

Title: Prenatal adversity alters the epigenetic profile of the prefrontal cortex: Sexually dimorphic effects of prenatal alcohol exposure and food-related stress

Authors: Alexandre A. Lussier<sup>1,2,3</sup>, Tamara Bodnar<sup>4</sup>, Michelle Moksa<sup>5</sup>, Martin Hirst<sup>5,6</sup>, Michael S. Kobor<sup>7,8,9,10</sup>, Joanne Weinberg<sup>4</sup>.

Affiliations:

<sup>1</sup> Psychiatric and Neurodevelopmental Genetics Unit, Center for Genomic Medicine, Massachusetts General Hospital, Boston, MA, USA.

<sup>2</sup> Department of Psychiatry, Harvard Medical School, Boston, MA, USA.

<sup>3</sup> Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, MA, USA.

<sup>4</sup> Department of Cellular and Physiological Sciences, Faculty of Medicine, Life Sciences Institute, University of British Columbia, Vancouver, British Columbia, Canada.

<sup>5</sup> Department of Microbiology and Immunology, Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada.

<sup>6</sup> Canada's Michael Smith Genome Sciences Centre, British Columbia Cancer Agency, Vancouver, BC, Canada.

<sup>7</sup> BC Children's Hospital Research Institute Vancouver, British Columbia, Canada.

<sup>8</sup> Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada.

<sup>9</sup> Centre for Molecular Medicine and Therapeutics, Vancouver, British Columbia, Canada.

<sup>10</sup> Program in Child and Brain Development, CIFAR, MaRS Centre, West Tower, 661 University Ave, Suite 505, Toronto, ON M5G 1M1, Canada.

Corresponding authors:

Alexandre A. Lussier: alussier[at]mgh.harvard.edu

Joanne Weinberg: joanne.weinberg[at]ubc.ca

Word count: 6017

Tables: 1

Figures 5

## **Abstract (199/200 words)**

Prenatal stressors or insults can have long-term consequences on developmental trajectories and health outcomes. Although the biological mechanisms underlying these effects are not fully understood, epigenetic mechanisms, such as DNA methylation, have the potential to link early-life environments to alterations in physiological systems, with long-term functional implications. We investigated the consequences of two prenatal insults, prenatal alcohol exposure (PAE) and a restricted feeding regimen (food-related stress), on DNA methylation profiles of the rat brain during early development. As these insults can have sex-specific effects on biological outcomes, we analyzed epigenome-wide DNA methylation patterns in the prefrontal cortex (PFC) of both male and female rats. We found both sex-dependent and sex-concordant influences of these insults on epigenetic patterns, highlighting the importance of including both sexes in studies of environmental influences. These alterations occurred in genes and pathways related to brain development and immune function, suggesting that PAE and food-related stress may reprogram neurobiological/physiological systems through central epigenetic changes, and may do so in a sex-dependent manner. Such epigenetic changes may reflect the sex-specific effects of prenatal insults on long-term functional and health outcomes and have important implications for understanding possible mechanisms underlying neurodevelopmental disorders beyond FASD, including autism spectrum disorder.

## INTRODUCTION

Neurodevelopmental and psychiatric disorders may be rooted in early-life environments, which can have profound influences on cognitive, neurobiological, and physiological outcomes. For instance, autism spectrum disorder (ASD) is influenced by both genetic mechanisms and environmental factors, such as prenatal maternal stress<sup>1, 2</sup>, medication use<sup>3</sup>, and maternal immune dysfunction<sup>2</sup>, among others (reviewed in <sup>4</sup>). By contrast, fetal alcohol spectrum disorder (FASD) has more clearly defined roots, with prenatal alcohol exposure (PAE) being the key etiological factor, although, as in ASD, environmental factors such as maternal nutrition, health, and stress can significantly influence outcome. Although the mechanisms linking environmental exposures to neurodevelopmental outcomes are not fully understood, one prevailing hypothesis is that the effects of early-life challenges become biologically embedded through epigenetic mechanisms, such as histone modifications, non-coding RNA expression, and DNA methylation (DNAm)<sup>5</sup>. The latter is the most commonly studied epigenetic modification and involves the addition of a methyl residue to the cytosines. Importantly, DNAm is relatively stable over time and perhaps most importantly, may be critical in capturing the effects of environmental exposures to modulate long-term gene expression, functional outcomes, and health<sup>6, 7</sup>.

FASD describes the wide range of cognitive, behavioral, adaptive, and physiological alterations that occur following PAE<sup>8</sup>. In addition to its direct teratogenic effects, PAE can program or sensitize key neurobiological and physiological systems, thus increasing later life vulnerabilities to adverse functional and health outcomes. Systems involved in regulation of the stress response, particularly, the hypothalamic-pituitary-adrenal (HPA) and immune systems, are altered by PAE and highly susceptible to programming. Indeed, in both animal model and clinical studies, PAE resulted in HPA dysregulation, including hyperresponsiveness to stressors<sup>9</sup>, alterations in diurnal HPA regulation<sup>10</sup>, increased physical and mental health problems, including metabolic disorders<sup>11</sup>, depression, and anxiety<sup>12</sup>, and deficits in immune system activity and regulation<sup>13</sup>. As early life stress or adversity can result in similar adverse outcomes in adulthood<sup>14, 15</sup>, it is in this context that PAE can be considered a type of prenatal stressor. Of particular relevance to the current study is the issue of sex differences in the adverse effects of PAE. Until recently, studies utilizing animal model often excluded or failed to analyze data from females. Nevertheless, of those studies that probed for sex-specific changes, differential effects of PAE on males and females were reported in both rodent and primate models, including differences in hippocampal microglia and cytokine expression<sup>16</sup>, hypothalamic-pituitary-adrenal (HPA) activity and regulation<sup>9</sup>, dopaminergic regulation<sup>17</sup>, immune responses<sup>18</sup>, social behavior (Holman; Sandra Kelly), and depressive- and anxiety-like behaviors<sup>12, 19-21</sup>. By contrast,

clinical research in the FASD field has typically included children of both sexes, and sex differences in prevalence of FASD, brain maturation, cognitive function, and mental health, among other outcomes, have been reported<sup>22-26</sup>. Although the biological mechanism that mediate altered developmental outcomes following PAE are not fully understood, several studies have revealed broad impacts of PAE on epigenetic patterns in the brain<sup>27</sup>. While the majority of studies limit their analyses to either male or female subjects, recent evidence from candidate gene analyses suggests that PAE may have sexually dimorphic effects on epigenetic profiles<sup>28-30</sup>. However, no studies have investigated whether there is a genome-wide sex-specific impact of PAE on the brain's epigenome, limiting our ability to identify the molecular mechanisms that may drive sexual dimorphisms associated with PAE, as well as their overlaps with other neurodevelopmental disorders such as ASD.

The present study was designed to fill this gap. We utilized a well-established rat model of PAE to examine the impact of two early-life exposures - PAE and restricted feeding - on genome-wide DNAm patterns of the prefrontal cortex (PFC), a key brain region for cognition and behavior. Our PAE model also includes not only an *ad libitum*-fed control diet group but also a secondary control, the pair-fed (PF) group. Pair-feeding is a standard procedure to control for the reduced food intake of animals consuming alcohol; PF animals get a reduced ration, matched to that of a PAE partner, and thus less than what would be consumed in a diet without alcohol. This results in hunger, abnormal feeding patterns (consuming most of the ration within a few hours of feeding and remaining food deprived for the remainder of the 24-hour period), and mild stress. A treatment in itself, pair-feeding can reprogram offspring behavior and physiological functions, such as alterations to stress system regulation<sup>31, 32</sup>, reproductive development and function<sup>33, 34</sup>, immune system development<sup>35</sup>, depressive- and anxiety-like behavior<sup>21</sup>, and cognitive function (Akitakea et al), among other outcomes. Studies on food scarcity or restriction in human populations have revealed parallel insights, showing that alterations to food access can have marked effects on the reprogramming of physiological systems<sup>36, 37</sup>, particularly if deficiencies occur during critical or sensitive periods of brain or organ development. To this end, several studies have investigated the effects of severe food scarcity on the developing fetus, particularly in the context of the Dutch Famine or Hunger Winter, identifying sexually-dimorphic effects on both physiological outcomes, such as metabolic disorders and brain function<sup>38</sup>, as well as DNAm patterns linked to growth and metabolism<sup>39</sup> that persist across the life course<sup>40</sup> and that are sex-specific<sup>41</sup>. We are among the first to investigate the impact of food-related stress at the epigenome-wide level in the brain, limiting our understanding of the long-term effects of food-related stress on developmental processes.

As such, the goals of the present study were to 1) identify *sex-specific* alterations to DNAm in response to prenatal stressors; 2) identify *sex-concordant* alterations to DNAm resulting from prenatal stressors; and 3) assess the shared etiology of genes influenced by PAE and food-related stress. Furthermore, we investigated the potential relevance of this impact for understanding neurodevelopmental disorders beyond FASD, specifically, ASD. To our knowledge, our study represents the first epigenome-wide analysis of sex differences in the brain in response to PAE and/or food-related stress. Importantly, these analyses provide insight into the biological pathways that influence the sexual dimorphic outcomes resulting from prenatal insults, such as alcohol exposure, stress, and food deprivation, while highlighting potential pathways driving the phenotypic overlaps between FASD and ASD.

