) and female (K) offspring. ✱p<0.05, ✱✱p<0.01, ✱✱✱p<0.001, ✱✱✱✱p<0.0001. See statistics table for sample size.

Next, we employed the novel object recognition test to assess cognition and memory. Essentially, the novel object recognition test is used to study innate exploratory behavior and has implications in several areas of the brain including the parahippocampal regions of the temporal lobe (Antunes & Biala, 2012; Ennaceur & Delacour, 1988; Hammond et al., 2004). The novel object recognition test is a multi-series assay that in our model consists of three phases (acclimation, training, and testing) separated by 24 hours. In the first phase the subject is acclimated to the testing chamber. During the second phase, 24 hours later, the subject re-enters the chamber with two like objects placed inside. On the following day, one of the objects is replaced with a new or "novel" object that is similar, but different than the object that it replaces. In order to recognize that the object is novel the subject must have more cognitive skills, or a higher level of cognition (Antunes & Biala, 2012). Previous paternal alcohol exposure models have reported impaired learning, but no changes in the novel object recognition task due to paternal alcohol at 8 weeks of age (Rathod et al., 2020). In the current study, we found increased exploration time in the paternal alcohol treatment male and female offspring during pubescent adolescence (Figure 5.4B-G). However, these effects did not persist in male offspring. For female offspring, we observed increased activity during the novel object recognition testing phase during immature adulthood and less distance traveled by mature adult maternal alcohol exposed group (Figure 5.4C,E). In order to evaluate cognition, we implemented two metrics, time spent in novel object quadrant and a discrimination index that depicts the subject's ability to distinguish the new object

from the old by factoring in the amount of time spent with each object (see materials and methods for details). Specifically, an increased discrimination index correlates to increased cognition. During pubescent adolescence, we observed an increase in the discrimination index, and thus an increase in cognition, in both male and female offspring from the dual parental alcohol exposure group (Figure 5.4H-K). Although paternal alcohol exposed pubescent adolescent females also displayed an increase in total time spent in novel object quadrant, this is likely due to increased total object exploration time, since the discrimination index did not show any significant changes (Figure 5.4I,K). Maternally exposed males displayed an increase in cognition in the sexually mature adolescent phase, while paternally exposed males displayed a decrease in cognition as immature adults (Figure 5.4H,J). No such later life changes were observed in females across treatments (Figure 5.4I,K). Collectively, these results reveal sex-specific, developmental age dependent changes in cognition in offspring exposed to maternal, paternal, and dual parental alcohol.

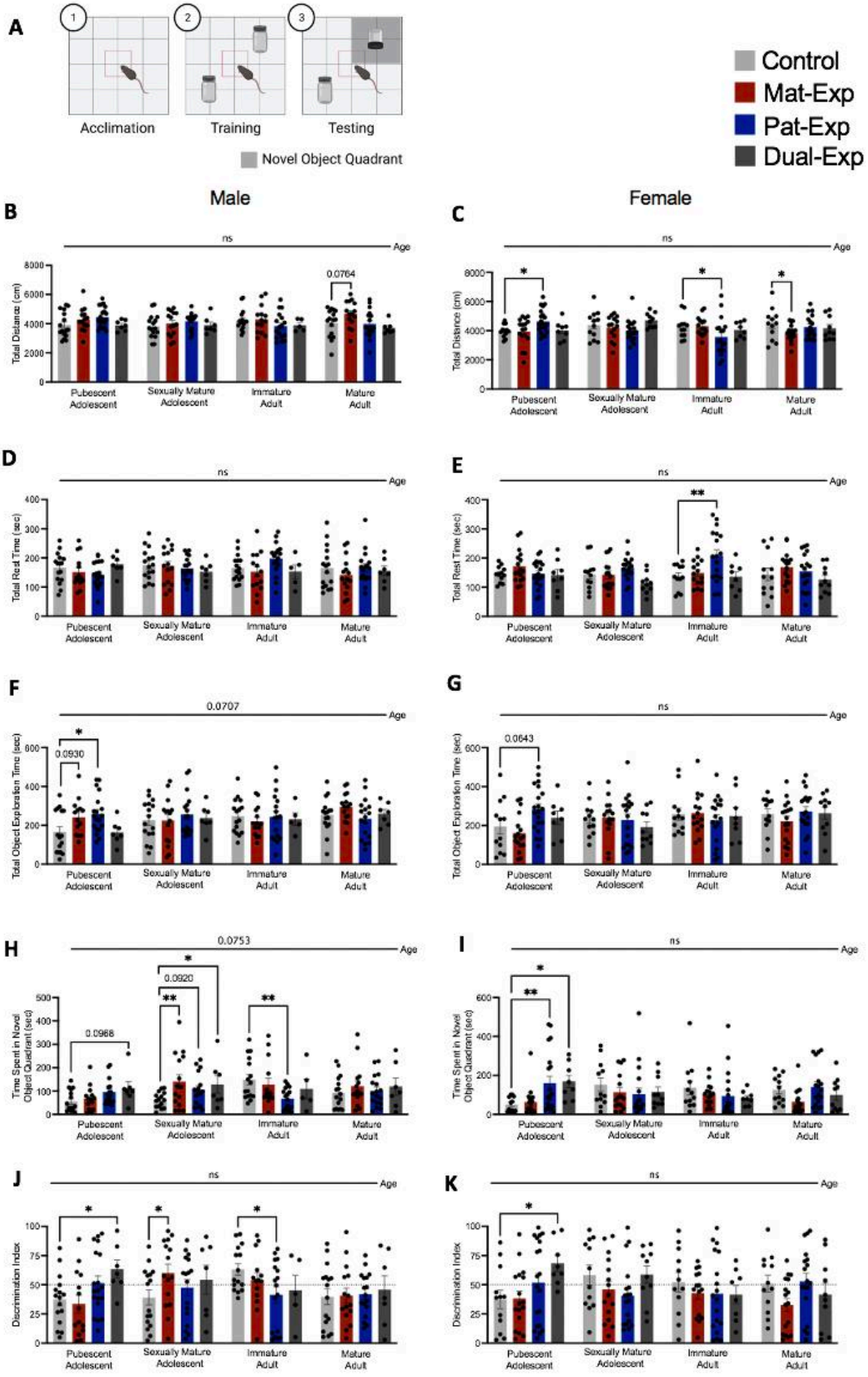
A
1
2
3
Acclimation
Training
Testing
Novel Object Quadrant
Control
Mat-Exp
Pat-Exp
Dual-Exp
Male
Female
B
C
D
E
F
G
H
I
J
K
ns
Age
0.0764
0.0707
0.0930
0.0643
0.0753
0.0920
0.0988
Total Distance (cm)
Total Rest Time (sec)
Total Object Exploration Time (sec)
Time Spent in Novel Object Quadrant (sec)
Discrimination Index
Pubescent Adolescent
Sexually Mature Adolescent
Immature Adult
Mature Adult

Figure 5.4. Sex-specific and developmental stage-dependent changes in cognition in offspring exposed to maternal, paternal, and dual parental alcohol. Novel object recognition paradigm (A). Two-way (treatment and developmental stage) ANOVA of total distance in male (B) and female (C) offspring. Two-way ANOVA of total rest time in male (D) and female (E) offspring. Two-way ANOVA of total object exploration time in male (F) and female (G) offspring. Two-way ANOVA of total time spent in novel object quadrant in male (H) and female (I) offspring. Two-way ANOVA of discrimination index in male (J) and female (K) offspring. ✱p<0.05, ✱✱p<0.01. See statistics table for sample sizes.

Finally, in addition to examining activity, anxiety, and cognition, we employed assays to assess compulsive-like behaviors and stress during the behavioral testing series. Specifically, we conducted the nestlet shredding test and marble burying test during the previously discussed developmental stages. Both the nestlet shredding test and marble burying test are assays used to assess repetitive, compulsive-like behaviors in mice and are often used in models of Obsessive-Compulsive Disorder (OCD) and Autism Spectrum Disorder (ASD) (Angoa-Pérez et al., 2013). Importantly, the rate of ASD is almost two times higher in FASD patients than the general population (Lange et al., 2018). Additionally, in order to examine stress, we conducted two nestlet shredding tests, a traditional test and one directly following a stressful situation (i.e., pre- and post-stress nestlet shredding tests). We used the open field maze as the stressor as mice have a natural aversion to large, brightly lit, open spaces (Seibenhener & Wooten, 2015).

