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Maternal upbringing and selective breeding for voluntary exercise behavior modify patterns of DNA methylation and expression of genes in the mouse brain

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1. Abstract

Selective breeding has been utilized to study the genetic basis of exercise behavior, but research suggests that epigenetic mechanisms, such as DNA methylation, also contribute to this behavior. In a previous study, we demonstrated that the brains of mice from a genetically selected high runner (HR) line have sex-specific changes in DNA methylation patterns in genes known to be genomically imprinted compared to those from a non-selected control (C) line. Through cross-fostering, we also found that maternal upbringing can modify the DNA methylation profiles of additional genes. Here, we identify an additional set of genes in which DNA methylation patterns and gene expression may be altered by selection for increased wheel-running activity and maternal upbringing. We performed bisulfite sequencing and gene expression assays of 14 genes in the brain and found alterations in DNA methylation and gene expression for *Bdnf*, *Pde4d*, and *Grin2b*. Decreases in *Bdnf* methylation correlated with significant increases in *Bdnf* gene expression in the hippocampus of HR compared to C mice. Cross-fostering also influenced the DNA methylation patterns for *Pde4d* in the cortex and *Grin2b* in the hippocampus, with associated changes in gene expression. We also found that the DNA methylation profiles for *Atrx* and *Oxtr* in the cortex and *Atrx* and *Bdnf* in the hippocampus were further modified by sex. Together with our previous study, these results suggest that DNA methylation and the resulting change in gene expression may interact with early-life influences to shape adult exercise behavior.

Keywords: bisulfite sequencing; DNA methylation; cross-fostering; brain; *Bdnf*; *Grin2b*; *Pde4d*; cyclic AMP response element-binding protein; exercise; wheel running; genetic selection.

2. Introduction

Selective breeding has been a powerful approach to studying the impact of genes on behavior (Wehner *et al.* 2001, Hill and Bünger 2004, Rhodes and Kawecki 2009, Hillis and Garland 2023). For example, previous studies have used selective breeding to enhance voluntary wheel-running behavior in mice (Swallow *et al.* 1998, Rhodes *et al.* 2005, Swallow *et al.* 2009). These studies involved selecting mice with the highest wheel-running distance during days 5 and 6 of a 6-day exposure to wheels as young adults and breeding them over successive generations. The four replicate high runner (HR) lines reached apparent selection limits between generations 17 and 27 (Careau *et al.* 2013), and HR mice from all four lines continue to run approximately 3-fold more revolutions per day than those from four non-selected control (C) lines through ~70 additional generations of selection (e.g., see Malisch *et al.* 2009, Garland *et al.* 2011, Acosta *et al.* 2015, Claghorn *et al.* 2016, Thompson *et al.* 2017, Cadney *et al.* 2021a).

As would be expected, this large difference in daily voluntary exercise is accompanied by evolved differences in both exercise abilities and motivation for (or reward received from) wheel running. For example, HR mice lines have improved capacity for aerobically supported exercise, as revealed by increased endurance (Meek *et al.* 2009) and maximal oxygen consumption (Cadney *et al.* 2021a and references therein) during forced treadmill exercise.

The neurobiological mechanisms contributing to the high activity levels of HR mice have received considerable attention but have yet to be fully elucidated. Recently, whole-genome sequencing (Hillis *et al.* 2020) and RNA sequencing (Saul *et al.* 2017, Zhang *et al.* 2018b) studies have revealed loci where allele frequencies have diverged between the HR and C lines, as well as differential expression of genes in key brain regions (Bronikowski *et al.* 2004). Differential expression has been identified for genes involved in motivation for exercise, reward-dependent processes, motor coordination, and various neurotransmitter signaling networks, including dopamine, serotonin, glutamate, and γ-aminobutyric acid (Kelly *et al.* 2012, Saul *et al.* 2017, Zhang *et al.* 2018b, Hillis *et*

al. 2020). The HR lines have also diverged in allele frequencies from C mice at loci that relate to the vomeronasal organ, a component of the olfactory system that activates neural circuits associated with motivation (Nguyen *et al.* 2020). In another study, mice were given wheel access for 6 days, but on day 7, wheel access was blocked such that half of the mice were prevented from running (Rhodes *et al.* 2003a). In mice that had continued wheel access, there was a strong positive correlation between running distance and expression of the immediate early gene c-Fos in the hippocampal dentate gyrus of C mice, but not in HR mice. In mice that were prevented from running, HR mice had increased c-Fos expression in brain regions implicated in arousal (lateral hypothalamus), natural reward (medial prefrontal cortex), and locomotion (caudate putamen), as compared with C mice. These data provide additional evidence of divergent genetic changes in the brains of the HR and C lines of mice.

Although previous studies of the HR lines of mice and other animal models clearly demonstrate a genetic basis for variation in physical activity, it is widely recognized that genetics cannot fully account for inter-individual variation in wheel-running behavior in laboratory rodents (Kelly *et al.* 2012, Kelly and Pomp 2013, Kostrzewa and Kas 2014) or physical activity in humans (Lightfoot *et al.* 2018, Wang *et al.* 2022). Environmental factors can significantly impact developmental processes (Waterland and Garza 1999, Garland *et al.* 2017), which in turn affect epigenetic modifications (Waterland and Michels 2007). Epigenetic modifications can chemically modify genes expressed in the brain and lead to behavioral changes, especially if they occur during critical periods of development (Waterland and Michels 2007, Suderman *et al.* 2012, Zhang *et al.* 2013, Waterland 2014). DNA methylation is one key epigenetic mechanism and is a likely candidate, as the establishment of DNA methylation marks during development are influenced by environmental factors, and they can be maintained throughout the lifespan, ensuring stability in the developmental programming of physical activity (Jirtle and Skinner 2007).

A well-known example of how DNA methylation in the brain can affect the behavior of parents and their offspring is demonstrated by the work of Meaney, Szyf, and

colleagues, who conducted an experiment using laboratory rats (Weaver *et al.* 2004). They observed that pups that were neglected by their mothers had increased circulating levels of corticosterone as adults, along with higher methylation of the promoter region of the glucocorticoid receptor (GR) gene in the hippocampus. This led to the repression of the gene and downregulation of GR expression, which resulted in diminished resilience to stress and increased anxiety-like behavior in adult rats. Adult rats with higher anxiety had lower levels of licking and grooming towards their own pups, indicating that the behavioral changes are transmitted to the next generation (Meaney and Szyf 2005a, Caldji *et al.* 2011). Notably, there was no biological transmission of DNA methylation and histone acetylation marks from the mother to the offspring. Instead, the behavior of the parent epigenetically altered the expression of a gene in the offspring, leading to similar levels of anxiety-like behaviors. Similarly, the maternal environment provided by HR mothers could present any number of opportunities for epigenetic alteration, either by behavioral reinforcement (as in the aforementioned GR study) or other means of direct chemical alterations, such as through breastmilk feeding (Pomar *et al.* 2021).

Because HR mice have distinct physiological and biochemical profiles from C mice, such as reduced body and fat mass (Swallow *et al.* 1999, 2001, Hiramatsu and Garland 2018), altered skeletal muscle size, fiber type composition, and enzyme activities (Houle-Leroy *et al.* 2000, Garland *et al.* 2002, Guderley *et al.* 2006, Bilodeau *et al.* 2009), increased plasma corticosterone concentrations (Girard and Garland 2002, Malisch *et al.* 2007, Malisch *et al.* 2009), changes in other hormones levels (Garland *et al.* 2016), and changes in monoamine neurotransmitters including dopamine (Rhodes *et al.* 2001, Rhodes and Garland 2003, Dishman *et al.* 2006), norepinephrine (Greenwood *et al.* 2005, Dishman *et al.* 2006, Waters *et al.* 2013), serotonin (5-hydroxytryptamine; Greenwood *et al.* 2003, Greenwood *et al.* 2005, Claghorn *et al.* 2016, Saul *et al.* 2017), and the endocannabinoid system (Keeney *et al.* 2008, Keeney *et al.* 2012, Thompson *et al.* 2017), we speculated that differences in the maternal environment might contribute to the differences in wheel-running behavior between adult HR and C mice. Supporting this possibility, we have recently shown that exercise behavior in the HR and C lines is

influenced by the early-life environment. For example, both HR and C mice given wheel access during their juvenile period, followed by a washout period of 52 days, demonstrated an increase in adult wheel running (Acosta *et al.* 2015). Other studies found that HR and C mice respond differentially to maternal or juvenile diets high in fat and simple sugars that are characteristic of "Western" diets (Hiramatsu *et al.* 2017, Cadney *et al.* 2021a, McNamara *et al.* 2021). Moreover, differences in body mass between C and HR dams have been previously documented, although these differences do not always exist (Keeney 2011, Hiramatsu *et al.* 2017, Cadney *et al.* 2021b). Thus, HR and C mice could also respond differently to mothers with different body masses and compositions, which has been demonstrated to influence offspring development and DNA methylation patterns (Godfrey *et al.* 2015, Sharp *et al.* 2015, Godfrey *et al.* 2017, Hieronimus and Ensenauer 2021). Taken together, these studies provide evidence that the early-life environment can interact with genetic makeup to influence adult activity levels.