## MATERIALS & METHODS

### Prenatal Treatments

All animal protocols were approved by the University of British Columbia Animal Care Committee and are consistent with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council 2011). Details of the procedures for breeding, feeding, and handling have been published previously<sup>31</sup>. Briefly, nulliparous Sprague-Dawley females (n=39) were pair-housed with a male, and vaginal lavage samples were collected daily for estrous cycle staging and to check for the presence of sperm, indicating gestation day 1 (GD1). Pregnant dams were then singly housed and assigned to one of three prenatal treatment groups: Prenatal alcohol exposure (PAE) - *ad libitum* access to liquid ethanol diet, 36% ethanol-derived calories, 6.37% v/v, n =13; Pair-fed (PF) - liquid-control diet, maltose-dextrin isocalorically substituted for ethanol, in the amount consumed by a PAE partner, g/kg body weight/GD), n =14; or Control (CON) - pelleted version of the liquid control diet, *ad libitum*, n =12. All animals had *ad libitum* access to water. Experimental diets (Weinberg/Kiever Liquid Ethanol Diet #710324, Weinberg/Kiever Liquid Control Diet #710109, and Pelleted Control Diet #102698, Dyets Inc., Bethlehem, PA) were provided from gestation days 1-21, and then replaced with standard laboratory chow (19% protein). Litters were weighed and culled at birth to 6 males and 6 females, when possible.

### Sample collection and DNA extraction

On postnatal day 22, female and male offspring (no more than 1 ♂ and 1 ♀ /litter to control for litter effects) were decapitated, and brains were removed and weighed; the prefrontal cortex (PFC) was then quickly dissected and frozen on dry ice in RNAlater (Qiagen, Hilden, Germany) (n=7-11/age/group; **Figure 1**). All tissue collected was left at 4°C for 1 day and then frozen at -80°C until

DNA extraction. Total DNA was extracted from the PFC (n=5/group/sex) using the RNA/DNA extraction kit (Qiagen, Hildren, Germany). Cells were mechanically lysed using the Omni Bead Ruptor Elite (Omni International, Kennesaw, GA). DNA concentration was assessed using Qubit Fluorometric Quantitation (Life Technologies, Carlsbad, USA).

### **Methylated DNA Immunoprecipitation and Next-Generation Sequencing**

Our methylated DNA immunoprecipitation following by next-generation sequencing (meDIP-seq) procedures were performed as previously described (Taiwo et al., 2012). To summarize briefly, we performed immunoprecipitation of 5-methylcytosine to obtain an enriched fraction of methylated DNA fragments. These fragments were then sequenced using the Illumina HiSeq by the Genome Science Center (Vancouver, BC) to identify regions of the genome enriched for DNAm.

### **Bioinformatic Analyses**

#### *Next-Generation Sequencing Quality Control*

Fastq files were aligned to the most current rat genome (Rn6, July 2014) using the Burrows-Wheeler Transform (BWA) tool to obtain .bam files (Li & Durbin, 2009). Bam files were filtered using *samtools* to remove duplicate reads, unpaired reads, and reads with a minimum quality score below 10.

#### *Peakset Generation*

Model-based analysis of ChIP-seq (MACS2; version 2.1.0.20140616) was used to identify enriched regions of DNAm across the genome<sup>42</sup>. The peak calling to identify peak regions (DNAm windows) was performed using the ‘callpeaks’ function on paired end bam files with no control input and the following options: –f BAMPE –m 5 50 –bw 300 –g 2.9e9 –q 0.05. Each sample was modeled individually, generating 30 total peaksets. These were imported into R using the DiffBind package. As all samples had slightly different predicted peaks, peaksets were combined into common regions using the dba.count function in DiffBind, which removed peaks found in less than 3 samples across the entire dataset and provided the total number of reads within each peak/sample. Reads mapping to sex chromosomes were removed for these analyses. The final dataset contained 358,773 meDIP-seq peaks.

#### *Data Preprocessing and Normalization*

Reads within each peak were converted to reads per kilobase per million (RPKM). Variation associated with batch effects due to sequencing chip were corrected using the ComBat method from the sva package (version 3.32.1), protecting the effects of prenatal treatment group and sex.

### *Differentially Methylated Region (DMR) Identification*

Linear modeling was performed using *edgeR* (version 3.26.8) to identify DMRs that were: 1) sex-concordant (~group+sex); 2) female-specific (~group; females only); or 3) male-specific (~group; males only). P-values were corrected for multiple-testing using the Benjamini-Hochberg method<sup>43</sup>. Significant DMRS at a false discovery rate (FDR) <0.05 were obtained for the following contrasts: PAEvCON, PAEvPF, and PFvCON (**Figure 2**). The final PAE-specific DMRs were significant in both PAEvCON and PAEvPF but not the PFvCON contrasts. The final PF-specific DMRs were statistically significant in both PFvCON and PAEvPF but not the PAEvCON contrasts. The final shared DMRs between PAE and PF were statistically significant in both the PAEvCON and PFvCON contrasts.

### *Genomic Enrichment*

A custom annotation was built for the peakset using the UCSC genome browser gene annotations. Briefly, genomic coordinates of all CpG islands, exons, introns, promoters (TSS -200bp and TSS -1500bp), 3' untranslated regions (UTR), 5' UTRs for the rn6 genome were obtained as bed files from the table browser. These were intersected with the meDIP-seq peaks uusing the intersectBed function from bedtools. The overlaps were concatenated into a single annotation set in R, where individuals peaks contained information for each potential genomic feature. Of note, regions spanning both introns and exons were deemed intron/exons boundaries and a given DMR could span multiple genomic features. P-values for genomic feature enrichment analyses were calculated by computing background levels of genomic features on 10,000 random subsets of DMRs, using the same number of PAE-specific, PF-specific, or shared DMRs.

### *Gene Ontology Analyses*

The gene-score resampling (GSR) tool of ErmineR (version 1.0.1.9) was used to identify gene function enrichment in the differentially methylated genes including the Gene Ontology (GO) annotations molecular function, biological process, and cellular component<sup>44, 45</sup>. The ermineR gene score resampling (GSR) tool was set with the following parameters: max gene set size = 2,000; min gene set size = 2; iterations = 10,000. Significant associations (FDR<0.05 and corrected multifunctionality p-value <0.05) were obtained for the following contrasts: PAEvCON, PAEvPF, and PFvCON. The final PAE-specific GO terms were statistically significant in both the PAEvCON and PAEvPF, but not the PFvCON contrasts. The final PF-specific GO terms were statistically significant in both the PFvCON and PAEvPF, but not the PAEvCON contrasts. The final shared GO terms

between PAE and PF were significant in both the PAEvCON and PFvCON contrasts.

## RESULTS

We performed three main sets of analyses, first focusing on identifying PAE-specific DMRs, followed by DMRs linked to food-related stress (PF group), and DMRs shared between PAE and PF. Within these main analyses, we further explored sex-concordant and sex-specific alterations to DNAm patterns within the prefrontal cortex (PFC).

### PAE caused sex-concordant alterations to DNAm patterns

To assess sex-concordant alterations to DNAm patterns following PAE, we performed linear modeling with both sexes included, utilizing a model that accounted for sex. Contrast analyses to identify PAE-specific alterations successfully identified 307 PAE-specific DMRs at an FDR <0.05. However, 14 of these overlapped with the male-specific DMRs and 5 overlapped with the females-specific DMRs. As these were likely driven by either males or females, respectively, they were not included in the sex-concordant DMRs (**Figure 3A**). As such, we found 288 sex-concordant DMRs that were consistent across both sexes and showed consistently different DNAm levels in PAE compared to CON and PF animals (**Figure 3B; Supplementary Table 1**).

Of these, 46 were up-methylated and 242 were down-methylated in PAE versus both CON and PF groups, with sizes ranging from 271 to 1,894bp (median=465bp). Furthermore, 193 of the DMRs showed at least 1.5-fold change in DNAm levels in PAE versus both CON and PF animals (**Supplementary Table 1**), suggesting that PAE could induce robust sex-concordant alterations to DNAm patterns.

Overall, 119 DMRs were located in genes, several of which were involved in potassium channel activity (*Kcnn1*, *Kcnn1*, *Kcnh5*, *Kcnip1*, *Kcnq1*) and ion signaling (*Grik1*, *Camk2d*, *Itpr2*, *Slc12a8*). Of note, five genes, *Camta1*, *Cpne4*, *Ephb1*, *Magi1*, and *Tmem178b*, had multiple DMRs (**Supplemental Table 1**). The majority of DMRs were found in intergenic regions, but also showed lower enrichment in these regions than by random chance ( $p= 0.0018$ ). By contrast, sex-concordant DMRs showed increased enrichment in exons ( $p= 0.026$ ) and introns ( $p= 0.0018$ ), which frequently spanned intron/exon boundaries.

Using gene-score enrichment, we identified 15 PAE-specific biological processes that were enriched in a sex-concordant manner. These included pathways involved in central nervous system development, metabolic processes, and the inflammatory response (**Supplemental Table 2**).

## **PAE caused sex-specific alterations to DNAm patterns**

Moving beyond sex-concordant alterations, we performed a sex-stratified analyses using linear modeling to identify sex-specific alterations following PAE. Contrast analyses revealed PAE-specific alterations at 18 DMRs in females and 59 DMRs in males at an FDR<0.05) (**Figure 3A; Supplemental Table 1**).

All 18 female-specific DMRs showed decreased DNAm in PAE compared to CON and PF animals, which ranged from 279 to 607bp in length (median=377bp). Of these, 7 DMRs were located in genes. Female-specific DMRs did not show any differences in genomic location enrichment compared to the background of the dataset. Five PAE-specific biological processes were identified, including those involved in acetylcholine and angiotensin receptor functions (**Supplemental Table 2**).

In males, 48 DMRs showed decreased DNAm and 11 showed increased DNAm in PAE compared to CON and PF animals. These male-specific DMRs ranged from 291 to 3,300 bp (median=417 bp), and 15 DMRs were located in genes. Again, no significant enrichment for genomic features was detected. Six PAE-specific biological processes included those involved in the regulation of hormone metabolism and other metabolic processes (**Supplemental Table 2**).

