ABSTRACT

Title of Document: Effects of chronic exercise on global DNA methylation and epigenetic factors in sperm and testes of mice.

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Epigenetic alterations of DNA affect DNA transcription and translation. These alterations occur frequently, however environmental exposures induce epigenetic changes to DNA that would otherwise remain in autoregulatory stasis. This study aimed to look at exercise as a possible environmental factor causing epigenetic change. The study also assessed global DNA methylation in sperm, which may transmit such epigenetic changes via the paternal germ line. Measurements were compared between groups of mice that engaged in chronic exercise or remained sedentary. This study also examined enzymes causing methylation shifts in sperm by comparing levels of mRNA expression of genes responsible for new DNA methylation – *DNMT3A, DNMT3B* and *DNMT3L* – in testes. These results were compared between exercise and sedentary cohorts, and in progeny to assess heritability of epigenetic change. The results showed a significant difference in global methylation in the sperm between exercise and sedentary cohorts and a concomitant increase in gene expression in multiple *DNMT3* genes.

EFFECTS OF CHRONIC EXERCISE ON GLOBAL DNA METHYLATION AND EPIGENETIC FACTORS IN SPERM AND TESTES OF MICE.

By

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Chapter 1: Introduction

General Introduction

The environmental conditions that an organism experiences are of paramount importance in regulating physiological processes. For example, it has been well established that application of the archetypical Mendelian dogma of predicting an organism's phenotype based only on its alleles, in many cases, is inadequate. Epigenetics as a field is centered on recognizing the environmental factors that contribute to these non-Mendelian methods of phenotype expression and elucidating the biological mechanisms that drive them. DNA methylation is one of these biological mechanisms.

DNA Methylation and Environmental Stimuli

DNA methylation, most typically involving the addition of a methyl group to the cytosine residue of cytosine-guanine dinucleotide sites in the genome (known as CpG sites), has been shown to affect the development of an organism by altering expression levels of certain genes[1]. CpG sites do not occur at regular intervals throughout the genome of an organism but in many cases are clustered closely together into areas called CpG islands, which are often present in the promoter regions of genes. The level of methylation of these CpG islands in gene promoter regions affects the degree to which those genes are expressed, with increased methylation generally leading to reduced transcription[2,3]. Although epigenetic effects such as DNA methylation have been well established in their ability to affect gene expression in one generation, recent studies have provided evidence that these epigenetic effects, much like an organism's genes

themselves, can be passed on to their progeny. Recently, there have been a number of studies aimed not only at how the living environment affects epigenetic factors within a given organism, but also at the transmission of these epigenetic markings to subsequent generations[4].

For example, much research has been conducted on the role of exposure to environmental stimuli and their effect on epigenetic alterations. Developing the canonical understandings of these effects has typically been accomplished through the study of the influence of maternal effects on offspring. Evidence exists suggesting that maternal-based influences including maternal in-utero environment[5], post-natal grooming[6] and lactation[7] can alter gene expression via epigenetic means in rodents. In contrast to the wealth of knowledge acquired through maternal-line study of epigenetic effects on offspring, paternal-line contribution studies remain, by comparison, limited.

Recently scientists have begun discovering stimuli that when applied to male mice, are able to induce phenotypic alterations in progeny without affecting the DNA sequence (i.e., via transmission of epigenetic factors). One such stimulus, prevalent particularly in studies highlighting paternal line-based studies, is the herbicide vinclozolin. In a number of these studies, pregnant mice exposed to vinclozolin have been shown to pass on methylation-based epigenetic alterations to two generations of offspring after exposure.[8,9] These alterations induced patent phenotypic changes in the male offspring directly exposed *in utero* to the vinclozolin by affecting fertility, and were perpetuated in further generations exclusively via the male germ line.