Motivation for physical activity is a complex trait, influenced by a variety of factors, including genetics and the early-life environment (Rowland 2016, Garland *et al.* 2017, Lightfoot *et al.* 2018, Dohnalova *et al.* 2022, Stults-Kolehmainen 2023). The goal of the present study was to quantify the DNA methylation profiles in the brains of HR mice, from a line bred for high wheel-running behavior, as compared with those from a non-selected C line. Using a full factorial cross-fostering paradigm, we have previously shown that the DNA methylation profiles for genes known to be genomically imprinted are altered in HR mice as compared with C, with additional imprinted genes also modified by maternal upbringing (Latchney *et al.* 2022). The paternally imprinted genes were particularly impacted, as HR mice had differential methylation patterns for *Rasgrf1* in the cortex and *Zdbf2* in the hippocampus compared with C mice. The methylation patterns of additional paternally imprinted genes, including *Peg1*, *Peg3*, *Igf2*, *Snrpn*, and *Impact,* were also modified with maternal upbringing (Latchney *et al.* 2022). Building on these findings, the current study investigates methylation changes in genes with known roles in nervous system development, epigenetic regulation, and stress physiology. Using bisulfite sequencing, our study reveals that both selective breeding for increased

wheel-running activity and alterations in maternal upbringing result in brain region-specific and sex-specific differential methylation patterns and gene expression changes. Our results provide valuable insights into how genetic and epigenetic factors may interact to influence inter-individual variations in physical activity levels.

3. Methods and Methods

3.1. Experimental Mice

The selective breeding experiment began in 1993 from outbred Hsd:ICR mice and has been described previously (Swallow *et al.* 1998, Careau *et al.* 2013). Within-family selection is based on average wheel revolutions run per day on days five and six of a six-day period of wheel access as young adults, and the experiment has resulted in four replicate high runner (HR) lines. Four additional lines are bred without regard to running as Control (C) lines. Mice are housed four per cage by sex except for breeding and wheel testing. Standard mouse chow (Teklad Rodent Diet W-8604) and tap water are provided ad libitum. Pregnant dams are given a breeder diet (Teklad S-2235 Mouse Breeder Sterilizable Diet 7004) through weaning. All experiments were approved by the University of California, Riverside IACUC.

A subset of virgin males and females from generation 89 was used to produce experimental mice of generation 90 (Latchney *et al.* 2022). One C line (Line 4) and one HR line (Line 7) were used because they represented extremes in body mass among their respective lines (Table 2 in Cadney *et al.* 2021b and reproduced in Supplemental Table 1). As described, it was hypothesized that differences in dam body size would lead to differential cross-fostering effects, including on wheel-running behavior of the pups. Thus, using lines with different body masses could serve as a positive control for offspring body masses at weaning. The wheel-running behavior of these lines was representative of their respective lines (Cadney *et al.* 2021b). The dams were typical of other line 4 and 7 breeders of the same generation in terms of both wheel-running and body mass (Cadney *et al.* 2021b). None of the dams had access to running wheels during pregnancy and lactation.

3.2. Experimental Design

Details of the experimental design and characteristics of the treatment groups and breeders used have been previously published (see Tables 1 and 2 in Cadney *et al.*

2021b, Latchney *et al.* 2022). Within 24 hours of birth, entire litters were fostered to another dam (no pup was returned to its biological mother) and litters were equalized to eight pups. Because sex could not be determined at birth, the litter sex ratio could not be controlled. Fostering only occurred between litters born within 24 hours of one another. During the 48 hours after fostering, all pups were checked regularly, and none were rejected by their foster mother. This factorial experimental design produced four groups (see Figure 1 in Latchney *et al.* 2022): C offspring raised by C dams (CC), C offspring raised by HR dams (CHR), HR offspring raised by C dams (HRC), and HR offspring raised by HR dams (HRHR). The number of male and female mice from each group used in this study is listed in Table 1.

Note that this design does not include a "control" group for the effects of fostering *per se*. This design was chosen to maximize the sample size in experimental groups sufficient to address specific hypotheses (i.e., the effects of cross-fostering HR and C mice), given logistical constraints on the total sample size. In particular, we wanted to determine whether rearing by an HR dam might be necessary for some proportion of the DNA methylation profiles observed in adult HR mice.

3.3 Brain Dissections

At eight weeks of age and one day after being removed from wheel access, mice were sacrificed via decapitation without anesthesia. The decision to avoid anesthesia was made to mitigate potential confounding effects on DNA methylation profiles and gene expression, as has been done in previous studies in our laboratory (Bronikowski *et al.* 2004, Saul *et al.* 2017, Zhang *et al.* 2018b) and others (Gomez-Pinilla *et al.* 2011, Intlekofer *et al.* 2013, Weaver *et al.* 2014, Sleiman *et al.* 2016, Lee and Soya 2017). Previous studies have also suggested that the use of anesthetics can damage DNA (Ni *et al.* 2017) and modify epigenetic marks (Dalla Massara *et al.* 2016, Joksimovic *et al.* 2018, Wu and Zhao 2018), gene expression profiles (Chastain-Potts *et al.* 2020), and intracellular signaling networks (Ni *et al.* 2015). These molecular changes can ultimately contribute to cognitive dysfunction (Ni *et al.* 2020). By avoiding anesthesia,

we aimed to minimize any potential interference with DNA methylation and gene expression, thereby preserving the integrity of our study and ensuring reliable data interpretation.

Brain dissections were performed on a pre-chilled stainless-steel surface surrounded by wet ice. With the ventral surface down, the cerebellum was gently removed with curved forceps. A midsagittal cut with a razor blade was then made to separate the cerebral hemispheres. From here, the hindbrain containing the pons and medulla was removed. With the medial side of a hemisphere facing up, the hypothalamus was removed using curved forceps. Following some clearing of lateral ventricular tissue, the hippocampus was rolled out. Lastly, curved forceps were used to separate the cortex from the corpus callosum. By following this protocol, we aimed to ensure accurate and consistent dissection of hippocampal and cortical regions (Meyerhoff *et al.* 2021). The hippocampus and cortex were snap-frozen on dry ice and stored at –80°C until further sequencing steps were performed.

3.4. *In-silico* Assay Design

Sequences for 14 genes were acquired from the Ensembl genome browser and annotated. When the study was first designed, genes were chosen based on previous studies in our HR model showing alterations in BDNF protein levels (Johnson *et al.* 2003, Rhodes *et al.* 2003b) and the glucocorticoid signaling system (Malisch *et al.* 2007, 2009) that would implicate such genes as *Crh*, *Fkbp5*, *Nr3c1*, and *Nr3c2*. However, the gene panel was expanded to include additional genes that have known roles in nervous system development (e.g., *Foxp2*, *Gfra1*, *Grin2b*, *Oxtr*, *Pde4d*, and *Sox1*) and epigenetic regulation (e.g., *Atrx*, *Ezh2*, *Mecp2*). These 14 genes were also part of a preselected panel of genes already designed by EpigenDx. The assay target sequences were re-evaluated against the UCSC mouse GRCm38 genome browser for repeat sequences, including LINE, SINE, LTR elements, and other DNA repeats. Sequences containing repetitive elements, low sequence complexity, high thymidine content, and overall CpG density were excluded from the *in-silico* design process.

Twenty-nine assays were designed to cover 169 CpG sites across 14 genes, and the percentage methylation of each CpG site was determined in each sample. A list of the genes, their respective coordinates, and the number of CpG sites analyzed in this study are provided in Table 2.

3.5. Bisulfite Sequencing and Data Analysis

As in our previous study (Latchney et al. 2022), bisulfite sequencing was performed on 92 samples (46 hippocampus and 46 cortex) by EpigenDx, Inc. (Hopkinton, MA). Tissue samples were digested using 500 µL of ZymoResearch M-digestion Buffer (Zymo, Irvine, CA) and 5-10 µL of protease K (20 mg/mL) with a final M-digestion concentration of 2X. The samples were incubated at 65°C for a minimum of 2 hours. 20 µL of the supernatant from the sample extracts were bisulfite modified using the ZymoResearch EZ-96 DNA Methylation-Direct Kit™ (cat# D5023) kit per the manufacturer's protocol with minor modification. The bisulfite-modified DNA samples were eluted using M-elution buffer in 46 µL.

All bisulfite-modified DNA samples were amplified using separate multiplex or simplex PCRs with Qiagen (Gaithersburg, MD) HotStar Taq. All PCR products were verified and quantified using the QIAxcel Advanced System. Prior to library preparation, PCR products from the same sample were pooled and purified using QIAquick PCR Purification Kit columns (Qiagen). In addition, PCR bias testing was performed using a standard curve of percent methylation controls (0% to 100%) for each CpG site assayed. From each standard curve, an R-squared (RSQ) value was calculated for each CpG site as well as the average RSQ value for each assay that was designed. All RSQ values were reported to be greater than 0.6. A summary of these data is provided in Supplemental Table 2.

Libraries were prepared using a custom Library Preparation method created by EpigenDx. Library molecules were then purified using Agencourt AMPure XP beads (Beckman Coulter) and quantified using the Qiagen QIAxcel Advanced System.

Barcoded samples were then pooled in an equimolar fashion before template preparation and enrichment were performed on the Ion Chef™ system (Thermo Fisher) using Ion 520™ & Ion 530™ ExT Chef reagents. Following this, enriched, template-positive library molecules were sequenced on the Ion S5™ sequencer using an Ion 530™ sequencing chip (Thermo Fisher).

FASTQ files from the Ion Torrent S5 server were aligned to the local reference database using open-source Bismark Bisulfite Read Mapper with the Bowtie2 alignment algorithm (https://www.bioinformatics.babraham.ac.uk/projects/bismark/; Krueger and Andrews 2011). Methylation levels were calculated in Bismark by dividing the number of methylated reads by the total number of reads, considering all CpG sites covered by a minimum of 30 total reads. CpG sites with fewer than 30 reads were excluded from analyses.