## **Prenatal food-related stress had both sex-concordant and sex-specific effects**

Next, we investigated the effects of pair-feeding, a restricted feeding paradigm that in itself induces prenatal stress related to hunger and disrupted feeding patterns. Importantly, this treatment may capture some elements of food insecurity or scarcity on DNAm patterns of the PFC. Using parallel approaches to the PAE analyses, we identified 129 sex-concordant, 8 female-specific, and 11 male-specific DMRs that were driven by pair-feeding effects (**Figure 4A; Supplemental Table 3**).

Of the 129 sex-concordant DMRs, 100 showed decreased DNAm and 29 showed increased DNAm in PF compared to CON and PAE animals (**Figure 4B**). 39 DMRs were located in genes, with most again being located in introns or intergenic regions. Of note, *Adarb2*, *Dgki*, and *Gpc5* contained two DMRs each, and perhaps most interestingly, one of the sex-concordant DMRs was located in an intronic region of *Nr3c1*, the glucocorticoid receptor (GR) gene. We also identified 22 PF-specific biological processes from the sex-concordant analysis, of which several were involved in metabolic processes (**Supplemental Table 4**).

Similar to previous analyses, that found a general decrease in DNAm from prenatal treatments; every sex-specific DMR identified here showed decreased DNAm in PF animals, suggesting that pair-feeding or food-related stress may also have generally inhibitory effects on DNAm patterns. Although few DMRs were located in genes (3/8 for females and 2/11 for males), we identified several PF-

specific biological pathways that displayed sex-specific effects (**Supplemental table 4**). In females, we identified 33 pathways that were involved in endocrine, immune, and metabolic processes, while in males, we identified 25 pathways, which were mainly involved in metabolic processes. Overall, these results suggest that the stress induced by pair-feeding is a distinct physiological stressor, which has broad and sexually-dimorphic effects on the regulatory mechanisms of the brain.

### **PAE and PF animals shared some sex-concordant and sex-specific effects on DNAm patterns**

Given that prenatal alcohol exposure and pair-feeding may share common pathways in the reprogramming of biological systems, we also investigated DMRs shared between these two prenatal exposures. Here, we found 733 sex-concordant, 197 female-specific, and 19 male-specific DMRs that were influenced by both PAE and PF (**Figure 5A; Supplemental table 5**).

Of the 733 sex-concordant, shared DMRs, 479 showed decreased DNAm and 254 showed increased DNAm in PAE and PF compared to CON animals (**Figure 4B**). Of these, 309 were located in genes, including the transcription start site of *Drd4*, the dopamine D4 receptor gene, which has previously been associated with PAE<sup>46-48</sup>. We also identified 33 PAE and PF-shared biological pathways from the sex-concordant analysis, of which several were involved in metabolic processes and hormone regulation (**Supplemental Table 6**).

In contrast to the PAE- and PF-specific DMRs, shared DMRs in females generally showed an increase in DNAm in PAE and PF animals compared to CON (114 of 197 DMRs), whereas in males, most DMRs showed lower DNAm in PAE and PF animals compared to CON (10 of 19 DMRs). Here, we identified 26 biological pathways that were enriched in females, including those involved in cellular stress and metabolism, and 10 biological pathways enriched in males, which were mainly involved in metabolic processes. These findings suggest that PAE and restricted feeding, both of which act in many respects as prenatal stressors, may influence common biological pathways, which may explain some of the overlap between their resulting phenotypes.

### **PAE-specific and shared DMRs overlapped with genes linked to autism spectrum disorder**

Finally, we assessed whether there were any overlaps of DMRs with genes previously implicated in autism spectrum disorder (ASD) from genome-wide association studies (GWAS)<sup>49</sup> and epigenome-wide association studies (EWAS) on peripheral<sup>50-52</sup> or central tissues<sup>53-55</sup> (**Table 1**).

Comparing results from the most recent GWAS of ASD<sup>49</sup>, we found one overlap with PAE-specific DMRs (*NEGR1*) and one overlap with shared DMRs (*MMS22L*). By contrast, we did not find any overlaps for PAE, PF, or shared DMRs with DNAm signatures of ASD in blood from EWAS

studies in human populations<sup>53, 54</sup>. However, we found one overlap between female-specific shared DMRs and a study of buccal epithelial cells from ASD cases (*NRG2*)<sup>55</sup>. Moreover, when we compared our current findings to a recent study of DNAm patterns in the PFC of individuals with ASD<sup>51</sup>, we found one overlap with PAE-specific DMRs (*CDH13*) and one overlap with shared DMRs (*PRKAR1B*). Importantly, *CDH13* was one of the few genes with multiple DMRs; in this instance, it contained two distinct DMRs that were identified in the male-specific and sex-concordant analyses. Findings from a cross-cortex analysis of ASD in the same study<sup>51</sup> also showed some overlaps with PAE-specific (*GRIK1*) and shared (*FRMD4A*) DMRs. Finally, we found another overlap between shared DMRs (*NEDD4L*) and an independent study of DNAm in nuclei isolated from the frontal cortex of men with AS<sup>50</sup>.

Of note, almost every overlapping gene was identified in the sex-concordant analyses, with the exception of *NRG2* and *CDH13*, as noted above. These findings suggest that the shared pathways between autism spectrum disorder and early life stressors may be agnostic to the effects of sex. Furthermore, we found no overlaps between PF-associated DMRs and ASD genes identified at either the genetic or epigenetic level, suggesting potentially distinct pathways between neurodevelopmental disorders and physiological changes induced by food-related stress.

## DISCUSSION

This manuscript highlights the sex-specific impact of prenatal insults/stressors such as alcohol and restricted feeding or food-related stress on developmental processes of the prefrontal cortex. For the first time, we show that PAE can cause both sex-concordant and sex-specific changes to DNAm patterns, which may explain some of the sexually dimorphic effects of PAE and phenotypic overlaps with neurodevelopmental disorders such as ASD. The pair-fed condition, which models food scarcity/insecurity, demonstrates that exposure to the maternal stress of hunger and disrupted feeding schedules can alter DNAm patterns of the PFC, which may have long-term consequences on brain function and downstream neurobiological, physiological, and behavioral processes.

### PAE-specific alterations

Utilizing a rat model of prenatal alcohol exposure, we have previously shown that PAE can alter the DNAm profile of the hypothalamus and white blood cells in females<sup>56</sup>. We note that none of the PAE-specific DMRs identified in that study overlapped with those identified in the present study, despite using animals from the same set of litters at the same age. However, we found similar alterations to biological processes involved in immune function and cellular metabolism, suggesting that common pathways are indeed influenced across tissues, even though specific regions of the epigenome may vary.

Importantly, we further extended our previous work by examining the sex-concordant and sex-specific alterations to DNAm patterns by PAE. Similar to most epigenetic studies of PAE, we observed a general hypomethylation in response to PAE, likely as a result of the effects of alcohol on one-carbon metabolism during development<sup>30</sup>. We note that these effects were present across all analyses, suggesting that they are not sex-specific. These results are in line with a study of DNAm and choline supplementation in PAE animals, which showed no significant differences between sexes<sup>57</sup>, suggesting that interventions to rescue one-carbon metabolism following PAE<sup>58, 59</sup> may be adequate in both males and females, without the need for sex-specific approaches. Similarly, the majority of DMRs in the PFC were linked to sex-concordant alterations, with a large proportion falling within potassium channel and ion signaling genes, which are closely linked to brain disorders<sup>60</sup>. Of note, potassium channels have recently been proposed as therapeutic targets for epilepsy and intellectual disability<sup>61</sup>, which are common co-morbidities of FASD<sup>62</sup>. As such, the high proportion of these genes in sex-concordant DMRs may point to an underlying mechanism in driving some of the phenotypic outcomes of PAE in human populations.

Beyond the broad and sex-concordant effects of PAE on DNAm, it is also possible that the sex-specific DMRs reflect some of the sexual dimorphisms observed for the cognitive and behavioral deficits linked to FASD. In particular, several genes that were linked to PAE in either males or females were involved in the regulation of cell adhesion and brain organization<sup>63, 64</sup>, such as *Cdh13* and *Itgb11*, as well as genes related to cortical development<sup>65, 66</sup>, such as *Tead1* and *Erbb4*. These findings may reflect overall sex-dependent structural differences in the PFC of PAE animals; indeed, structural differences between male and female brains have been reported in several brain imaging studies of individuals with FASD. For example, boys with PAE display larger differences in cortical volume than girls compared to their control counterparts across development<sup>67</sup>, and sex differences in cortical thickness and brain volume in childhood have also been reported<sup>68</sup>. Adolescents with FASD also show sex-specific differential activation of the frontal, medial, and temporal cortices compared to controls, further suggesting that PAE-induced epigenetic alterations may have important and sex-dependent downstream effects on behavior and cognition<sup>24</sup>. In addition to this potential relationship with structural alterations, one of the female-specific DMRs was located in *Stk3*, a gene previously associated with intellectual disability<sup>69</sup>. By contrast, male-specific DMRs showed alterations to hormonal regulation and metabolic processes, pointing to key differences in the reprogramming of broader physiological and cognitive functions of the PFC across sex. Taken together, these findings highlight the potential role of epigenetic modifications in the PFC to drive the sex differences identified in individuals with FASD across multiple domains of cognitive, behavioral, and mental function.