Previous studies of paternal alcohol exposure reported no differences in marble burying test due to alcohol in 8-week-old offspring (Rathod et al., 2020). In the current study, we found a decrease in the number of marbles buried in both male and female paternal alcohol exposed group as well as maternal and dual parental exposed females during pubescent adolescence (Figure 5.5B,C). No further differences were observed in later life male offspring, although female offspring displayed an increase in marbles buried as immature adults relative to the controls (Figure 5.5B,C). These results are intriguing as other assays also showed inversion of phenotype across development, suggesting a bounce back, or resiliency effect and subsequent overcompensation. Next, we

employed the nestlet shredding test, and found that males and females in the paternal alcohol exposure as well as males in the maternal alcohol exposed group displayed an increased amount of nestlet shredded (Figure 5.5D,E). However, no differences in amount shredded were observed in later stages of development (Figure 5.5D,E). Finally, we conducted a post-stress nestlet shredding test and found that only pubescent adolescent males in the maternal and paternal alcohol exposed groups displayed increased amounts of nestlet shredding (Figure 5.5F,G). To evaluate the effects of stress, we divided the amount of nestlet shredded in the post-stress test by the total amount shredded in both tests and multiplied by 100 to acquire a stress index. Interestingly, the pubescent adolescent maternally exposed males displayed an increase in the stress index and immature adult dual parentally exposed males displayed a decrease (Figure 5.5H). No difference in stress index were observed in females across the developmental windows examined (Figure 5.5I). It is important to note, that although the literature often classifies the marble burying and nestlet shredding tests as measuring similar behavioral phenotypes, our data suggest that they are indeed different. The discrepancy observed is possibly due to novelty of the test objects, marbles and nestlets. Mice routinely receive fresh nestlets as part of commonplace animal husbandry measures, while marbles are absent from home cage environments. Another possibility could be the inherent properties of the objects themselves. Marbles are cold round objects, while nestlets are soft and provide warmth and protection when dissociated and used to build nests. Regardless, these results reveal sex-specific, developmental stage dependent changes in compulsive-like behaviors and stress due to maternal, paternal, and dual parental alcohol exposures.

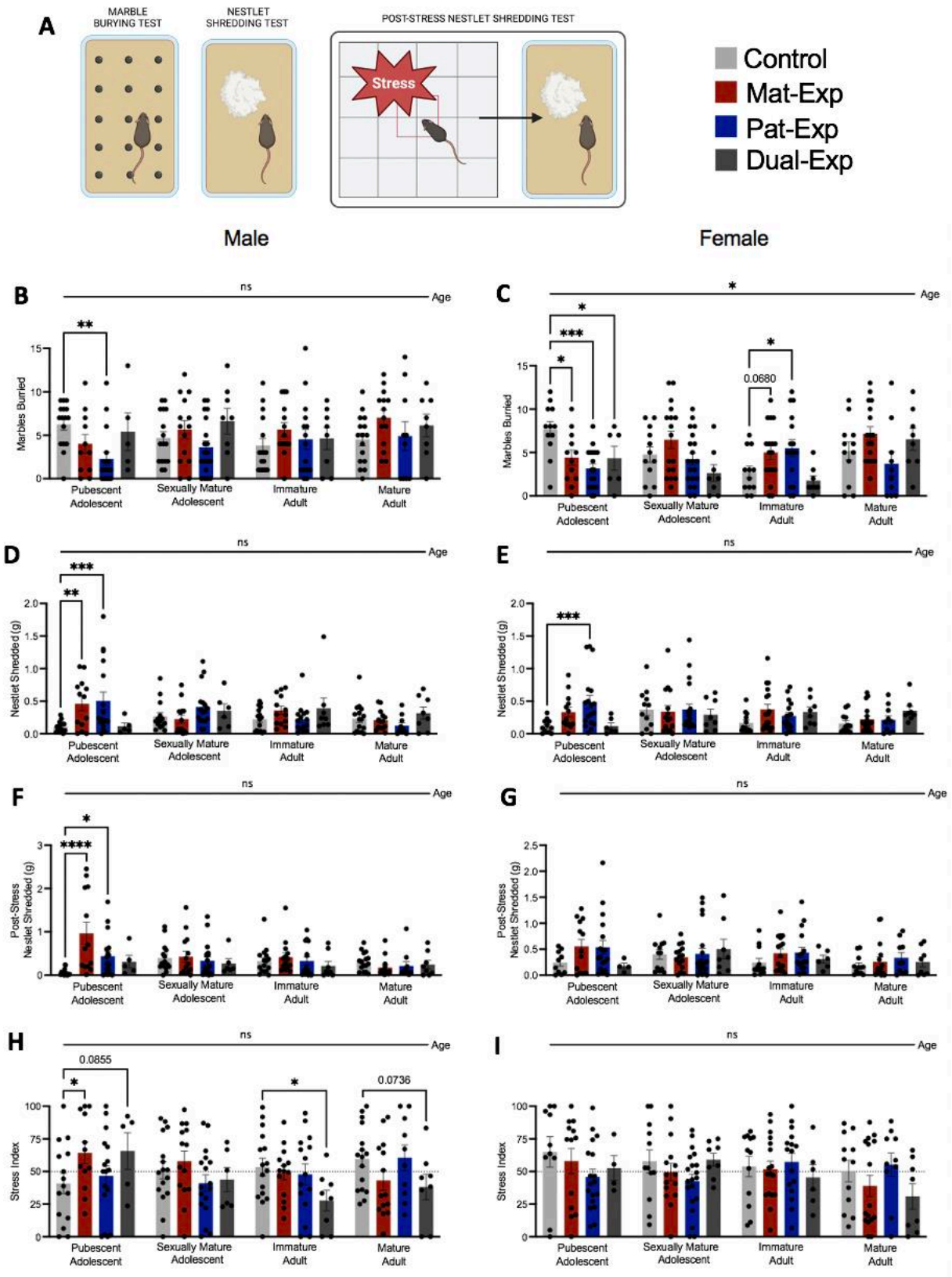
A
MARBLE BURYING TEST
NESTLET SHREDDING TEST
POST-STRESS NESTLET SHREDDING TEST
Stress
Control
Mat-Exp
Pat-Exp
Dual-Exp
Male
Female
B
C
D
E
F
G
H
I
ns
Age
*
Marbles Buried
Nestlet Shredded (g)
Post-Stress Nestlet Shredded (g)
Stress Index
0.0680
0.0855
0.0736
Pubescent Adolescent
Sexually Mature Adolescent
Immature Adult
Mature Adult

Figure 5.5. The effects parental alcohol on compulsive-like behaviors and stress due in offspring. Marble burying and nestlet shredding test paradigm (A). Two-way (treatment and developmental stage) ANOVA of marbles buried in male (B) and female (C) offspring. Two-way ANOVA of amount of nestlet shredded in the pre-stress nestlet shredding test in male (D) and female (E) offspring. Two-way ANOVA of amount of nestlet shredded during the post-stress nestlet shredding test in male (F) and female (G) offspring. Two-way ANOVA of the nestlet shredding test stress index in male (H) and female (I) offspring. ✱p<0.05, ✱✱p<0.01, ✱✱✱p<0.001, ✱✱✱✱p<0.0001. See statistics table for sample sizes.

3.3. Voluntary physical activity as a measure of health

The life expectancy for individuals with FAS is 34 years, which is approximately half the lifespan of the general population (Thanh & Jonsson, 2016). In individuals with FAS, the leading causes of death, not including external causes were diseases of the nervous system (8%), respiratory system (8%), digestive system (7%), and circulatory system (4%), as well as congenital malformations (7%), and behavioral disorders (4%) (Thanh & Jonsson, 2016). Unfortunately, research on the association between FASD and later life health consequences is poorly lacking (Moore & Riley, 2015).