3.6. Gene Expression by RT-qPCR

RNA was extracted from cortical or hippocampal tissue using the RNeasy Mini Plus kit (Qiagen) according to the manufacturer's instructions. RNA was quantified using the NanoDrop spectrophotometer and 1 µg of RNA was reverse transcribed to cDNA using Superscript IV reverse transcriptase and random hexamers (Invitrogen). To test that RNA was free of genomic DNA contamination, a control without reverse transcriptase was included. The resulting cDNA was amplified using TaqMan Gene Expression Assays (Life Technologies) and TaqMan Gene Expression PCR Master Mix (Life Technologies) according to the manufacturer's protocol. Amplification was performed with QuantStudio3 in duplicates. TaqMan probes used are listed in Table 3. Transcripts evaluated in cortical samples were: *Crh*, *Ezh2*, *Fkbp5*, *Foxp2*, *Oxtr*, and *Pde4d*. Transcripts evaluated in hippocampal samples included: *Bdnf*, *Ezh2*, and *Grin2b*. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), and measurements were normalized to the average expression of three reference genes, *Gapdh*, *beta-actin*, and *Eif2b1*.

3.7. Statistical Analysis

As in our previous study (Latchney et al. 2022), data were analyzed as mixed models in SAS 9.1.3 (SAS Institute, Cary, NC) Procedure Mixed, with REML estimation and Type III Tests of Fixed Effects. Line (selected line 7 vs. non-selected line 4), foster-line, and sex were fixed effects, while dam ID (n = 28) was a random effect nested within line × foster-line. Separate mixed models were created to analyze the percent methylation levels for CpG sites across the entire genomic region for each gene (Supplemental Table 3) and for those genes that were subsequently analyzed by RT-qPCR (Supplemental Table 4).

In all tables, we present least squares means (L.S. mean) and standard errors (S.E.) for each gene for both brain regions (cortex and hippocampus) and the results of each F-statistic. Supplemental Tables 3 and 4 also present the p-values for the differences in the L.S. means between the in-fostered and cross-fostered groups (i.e., the effect of cross-fostering between the HR and C lines by sex). Supplemental material can also be referenced for main effects, as well as interactions (line × foster-line, line × sex, foster-line × sex, line × foster-line × sex).

In all analyses, outliers were iteratively removed when the standardized residuals exceeded ~3. As was done in our prior publications (Cadney *et al.* 2021b, Latchney *et al.* 2022), statistical significance was judged at $p < 0.05$. Supplemental Tables 3 and 4 include 259 p-values combined. Of these 259 p-values, 33 were nominally significant at $p < 0.05$. If all null hypotheses were true, then one would expect ~13 p-values (0.05×259) to be < 0.05 by chance alone. To compensate for multiple related tests, we used the False Discovery Rate (FDR) procedure as implemented in PROC MULTTEST in SAS version 9.1.3. Based on this procedure, an adjusted critical value of 0.0055 would be appropriate for controlling the false discovery rate at a 10% Type I error rate. P-values < 0.0055 based on FDR are referred to as significant, whereas those with p-value < 0.05 are referred to as nominally significant. Because small changes in DNA methylation that are deemed weakly significant can produce large changes in gene

expression, we report nominally significant p-values in addition to those that passed the FDR procedure. In addition to reporting significant and nominally significant p-values, effect sizes, presented as Hedges' g values, were also calculated for group comparisons (CC vs. HRHR, CC vs. CHR, and HRHR vs. HRC). Hedges' g values greater than ± 0.8 were considered to be a large effect (Lakens 2013, Breton *et al.* 2017, Latchney *et al.* 2022).

4. Results

4.1. Line and foster-line influences on DNA methylation and gene expression in the cortex.

The genetic line (line) did not yield any significant main effects on DNA methylation levels for the 14 genes analyzed in the cortex (Supplemental Table 3). However, maternal upbringing (foster-line) had numerous effects on the percent DNA methylation and gene expression for three genes: *Pde4d*, *Crh*, and *Fkbp5* (Figure 1). We report these data in detail below.

Phosphodiesterase 4D (*Pde4d*) is an enzyme involved in the regulation of intracellular levels of cyclic adenosine monophosphate (cAMP), a critical second messenger molecule in many cell communication pathways. To control the activity of cAMP, phosphodiesterases (PDEs) catalyze the hydrolysis of cAMP to generate 5′ AMP. Cross-fostering had a nominally significant effect on the DNA methylation profile for *Pde4d* (Fline: $F_{1,37} = 4.81$; $p = 0.035$) but significantly interacted with genetic line (Line × Fline: $F_{1,37} = 9.22$; $p = 0.004$; Figure 1A). The least squares mean for C pups raised by HR dams were larger than that of C pups raised by C dams (CC vs. CHR combined-sex p-value = 0.0006; Supplemental Table 3), and this increase was statistically significant for males (CC vs. CHR separate-sex p-value = 0.008; Figure 1A). An analysis of effect size between CC vs. CHR (cross-foster), HRHR vs. HRC (cross-foster), and CC vs. HRHR (in-foster) groups revealed a large effect size with a Hedges' g value of 1.00 for CHR females compared to CC females and 2.31 for CHR males compared to CC males (Figure 1A). Notably, there was no cross-fostering effect between HR pups raised by C or HR dams, indicating differential cross-fostering effects between C and HR lines. Quantification of *Pde4d* gene expression in all fostered mice revealed a nominally significant effect of genetic line (Line: $F_{1,38} = 7.29$; $p = 0.010$) and a nominally significant interaction between genetic line and foster line (Line × Fline: $F_{1,38} = 4.71$; $p = 0.036$; Figure 1B). C mice reared by HR dams had reduced gene expression (CC vs. CHR combined-sex p-value = 0.008), with male CHR exhibiting a greater decrease in *Pde4d* expression than females (Male CC vs. CHR p-value = 0.038; Female CC vs. CHR p-

value = 0.080; Supplemental Table 4). An analysis of effect size revealed a larger effect size for CHR males compared to CC males (Hedges' g value = -1.31) than for CHR females compared to CC females (Hedges' g value = -1.16; Figure 1B). These results are consistent with the expected relationship between increased DNA methylation and decreased gene expression.

The corticotropin-releasing hormone (*Crh*) gene encodes a member of the corticotropin-releasing factor family. In response to stress, CRH is secreted by the paraventricular nucleus of the hypothalamus and binds to corticotropin-releasing hormone receptors to stimulate the release of adrenocorticotropic hormone from the pituitary gland (Kageyama *et al.* 2021). Cross-fostering alone had no statistical influence on *Crh* DNA methylation (Fline: $F_{1,38} = 0.05$; $p = 0.831$), but there was a nominally significant line × foster-line interaction (Line × Fline: $F_{1,38} = 6.57$; $p = 0.015$; Figure 1C). Although the combined-sex analysis for the least squares means for C pups raised by HR dams was not significant (Male CC vs. CHR combined-sex p-value = 0.105; Supplemental Table 3), male pups raised by HR dams had a significant increase in *Crh* methylation (Male CC vs. CHR p-value = 0.041; Figure 1C). Additionally, although not statistically significant, there was a trend for increased *Crh* methylation for HR pups raised by C dams compared to HR pups raised by HR dams (HRHR vs. HRC combined-sex p-value = 0.057), and this increase was more pronounced in males (Male HRHR vs. HRC p-value = 0.055; Supplemental Table 3). An analysis of effect size between key groups revealed a large effect size for HRHR females compared to CC females, CHR males compared to CC males, and HRC males compared to HRHR males (Figure 1C), suggesting variable sex-specific effects of cross-fostering on *Crh* methylation levels in HR mice. When *Crh* gene expression was quantified in all fostered groups, there was a significant effect of genetic line (Line: $F_{1,38} = 72.3$; $p < 0.001$; Figure 1D). Combined-sex analysis revealed an increase in *Crh* gene expression in the HRHR group compared to the CC group (CC vs. HRHR combined-sex p-value < 0.001; Figure 1D), with the female HRHR group having a larger increase in *Crh* gene expression than the male HRHR group (Female CC vs HRHR p-value < 0.001; Male CC vs. HRHR p-value = 0.058; Supplemental Table 4). There was also a statistical effect of sex on *Crh* gene

expression (Sex: $F_{1,38} = 15.57$; p-value < 0.001), which interacted with genetic line (Line × Sex: $F_{1,38} = 10.85$; p-value = 0.002; Figure 1D). Compared to female CC mice, female HRHR mice displayed a larger Hedges' g value than male HRHR vs. CC mice, supporting the result that female HRHR mice demonstrated a larger increase in *Crh* gene expression (Figure 1D).