### Prenatal food-related stress-induced alterations

We also identified a unique epigenetic signature of pair-feeding effects in the PFC. As noted, the pair-fed group in the PAE model is the standard control for the effects of alcohol on food intake<sup>70</sup>. However, compared to the PAE group that, albeit eating less, eats *ad libitum*, pair-feeding is a treatment in itself, with the PF dams receiving a restricted ration, which results in both hunger and a disrupted feeding schedule. These stress-related effects could potentially parallel or model food scarcity or food insecurity in human populations. As such, the altered epigenetic patterns we observed in the PFC may provide insight into possible alterations that could result from such stressors during development in children. Similar to previous studies of famine in humans and food deprivation in animal models, we observed more DMRs that showed decreased DNAm than increased in PF animals, suggesting that food-related stress may also interfere with one-carbon metabolism and the pathways that deposit DNAm. We also identified a sex-concordant DMR that showed decreased DNAm in PF animals in the glucocorticoid receptor *Nr3c1*, which plays a key role in stress responsivity and may

reflect a reprogramming of the stress response. Of note, a previous study of offspring from dams fed an isocaloric protein-deficient diet before pregnancy found a similar decrease in DNAm in *Nr3c1*, suggesting that these effects may be due to the stress of reduced food intake, which results following any nutrient deficiency in the diet, rather than the nutritional deficits previously described<sup>71</sup>. This result is in line with previous studies that have shown that pair-feeding is a considerable stressor on dams, with lasting consequences on the behavior, physiology, and cognitive processes of their offspring. As such, altered DNAm of this key HPA axis gene may reflect broader alterations to stress response systems, which may in turn, influence the programming of numerous physiological systems linked to the stress response, including immune function, metabolic processes, and circadian rhythms. Indeed, we observed an association between pair feeding and altered DNAm in biological processes related to these same pathways, further suggesting that exposure to chronic food-related stress during development can have widespread effects on physiological processes.

We also found that the effects of prenatal restricted feeding differed between males and females, with potentially long-term consequences on the functioning of biological systems and disease risk. Previous studies of the offspring of women pregnant during the Dutch Hunger Winter also identified sex-specific effects of food insufficiency on DNAm patterns<sup>41</sup>, alongside sex-specific alterations to brain size<sup>38</sup>, increased risk of affective disorders in males<sup>72</sup>, and altered lipid profiles in females<sup>36</sup>. The latter is of particular note, as female-specific DMRs in the present study showed an enrichment for metabolic processes related to lipid biosynthesis. Although the PFC is not primarily involved in lipid metabolism, alterations to epigenetic patterns of the brain may point to a broader physiological response to prenatal food-related stress that influences tissues throughout the body. In contrast to females, males showed an enrichment of processes related to carbohydrate processing, suggesting fundamental differences in the pathways influenced by food-related stress or disordered eating patterns between sexes. As brain activity and cognitive performance are closely tied to metabolism<sup>73</sup>, these metabolic alterations may reflect profound changes in PFC function, which may ultimately influence the neurobiological and behavioral effects of prenatal food scarcity and stress.

### **Common impacts of prenatal stressors**

Beyond the specific impacts of PAE and food-related stress, our results point to common effects of prenatal stressors on epigenomic pathways of the brain, which may highlight pathways underlying more general responses to stressors. It is noteworthy that prenatal alcohol exposure and pair-feeding can have overlapping effects on aspects of development. Similar to pair-feeding, PAE results in reduced food intake, which can alter aspects of HPA activity and regulation<sup>74</sup>, reproductive

development and function<sup>33</sup>, development and activity of the immune system<sup>35, 75</sup>, and depressive- and anxiety-like behavior<sup>12, 21</sup>, and cognitive function (Akitakea). Thus, while the PAE and PF conditions differ in the type of early life challenge they represent, these early life stressors or adversities may target similar aspects of brain and organ development and thus result in parallel outcomes that, in many instances, may be sex-dependent or sexually dimorphic. Importantly, both PAE and pair-feeding can result in HPA dysregulation, albeit possibly through different mechanisms<sup>32</sup>, which can have widespread programming effects on both epigenetic and physiological processes during development. As our analyses parsed out the specific effects of PAE and food-related stress, our results likely reflect broader alterations caused by alterations in endocrine and immune pathways during prenatal development.

Previous studies have shown that maternal stress during development can have profound effects on offspring physical and mental health<sup>76</sup>, as well as epigenetic processes<sup>77</sup>. Similarly, we found sex-concordant DNAm alterations in several risk genes involved in mental disorders. For instance, *CACNA1C* is a gene involved synaptic plasticity that has been linked to bipolar disorder, schizophrenia, major depressive disorder, and ASD<sup>78</sup>. There is also evidence that *CACNA1C* interacts with stress to cause depressive symptoms<sup>79</sup>, which, combined with evidence of increased depressive symptoms in PAE and PF animals, suggests that the DNAm alterations observed following prenatal stress may prime or sensitize the organism, increasing vulnerability to adverse mental outcomes. In addition to *CACNA1C*, we found several DMRs in *Pcdh9*, another susceptibility gene for depression<sup>80</sup>, further highlighting that the shared pathways between prenatal stressors may reprogram key biological systems involved in mental health. Finally, we identified a DMR in two genes involved in the dopaminergic system, *Nrg2* and *Drd4*, suggestive of stress-induced alterations to dopamine regulation and reward pathways. Importantly, *Drd4* was previously linked to PAE in a study of DNAm in the rat hypothalamus<sup>56</sup>, as well as three prior studies of FASD in humans<sup>46-48</sup>. This finding suggests that the shared effects of prenatal stressors may vary based on the brain region or tissue examined. These results also emphasize the inexorable link between PAE and early (both pre- and postnatal) life adversity, experienced disproportionately by individuals with FASD, and that cannot be fully disentangled<sup>10</sup>. However, these findings may also point to potential genes that can be targeted through therapeutic interventions to reduce the overall impact of prenatal stressors on well-being and risk for disease.

## Overlaps between prenatal stressors and autism spectrum disorder

Despite differences in the core phenotypic characteristics of FASD and ASD, these

neurodevelopmental disorders share several phenotypic characteristics<sup>81</sup>, which include deficits in social and communicative functioning<sup>82</sup>, socially inappropriate behaviors and difficulty with peers<sup>81</sup>, as well as hyperactivity, impulsivity, emotional lability, and difficulty changing strategies or inflexibility<sup>83</sup>. Moreover, co-morbidity between PAE/FASD and ASD or autism-like symptoms has been reported by several groups<sup>62, 84</sup>. Case reports on children from the toddler years up to 15 years of age<sup>83, 85</sup> were among the first publications to provide data on comorbidity, identifying behavioral alterations characteristic of ASD in children diagnosed with FASD, such as impaired social behavior, peer relationships and social reciprocity; delays or deficits in verbal and nonverbal communication; lack of make believe and social imitative play; restricted repertoires of activities and interests; resistance to change; tactile defensiveness/ abnormal sensory responses; and stereotyped motor behaviors. Studies on larger cohorts of individuals with FASD, ASD, and other neurodevelopmental disorders also support an association between heavy PAE and ASD. For example, exploratory data from a diagnostic clinic found that of 21 individuals with FASD, 16 (72%) met ICD-10 criteria for childhood autism<sup>86</sup>. Together, these findings underscore the fact that ASD can be comorbid with FASD and suggest that common pathways may underlie ASD and FASD. The fact that these comorbidities are not widely recognized may suggest that for some individuals, a diagnosis of FASD precludes secondary diagnoses, such as autism. The opposite is also possible - children diagnosed with ASD typically are not investigated for possible FASD<sup>85</sup>. This points to the need for more comprehensive approaches to diagnosis for both FASD and ASD.

Importantly, our current and prior findings suggest a link between the epigenomic mechanism that may underlie these disorders. In our recent epigenome-wide study of individuals with FASD, we found an enrichment of ASD-related genes in these individuals<sup>47</sup>, highlighting a potential link between FASD and ASD phenotypes and underlying biological pathways. Similarly, we observed some overlapping genes between FASD and ASD in the present study. Of note, one of the PAE DMRs overlapped with one of the strongest genetic signals of ASD in human populations, *NEGR1*, which was one of four replicated genes in the largest genome-wide association study to date (N=18,381 ASD cases)<sup>49</sup>. This gene is an adhesion protein that modulates synapse formation and plasticity in the hippocampus and cortex<sup>87</sup>. Importantly, *NEGR1* has also been linked to other psychiatric disorders, such as schizophrenia, depression, and Alzheimer's disease, as well as human intelligence and dyslexia. Collectively, these findings point to an important role for *NEGR1* in neural function and mental health disorders, and highlight the overlapping phenotypes and deficits present in individuals with ASD and FASD. Additional overlaps of DNA methylation profiles with those in the brains of individuals with ASD were also observed; in particular, several genes linked to DMRs shared between PAE and

PF, further emphasizing potential common pathways between ASD, FASD, and developmental outcomes linked to prenatal stressors. As well, we identified common alterations to *Nrg2*, a gene involved in the dopaminergic system, which is dysregulated in FASD<sup>17</sup>, and may highlight common etiologies between prenatal stressors and ASD. By contrast, we found no overlaps with DMRs associated with pair-feeding or food-related stress alone. Although there were fewer genes in this subset, this result may point to a more general role for stress in the common pathways to neurodevelopmental outcomes, with fewer effects observed when narrowing in on specific subtypes of stress, such as in the case of pair-feeding. Perhaps most surprisingly, almost every gene overlapping with studies of ASD was linked to sex-concordant alterations in the PFC, despite ASD primarily affecting males. This finding suggests that the pathways underlying the overlapping phenotypes between ASD and those resulting from prenatal stressors may be agnostic to the effects of sex, though they may manifest through varying phenotypes between males and females.