Previous studies have shown that exposures to metabolic stress, be they transient- as in a brief fasting period prior to copulation[10]; or chronic – such as

chronic high fat diet[11]; can yield observable phenotypic alterations in offspring that are carried epigenetically through the paternal germ line. In fact, Ng et al.[11] demonstrated that these phenotypic alterations can coincide with varying levels of methylation at certain differentially methylated regions (DMRs) across the genome of the offspring. For example, adult male rats fed a high fat diet yielded offspring with significantly reduced methylation at a CpG site in the *Il13ra2* gene, which in turn coincided with alterations in gross morphology and function of pancreatic islet cells.[11]

Like exposure to vinclozolin[12], transient fasting, and high-fat diet – chronic exercise is a physiological stressor, and thus has the potential to leave a lasting epigenetic impression upon an organism and its progeny across generations. One recent study, published by Zhang et al. in 2011, showed a correlation between increasing levels of physical activity and levels of methylation across all genomic LINE-1 sequences in women of varying ages and races[13]. That study in particular has lent credence to the hypothesis that chronic exercise contributes to alterations in DNA methylation patterns. Another study, published by Barres et al. in 2012, provided direct evidence that an acute bout of exercise can induce significant methylation remodeling on a global scale as well as at individual genes related to metabolic regulation.[14] In spite of these groundbreaking studies, the question of transmittability of such altered patterns in DNA methylation to the next generation has not been addressed.

Transgenerational Effects of Exercise

There exists a substantial body of research suggesting that the metabolic stress engendered by physical activity on an organism has the potential to foster phenotypic

alterations in its offspring. More specifically, certain studies have suggested that pregnant mothers who exercise during pregnancy give birth to offspring with enhanced insulin sensitivity[15] but also lower birth weight and slower neonatal growth rates, in some cases across generations[16]. In humans, exercising mothers have even been shown to produce offspring with lower adiposity and enhanced linguistic proficiency early in life[17]. Thus, considerable evidence points to a role for maternal exercise in altering phenotypes in multiple generations of offspring. We are aware of no human work examining paternal influences. Since as early as 1998, studies have put forth evidence that chromosomal markings in an organism may retain an epigenetic "memory" from the previous generation, and that they are carried through *both* the maternal and paternal germ lines[18,19]. As metabolic stress, for example exercise or diet, can result in transmittable epigenetic change[20], the need to study paternal-line epigenetic contributions emerges when attempting to understand the epigenomic profile of an organism.

Recent, heretofore unpublished studies from our research group's laboratory have provided evidence of an effect on metabolic and gross morphological phenotypes in the future generations of offspring in chronically exercising versus sedentary mice.[21,22] This transgenerational phenotype also coincided with differing expression levels of genes that govern a number of metabolic pathways[23]. As these studies were conducted in inbred, genetically identical strains of mice, the observed effects suggest transgenerational propagation through an epigenetic – not genetic – mechanism.

In summary, emerging evidence suggests that: 1) chronic exercise can induce changes in DNA methylation; 2) chronic exercise prior to and during conception can impact phenotypes in mice offspring; 3) DNA methylation patterns can be transmitted across generations and have an impact on phenotypes. The present study aimed to examine the paternal intersection of these lines of evidence by examining the role of chronic exercise in altering DNA methylation patterns in the sperm of exercised and sedentary mice in multiple generations.

Purpose

There exists a large body of evidence clarifying the maternal role in the epigenetic state of progeny, while in comparison the paternal role remains under-studied. The purpose of the present thesis was to assess the effect of long-term chronic exercise on levels of DNA methylation in mature sperm as well as mRNA expression levels of genes responsible for DNA methylation – *Dnmt* genes – in the testes of mice across multiple generations.

For this project, the analyses were performed on both mature sperm cells and whole testicle samples. Sperm cells were chosen because of their transmitting role in linking the epigenetics of an animal in one generation to its progeny. In line with this, testicular samples were required for analysis as this is the site of synthesis of the aforementioned sperm cells. Using these tissues, we measured mRNA levels of various isoforms of de novo DNA methylation genes *Dnmt3a, Dnmt3b,* and *Dnmt3l*, as well as the maintenance DNA methylation gene *Dnmt1*. In doing so, we sought to observe how members of the *Dnmt1* and *Dnmt3* gene families may moderate global

DNA methylation in sperm, and how the expression of these genes is affected by exercise ancestry.

Hypotheses

We hypothesized that global DNA methylation levels in sperm obtained from exercise (wheel-running) would be significantly higher compared to sedentary cohorts. What is more, we hypothesized that between exercise and sedentary cohorts there would be a significant difference in expression of at least one gene in the *Dnmt* gene family in testis tissue, which would then emerge as a primary suspect as a contributing factor to exercise-induced de novo DNA methylation. Evidence from a previous study demonstrating a conservation of methylation markings between somatic and germ cells[21,24] suggests that analysis of sperm cells provides an opportunity to view the methylation state of the current generation as well as the methylation markings being passed on to progeny. As a result, we also hypothesized that these effects of differential *Dnmt* gene expression from the previous generation would be recapitulated in offspring, showing an epigenetic maintenance of the phenotype between generations.