FK506 binding protein 5 is a protein that encodes for the *Fkbp5* gene and functions as a co-chaperone to regulate glucocorticoid receptor activity in response to stress (Zannas *et al.* 2016). Neither genetic line nor cross-fostering significantly modified *Fkbp5* DNA methylation, but the two variables produced a nominally significant interaction (Line × Fline: $F_{1,38} = 4.46$; p = 0.041; Figure 1E). Combined-sex analysis revealed a decrease in the least squares means of the HRHR group compared to the CC group (CC vs. HRHR combined-sex p-value = 0.047; Supplemental Table 3). In addition, the least squares means of C pups raised by HR dams was significantly less than the CC group (CC vs. CHR combined-sex p-value = 0.012), with males showing a greater decrease than females (Male p-value = 0.038; Figure 1E). These genetic line × foster-line interactions produced large effect sizes between the CC and CHR groups and between the CC and HRHR groups for both females and males (Figure 1E). However, the percent DNA methylation between HR mice raised by C vs. HR mothers did not differ, similar to observations made for *Pde4d* (Figure 1A, B), suggesting that C mice may respond to cross-fostering differently than HR mice. When *Fkbp5* gene expression was quantified, there was a nominally significant effect of genetic line (Line: $F_{1,38} = 8.25$; p = 0.007) but a significant effect of foster-line (Fline: $F_{1,38} = 14.01$; p < 0.001) and a significant interaction between genetic line and foster-line (Line × Fline: $F_{1,38} = 14.32$; p < 0.001). The interaction between sex and cross-fostering was also nominally significant (Fline × Sex: $F_{1,38} = 4.99$; p = 0.032; Figure 1F). The least squares means for C pups raised by HR dams were smaller than those raised by C dams (CC vs. CHR combined-sex p-value < 0.001), and this decrease was observed in both male (p-value = 0.0001) and female (p-value = 0.002) pups that were raised by HR dams (Figure 1F). The HRHR group was also smaller than the CC group (CC vs. HRHR p-value < 0.001), particularly in males (p-value < 0.001). An analysis of effect size also revealed large

effect sizes for *Fkbp5* gene expression for all fostered groups (Figure 1F), supporting the complex and multifactorial interaction of genetic line, cross-fostering, and sex on *Fkbp5* gene expression.

4.2. Influence of sex on DNA methylation and gene expression in the cortex.

Although sex differences are not a primary question for this study, sex-specific cross-fostering effects were previously found within this cohort (Cadney *et al.* 2021b, Latchney *et al.* 2022) and have been reported for numerous traits in other studies (Bester-Meredith and Marler 2001, Li *et al.* 2013, Zhu *et al.* 2016). Therefore, we also considered sex-specific cross-fostering effects on DNA methylation only. Within the cortex, a significant main effect of sex was observed for the DNA methylation profiles for *Atrx* (Sex: $F_{1,38} = 1{,}881$; $p < 0.0001$; Figure 2A) and *Oxtr* (Sex: $F_{1,38} = 14.8$; $p < 0.001$; Figure 2B). For these genes, genetic line and cross-fostering had the strongest effects on females. The least squares means for *Atrx* methylation in the female HRHR group was smaller than the CC group (Female CC vs. HRHR p-value = 0.044). Effect sizes were -1.32 for the female HRHR group compared to the female CC group for *Atrx* and -0.96 for the female HRHR group compared to female CC group for *Oxtr*, also demonstrating that cross-fostering reduced DNA methylation levels for these three genes. Sex also had a nominally significant effect on *Nr3c2* methylation (Sex: $F_{1,38} = 8.41$; $p = 0.006$; Figure 2C) and had a nominally significant interaction with foster-line for *Grin2b* (Fline × Sex: $F_{1,37} = 5.16$; $p = 0.029$; Figure 2D), *Mecp2* (Fline × Sex: $F_{1,38} = 3.73$; $p = 0.041$; Figure 2E), and *Gfra1* (Fline × Sex: $F_{1,37} = 6.68$; $p = 0.014$; Figure 2F). Together, these sex-specific effects are consistent with our prior work demonstrating that early-life effects often interact with sex (Cadney *et al.* 2021b, Latchney *et al.* 2022).

4.3. Line and foster-line influences on DNA methylation and gene expression in the hippocampus.

Brain-derived neurotrophic factor (*Bdnf*) plays many roles in promoting neuronal growth, differentiation, survival, and plasticity (Miranda *et al.* 2019). Of the 14 genes analyzed in the hippocampus, only *Bdnf* DNA methylation displayed a nominally significant effect

by genetic line (Line: $F_{1,38} = 4.86$; $p = 0.034$; Figure 3A). Although cross-fostering did not yield any significant main effects, there was a three-way interaction among line, foster-line, and sex that was nominally significant (Line × Fline × Sex: $F_{1,38} = 4.00$; $p = 0.034$). In particular, the male HRHR group exhibited a trend for reduced DNA methylation compared to the male CC group (Male CC vs. HRHR p-value = 0.059; Supplemental Table 3). An analysis of effect size between key groups revealed several large effect sizes. Females had a Hedges' g value of 1.00 for the CHR group compared to the CC group, while males had a value of -1.10 for the CHR group compared to the CC group and -1.23 for the HRHR group compared to the CC group. When *Bdnf* gene expression was quantified, the HR line had a significant increase in gene expression compared to the C line (Line: $F_{1,38} = 72.3$; $p < 0.001$; Figure 3B). There was also a main effect of sex (Sex: $F_{1,38} = 15.57$; $p < 0.001$), which was further modified by genetic line (Line × Sex: $F_{1,38} = 10.85$; $p = 0.002$). Although both female and male HRHR groups had higher expression of *Bdnf* than the CC group (CC vs. HRHR combined-sex p-value < 0.001), separate-sex analysis revealed that the increase was significant in HRHR females (Male CC vs. HRHR p-value = 0.058; Female CC vs. HRHR p-value < 0.001; Supplemental Table 2). Female HRHR mice had a 5-fold increase in *Bdnf* gene expression compared to female CC mice, whereas male HRHR mice had a 2.6-fold increase compared to male CC mice. Additional analysis revealed a larger effect size for HRHR females compared to CC females (4.64) than for HRHR males compared to CC males (1.30; Figure 3B), supporting a greater effect of selective breeding on *Bdnf* gene expression in females.

Grin2b is a gene that encodes the GluN2B subunit of N-methyl-D-aspartate (NMDA) receptors (Endele *et al.* 2010). Within the hippocampus, *Grin2b* was the only gene whose DNA methylation profile had a nominally significant effect of cross-fostering (Fline: $F_{1,38} = 5.88$; $p = 0.020$; Figure 3C). Pups raised by HR dams had reduced DNA methylation compared to those raised by C dams (CC vs. CHR combined-sex p-value = 0.019; Supplemental Table 3). This is reflected by an effect size of -1.27 for the female CHR group compared to female CC. Similar effects are observed in male CHR mice compared to male CC mice (Hedges' g: -1.23), demonstrating that being raised by an

HR dam, regardless of biological sex, produces large effects on *Grin2b* DNA methylation in C mice. In addition, the HRHR group also had decreased *Grin2b* methylation compared to the CC group (CC vs. HRHR combined-sex p-value = 0.016), with male HRHR mice demonstrating the most significant decrease (Male p-value = 0.012). Similar results were observed for *Grin2b* gene expression in that cross-fostering C pups with HR dams increased gene expression (Fline: $F_{1,38} = 29.79$; $p < 0.001$; Figure 3D). Pups raised by HR dams had increased *Grin2b* gene expression compared to those raised by C dams (CC vs. CHR combined-sex p-value < 0.0001), and this was observed in both males and females (Male p-value = 0.0003; Female p-value = 0.009). On the other hand, HR pups raised by C dams had decreased gene expression compared to those raised by HR dams (HRHR vs. HRC combined-sex p-value = 0.005), demonstrating differential effects of cross-fostering between HR and C dams. The female HRC group had the most significant decrease in DNA methylation (Female HRHR vs. HRC p-value = 0.011). This is also reflected by large effect sizes among all cross-fostered groups (Figure 3D), further supporting the robust effects cross-fostering had on *Grin2b* gene regulation.

The Enhancer of Zeste Homolog 2 (*Ezh2*) gene encodes a catalytic subunit of the polycomb-group family that provides instructions for making histone methyltransferases. By methylating histones, particularly trimethylating H3K27, it functions as an epigenetic suppressor to transcriptionally repress gene expression (Buontempo *et al.* 2022). The *Ezh2* gene within the hippocampus was the only gene where genetic line and foster-line produced a nominally significant interaction (Line × Fline: $F_{1,38} = 4.69$; $p = 0.037$; Figure 3E). However, when *Ezh2* gene expression was quantified, there was no significant effect of any individual variable or interaction (Figure 3F).

4.4. Influence of sex on DNA methylation and gene expression in the hippocampus.

In addition to the sex-specific effects observed for *Bdnf* in the hippocampus (Figures 3A and 3B), there was also a significant effect of sex for *Atrx* methylation ($F_{1,38} = 3771$; $p < 0.001$; Figure 4A), whereas *Foxp2* ($F_{1,38} = 8.91$; $p = 0.005$; Figure 4B) and *Oxtr* ($F_{1,38} =$

5.48; p = 0.025; Figure 4C) methylation demonstrated nominally significant effects of sex. Although the combined-sex analysis for the least squares means for the CC vs. HRHR, CC vs. CHR, or HRHR vs. HRC groups were not significant for *Atrx, Foxp2, or Oxtr*, separate-sex analyses of cross-fostering (CC vs. CHR and HRHR vs. HRC) and genetic selection (CC vs. HRHR) yielded significant sex-specific effects in *Atrx* methylation (Figure 4A, Supplemental Table 3).