## Limitations

Our study had some limitations. First, due to the nature of meDIP-seq, we could not quantitatively assess the change in DNAm between exposure groups. Instead, we observed changes in enrichment patterns of DMRs; nonetheless these findings provide insight into larger-scale alterations to the DNA methylome as a result of prenatal exposures. Second, we cannot rule out that some of the observed differences were due to changes in cell type composition resulting from prenatal adversity/stress. Although we narrowed our focus to a specific brain region, as opposed to the entire brain, to limit such effects, future studies could aim to measure epigenetic modifications in specific cell types or move toward single-cell analyses to fully uncover the neurobiological mechanisms influenced by PAE and other stressors. Third, although our pair-feeding paradigm models food-related stress and disordered eating in humans, it remains an imperfect measure, which limits these comparisons. Nevertheless, we observed findings similar to those observed in human populations that experience food scarcity, suggesting that we tapped into common biological pathways related to food scarcity; more targeted and refined studies are needed to uncover more specifically the biological impacts of food-related stress on the brain. Finally, our sample size was relatively small, limiting our ability to detect sex-specific effects, as exemplified by the lower number of DMRs in the sex-specific analyses. However, an alternative explanation for this finding is that we examined animals at weaning, which is 22 days of age, well before the onset of puberty, when sex differences begin to fully emerge. As such, subsequent studies should examine epigenetic changes before and after pubertal onset to gain a deeper understanding of PAE-induced sexual dimorphisms.

## **CONCLUSIONS**

This study highlights the complex network of neurobiological pathways that respond to prenatal adversity/stressors and that modulate the differential effects of early life insults on functional and health outcomes. Our results also point to some key genes that may drive the phenotypic and biological overlaps between FASD and ASD, pinpointing genes that may influence the manifestation of symptoms present in both disorders. Identifying common neurobiological pathways may provide insight into the biological underpinnings common to FASD and ASD, as well as the downstream consequences of prenatal adversity or stress. Finally, the study of these exposures provides a unique opportunity to investigate the sex-specific effects of prenatal stressors on epigenetic mechanisms, as the possible biological mechanisms underlying sex-specific responses to prenatal insults are understudied and remain largely unknown. Taken together, the novel insights provided by our data may ultimately help to identify novel therapeutic targets for the prevention of the adverse consequences of prenatal adversity and the treatment of neurodevelopmental disorders.

## REFERENCES

1. Ronald A, Pennell C, Whitehouse A. Prenatal Maternal Stress Associated with ADHD and Autistic Traits in early Childhood. 10.3389/fpsyg.2010.00223. *Front Psychol.* 2011;1:223.
2. Beversdorf DQ, Stevens HE, Margolis KG, Van de Water J. Prenatal Stress and Maternal Immune Dysregulation in Autism Spectrum Disorders: Potential Points for Intervention. *Curr Pharm Des.* 2019;25(41):4331-4343. doi:10.2174/1381612825666191119093335
3. Andalib S, Emamhadi MR, Yousefzadeh-Chabok S, et al. Maternal SSRI exposure increases the risk of autistic offspring: A meta-analysis and systematic review. *Eur Psychiatry.* 2017;45:161-166. doi:10.1016/j.eurpsy.2017.06.001
4. Karimi P, Kamali E, Mousavi SM, Karahmadi M. Environmental factors influencing the risk of autism. *Journal of research in medical sciences : the official journal of Isfahan University of Medical Sciences.* 2017;22:27-27. doi:10.4103/1735-1995.200272
5. Bird A. Perceptions of epigenetics. *Nature.* May 24 2007;447(7143):396-8. doi:10.1038/nature05913
6. Boyce WT, Kobor MS. Development and the epigenome: the 'synapse' of gene-environment interplay. *Dev Sci.* Jan 2015;18(1):1-23. doi:10.1111/desc.12282
7. Aristizabal MJ, Anreiter I, Halldorsdottir T, et al. Biological embedding of experience: A primer on epigenetics. *Proceedings of the National Academy of Sciences.* 2020;117(38):23261. doi:10.1073/pnas.1820838116
8. Bertrand J, Floyd L, Weber MK. Fetal alcohol syndrome. Guidelines for referral and diagnosis. 2005;
9. Weinberg J, Sliwowska JH, Lan N, Hellemans KGC. Prenatal alcohol exposure: Foetal programming, the hypothalamic-pituitary-adrenal axis and sex differences in outcome. *J Neuroendocrinol* 2008. p. 470-488.
10. McLachlan K, Rasmussen C, Oberlander TF, et al. Dysregulation of the cortisol diurnal rhythm following prenatal alcohol exposure and early life adversity. *Alcohol* 2016. p. 9-18.
11. Kable JA, Mehta PK, Coles CD. Alterations in Insulin Levels in Adults with Prenatal Alcohol Exposure. <https://doi.org/10.1111/acer.14559>. *Alcoholism: Clinical and Experimental Research.* 2021/03/01 2021;45(3):500-506. doi:<https://doi.org/10.1111/acer.14559>
12. Hellemans KG, Sliwowska JH, Verma P, Weinberg J. Prenatal alcohol exposure: fetal programming and later life vulnerability to stress, depression and anxiety disorders. *Neurosci Biobehav Rev.* May 2010;34(6):791-807. doi:10.1016/j.neubiorev.2009.06.004
13. Bodnar TS, Weinberg J. Prenatal Alcohol Exposure : Impact on Neuroendocrine – Neuroimmune Networks. 2013. p. 307-357.
14. Glover V. Maternal depression, anxiety and stress during pregnancy and child outcome; what needs to be done. *Best Pract Res Clin Obstet Gynaecol.* Jan 2014;28(1):25-35. doi:10.1016/j.bpobgyn.2013.08.017
15. Van den Bergh BRH, van den Heuvel MI, Lahti M, et al. Prenatal developmental origins of behavior and mental health: The influence of maternal stress in pregnancy. *Neurosci Biobehav Rev.* Oct 2020;117:26-64. doi:10.1016/j.neubiorev.2017.07.003
16. Ruggiero MJ, Boschen KE, Roth TL, Klintsova AY. Sex Differences in Early Postnatal Microglial Colonization of the Developing Rat Hippocampus Following a Single-Day Alcohol Exposure. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology.* 2018;13(2):189-203. doi:10.1007/s11481-017-9774-1
17. Uban KA, Comeau WL, Ellis LA, Galea LA, Weinberg J. Basal regulation of HPA and dopamine systems is altered differentially in males and females by prenatal alcohol exposure and chronic variable stress. *Psychoneuroendocrinology* 2013.

18. Lee S, Rivier C. Gender differences in the effect of prenatal alcohol exposure on the hypothalamic-pituitary-adrenal axis response to immune signals. *Psychoneuroendocrinology* 1996; p. 145-155.
19. Caldwell KK, Sheema S, Paz RD, et al. Fetal alcohol spectrum disorder-associated depression: evidence for reductions in the levels of brain-derived neurotrophic factor in a mouse model. *Pharmacol Biochem Behav* 2008; p. 614-624.
20. Raineiki C, Hellemans KGC, Bodnar T, et al. Neurocircuitry underlying stress and emotional regulation in animals prenatally exposed to alcohol and subjected to chronic mild stress in adulthood. *Front Endocrinol (Lausanne)* 2014; p. 1-14.
21. Lam VYY, Raineiki C, Ellis L, Yu W, Weinberg J. Interactive effects of prenatal alcohol exposure and chronic stress in adulthood on anxiety-like behavior and central stress-related receptor mRNA expression: Sex- and time-dependent effects. *Psychoneuroendocrinology*. Nov 2018;97:8-19. doi:10.1016/j.psyneuen.2018.06.018
22. Thanh NX, Jonsson E, Salmon A, Sebastianski M. Incidence and prevalence of fetal alcohol spectrum disorder by sex and age group in Alberta, Canada. *J Popul Ther Clin Pharmacol*. 2014;21(3):e395-404.
23. Woods KJ, Thomas KGF, Molteno CD, Jacobson JL, Jacobson SW, Meintjes EM. Prenatal alcohol exposure affects brain function during place learning in a virtual environment differently in boys and girls. *Brain Behav*. Nov 2018;8(11):e01103. doi:10.1002/brb3.1103
24. Tesche CD, Kodituwakku PW, Garcia CM, Houck JM. Sex-related differences in auditory processing in adolescents with fetal alcohol spectrum disorder: A magnetoencephalographic study. *Neuroimage Clin*. 2015;7:571-87. doi:10.1016/j.nicl.2014.12.007
25. Uban KA, Herting MM, Wozniak JR, Sowell ER. Sex differences in associations between white matter microstructure and gonadal hormones in children and adolescents with prenatal alcohol exposure. *Psychoneuroendocrinology*. Sep 2017;83:111-121. doi:10.1016/j.psyneuen.2017.05.019
26. Sayal K, Heron J, Golding J, Emond A. Prenatal alcohol exposure and gender differences in childhood mental health problems: a longitudinal population-based study. *Pediatrics*. Feb 2007;119(2):e426-34. doi:10.1542/peds.2006-1840
27. Lussier AA, Weinberg J, Kobor MS. Epigenetics studies of fetal alcohol spectrum disorder: where are we now? *Epigenomics*. Mar 2017;9(3):291-311.
28. Petrelli B, Weinberg J, Hicks GG. Effects of prenatal alcohol exposure (PAE): insights into FASD using mouse models of PAE. Biochemistry and cell biology = Biochimie et biologie cellulaire. 2018/01/25 ed2018. p. 131-147.
29. Schaffner SL, Lussier AA, Baker JA, Goldowitz D, Hamre KM, Kobor MS. Neonatal Alcohol Exposure in Mice Induces Select Differentiation- and Apoptosis-Related Chromatin Changes Both Independent of and Dependent on Sex. *Front Genet*: Frontiers Media S.A.; 2020. p. 35.
30. Ngai YF, Sulistyoningrum DC, O'Neill R, Innis SM, Weinberg J, Devlin AM. Prenatal alcohol exposure alters methyl metabolism and programs serotonin transporter and glucocorticoid receptor expression in brain. *American journal of physiology Regulatory, integrative and comparative physiology* 2015; p. R613-22.
31. Glavas MM, Ellis L, Yu WK, Weinberg J. Effects of prenatal ethanol exposure on basal limbic-hypothalamic-pituitary-adrenal regulation: role of corticosterone. *Alcoholism: clinical and experimental research* 2007; p. 1598-1610.
32. Lan N, Chiu MPY, Ellis L, Weinberg J. Prenatal alcohol exposure and prenatal stress differentially alter glucocorticoid signaling in the placenta and fetal brain. *Neuroscience* 2017. p. 167-179.