Physical activity is linked to health and longevity and is associated with higher life expectancy (Reimers et al., 2012). While a sedentary lifestyle increases all-cause mortality and the risk of cardiovascular disease, diabetes, and cancer (Park et al., 2020). The association of sedentary or active behavior in association with congenital disorders is lacking, although some studies use animal models and simple behavioral tests to analyze activity in relation to different disease models. However, these studies are typically conducted in inorganic settings such as the open field maze and may not accurately reflect the subject's true level of activity. For instance, the open field test may be confounded by the element of stress, as mice have an inherent aversion to brightly lit open environments (Seibenhener & Wooten, 2015). In order to assess activity without these confounders, we implemented running wheels placed in the subject's home cage for an extended period of time. Specifically, 16-week adult mice received a wireless running wheel in their home

cage for two weeks, with the first week being the acclimation period, meaning the data from this time frame was not assessed.

In the current study, we observed a decrease in voluntary physical activity in mature adult male offspring born to dual parental alcohol exposure only (Figure 5.6B). Additionally, we accounted for circadian rhythm by separating the active and resting phase and dividing each phase into 4-hour blocks (RP1-3 and AP1-3). It is important to note that mice are nocturnal, so the active phase is during the dark cycle and the resting phase occurs during the light cycle. We found that adult male dual parental alcohol exposed male offspring were less active in the second and third active phases (Figure 5.6C). We did not observe any differences due to alcohol in the female offspring (Figure 5.6D). Collectively, this data suggests that only male offspring from dual parental alcohol exposure are susceptible to adult sedentary behavior that may be associated with early onset of disease.

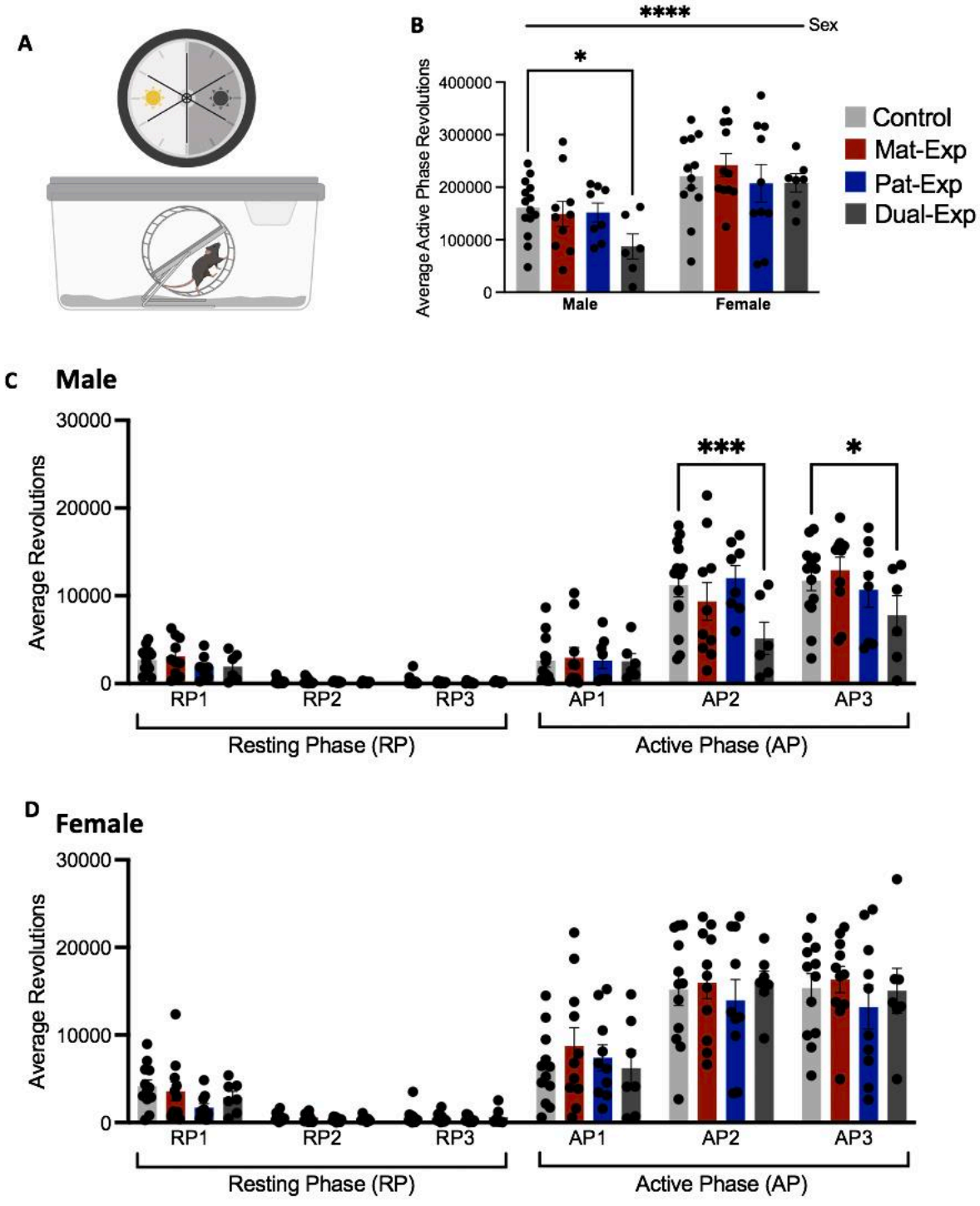
A
B
****
Sex
*
Average Active Phase Revolutions
400000
300000
200000
100000
0
Male
Female
Control
Mat-Exp
Pat-Exp
Dual-Exp
C
Male
30000
20000
10000
0
Average Revolutions
***
*
RP1
RP2
RP3
AP1
AP2
AP3
Resting Phase (RP)
Active Phase (AP)
D
Female
30000
20000
10000
0
Average Revolutions
RP1
RP2
RP3
AP1
AP2
AP3
Resting Phase (RP)
Active Phase (AP)

Figure 5.6. Adult voluntary physical activity due to parental alcohol exposure. Home cage running wheel as voluntary physical activity paradigm (A). Two-way ANOVA (treatment and sex) of average active phase revolutions of adult male and female offspring (B). Two-way ANOVA (treatment and time) of average revolutions for male (C) and female (D) adult offspring. ✱p<0.05, ✱✱✱p<0.001, ✱✱✱✱p<0.0001. See statistics table for sample sizes.

3.4. Voluntary physical activity as a behavioral intervention

Physical exercise is emerging as a promising intervention for central nervous system (CNS) dysfunction (Morgan et al., 2015). Although the mechanism by which exercise positively affects CNS function remains unclear, studies point to increases in brain derived neurotrophic factor (BDNF) and decreases in oxidative stress (Camiletti-Moirón et al., 2013; Cotman & Berchtold, 2002; Cotman & Engesser-Cesar, 2002; Dishman et al., 2006). Increases in BDNF due to exercise is a logical cause of the positive effects of exercise on CNS function due to its role in neurogenesis, cognitive function, and protection from cognitive decline (Cotman & Engesser-Cesar, 2002). In fact, decreased BDNF has been linked to anxiety and depression (Anderson & Shivakumar, 2013).

Previous studies have shown a decrease in cortex BDNF in offspring due to paternal alcohol exposure (Ceccanti et al., 2016). Since paternal alcohol exposed offspring have been shown to have lower levels of BDNF, and BDNF can be increased via exercise, we hypothesized that voluntary physical activity had the potential to rectify some of the behavioral changes seen in our model. In the current study, we initiated voluntary physical activity via running wheels for two weeks beginning at 16 weeks of age (mature adulthood). At 18 weeks of age, we conducted the behavioral testing series depicted in Figure 1, in order to examine the effects of voluntary physical activity on parental alcohol induced behavioral changes in adulthood. Within each litter of exposed offspring one male and one female were randomly assigned to the voluntary physical activity group.

First, we conducted the open field maze with littermates that received voluntary physical activity (VPA) and ones that did not (NPA). For total distance we did not observe any changes in male offspring (Figure 5.7C), while females had an overall increase in total distance traveled after receiving running wheels, with the maternal alcohol exposed females significantly increasing total distance relative to the controls (Figure 5.7D). Additionally, total rest time in paternal alcohol exposed males was drastically reduced by the implementation of VPA (Figure 5.7E). In females, total rest time was significantly decreased overall for those that received VPA, and paternal alcohol exposed females saw a significant decrease in total rest time from NPA littermates (Figure 5.7F). We did not observe any changes in center entries, center distance, or time in center in males due to VPA (Figure 5.7G,I,K). In females VPA had a significant overall effect on center entries and center distance, with no effect on time in center (Figure 5.7H,J,L). We observed that paternal alcohol exposed females had increased center entries and center distance relative to their NPA littermates (Figure 5.7H,J), suggesting that they were less anxious. Overall, these results reveal that voluntary physical activity can modulate the activity and anxiety-like behavioral effects of paternal and maternal alcohol consumption in a sex-dependent manner, with females being more likely to benefit from the positive effects.