5. Discussion

Inter-individual variation in physical activity levels in both humans (Lightfoot *et al.* 2018, Wang *et al.* 2022) and rodents (Kostrzewa and Kas 2014, Hillis *et al.* 2020, Hillis and Garland 2023) are widely known to have a significant genetic component. However, a growing body of literature supports the hypothesis that epigenetic mechanisms and maternal care (Francis and Meaney 1999, Liu *et al.* 2000, Weaver *et al.* 2004, Meaney and Szyf 2005a, b, Weaver *et al.* 2006, Caldji *et al.* 2011, Suderman *et al.* 2012, Zhang *et al.* 2013, Garland *et al.* 2017) can also substantially influence early-life behaviors, with lasting effects into adulthood. Our previous work has shown that selective breeding for elevated physical activity across many generations results in significant genetic (Hillis *et al.* 2020, Hillis and Garland 2023) and epigenetic (Latchney *et al.* 2022) contributions to increased wheel-running in the HR lines of mice. In the present study, we expand on our previous work (Latchney *et al.* 2022) by identifying an additional set of neurobiologically relevant genes that exhibited changes in DNA methylation patterns and gene expression as a result of both genetic selection history and maternal upbringing. Specifically, mice from a line bred for increased wheel-running activity exhibited brain region-specific changes in both the methylation patterns and gene expression of *Bdnf* in the hippocampus, with female HR mice having a more robust increase in *Bdnf* expression compared to male HR mice. In addition, cross-fostering alone modified the methylation patterns of *Pde4d* in the cortex and *Grin2b* in the hippocampus and further interacted with genetic line to modulate the methylation and expression of additional genes, including *Crh* and *Fkbp5* in the cortex and *Ezh2* in the hippocampus. Below, we provide an in-depth analysis of the importance of each of these genes and how their effects may converge to regulate intracellular signaling pathways in the brain (Basso and Suzuki 2017, Fernandes *et al.* 2017). We also discuss the influence of maternal effects and sex on physical activity levels. Our findings, combined with our prior study (Latchney *et al.* 2022), provide insights into the epigenetic regulation of genes associated with physical activity and highlight the potential role of environmental factors in shaping gene expression.

The primary objective of the present study was to identify genes that are epigenetically modified in mice from a line that has been selectively bred for increased wheel-running activity. Of the 14 genes analyzed, only *Bdnf* DNA methylation was modified by selection, and it was paralleled by a significant upregulation of *Bdnf* gene expression in HR mice, particularly females, regardless of cross-fostering (Figure 3A and 3B). Although the change in DNA methylation did not pass the FDR procedure, it is not uncommon to observe small percentage changes in methylation levels that correspond with large effect sizes and robust changes in gene expression (Breton *et al.* 2017, Latchney *et al.* 2022). Moreover, this change was brain region-specific, as it was only observed in the hippocampus but not the cortex. BDNF signals through the tropomyosin receptor kinase (Trk) B receptor and has well-established roles in neurodevelopment, neuronal survival, and synaptic plasticity. Previous studies have demonstrated that acute exercise can increase the transcription of *Bdnf* through DNA methylation (Gomez-Pinilla *et al.* 2011, Sleiman *et al.* 2016), histone modifications (Abel and Rissman 2013, Intlekofer *et al.* 2013, Ieraci *et al.* 2015), and microRNAs (Bao *et al.* 2014, Cosin-Tomas *et al.* 2014, Hu *et al.* 2015, Pan-Vazquez *et al.* 2015), resulting in significant increases in hippocampal *Bdnf* levels (Soya *et al.* 2007, Lee and Soya 2017). Upregulation of hippocampal BDNF has been linked to improved performance in behavioral tasks related to learning and memory (Vaynman *et al.* 2004), increased hippocampal neurogenesis (Rhodes *et al.* 2003b, Soya *et al.* 2007), and enhanced excitatory transmission (Zafra *et al.* 1990, Patterson *et al.* 1992, Castren *et al.* 1993, Canossa *et al.* 1997). In our selective breeding experiment, we have previously shown that HR mice from generation 25 had significantly higher levels of hippocampal BDNF after seven nights of wheel running, and hippocampal BDNF levels correlated with distance run (Johnson *et al.* 2003). In addition to the aforementioned studies demonstrating that acute exercise can epigenetically regulate the expression of *Bdnf*, our current results show for the first time that genetic upregulation of exercise behavior across multiple generations can also epigenetically regulate *Bdnf* expression.

Because exercise activates neurons (Oladehin and Waters 2001, Clark *et al.* 2010), our findings are also consistent with the view that DNA methylation and histone post-

translational modifications regulate *Bdnf* transcription in an activity-dependent manner (Martinowich *et al.* 2003, Lubin *et al.* 2008, Roth *et al.* 2009, Guo *et al.* 2011). It is possible that the decreased methylation of *Bdnf* and corresponding upregulation of *Bdnf* transcript in HR mice could be a result of increased neuronal activation. However, support for this hypothesis in our HR mouse model remains elusive. As discussed earlier, HR and C mice display differential gene activity when they have access to running wheels, such that C mice show a significant correlation between distance run and c-Fos immunoreactivity in the hippocampal dentate gyrus, while HR mice do not (Rhodes *et al.* 2003a). Physical exercise also potently stimulates hippocampal neurogenesis (van Praag *et al.* 1999a, van Praag *et al.* 1999b) and increases hippocampal BDNF levels (Johnson *et al.* 2003), but when mice from generations 25 and 27 were housed with wheel access for 40 days, only C mice showed a strong correlation between BDNF concentration and levels of neurogenesis, while HR mice did not (Rhodes *et al.* 2003b). We postulated that there might be a limit to the number of new neurons that can be made and activated in response to exercise, and that a plateau exists in HR mice (Rhodes *et al.* 2003a, Rhodes *et al.* 2003b). Although it is reasonable to hypothesize that reduced levels of *Bdnf* methylation could be a component of the HR phenotype, further investigation is needed to fully understand the relationship between wheel running, neuronal activity, and epigenetic regulation of *Bdnf* in our HR model.

In addition to genetic factors, earlier research on HR mice has demonstrated differential responses to a variety of early-life influences, including a “Western” diet (Meek *et al.* 2010, Hiramatsu *et al.* 2017, Cadney *et al.* 2021a, McNamara *et al.* 2021), cross-fostering (Cadney *et al.* 2021b), and exercise (Acosta *et al.* 2015, Cadney *et al.* 2021a). Here, we observed brain region-specific alterations in DNA methylation levels and gene expression of *Pde4d* (cortex) and *Grin2b* (hippocampus) in response to cross-fostering, although there seemed to be a dissociation between the extent of methylation changes and the extent of gene expression changes. In the cortex, C mice raised by HR mothers had elevated *Pde4d* gene expression compared to the CC group. However, cross-fostering did not seem to influence *Pde4d* gene expression in HR mice (Figure

1B). In the hippocampus, C mice raised by HR mothers had elevated *Grin2b* gene expression compared to the CC group. In contrast, HR mice raised by C dams had reduced gene expression compared to the HRHR group (Figure 3D). Additional cross-fostering effects in which HR mice responded differently than C mice were observed for *Crh* and *Fkbp5* in the cortex (Figure 1) and *Ezh2* in the hippocampus (Figure 3). These results suggest differential effects of cross-fostering between HR and C dams. One explanation for these differential effects may be that the HR phenotype confers trade-offs with early-life resilience (Garland *et al.* 2022). This idea is supported by past research demonstrating that alterations in gene expression via DNA methylation can modulate stress responses (Weaver *et al.* 2006). In our study, we also observed cross-fostering effects on the methylation level and gene expression of *Crh* and *Fkbp5* in the cortex (Figure 1), both of which have known roles in modulating glucocorticoid activity and stress responses (Zannas *et al.* 2016, Kageyama *et al.* 2021). Therefore, it is possible, for example, that C mice possess robust epigenetic responses to fostering, which may be an early-life stressor in itself (Weaver *et al.* 2004, Weaver *et al.* 2006, Malkesman *et al.* 2008, Lu *et al.* 2009, Plyusnina *et al.* 2009, Roth *et al.* 2009), whereas these responses are blunted in HR mice. Although the source and mechanisms for the differential cross-fostering effects that we observed remain unknown, our *Pde4d, Grin2b, Crh,* and *Fkbp5* data suggest environmental factors (e.g., some aspect of maternal care, maternal body mass, or milk composition) may modulate gene activity. This is consistent with studies showing that postnatal maternal care can enhance novel object recognition and increase hippocampal *Bdnf* and *Grin2b* gene expression in male rat offspring (Liu *et al.* 2000, Champagne and Meaney 2007).

Bdnf, *Pde4d*, and *Grin2b* are essential intracellular signaling molecules that are epigenetically regulated in response to environmental factors, including exercise and maternal care (Lee *et al.* 2008, Qiang *et al.* 2010, Masrour *et al.* 2018). Physical exercise activates signaling cascades that trigger a wave of phosphorylation and other post-translational modifications that reach the nucleus and engage epigenetic mechanisms to alter gene expression (Vaynman *et al.* 2003, 2004, Muller *et al.* 2008, Gomez-Pinilla *et al.* 2011). Related to our current work, exercise is a potent inducer of

BDNF expression and facilitates synaptic plasticity, at least in part, by stimulating NMDA receptors (Vaynman *et al.* 2003, Farmer *et al.* 2004). Exercise-induced activation of NMDA receptors leads to an increase in intracellular calcium and subsequent stimulation of signaling pathways, including calcium/calmodulin-dependent protein kinase IV (CaMKIV), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), and CREB-binding protein (CBP). These signaling cascades eventually lead to the phosphorylation of cAMP response element-binding protein (CREB; Nguyen and Woo 2003, Xia and Storm 2012, Esvald *et al.* 2020). Although CREB activation is commonly associated with neuronal activation, the effect of CREB stimulation on neurons depends on the nature of the stimulus and its cellular context. For example, activation of NMDA receptors leads to dephosphorylation of CREB in extrasynaptic neurons, whereas in synaptic sites, it leads to phosphorylation of CREB and CREB-dependent gene expression (Ghiani *et al.* 2007).