Chapter 2: Methods

Overview and Animal Care

This study was approved by the University of Maryland IACUC, approval #R-10-93. Twenty male and twenty female C57BL/6J strain mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and were randomly assigned to be housed in cages either containing running wheels – representing the exercise cohort – or in sedentary cages without running wheels. All mice were housed with a room temperature maintained at ~71ºF with a 12 hour light/dark cycle. This first generation of mice – the F0 generation - was assigned to either exercise or sedentary cohorts at eight weeks of age. At 20 weeks of age mice from this generation were assigned to breeding pairs with other mice of the same cohort. Exercise females continued to run on running wheels during breeding, while pregnant and while nursing offspring. The resultant litters yielded the F1 cohort, which in turn were all placed in sedentary cages and at eight weeks, 10 males and 10 females of each cohort were placed in breeding pairs of the same cohort (parental intervention) to produce the F2 generation. Mice not placed in breeding pairs were sacrificed at either eight or 28 weeks of age for examination of dependent variables.

Mice from all cohorts were changed into fresh cages once per week. For mice in wheel cages, total wheel running (revolutions) was recorded for 24 hours per day using a computerized running wheel monitoring system (Lafayette Instruments, Lafayette, IN), for the first three days of each week following cage changing.

Sperm and testes samples were isolated from F0 28-wk (n=20), F1 8-wk (n=12) and 28-wk F1 (n=20), and F2 8-wk (n=13) and 28-week F2 (n=20) C57Bl/6 mice. An F0 control group (n=20) that was raised in identical conditions to F0 generation but did not breed was used to account for the effect of pregnancy in females analyzed in a related study. The ability of exercise to affect global DNA methylation in mature male gametes was evaluated by MethylFlash colorimetric methylation analysis of genomic DNA isolated from mature male sperm

Tissue Preparation

Testes and sperm were isolated during the dissection and sacrifice of mouse cohorts. Mice were moved to a ventilatory hood 48 hours prior to sacrifice with wheel cages removed to prevent the influence of acute exercise. Six hours prior to sacrifice, food was removed from the cages. Mice were anaesthetized via isoflurane vapor exposure and euthanized by exsanguination and subsequent excision of the heart. Next, the mouse testes, caudal epididymi, and ductus deferens were dissected in one uniform complex, rinsed in deionized water and placed in a small cell culture dish containing 1.4 mL Delbucco's Modified Eagle's Medium (DMEM). Next, a 22 gauge, 1.5-inch long needle was bent at a right angle using a metal clamp. Using the bent, transverse edge of the needle, the contents of the ductus deferens were expressed into the cell culture dish. Then, the ductus deferens were excised and the caudal epididymi were punctured and, through the aperture created, the contents were also expressed into the cell culture dish. With all sperm removed from the ductus deferens and caudal epididymi, both were excised and the testes were weighed and

transferred to 2.0 mL screw cap tubes and frozen in liquid nitrogen. The time elapsed between the moment in which the animal was sacrificed and the testis samples in tubes were placed in liquid nitrogen was approximately 8-10 minutes. The sperm suspended in DMEM was then transferred to a 1.7 mL microfuge tube using a transfer pipet. Once in microfuge tubes, the samples were centrifuged at low velocity (2000x g) for one minute to pellet the sperm, and the DMEM medium was removed via pipetting, and in its place 100µL of cell lysis solution was pipetted into the tube. Samples in cell lysis solution were mix pipetted until the solution appeared homogeneous and were stored at 4ºC.