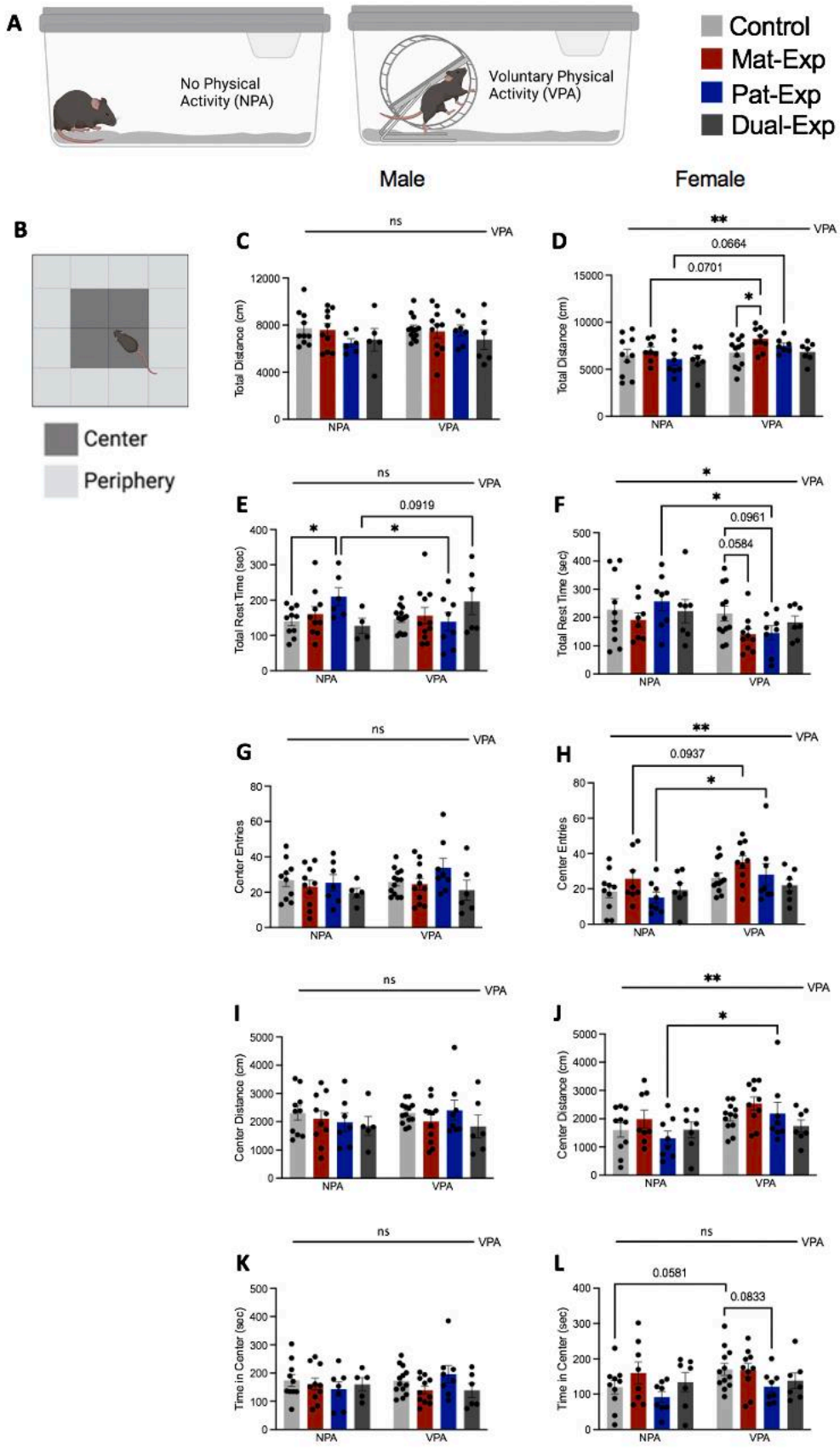
A
No Physical Activity (NPA)
Voluntary Physical Activity (VPA)
Control
Mat-Exp
Pat-Exp
Dual-Exp
Male
Female
B
Center
Periphery
C
D
E
F
G
H
I
J
K
L
Total Distance (cm)
Total Rest Time (sec)
Center Entries
Center Distance (cm)
Time in Center (sec)
NPA
VPA
ns
0.0664
0.0701
0.0919
0.0961
0.0584
0.0937
0.0581
0.0833

Figure 5.7. Voluntary Physical Activity as a behavioral intervention for parental alcohol exposed offspring activity and anxiety. Behavioral intervention paradigm (A). Open field maze paradigm (B). Two-way (treatment and behavioral intervention) ANOVA of total distance for male (C) and female (D) offspring. Two-way ANOVA of total rest time for male (E) and female (F) offspring. Two-way ANOVA of center entries for male (G) and female (H) offspring. Two-way ANOVA of center distance for male (I) and female (J) offspring. Two-way ANOVA of time in center for male (K) and female (L) offspring. ✱p<0.05, ✱✱p<0.01. See statistics table for sample sizes.

Next, we explored the effects of physical activity on cognition. There is much evidence to support physical exercise as having an enormous benefit on cognitive functioning (Mandolesi et al., 2018). In humans, physical exercise has been shown to increase academic achievement and cognition, and prevent cognitive decline (Basso & Suzuki, 2017). Importantly, individuals with FASD have a lower IQ, with an average IQ of 65.9 for individuals with FAS and 79.5 for FASD patients, with the normal population average range between 90 and 109 (Fadeeva & Nenasteva, 2022). As physical activity has been shown to enhance cognition, we next examined the ability of voluntary physical activity to modulate changes in cognition induced by parental alcohol exposure. After two weeks of home cage voluntary physical activity via wireless running wheels, we administered the novel object recognition test to both voluntary physical activity (VPA) and no physical activity (NPA) male and female littermates (Figure 5.8). We did not observe any differences in total distance or test rest time (Figure 5.8C-D). Next, we examined total object exploration time and found no difference in adult male offspring (Figure 5.8G). However, we found that dual parental alcohol exposed NPA females had an increased total object exploration time relative to the NPA controls, although they were not significantly different from VPA Dual-Exp females (Figure 5.8H). Next, we analyzed time spent in novel object quadrant and the discrimination index and found that adult male Dual-Exp offspring benefited from VPA, with increased amount of time in novel object quadrant and increased discrimination index between NPA and VPA Dual-Exp (Figure 5.8I,K). We did not observe any differences in time spent in novel object quadrant or discrimination index in female offspring (Figure 5.8J,L). Taken together,

this data suggests that voluntary physical activity could increase cognition in adult male offspring in which both parents consume alcohol.