Given this, CREB activation is widely considered to be a convergence point for various intracellular signaling molecules (Wang *et al.* 2018), and its transcriptional activity depends on its phosphorylation status, which is determined by the opposing actions of protein kinases and phosphatases (Ortega-Martinez 2015). Related to BDNF, there is a reciprocal relationship between BDNF and CREB, as BDNF treatment leads to CREB phosphorylation in primary cultures of rat cortical neurons as well as hippocampal slice cultures (Finkbeiner *et al.* 1997, Pizzorusso *et al.* 2000, Esvald *et al.* 2020). Conversely, blocking CaMKII and MAPK in the hippocampus reduces CREB and *Bdnf* mRNA and abrogates the cognitive enhancement induced by exercise (Vaynman *et al.* 2003, 2004, 2007). PDE4 and CREB are also closely related in that inhibition of PDE4 results in increased cAMP levels and subsequently increases the CREB phosphorylation and BDNF levels (Monti *et al.* 2006). These cascades of changes are associated with improvements in memory performance in mouse models of various cognitive impairments, including Alzheimer's disease and Rubinstein-Taybi syndrome (Monti *et al.* 2006, Xu *et al.* 2018, Zhang *et al.* 2018a, Cui *et al.* 2019, Wimmer *et al.* 2020) and psychiatric disorders, including depression, schizophrenia, Rett syndrome, and fragile X syndrome (Bourtchouladze *et al.* 2003, Wiescholleck and Manahan-

Vaughan 2012). CREB phosphorylation is also facilitated by NMDA receptors (Ghiani *et al.* 2007, Palomer *et al.* 2016) and GRIN2B expression is influenced by voluntary exercise (Masrour *et al.* 2018). Although the exact signaling cascades have not been elucidated in our HR mouse model, it is possible, for example, that increased *Grin2b* gene expression, as we have observed in C mice reared by HR dams (Figure 3D), could be connected to reduced gene expression for downstream signaling molecules that serve to negatively regulate CREB activity, such as *Pde4d* (Figure 1D). Collectively, the epigenetic regulation of key neurobiological genes could potentially be at the crossroads of signaling pathways involving cAMP and neurotrophic factors like BDNF. Although each of these signaling molecules has distinct molecular targets, their pathways culminate in the phosphorylation and activation of CREB, making it a potential convergence point for multiple intracellular signaling cascades in our HR mouse model.

Sex-specificity of various early-life experiences, including cross-fostering, on later developmental outcomes has been reported previously (Bester-Meredith and Marler 2001, Li *et al.* 2013, Baker *et al.* 2015, Zhu *et al.* 2016, Rosenfeld 2017). Although sex-specific effects were not a primary focus of this study, we observed sex-specific cross-fostering effects on DNA methylation profiles for various genes in the cortex and hippocampus. Significant main effects of sex were observed for *Atrx* and *Oxtr* in the cortex and *Atrx* in the hippocampus. We also observed a main effect of sex for *Bdnf* gene expression, with female HR mice exhibiting a larger increase in *Bdnf* expression than male HR mice compared to sex-matched C mice (Figure 3B). These findings align with numerous previous studies that revealed sex as a significant factor in shaping the main effects of linetype on several traits, including body composition and mass, and adult wheel-running behavior (Garland *et al.* 2011, Hiramatsu *et al.* 2017, Hiramatsu and Garland 2018, Cadney *et al.* 2021b), and stress responses (Malisch *et al.* 2007). It is also particularly striking that our observed cross-fostering effects on females, such as the increased *Bdnf* expression, align with their robust increase in wheel-running behavior as compared with non-selected C mice. Given the sex-specific cross-fostering effects observed in this study and our prior studies (Cadney *et al.* 2021b, Latchney *et al.* 2022), as well as documented sex-specific heterosis in crosses of HR lines (Hannon *et*

al. 2011), future studies should consider how sex may shape the trajectory of adult exercise behavior (Ross and Desai 2005, Hiramatsu *et al.* 2017, Tao *et al.* 2019, Conner *et al.* 2020).

Our findings regarding the impact of cross-fostering on DNA methylation provide compelling evidence for the enduring effects of the early-life environment on epigenetic regulation of gene expression. The observed alterations in DNA methylation patterns suggest that early-life experiences can shape epigenetic modifications, which in turn may contribute to the persistence of phenotypic traits into adulthood.

These findings have potential translational relevance to human development, as studies have highlighted the critical role of early-life experiences, including maternal care and environmental factors, in shaping various aspects of human development and health outcomes (Meaney 2001, Weaver *et al.* 2004, Weaver *et al.* 2006, Barker 2007, Waterland and Michels 2007). Our results support these observations and suggest that DNA methylation changes, influenced by early-life environments, may contribute to the long-term consequences observed in humans.

The C × HR cross in our study also provides an interesting parallel to human development. In humans, the concept of cross-fostering can be analogized to situations where children are raised in different environments or by adoptive parents. These circumstances allow us to examine the impact of early-life experiences on individuals with different genetic backgrounds. By considering the C × HR cross in this context, our findings shed light on the potential interplay between genetics and environment and its relevance to human phenotypic variation.

Although the current study presents novel insights into potential epigenetic mechanisms underlying the developmental origins of exercise and physical activity, some limitations should be considered in future studies. We note that these limitations have been identified in our previous publication (Latchney *et al.* 2022) because they pertain to the same cohort of mice.

Firstly, the cross-fostering experimental design used in the current study did not include non-fostering controls, where C offspring are reared by their biological C dam and HR offspring are reared by their biological HR dam. This limitation was intentionally designed to achieve the experimental aims of the first study that utilized this cohort of mice (Cadney *et al.* 2021b). Resource constraints and early pandemic restrictions also posed limitations, resulting in a sample size that was restricted to 20 litters.

Secondly, the DNA used in this study was extracted from bulk hippocampal and cortical tissue, which consists of a heterogeneous collection of cells. Importantly, changes of interest in DNA methylation may only occur in specific subpopulations of cells, and cell-type heterogeneity within tissues can confound statistical analyses (Rahman and McGowan 2022). Thus, when DNA is not obtained from sorted cells, adjustment for cell-type percentages in the main model or in subsequent analyses could increase the reliability of our findings. Future studies using single-cell transcriptomics and epigenomics approaches will also help to address issues of tissue-specific cellular heterogeneity and composition (Jaffe and Irizarry 2014, Hui *et al.* 2018).

Thirdly, only 14 genes were chosen for this study, rather than an unbiased genome-wide screen for expression and methylation changes. Almost certainly, more genes are differentially methylated in response to differences in maternal upbringing and physical activity levels. Additionally, it is important to differentiate between 5-methylcytosine and 5-hydroxymethylcytosine in future investigations, as bisulfite sequencing does not distinguish between these two epigenetic modifications. Given the enrichment of 5-hydroxymethylcytosine in the brain, understanding the roles of both 5-methylcytosine and 5-hydroxymethylcytosine in mediating alterations in DNA methylation associated with physical activity and early-life programming factors will be important (Kinney *et al.* 2011, Sherwani and Khan 2015).

Lastly, in several cases, changes in DNA methylation did not directly correlate with changes in gene expression, such as *Crh* and *Fkbp5* in the cortex (Figure 1) and *Bdnf*

and *Grin2b* in the hippocampus (Figure 3). In these cases, nominally significant changes in DNA methylation were associated with significant changes in gene expression. Although this dissociation could simply represent differences in statistical power to detect the two types of changes (e.g., caused by differences in measurement error), such uncoupling of DNA methylation and gene expression has been previously documented (Murphy *et al.* 2012, Kile *et al.* 2013, Kochmanski *et al.* 2017, Montrose *et al.* 2017) and warrants exploration of additional epigenetic mechanisms, such as histone modifications (Abel and Rissman 2013, Intlekofer *et al.* 2013, Ieraci *et al.* 2015), microRNAs (Bao *et al.* 2014, Cosin-Tomas *et al.* 2014, Hu *et al.* 2015, Pan-Vazquez *et al.* 2015), and long noncoding RNAs (Aliperti and Donizetti 2016).

6. Conclusions

Our study highlights the role of DNA methylation in exercise behavior and its potential as an epigenetic mechanism causing inter-individual differences in response to cross-fostering. In addition to the 16 genes analyzed in our previous study (Latchney *et al.* 2022), we analyzed the DNA methylation patterns for an additional 14 genes in a genetically selected line of high-running mice and a non-selected control line whose offspring were fostered in a full factorial experimental design. Our results suggest that these two genetically differentiated lines differed in DNA methylation patterns and expression of genes that are crucial for nervous system development. Moreover, many of these gene expressions differed between the cortex and hippocampus. These results emphasize the importance of considering both genetic and epigenetic factors in studies of voluntary physical activity and, more generally, highlight the need to better understand the complex interactions between genes and the environment in shaping behavior. Overall, our study contributes to a growing body of research that seeks to elucidate the role of epigenetic mechanisms in the regulation of exercise behavior and its potential as a target for interventions aimed at improving health outcomes.

7. Declarations

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9. Table

Table 1. Litter parameters for bisulfite sequencing and RT-qPCR analyses. Cortex and hippocampal tissue from a subset of offspring were used for DNA methylation and gene expression analyses. All pups were maintained until sacrifice at 59 days of age.