33. Sliwowska JH, Comeau WL, Bodnar TS, Ellis L, Weinberg J. Prenatal Alcohol Exposure and Pair Feeding Differentially Impact Puberty and Reproductive Development in Female Rats: Role of the Kisspeptin System. *Alcohol Clin Exp Res*. 2016;40(11):2368-2376. doi:10.1111/acer.13233
34. Gawałek M, Sliwowska JH. Neuronal basis of reproductive dysfunctions associated with diet and alcohol: From the womb to adulthood. *Reprod Biol*. Jun 2015;15(2):69-78. doi:10.1016/j.repbio.2015.04.001
35. Bodnar TS, Hill LA, Weinberg J. Evidence for an immune signature of prenatal alcohol exposure in female rats. *Brain Behav Immun*. Nov 2016;58:130-141. doi:10.1016/j.bbi.2016.05.022
36. Lumey LH, Stein AD, Kahn HS, Romijn JA. Lipid profiles in middle-aged men and women after famine exposure during gestation: the Dutch Hunger Winter Families Study. *Am J Clin Nutr*. Jun 2009;89(6):1737-43. doi:10.3945/ajcn.2008.27038
37. Dearden L, Bouret SG, Ozanne SE. Sex and gender differences in developmental programming of metabolism. *Molecular Metabolism*. 2018/09/01/ 2018;15:8-19. doi:<https://doi.org/10.1016/j.molmet.2018.04.007>
38. de Rooij SR, Caan MWA, Swaab DF, et al. Prenatal famine exposure has sex-specific effects on brain size. *Brain* 2016. p. 2136-2142.
39. Tobi EW, Goeman JJ, Monajemi R, et al. DNA methylation signatures link prenatal famine exposure to growth and metabolism. *Nature Communications*. 2014/11/26 2014;5(1):5592. doi:10.1038/ncomms6592
40. Heijmans BT, Tobi EW, Stein AD, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A* 2008. p. 17046-9.
41. Tobi EW, Lumey LH, Talens RP, et al. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. *Hum Mol Genet*. 2009/08/04 ed: Oxford University Press; 2009. p. 4046-4053.
42. Zhang Y, Liu T, Meyer Ca, et al. Model-based analysis of ChIP-Seq (MACS). *Genome Biol* 2008. p. R137.
43. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B (Methodological)*. 1995 1995;57(1):289 - 300. doi:10.2307/2346101
44. Gillis J, Mistry M, Pavlidis P. Gene function analysis in complex data sets using ErmineJ. *Nat Protoc*. Department of Psychiatry, University of British Columbia, Vancouver, British Columbia, Canada. 2010. p. 1148-1159.
45. Lee HK, Braynen W, Keshav K, Pavlidis P. ErmineJ: tool for functional analysis of gene expression data sets. *BMC Bioinformatics* 2005. p. 269.
46. Lussier AA, Morin AM, MacIsaac JL, et al. DNA methylation as a predictor of fetal alcohol spectrum disorder. *journal article. Clin Epigenetics*. January 12 2018;10(1):5. doi:10.1186/s13148-018-0439-6
47. Portales-Casamar E, Lussier AA, Jones MJ, et al. DNA methylation signature of human fetal alcohol spectrum disorder. *Epigenetics & Chromatin* 2016. p. 25.
48. Fransquel PD, Hutchinson D, Olsson CA, et al. Perinatal maternal alcohol consumption and methylation of the dopamine receptor DRD4 in the offspring: the Triple B study. *Environmental Epigenetics* 2016. p. dvw023-dvw023.
49. Grove J, Ripke S, Als TD, et al. Identification of common genetic risk variants for autism spectrum disorder. *Nat Genet*. 2019;51(3):431-444. doi:10.1038/s41588-019-0344-8
50. Nardone S, Sams DS, Zito A, Reuveni E, Elliott E. Dysregulation of Cortical Neuron DNA Methylation Profile in Autism Spectrum Disorder. *Cereb Cortex*. 2017;27(12):5739-5754. doi:10.1093/cercor/bhw250

51. Wong CCY, Smith RG, Hannon E, et al. Genome-wide DNA methylation profiling identifies convergent molecular signatures associated with idiopathic and syndromic autism in post-mortem human brain tissue. *Hum Mol Genet*. 2019;28(13):2201-2211. doi:10.1093/hmg/ddz052
52. Ladd-Acosta C, Hansen KD, Briem E, Fallin MD, Kaufmann WE, Feinberg AP. Common DNA methylation alterations in multiple brain regions in autism. *Mol Psychiatry*. 2014;19(8):862-871. doi:10.1038/mp.2013.114
53. Hannon E, Schendel D, Ladd-Acosta C, et al. Elevated polygenic burden for autism is associated with differential DNA methylation at birth. *Genome Med*. 2018/03/28 2018;10(1):19. doi:10.1186/s13073-018-0527-4
54. Andrews SV, Sheppard B, Windham GC, et al. Case-control meta-analysis of blood DNA methylation and autism spectrum disorder. *Molecular Autism*. 2018/06/28 2018;9(1):40. doi:10.1186/s13229-018-0224-6
55. Berko ER, Suzuki M, Beren F, et al. Mosaic Epigenetic Dysregulation of Ectodermal Cells in Autism Spectrum Disorder. *PLOS Genetics*. 2014;10(5):e1004402. doi:10.1371/journal.pgen.1004402
56. Lussier AA, Bodnar TS, Mingay M, et al. Prenatal Alcohol Exposure: Profiling Developmental DNA Methylation Patterns in Central and Peripheral Tissues. *Front Genet*. 2018;9:610-610. doi:10.3389/fgene.2018.00610
57. Otero NKH, Thomas JD, Saski CA, Xia X, Kelly SJ. Choline Supplementation and DNA Methylation in the Hippocampus and Prefrontal Cortex of Rats Exposed to Alcohol During Development. *Alcoholism: Clinical and Experimental Research* 2012. p. 1701-1709.
58. Wozniak JR, Fink BA, Fuglestad AJ, et al. Four-year follow-up of a randomized controlled trial of choline for neurodevelopment in fetal alcohol spectrum disorder. *J Neurodev Disord*. 2020/03/12 2020;12(1):9. doi:10.1186/s11689-020-09312-7
59. Wozniak JR, Fuglestad AJ, Eckerle JK, et al. Choline supplementation in children with fetal alcohol spectrum disorders: a randomized, double-blind, placebo-controlled trial. *Am J Clin Nutr*. Nov 2015;102(5):1113-25. doi:10.3945/ajcn.114.099168
60. Kumar P, Kumar D, Jha SK, Jha NK, Ambasta RK. Chapter Three - Ion Channels in Neurological Disorders. In: Donev R, ed. *Adv Protein Chem Struct Biol*. Academic Press; 2016:97-136.
61. Noh W, Pak S, Choi G, Yang S, Yang S. Transient Potassium Channels: Therapeutic Targets for Brain Disorders. Review. *Front Cell Neurosci*. 2019-June-13 2019;13(265)doi:10.3389/fncel.2019.00265
62. Popova S, Lange S, Shield K, et al. Comorbidity of fetal alcohol spectrum disorder: a systematic review and meta-analysis. *Lancet*: Elsevier; 2017. p. 1-10.
63. Hirano S, Takeichi M. Cadherins in Brain Morphogenesis and Wiring. *Physiol Rev*. 2012/04/01 2012;92(2):597-634. doi:10.1152/physrev.00014.2011
64. Schmid RS, Anton ES. Role of Integrins in the Development of the Cerebral Cortex. *Cereb Cortex*. 2003;13(3):219-224. doi:10.1093/cercor/13.3.219
65. Mukhtar T, Breda J, Grison A, et al. Tead transcription factors differentially regulate cortical development. *Sci Rep*. 2020;10(1):4625-4625. doi:10.1038/s41598-020-61490-5
66. Wang H, Liu F, Chen W, et al. Genetic recovery of ErbB4 in adulthood partially restores brain functions in null mice. *Proceedings of the National Academy of Sciences*. 2018;115(51):13105. doi:10.1073/pnas.1811287115
67. Lebel C, Mattson SN, Riley EP, et al. A longitudinal study of the long-term consequences of drinking during pregnancy: heavy in utero alcohol exposure disrupts the normal processes of brain development. *J Neurosci*. Oct 31 2012;32(44):15243-51. doi:10.1523/jneurosci.1161-12.2012