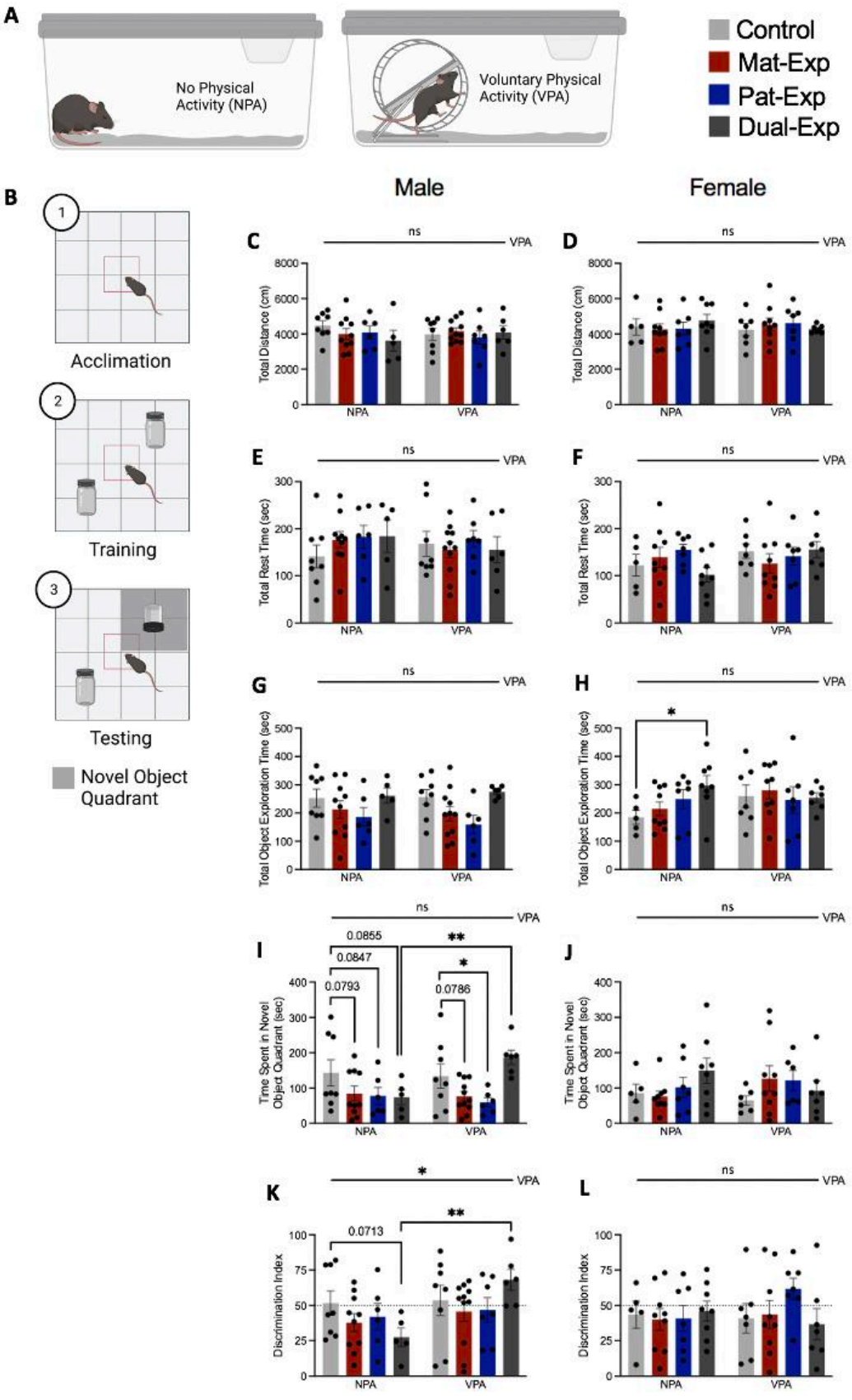
A
No Physical Activity (NPA)
Voluntary Physical Activity (VPA)
Control
Mat-Exp
Pat-Exp
Dual-Exp
B
1
Acclimation
2
Training
3
Testing
Novel Object Quadrant
Male
Female
C
D
E
F
G
H
I
J
K
L
ns
VPA
NPA
Total Distance (cm)
Total Rest Time (sec)
Total Object Exploration Time (sec)
Time Spent in Novel Object Quadrant (sec)
Discrimination Index
0.0855
0.0847
0.0793
0.0786
0.0713
*
**

Figure 5.8. Voluntary Physical Activity as a behavioral intervention for parental alcohol exposed offspring cognition and memory. Home cage wireless running wheels as a behavioral intervention paradigm (A). Novel object recognition test as a measure of cognition experimental paradigm (B). Two-way (treatment and voluntary physical activity) ANOVA of total distance in male (C) and female (D) offspring. Two-way ANOVA of total rest time in male (E) and female (F) offspring. Two-way ANOVA of total object exploration time in male (G) and female (H) offspring. Two-way ANOVA of time spent in novel object quadrant in male (I) and female (J) offspring. Two-way ANOVA of discrimination index in male (K) and female (L) offspring. ✱$p<0.05$, ✱✱$p<0.01$. See statistics table for sample sizes.

Finally, we investigated the ability of voluntary physical activity to modulate compulsive-like behaviors and stress. Although there is a debate in the literature on the efficacy of exercise and OCD improvement, some studies indicate that physical activity may help to improve OCD symptoms (Freedman & Richter, 2021). Importantly, stronger evidence exists for the benefits of exercise on stress management (Jackson, 2013). Indeed, in healthy individuals, regular exercise has been shown to be protective against the negative emotional consequences of stress (Childs & de Wit, 2014). Importantly, FASD patients have an increased vulnerability to stress in adulthood (Hellemans et al., 2008).

In the current study, we tested the ability of voluntary physical activity to alter repetitive-like behaviors and the stress response in adult offspring exposed to parental alcohol before birth. We first examined marbles buried and found that in males the Dual-Exp NPA group buried more marbles than NPA controls, and that upon VPA exposure this difference did not persist (Figure 5.9C). In females, both Mat-Exp and Dual-Exp NPA groups buried more marbles than the NPA controls, but within VPA littermates, only the Dual-Exp group was significantly increased relative to the VPA control (Figure 5.9D). Next, we analyzed results from the nestlet shredding test and found an increase in amount shredded in both pre- and post-stress nestlet shredding tests in the Mat-Exp NPA male offspring relative to NPA controls (Figure 5.9E,G). Interestingly, within the VPA males no differences were recorded in amount of nestlet shredded (Figure 5.9E,G). We did not observe any differences in NPA or VPA groups in female offspring during the nestlet shredding

tests (Figure 5.9F,H). Finally, we calculated the stress index, and found a significant decrease in stress in the Pat-Exp VPA male offspring, and a trending decrease in both male and female Dual-Exp VPA offspring (Figure 5.9I,J). Overall, these results provide support for the positive benefits of voluntary physical activity on repetitive-like behaviors and stress induced by parental alcohol exposure.

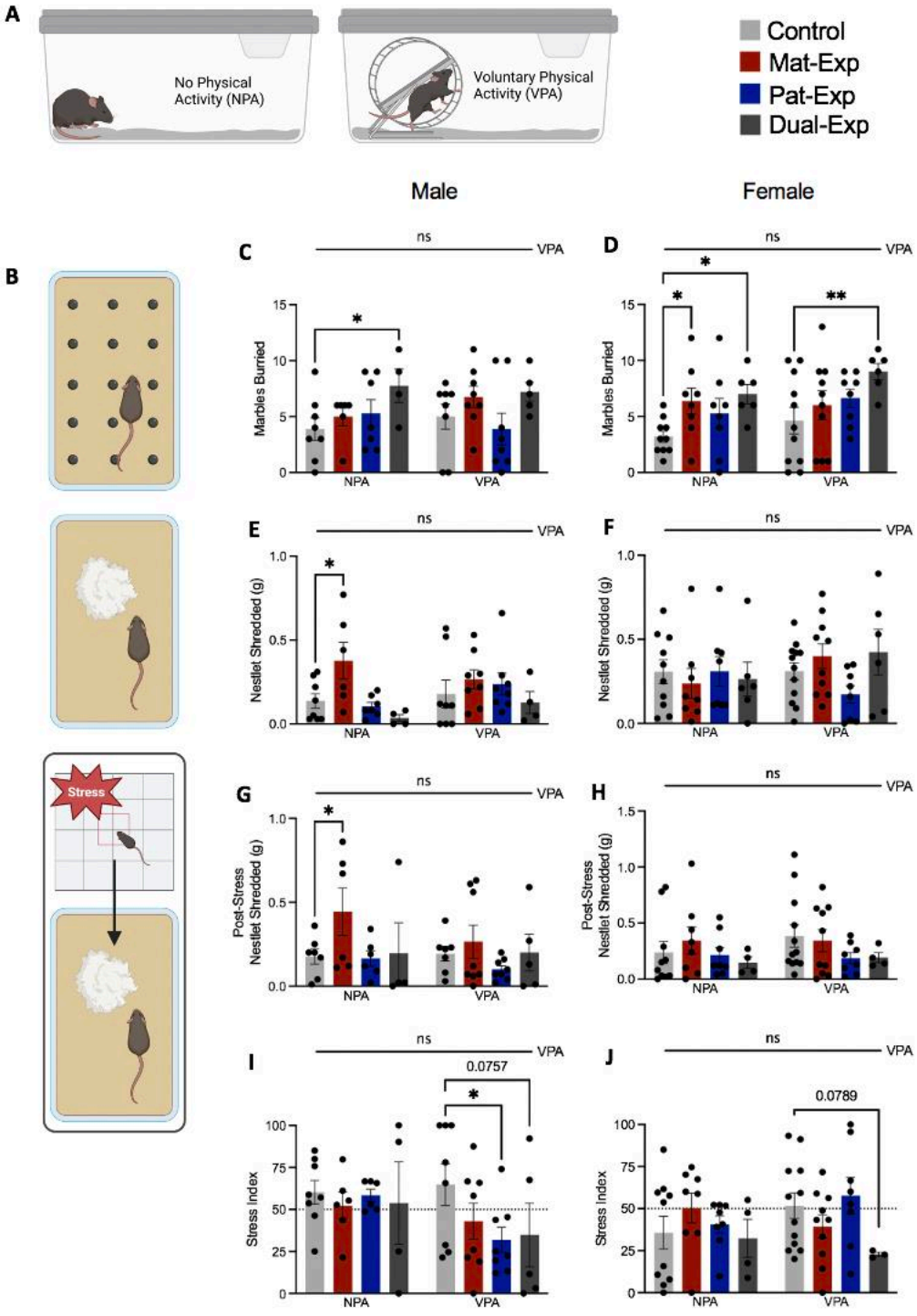
A
No Physical Activity (NPA)
Voluntary Physical Activity (VPA)
Control
Mat-Exp
Pat-Exp
Dual-Exp
Male
Female
B
Stress
C
D
E
F
G
H
I
J
ns
VPA
NPA
Marbles Buried
Nestlet Shredded (g)
Post-Stress Nestlet Shredded (g)
Stress Index
0.0757
0.0789