Group	Litter	Female	Male	Pups
CC	5	6	5	11
CHR	5	6	6	12
HRC	5	6	6	12
HRHR	5	6	5	11
Total	***20***	***24***	***22***	***46***

Table 2. Coordinates, genomic context, and number of CpG sites analyzed for 14 genes analyzed by bisulfite sequencing. TSS indicates relative to the ATG transcription start codon. Negative sign indicates a location upstream of ATG; positive sign indicates a location downstream of ATG.

Gene	Coordinates (GRCm38)	From TSS	# Assays	Genomic Context	# CpG sites
Atrx	ChrX: 105929666; ChrX: 105929641	-263; -238	1	5' Upstream	2
Bdnf	Chr2:109692087 - 109692500	17388 to 17801	4	Intron 1	14
Crh	Chr3: 19695567 - 19695541	-171 to -145	1	5' Upstream	4
	Chr3: 19694687 - 19694626	710 to 771	1	Intron 1	7
Ezh2	Chr6: 47595049; Chr6: 47595003	-19; -13	1	5' Upstream	2
	Chr6: 47595029 - 47595003	2 to 28	1	5' UTR	7
	Chr6: 47594301 - 47593767	730 to 1264	1	Intron 1	11
Fkbp5	Chr17: 28441899 - 28441244	75626 to 76281	2	Intron 4	6
Foxp2	Chr6: 15181562 - 15181598	280214 to 280250	1	Intron 3	4
Gfra1	Chr19: 58455297 - 58455243	110 to 156	1	5' UTR	5
Grin2b	Chr6: 136172324 - 136172228	802 to 898	1	Intron 1	12
Mecp2	ChrX:74108970	26394	1	Intron 1	1
Nr3c1	Chr18: 39490238 - 39490064	437 to 611	2	Intron 1	26
	Chr18: 39441359	49316	1	Intron 2	1
Nr3c2	Chr8: 77097784	198013	1	Intron 3	1
Oxtr	Chr6: 112491157 - 112490655	-1214 to -712	2	5' Upstream	17
	Chr6: 112489883 - 112489806	61 to 138	1	5' UTR	11
	Chr6: 112489787 - 112489026	157 to 918	2	Exon 1	16
Pde4d	Chr13: 109685958 - 109686008	1031782 to 1031832	1	Intron 3	5
Sox1	Chr8: 12394096 - 12394938	-1199 to -357	2	5' Upstream	10
	Chr8: 12396508 to 12396559	1214 to 1265	1	Exon 1	7
				Total # CpG sites analyzed	***169***

Table 3. List of genes and corresponding TaqMan assays used for RT-qPCR.

Gene	TaqMan Assay ID	GRCm38 Chromosome Location	Exon Boundary
Actb: actin, beta	Mm00607939_s1	Chr5: 142903116 - 142906724	6
Bdnf: brain derived neurotrophic factor	Mm01334047_m1	Chr2: 109674700 - 109727043	1 - 2
Crh: corticotropin releasing hormone	Mm04206019_m1	Chr3: 19693401 - 19695396	1 - 2
Eif2b: eukaryotic translation initiation factor 2B, subunit 1 (alpha)	Mm01199614_m1	Chr5: 124570214 - 124579057	7 - 8
Ezh2: enhancer of zeste homolog 2	Mm00468464_m1	Chr6: 47530274 - 47595340	19 - 20
Fkbp5: FK506 binding protein 5	Mm00487406_m1	Chr17: 28398753 - 28486149	10 - 11
Foxp2: forkhead box P2	Mm00475030_m1	Chr6: 14901226 - 15441977	6 - 7
Grin2b: glutamate receptor, ionotropic, NMDA2B (epsilon 2)	Mm00433820_m1	Chr6: 135715272 - 136173819	13 - 14
Oxtr: oxytocin receptor	Mm01182684_m1	Chr6: 112473684 - 112491308	1 - 2
Pde4d: phosphodiesterase 4D, cAMP specific	Mm00456879_m1	Chr13: 108654177 - 109955969	6 - 7
rn18s: 18S ribosomal RNA	Mm03928990_g1	N/A	N/A

10. Figure Legends

Figure 1. Effects of selection and cross-fostering on DNA methylation (A, C, E) and gene expression (B, D, F) for genes in the cortex that demonstrated a main effect and/or interaction of line and/or foster-line. Line, fline, sex, line × fline, line × sex, fline × sex, line × fline × sex were included as terms in all models. Separate models were run for each gene. N = 46 mice. F-statistic and associated p-values for each gene are reported alongside each graph. p-values for significant differences of least squares means from SAS Procedure Mixed are shown (* p < 0.05, ** p < 0.01; *** p < 0.001). Hedges' g value from key comparisons is also reported. Values ± 0.8 or greater (indicated in bold) were viewed as large effect sizes. See Supplemental Tables 3 and 4 for complete statistical results, including combined-sex analyses.

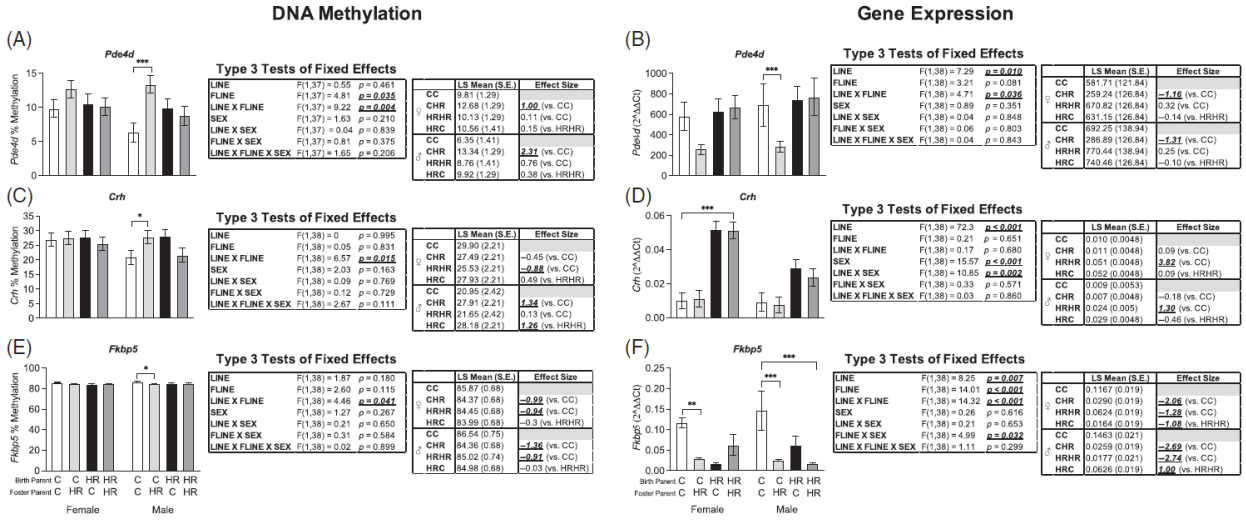


Figure 2. Effects of sex on percent DNA methylation for genes in the cortex. Line, fline, sex, line × fline, line × sex, fline × sex, line × fline × sex were included as terms in all models. Separate models were run for each gene. N = 46 mice. F-statistic and associated p-values for each gene are reported alongside each graph. p-values for significant differences of least squares means from SAS Procedure Mixed are shown (* $p < 0.05$). Hedges' g value from key comparisons is also reported. Values ± 0.8 or greater (indicated in bold) were viewed as large effect sizes. See Supplemental Table 3 for complete statistical results, including combined-sex analyses.

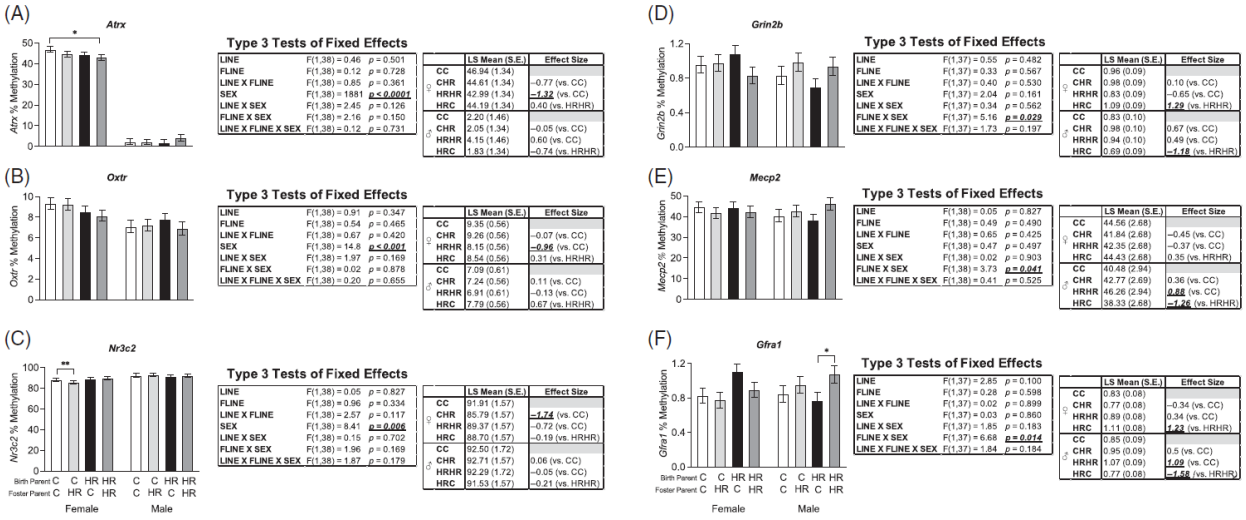


Figure 3. Effects of selection and cross-fostering on DNA methylation (A, C, E) and gene expression (B, D, F) for genes in the hippocampus that demonstrated a main effect and/or interaction of line and/or foster-line. Line, fline, sex, line × fline, line × sex, fline × sex, line × fline × sex were included as terms in all models. Separate models were run for each gene. N = 46 mice. F-statistic and associated p-values for each gene are reported alongside each graph. p-values for significant differences of least squares means from SAS Procedure Mixed are shown (* p < 0.05, ** p < 0.01; *** p < 0.001). Hedges' g value from key comparisons is also reported. Values ± 0.8 or greater (indicated in bold) were viewed as large effect sizes. See Supplemental Tables 3 and 4 for complete statistical results, including combined-sex analyses.