68. Treit S, Chen Z, Zhou D, et al. Sexual dimorphism of volume reduction but not cognitive deficit in fetal alcohol spectrum disorders: A combined diffusion tensor imaging, cortical thickness and brain volume study. *Neuroimage Clin.* 2017;15:284-297. doi:10.1016/j.nicl.2017.05.006
69. Gambin T, Yuan B, Bi W, et al. Identification of novel candidate disease genes from de novo exonic copy number variants. *Genome Med.* 2017;9(1):83-83. doi:10.1186/s13073-017-0472-7
70. Weinberg J. Nutritional issues in perinatal alcohol exposure. *Neurobehav Toxicol Teratol* 1984.
71. Burdge GC, Slater-Jeffries J, Torrens C, Phillips ES, Hanson MA, Lillycrop KA. Dietary protein restriction of pregnant rats in the F0 generation induces altered methylation of hepatic gene promoters in the adult male offspring in the F1 and F2 generations. *The British journal of nutrition* 2007. p. 435-439.
72. Brown AS, Susser ES, Lin SP, Neugebauer R, Gorman JM. Increased risk of affective disorders in males after second trimester prenatal exposure to the Dutch hunger winter of 1944-45. *Br J Psychiatry*. May 1995;166(5):601-6. doi:10.1192/bj.p.166.5.601
73. Dienel GA. Brain Glucose Metabolism: Integration of Energetics with Function. *Physiol Rev*. Jan 1 2019;99(1):949-1045. doi:10.1152/physrev.00062.2017
74. Weinberg J. Effects of Ethanol and Maternal Nutritional Status on Fetal Development. *Alcoholism: Clinical and Experimental Research* 1985. p. 49-55.
75. Bodnar TS, Raineki C, Wertelecki W, et al. Altered maternal immune networks are associated with adverse child neurodevelopment: Impact of alcohol consumption during pregnancy. *Brain Behav Immun*. May 5 2018;doi:10.1016/j.bbi.2018.05.004
76. Kinsella MT, Monk C. Impact of maternal stress, depression and anxiety on fetal neurobehavioral development. *Clin Obstet Gynecol*. 2009;52(3):425-440. doi:10.1097/GRF.0b013e3181b52df1
77. Cao-Lei L, Laplante DP, King S. Prenatal Maternal Stress and Epigenetics: Review of the Human Research. *Current Molecular Biology Reports*. 2016/03/01 2016;2(1):16-25. doi:10.1007/s40610-016-0030-x
78. Moon AL, Haan N, Wilkinson LS, Thomas KL, Hall J. CACNA1C: Association With Psychiatric Disorders, Behavior, and Neurogenesis. *Schizophr Bull*. 2018;44(5):958-965. doi:10.1093/schbul/sby096
79. Dedic N, Pöhlmann ML, Richter JS, et al. Cross-disorder risk gene CACNA1C differentially modulates susceptibility to psychiatric disorders during development and adulthood. *Mol Psychiatry*. Mar 2018;23(3):533-543. doi:10.1038/mp.2017.133
80. Xiao X, Zheng F, Chang H, et al. The Gene Encoding Protocadherin 9 (PCDH9), a Novel Risk Factor for Major Depressive Disorder. *Neuropsychopharmacology*. 2018/04/01 2018;43(5):1128-1137. doi:10.1038/npp.2017.241
81. Bishop S, Gahagan S, Lord C. Re-examining the core features of autism: a comparison of autism spectrum disorder and fetal alcohol spectrum disorder. *J Child Psychol Psychiatry*. Nov 2007;48(11):1111-21. doi:10.1111/j.1469-7610.2007.01782.x
82. Stevens SA, Nash K, Koren G, Rovet J. Autism characteristics in children with fetal alcohol spectrum disorders. *Child Neuropsychol*. 2013;19(6):579-87. doi:10.1080/09297049.2012.727791
83. Harris SR, MacKay LL, Osborn JA. Autistic behaviors in offspring of mothers abusing alcohol and other drugs: a series of case reports. *Alcohol Clin Exp Res*. Jun 1995;19(3):660-5. doi:10.1111/j.1530-0277.1995.tb01564.x
84. Lange S, Rehm J, Anagnostou E, Popova S. Prevalence of externalizing disorders and Autism Spectrum Disorders among children with Fetal Alcohol Spectrum Disorder: systematic review and meta-analysis. *Biochem Cell Biol*. Apr 2018;96(2):241-251. doi:10.1139/bcb-2017-0014
85. Nanson JL. Autism in fetal alcohol syndrome: a report of six cases. *Alcohol Clin Exp Res*. Jun 1992;16(3):558-65. doi:10.1111/j.1530-0277.1992.tb01417.x

86. Mukherjee R, Layton M, Yacoub E, Turk J. Autism and autistic traits in people exposed to heavy prenatal alcohol: data from a clinical series of 21 individuals and nested case control study. *Advances in Mental Health and Intellectual Disabilities*. 2011;5(1):42-49. doi:10.5042/amhid.2011.0015
87. Singh K, Jayaram M, Kaare M, et al. Neural cell adhesion molecule Negr1 deficiency in mouse results in structural brain endophenotypes and behavioral deviations related to psychiatric disorders. *Sci Rep*. 2019/04/01 2019;9(1):5457. doi:10.1038/s41598-019-41991-8

**TABLES**
**Table 1. Overlap of genes linked to autism spectrum disorder with differentially methylated regions**

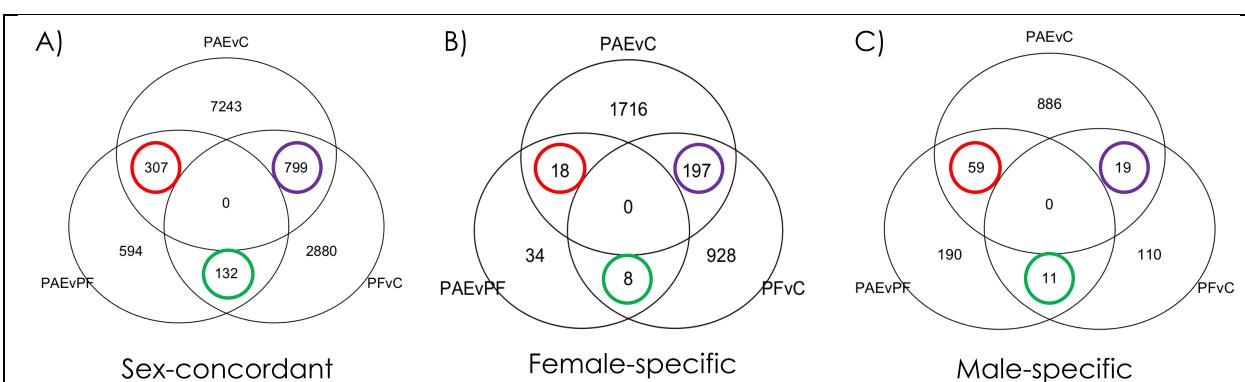
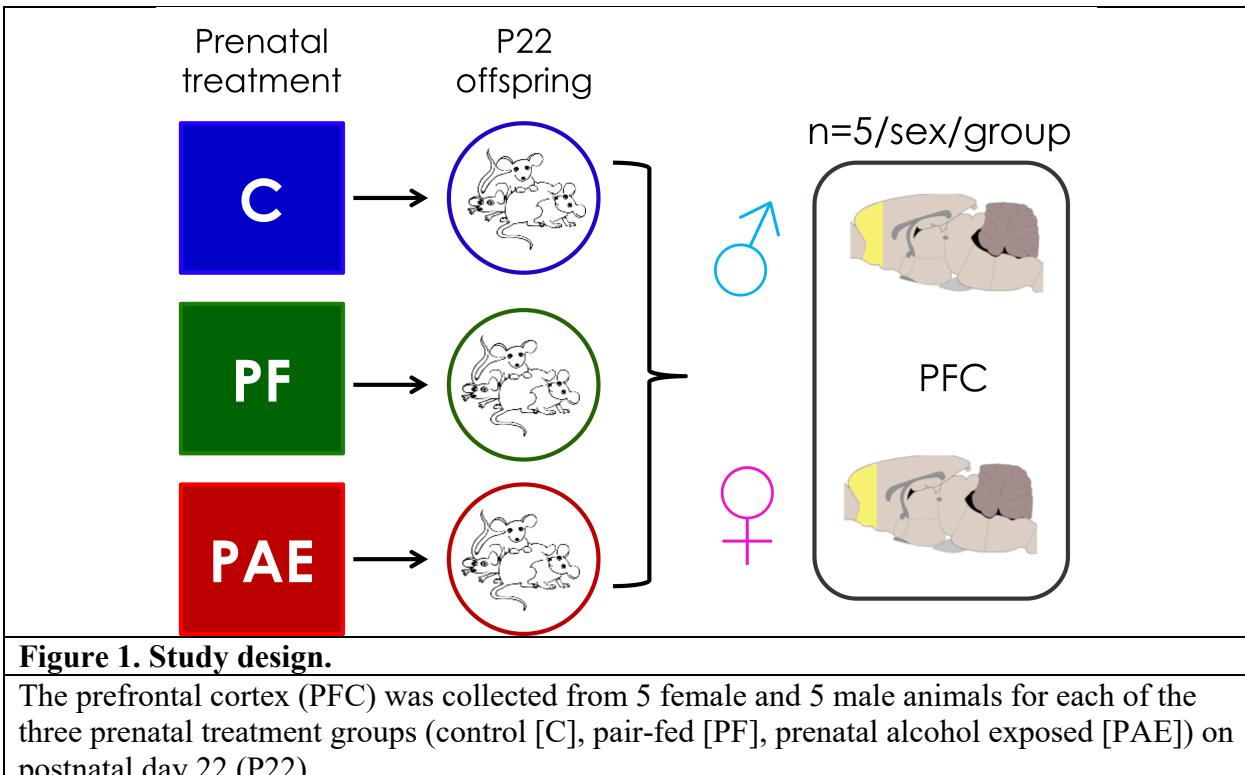
Study	Sample	Age at collection	Tissue	Overlapping genes		
				PAE	PF	Shared
<i>Genome-wide association study (GWAS)</i>						
Grove 2019	18,381 ASD cases 27,969 controls			NEGR1*		MMS22L
<i>Epigenome-wide association studies (EWAS)</i>						
Andrews 2019	796 ASD cases 858 controls	4-18 years	Blood			
Berko 2014	47 ASD cases 48 controls	2-17 years	Buccal epithelial cells			NRG2 <sup>+</sup>
Hannon 2018	629 ASD cases 634 controls	Birth	Neonatal blood spots			
Ladd Acosta 2014	19 autism cases 21 controls	2-51 years	Prefrontal cortex; Temporal cortex; Cerebellum			
Nardone 2017	16 male ASD cases 15 male controls	17-68 years	Frontal cortex			NEDD4L
Wong 2019	36 ASD cases 33 controls	29.3 years (±28.2)	Prefrontal cortex	CDH13 <sup>+</sup>	PRKAR1B	
	33 ASD cases 38 controls	29.1 years (±18.6)	Temporal cortex			
	34 ASD cases 29 controls	30.6 years (±21.2)	Cerebellum			
	30 ASD cases 29 controls	29 .0 years (±18.9)	Prefrontal and temporal cortex, analyzed together		GRIK1	FRMD4A

ASD = autism spectrum disorder; PAE = prenatal alcohol exposed; PF = pair-fed (exposed to food-related stress); shared = DMRs shared between PAE and PF compared to controls.