Figure 5.9. Voluntary Physical Activity as a behavioral intervention for parental alcohol exposed offspring repetitive-like behaviors and stress. Home cage wireless running wheels as a behavioral intervention paradigm (A). Marble burying test (top), nestlet shredding test (middle), and post-stress nestlet shredding test (bottom) experimental paradigm (B). Two-way (treatment and voluntary physical activity) ANOVA of marbles buried in male (C) and female (D) offspring. Two-way ANOVA of amount of nestlet shredded in male (E) and female (F) offspring. Two-way ANOVA of post-stress amount of nestlet shredded in male (G) and female (H) offspring. Two-way ANOVA of the calculated stress index in male (I) and female (J) offspring. ✱$p<0.05$, ✱✱$p<0.01$. See statistics table for sample sizes.

3.5. Parental alcohol exposure before birth and alterations in adult offspring physiology

As the life expectancy for individuals with FAS is only 34 years, and many of the leading causes of death include diseases of major organ systems, we next sought to examine physiology within the adult offspring exposed to parental alcohol (Thanh & Jonsson, 2016). First, we conducted dual energy X-ray absorptiometry (DXA) scans and analyzed broad metrics of body composition. We did not find any differences in the fat to lean weight ratio due to parental alcohol exposure (Figure 5.10A). Next, we analyzed bone area and found a significant decrease in male Dual-Exp offspring (Figure 5.10B). With this in mind we next analyzed bone mineral density and found an increase in adult male Mat-Exp (Figure 5.10C). Taken together, parental alcohol exposures have the ability to modulate adult offspring body composition metrics.

We next investigated major organs within the adult parental alcohol exposed offspring. Specifically, we dissected and weighed the heart, liver, kidneys, adrenal glands, thymus, and spleen. We did not observe any significant differences in heart, kidney, thymus, or adrenal gland to bodyweight ratios (Figure 5.10D-G). However, we did find a significant increase in spleen to body weight in male Dual-Exp offspring (Figure 5.10H) and significant decreases in liver to body weight in male Mat-Exp, Pat-Exp, and Dual-Exp adult offspring (Figure 5.10I). Collectively, these results indicate that parental alcohol exposure leaves long lasting impacts on offspring physiology.

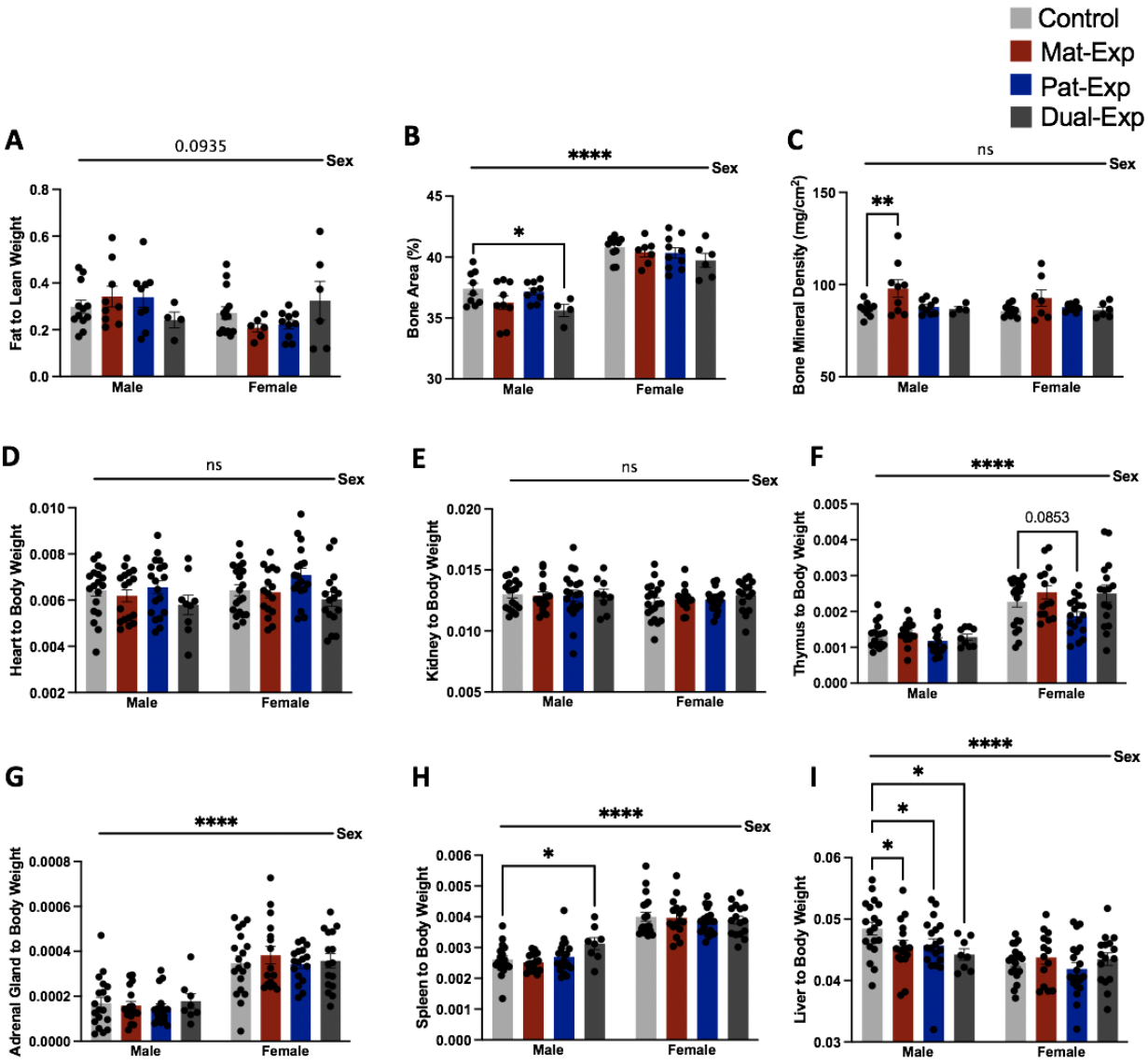

Figure 5.10. Changes in mature adult offspring physiology due to parental alcohol exposure. Two-way (treatment and sex) ANOVAs of DXA measurements of fat to lean weight (A), bone area (B), and bone mineral density (C). Two-way (treatment and sex) ANOVAs of organ to body weight for the heart (D), kidney (E), thymus (F), adrenal glands (G), spleen (H), and liver (I). ✱p<0.05, ✱✱p<0.01, ✱✱✱✱p<0.0001. See statistics table for sample sizes.