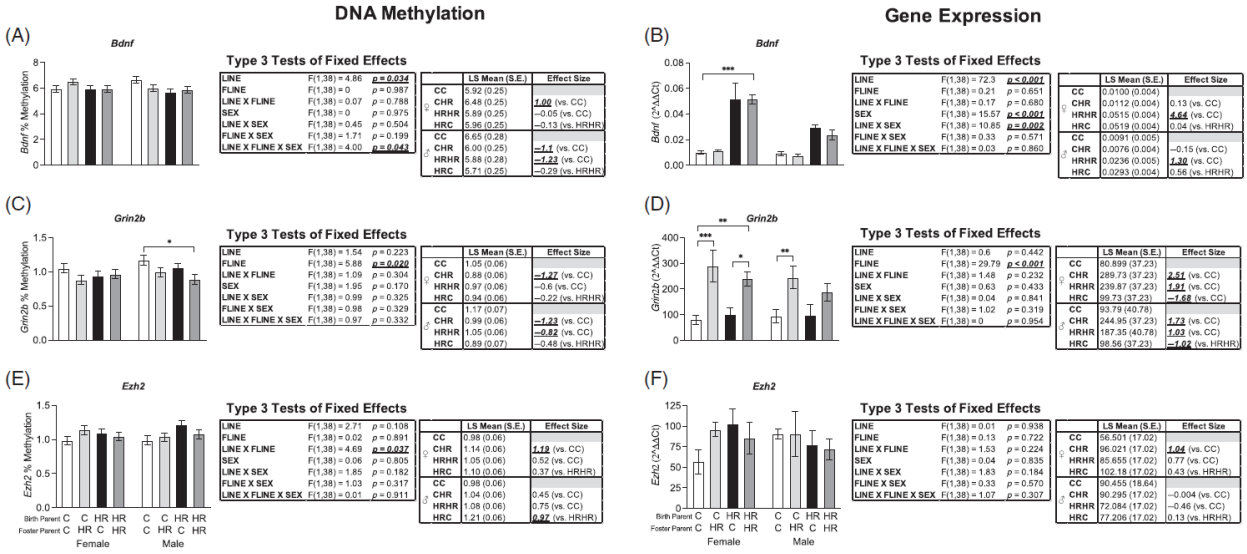
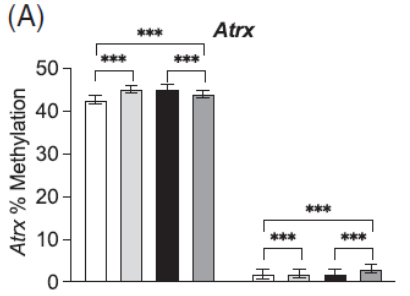


Figure 4. Effects of sex on DNA methylation for genes in the hippocampus. Line, fline, sex, line × fline, line × sex, fline × sex, line × fline × sex were included as terms in all models. Separate models were run for each gene. N = 46 mice. F-statistic and associated p-values for each gene are reported alongside each graph. p-values for significant differences of least squares means from SAS Procedure Mixed are shown (*** p < 0.001). Hedges' g value from key comparisons is also reported. Values ± 0.8 or greater (indicated in bold) were viewed as large effect sizes. See Supplemental Table 3 for complete statistical results, including combined-sex analyses.

(A)

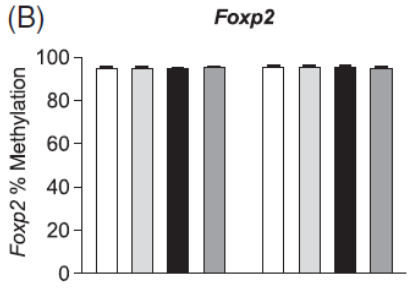


Type 3 Tests of Fixed Effects

LINE	F(1,38) = 1.03	$p = 0.318$
FLINE	F(1,38) = 0.71	$p = 0.404$
LINE X FLINE	F(1,38) = 0.96	$p = 0.334$
SEX	F(1,38) = 3771	***p < 0.001***
LINE X SEX	F(1,38) = 0	$p = 0.991$
FLINE X SEX	F(1,38) = 0.98	$p = 0.981$
LINE X FLINE X SEX	F(1,38) = 2.83	$p = 0.100$

		LS Mean (S.E.)	Effect Size
♀	CC	42.67 (0.94)	
	CHR	45.08 (0.94)	***1.15*** (vs. CC)
	HRHR	43.96 (0.94)	0.61 (vs. CC)
	HRC	45.19 (0.94)	0.59 (vs. HRHR)
♂	CC	1.87 (1.03)	
	CHR	1.95 (0.94)	0.04 (vs. CC)
	HRHR	2.07 (0.94)	0.09 (vs. CC)
	HRC	3.11 (1.03)	0.47 (vs. HRHR)

(B)

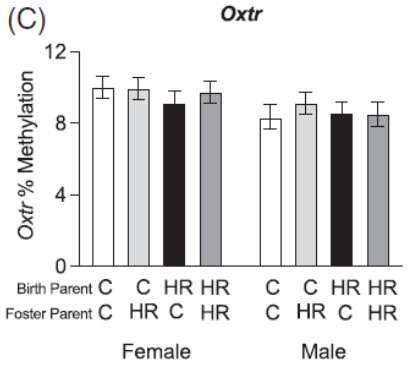


Type 3 Tests of Fixed Effects

LINE	F(1,38) = 2.29	$p = 0.139$
FLINE	F(1,38) = 0.06	$p = 0.807$
LINE X FLINE	F(1,38) = 0.07	$p = 0.789$
SEX	F(1,38) = 8.91	***p = 0.005***
LINE X SEX	F(1,38) = 0.68	$p = 0.416$
FLINE X SEX	F(1,38) = 2.59	$p = 0.116$
LINE X FLINE X SEX	F(1,38) = 1.45	$p = 0.235$

		LS Mean (S.E.)	Effect Size
♀	CC	95.60 (0.20)	
	CHR	95.73 (0.20)	0.29 (vs. CC)
	HRHR	95.77 (0.20)	0.38 (vs. CC)
	HRC	95.36 (0.20)	***0.92*** (vs. HRHR)
♂	CC	96.21 (0.22)	
	CHR	96.23 (0.20)	0.04 (vs. CC)
	HRHR	95.67 (0.22)	***−1.1*** (vs. CC)
	HRC	96.09 (0.20)	***0.89*** (vs. HRHR)

(C)



Type 3 Tests of Fixed Effects

LINE	F(1,38) = 0.65	$p = 0.424$
FLINE	F(1,38) = 0.46	$p = 0.504$
LINE X FLINE	F(1,38) = 0.01	$p = 0.911$
SEX	F(1,38) = 5.48	***p = 0.025***
LINE X SEX	F(1,38) = 0.13	$p = 0.725$
FLINE X SEX	F(1,38) = 0.01	$p = 0.925$
LINE X FLINE X SEX	F(1,38) = 0.65	$p = 0.426$

		LS Mean (S.E.)	Effect Size
♀	CC	9.99 (0.63)	
	CHR	9.94 (0.63)	−0.04 (vs. CC)
	HRHR	9.72 (0.63)	−0.19 (vs. CC)
	HRC	9.14 (0.63)	−0.41 (vs. HRHR)
♂	CC	8.34 (0.69)	
	CHR	9.11 (0.63)	0.52 (vs. CC)
	HRHR	8.49 (0.69)	0.10 (vs. CC)
	HRC	8.55 (0.63)	0.04 (vs. HRHR)

Supplemental Table 1. Characteristics of generation 89 breeder females as published in Cadney et al. (2021b; see Table 2). Average daily revolutions on days 5 and 6 (n = 126) and subsequent body mass (n = 134) at the time their pups were weaned are shown as least squares means and standard errors from SAS Procedure Mixed. Shown in parentheses are the corresponding values for a separate set of breeders used in the Cadney et al. (2021b) study.

Supplemental Table 2. Summary of the target loci, number of assays and CpG sites, average number of reads, and PCR bias testing results (shown as average R-squared value, RSQ).

Supplemental Table 3. Percent methylation levels for CpG sites across the entire genomic region for each gene. p-values represent differences in the least squares means between the in-fostered and cross-fostered groups and include main effects and interactions (line × foster-line, line × sex, foster-line × sex, line × foster-line × sex).

Supplemental Table 4. RT-qPCR results for select genes that exhibited a main effect of line and/or foster-line for DNA methylation. p-values represent differences in the least squares means between the in-fostered and cross-fostered groups and include main effects and interactions (line × foster-line, line × sex, foster-line × sex, line × foster-line × sex).