Genomic DNA was isolated from all sperm samples using the PureGene DNA Purification System (Gentra Systems, Minneapolis, Minnesota). This began with an incubation of sperm samples in cell lysis buffer at 65°C for one hour, followed by the addition of 5 µL Proteinase K to each sample, which was then inverted 50 times and left to incubate overnight at 55°C. The following day samples were incubated on ice for 5 minutes or until they reached room temperature, after which 50 µL Protein Precipitate solution was added to the each lysate, which was then vortexed for 20 seconds then centrifuged for 17,200 x g for five minutes. The resulting supernatant was poured into a new microfuge tube containing 100 µL isopropanol and inverted 50 times to mix, then centrifuged at 17,500 x g for five minutes to visualize the DNA pellet. The isopropanol was then discarded and microfuge tubes were drained briefly on absorbent paper. Next 100 µL of 70% ethanol was added to each tube, which was then centrifuged at 17,200 x g for two minutes. Each tube was then drained of ethanol and dried on absorbent paper to

desiccate the DNA pellet, which was then rehydrated in ultrapure water. Prior to analysis, rehydrated DNA was incubated overnight at room temperature. DNA samples were assessed for purity and concentration using a NanoDrop 3300 fluorospectrometer (ThermoScientific, Wilmington, DE), and samples with a 260/280 ratio under 1.7 were repurified and reanalyzed. Prior to use DNA isolated from sperm samples was stored at 4°C.

Total RNA for mRNA expression of *Dnmt3* family genes was isolated from testis samples homogenized in Trizol. To do this, one mL Trizol was added to a 10 mL Falcon Tube which was then placed in an ice bath. A single testis sample was placed into the Falcon Tube, and the bladed end of a drill homogenizer was lowered slowly into the tube. The homogenizer was turned on to a low setting and the Falcon Tube was rotated slowly so as to ensure complete homogenization of the tissue. While doing this, the Falcon Tube was kept in the ice bath and the homogenizer was never left on for over fifteen seconds to prevent RNA denaturing. The homogenate was then transferred to a 1.7 mL microfuge tube and stored at -80°C prior to RNA extraction.

After homogenizing testis samples in Trizol, RNA was isolated first by adding 200 µL of chloroform per mL of testis homogenate, which then was rotated to mix gently for 10 minutes. This mixture was then centrifuged at 12,000 xg for 12 minutes at 4°C, after which the upper aqueous phase was carefully pipetted out and into a fresh 1.7 mL microfuge tube. With the aqueous phase in a new tube, 500 µL isopropanol per original 1 mL of Trizol was added, then this was rotated to mix gently for 10 minutes and centrifuged at 12,000 xg for 8 minutes at 4°C. After

centrifuging the supernatant was carefully removed and 1.5 mL of ethanol per original 1 mL Trizol was added to the tube to wash the pellet. The tube was briefly vortexed, then incubated for 5 minutes at room temperature, then centrifuged at 7,500 xg for 5 minutes at 4°C. Finally, the ethanol was carefully removed, the tubes were left open to dry the pellet for approximately 10 minutes, and 50 µL of TE buffer was added to each to dissolve the RNA pellet. Purity and quantity of isolated RNA was determined using a NanoDrop 3300 fluorospectrometer (ThermoScientific, Wilmington, DE). Prior to use, RNA samples were stored at -80°C.

Global Methylation

Global methylation of sperm DNA was measured using the MethylFlash kit (Epigentek, Farmingdale, New York). The MethylFlash assay is an ELISA-like colorimetric assay that comes complete with universal positive and negative controls that are utilized to establish a standard curve to which samples of genomic DNA were compared for levels of global methylation, measured quantitatively by the color emitted by the assay reaction. In each 96-well plate positive controls to establish a standard curve, the negative control sample, as well as a cDNA sample (used to compare to the negative control for validation purposes) and a sample from an adult male not used in the study were measured in duplicate. Sperm DNA samples of all cohorts in this study were measured in triplicate.

To use the MethylFlash kit, a 10X wash buffer was diluted according to manufacturer's protocols for later washing of samples in the 96-well plate. Next, using a multichannel pipette, 80 µL of manufacturer-provided Binding Solution was added to each well. Then, 5 µL of positive control sample was diluted in 5 µL TE

buffer to make a 10 ng/μL solution. This solution was then diluted into 5, 2, 1 and 0.5 ng/μL solutions, of which 1 μL of all dilutions – including 10 ng/μL – were added to wells in duplicate. Similarly, 1 μL of Negative Control was added to two separate wells, as were 100 ng DNA samples of cDNA and genomic DNA from an unused cohort. After standard and control samples were added to the plate in duplicate, 100 ng of each sample was added to wells in triplicate as long as the sample solution added to each well was between 1-8 mL. Samples in solution where 100 ng DNA could be added in less than 1 mL were diluted at a 1:10 dilution factor. Samples with 100 ng DNA requiring the addition of over 8 mL were not used.