\*NEGR1 was one of four genes replicated in an independent sample by Grove et al. (2019) and is one of the strongest loci for ASD.

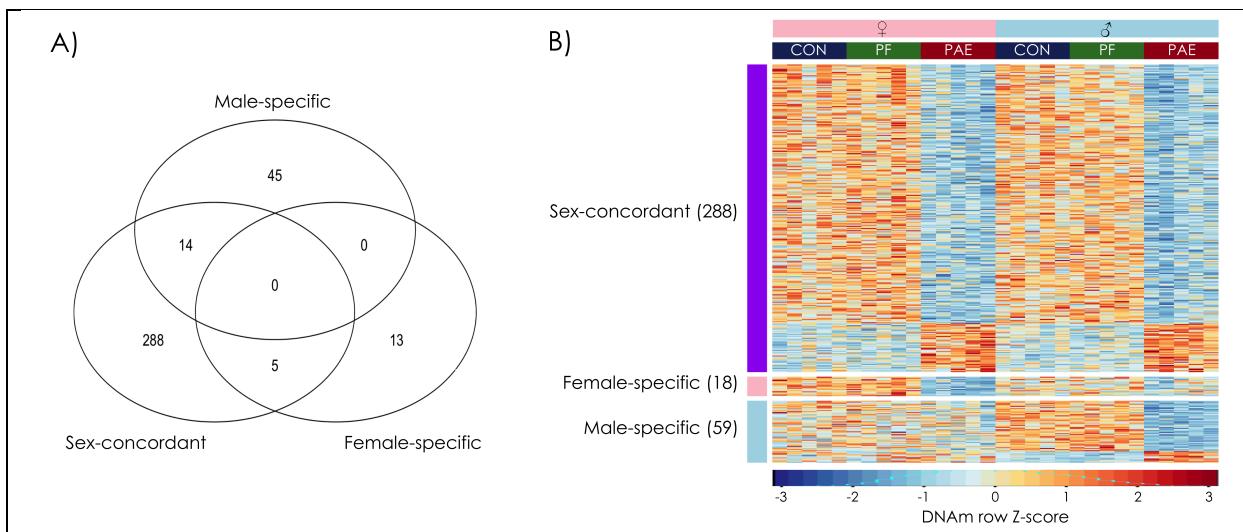
<sup>+</sup> All overlapping genes were linked to sex-concordant differentially methylated regions (DMRs), with the exception of CDH13, which had both sex-concordant and male-specific DMRs in response to PAE, and NRG2, which had a female-specific DMR.

## FIGURES



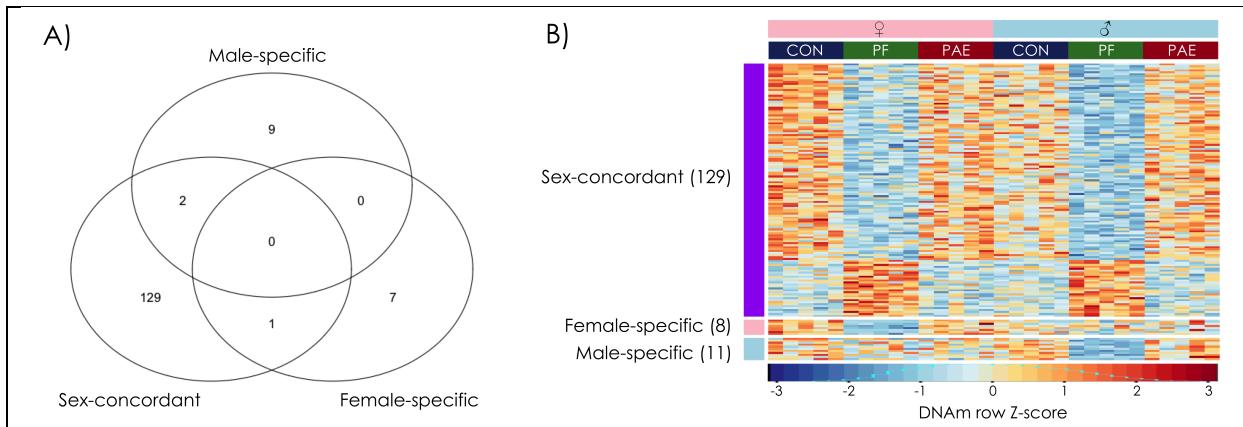
**Figure 2. Identification of PAE and PF-specific hits across analyses at a false-discovery rate <0.05.**

**A**) Analysis of sex concordant effects (DNA $\sim$  group + sex) revealed 307 PAE-specific DMRs (red), 132 PF-specific DMRs (green), and 799 DMRs shared between PAE and PF (purple). **B**) Analysis of female-specific DMRs (DNA  $\sim$  group; females only) revealed 18 PAE-specific, 8 PF-specific DMRs, and 197 DMRs shared between PAE and PF (purple). **C**) Analysis of male-specific DMRs (DNA  $\sim$  group; males only) revealed 59 PAE-specific, 11 PF-specific DMRs, and 19 DMRs shared between PAE and PF (purple). Diagram circles represent the three contrasts performed for each analysis.



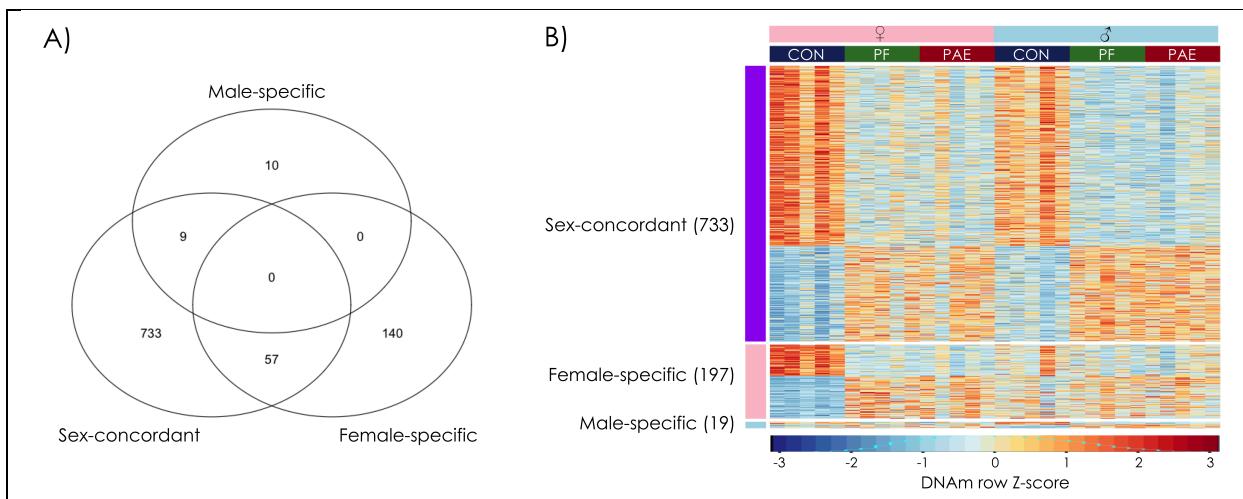
**Figure 3. PAE-specific differentially methylated regions.**

**A)** Venn diagram showing the overlap among the three sets of PAE-specific DMRs at a false-discovery rate <0.05. 307 DMRs were identified in the analysis of both sexes together, with 5 driven primarily by females and 14 primarily by males. As such, 18 DMRs were categorized as female-specific and 59 as male-specific. **B)** Heatmap of the DMRs, where each row is a DMR, scaled to Z-score of DNAm, and each column is a different animal. Most DMRs showed a decrease in the PAE (red) compared to the CON (blue) and PF (green) animals.



**Figure 4. PF-specific differentially methylated regions.**

**A)** Venn diagram showing the overlap among the three sets of PF-specific DMRs. 132 DMRs were identified in the analysis of both sexes together, with 1 driven primarily by females and 2 primarily by males. As such, 8 DMRs were categorized as female-specific and 11 as male-specific. **B)** Heatmap of the DMRs, where each row is a DMR, scaled to Z-score of DNAm, and each column is a different animal. Most DMRs showed a decrease in the PF (green) compared to the CON (blue) and PAE (red) animals.



**Figure 5. Differentially methylated regions shared between PAE and PF.**

**A)** Venn diagram showing the overlap among the three sets of differentially methylated regions (DMRs) shared between PAE and PF animals. 799 DMRs were identified in the analysis of both sexes together, with 57 driven primarily by females and 9 driven primarily by males. As such, 197 DMRs were categorized as female-specific and 19 were categorized as male-specific. **B)** Heatmap of the DMRs, where each row is a DMRs, scaled to Z-score of DNAm, and each column is a different animal. Most DMRs showed a decrease in the PF (green) and PAE (red) compared to the CON (blue) animals.