4. Discussion

Although often thought of as a pediatric disorder, FASD is a lifelong debilitating condition. Importantly, FASD is completely preventable with lifestyle changes, but conflicting health messaging not only hinders prevention, but can also be misleading and result in negative lifelong health consequences in the next generation. Many factors play a role in the severity of FASD outcomes, such as dose and timing of alcohol consumption, and as of yet these factors are not fully understood. Within this study, we present evidence for the role of maternal, paternal, and dual parental alcohol exposure as having important implications on offspring health and behavior across the lifespan. Although largely ignored, paternal alcohol consumption is a significant modulator of offspring health and behavior. This body of evidence urges for the immediate implementation of healthcare messaging that encompasses the complete picture of the inheritance of environmental exposures from both parents.

CHAPTER VI

SUMMARY & CONCLUSIONS

1. Summary

In chapter I we described the foundational knowledge of FASD and that vast variation of presenting symptoms. We discussed how early life exposures, specifically during gestation, could have negative consequences for development and how the placenta plays an important role in the health of the pregnancy. Next, we described how alcohol has biphasic effects both on the CNS and male reproductive tract. We discussed mouse models as a critical tool for FASD models but warn of potential confounders between different models that could lead to discrepancies. Finally, we defined epigenetics and briefly discussed epigenetic inheritance of paternal alcohol exposure.

In chapter II we established a crosstalk between paternal environmental exposure and maternal genetics. Specifically, our results indicated that maternal factors could modulate paternal alcohol exposure induced changes in fetoplacental growth. We proposed that the placental adaptations observed in hybrid crosses helped to alleviate fetal growth restriction due to paternal alcohol.

In chapter III we evaluated the effects of paternal alcohol dose on fetoplacental changes. Using clinically relevant concentrations and corresponding doses, we reported a biphasic effect of paternal alcohol on fetoplacental growth. With lower doses resulting in fetoplacental overgrowth and higher doses leading to fetoplacental growth restriction. These indirect effects on offspring growth mirror the direct effects observed by others in many organ systems.

In chapter IV we implemented and dual parental model of alcohol exposure and examined the effects of maternal, paternal, and dual parental alcohol exposure on fetal craniofacial growth.

Interestingly, we found that each exposure programmed distinct craniofacial phenotypes and altered neurological development. Importantly, paternal alcohol consumption could induce craniofacial changes in the absence of maternal alcohol intake.

Finally, in chapter V, we move beyond the fetal window and investigate the effects of maternal, paternal, and dual parental alcohol exposure across the lifespan. In support of the Developmental Origins of Health and Disease (DOHaD) hypothesis, we found that parental alcohol exposure leaves lasting impacts across the lifespan. However, we suggest that these parental alcohol induced changes could be modified through voluntary physical activity as a behavioral intervention.

In summary, the results presented fill a large gap in our understanding of the penetrance of FASD and helps to describe the wide variation seen in presenting symptoms. Importantly, this body of work identifies paternal alcohol consumption as a significant inducer and contributor to FASD phenotypes. Moreover, these results redress the stigma that maternal alcohol is solely to blame for FASD. Given this data, we urge for the immediate issuance of pre-pregnancy health messaging that directly acknowledges the father's role in the health of the pregnancy and responsibility for FASD diagnosis.

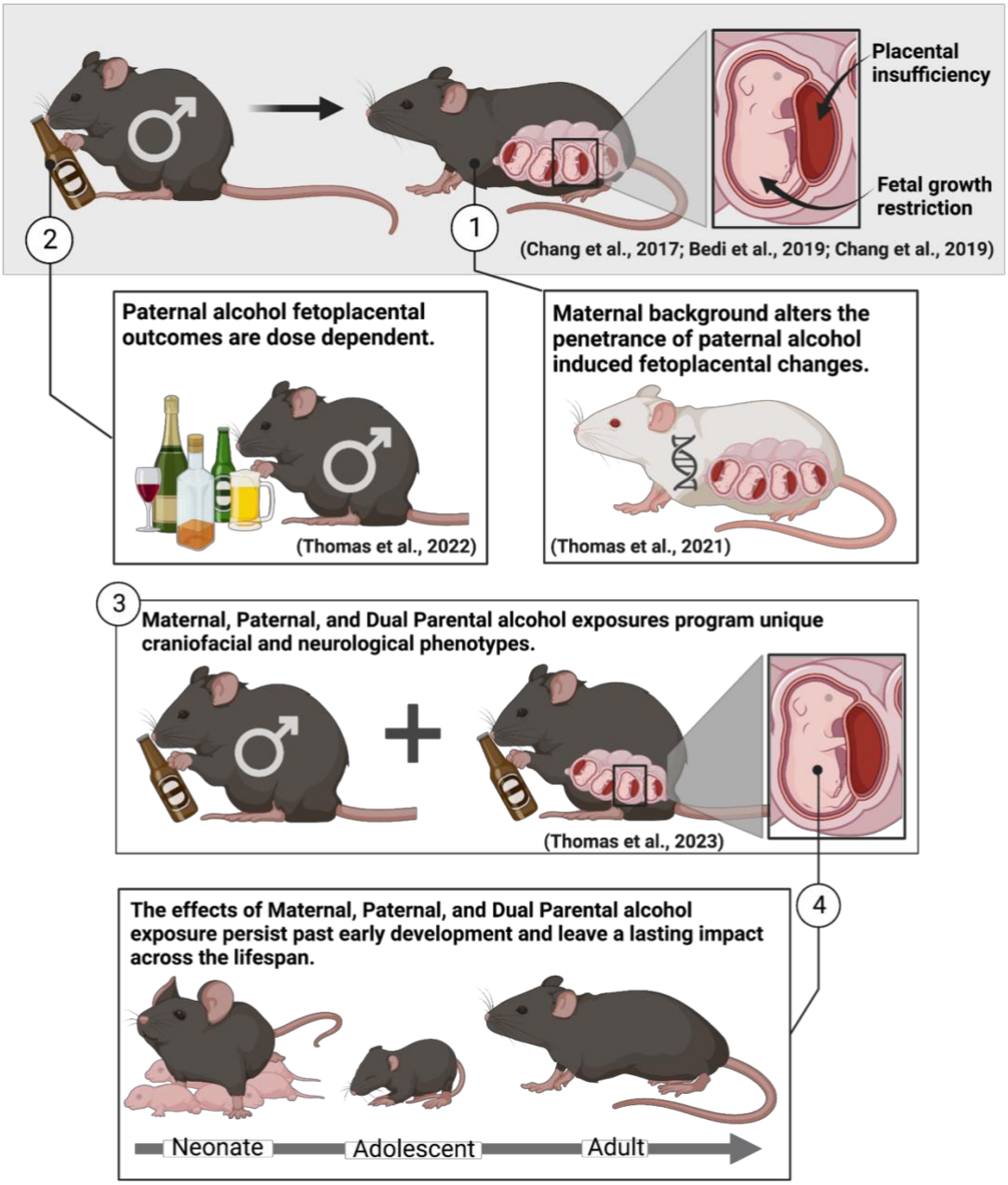

Figure 6.1. Infographic summary of experimental results

3. Conclusions

FASD is an umbrella term that encompasses many diagnoses, such as Fetal Alcohol Syndrome (FAS), partial FAS, Alcohol Neurodevelopmental Disorder, and Alcohol Related Birth Defects. FASDs present with a wide range of developmental abnormalities that affect growth, craniofacial patterning, neurological development, and behavior. Fetal Alcohol Syndrome was first described in the clinic in 1973. By 1981, the US surgeon general issued a warning to limit alcohol during pregnancy. Eventually, in 2005 the surgeon general updated this warning to avoid alcohol altogether during pregnancy. Significantly, this year, 2023 marks the 50th anniversary of the first FAS clinical description. Although FASD first reached clinical reports in 1973, this area of research is still reported to be in its infancy. In fact, within the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), FASD is included among conditions that need more research.

Traditionally, FASD research has focused exclusively on maternal alcohol exposure, and public health messaging has solely targeted women who may be expecting. Such messaging can be seen in the screenshot of the CDC webpage from 2021 (Figure 6.2), that states FASDs "occur in a person whose mother drank alcohol during pregnancy" and "FASDs are caused by a woman drinking alcohol during pregnancy."

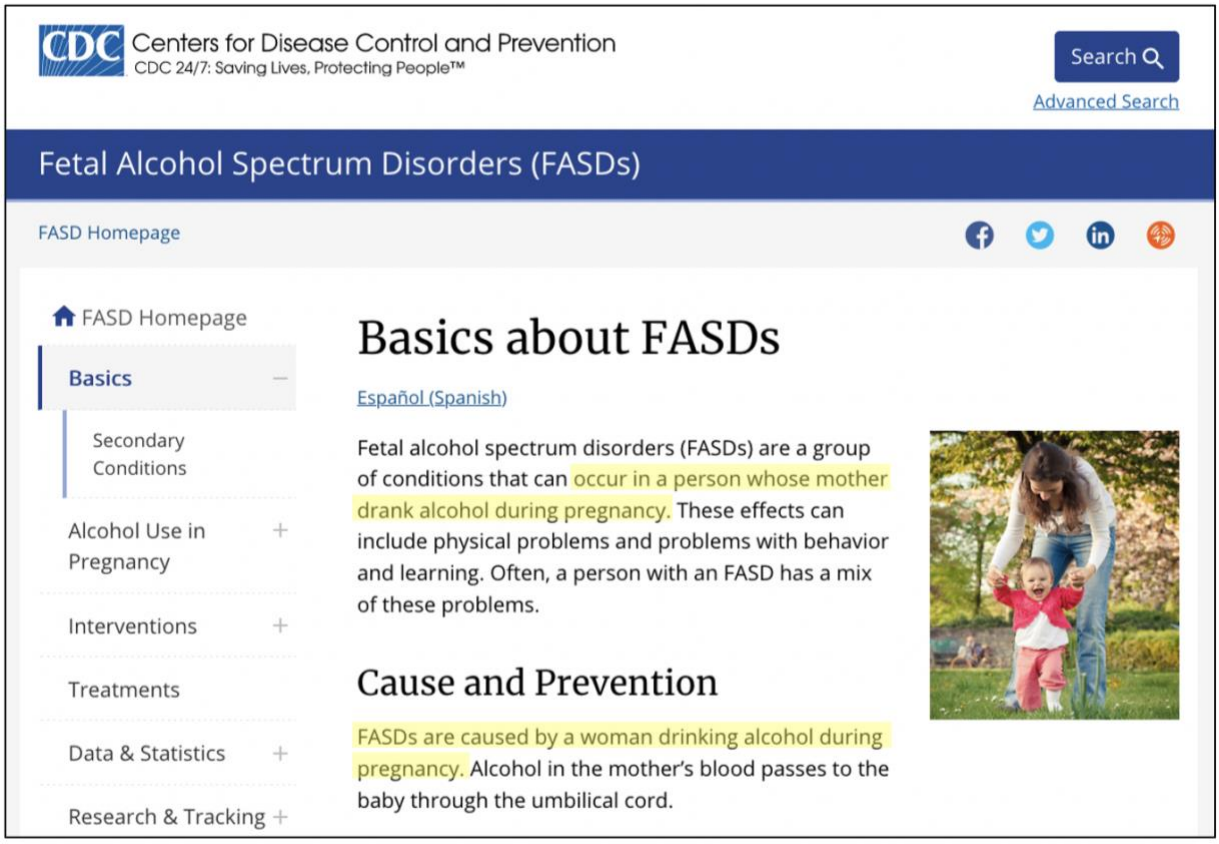

Figure 6.2. Screenshot of CDC webpage in 2021 [https://www.cdc.gov/ncbddd/fasd/facts.html#:~:text=Fetal%20alcohol%20spectrum%20disorders%20(FASDs)%20are%20a%20group%20of%20conditions,a%20mix%20of%20these%20problems]

But, What about Dad? We know that men drink more than women. And importantly, of the children with FASD, 75% have alcoholic fathers. Moreover, in a clinical study of almost 70,000 mother-father-offspring trios it was discovered that there was increased microcephaly in children born to higher paternal alcohol consumption. To challenge the maternal centric focus for the cause of FASD, previous work by the Golding Lab designed and implemented a chronic limited access, voluntary alcohol consumption mouse model. Using this model, they found that preconception paternal alcohol consumption resulted in the FASD-like outcomes of fetal growth restriction that was accompanied by placental insufficiency.

To build upon the lab's previous work, my PhD research found that maternal background alters the penetrance of paternal alcohol induced fetoplacental changes, paternal alcohol induced fetoplacental changes are dose dependent and lead to altered craniofacial development, and that the paternal alcohol induced developmental programming leads to long lasting changes in health and behavior in adolescent and adult offspring. Collectively, these findings have had a significant impact on the field of paternal alcohol exposure. Importantly, this research has aided in the broadening of health messaging to encompass the lifestyle of both parents. For example, current health messages concerning FASD as seen on the CDC webpage (Figure 6.3), now states that FASDs occur in a *person who was exposed to alcohol before birth*, rather than placing blame exclusively on the mother.

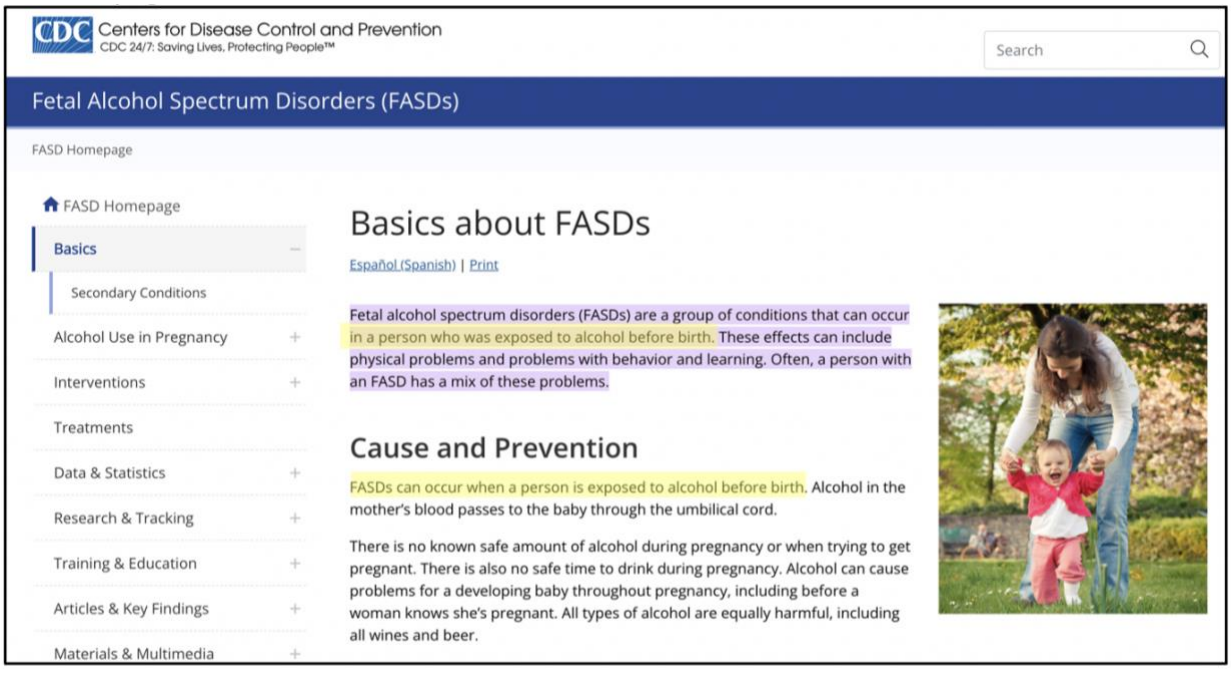

Figure 6.3. Screenshot of CDC webpage in 2023 [https://www.cdc.gov/ncbddd/fasd/facts.html#:~:text=Fetal%20alcohol%20spectrum%20disorders%20(FASDs)%20are%20a%20group%20of%20conditions,a%20mix%20of%20these%20problems]

Although the messaging does not specifically implicate Dad in the presentation of FASD, it does come a long way to alleviate the maternal central focus for the fault of FASD. It is my hope that health messaging will continue to evolve to encompass the complete picture of developmental health, and that both the mother and father share in the responsibility of the health of the pregnancy.

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