After adding samples to wells, the plate was sealed with parafilm and incubated at 37°C for 90 minutes. Upon finishing the incubation, the parafilm was removed, the solution poured out of all wells in the plate and each well was then washed three times with 150 μL wash buffer using a multichannel pipette. From there, manufacturer-provided Capture Antibody was diluted in wash buffer to a 1:1000 dilution, and 50 μL of this solution was added to each well using a multichannel pipette. The plate was then sealed with parafilm and incubated at room temperature for 60 minutes. As in the previous step, the solution was then removed from all wells and the wells were washed three times with 150 μL of wash buffer. Next, manufacturer-provided Detection Antibody was diluted in wash buffer at a 1:2000 dilution, of which 50 μL were added to each well. The plate was then sealed in parafilm and allowed to incubate at room temperature for 30 minutes. The solution was then decanted, and all wells were washed again as before but four times in this instance, with 150 μL wash buffer each time. Then, manufacturer-provided Enhancer

Solution was diluted in wash buffer at a 1:5000 dilution, of which 50 µL was added to each well. The plate was then sealed in parafilm and allowed to incubate for 30 minutes at room temperature. After the incubation, the solution was decanted from all wells and each well was washed five times with 150 µL wash buffer. Next, away from light, 100 µL of photosensitive Developer Solution was added to each well using a multichannel pipette and allowed to incubate for between 2-3 minutes, until positive controls changed to a light blue color, at which point the colorimetric development was halted by the addition of 50 µL of proprietary Stop Solution to each well. The plate was then covered with aluminum foil and transferred to a plate reader for analysis.

Measurement of colorimetric reactions was done at λ= 450 nm and carried out in a Molecular Devices Emax Precision Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA) and analyzed using SOFTmax PRO proprietary software (Molecular Devices Corporation, Sunnyvale, CA).

Analysis of raw absorbance values began by plotting the absorbance values of the standard curve constructed by the positive control dilutions on a scatter plot, then calculating a line of best fit of those values. Sample absorbance values were converted to amount and percentage of methylated DNA by subtracting the absorbance value of the negative control from the absorbance of the sample. This value was then divided by two times the slope the best-fit line of the standard curve (as the positive control solution contained only 50% methylated DNA).

We followed the manufacturer instructions for all conditions and establishing quality control and precision metrics through ratios of no-template

controls and appropriate ranges of slope of the positive control sample dilutions. All sample absorbance values were found to be within the bounds of the positive control standard curve.

Gene Expression

cDNA, or complimentary DNA, is DNA used for gene expression analysis and is made by polymerizing a second single strand of complimentary DNA onto single stranded RNA isolated from a sample in a process known as reverse transcription. Thus, cDNA was produced using industry-standard techniques of reverse transcription, using 1 μg of RNA isolated from the aforementioned testis samples. This cDNA was then used in PCR (polymerase chain reaction) reactions for genes *Dnmt3a, Dnmt3b,* and *Dnmt3l*, which was separated using an agarose gel by means of gel electrophoresis. The bands of cDNA amplicons (amplified sequences) formed in this process were used to assess gene expression of these genes, as quantified via brightness of the bands as measured using NIH Image/J software.

Primers for *Dnmt3a* and *Dnmt3a2* were designed specifically to span exons unique to target genes. These primers were derived in part from published work by Chen et al. 2002.[24] Primers for *Dnmt3b* were designed using parameters first established by Okano et al. 1998.[25] Primers for *Dnmt3l* splice variants were derived from Shovlin et al. 2007, which showed the variants of the gene expressed exclusively in round spermatids and pachytene spermatocytes located in the post-natal testis.[26] All primer sequences can be found in the Appendices section of this document.

Optimal PCR conditions for each variant were optimized first using a temperature gradient MJ Research PTC-200 Thermo Cycler (Bio-Rad Laboratories, Hercules, CA) and optimized for annealing temperature and buffer type. The optimized conditions for the gene variants used in this study are listed in the appendices of this document. The PCR products were then electrophoresed through a 300 mL 1.5% agarose gel infused with 15 μL ethidium bromide and imaged under ultraviolet light. The resultant bands were assessed for uniformity and resolution: they should be uniform and tight, without streaks or aberrant bands that when compared to a 100-base pair ladder were not of product sizes smaller or larger than the target product size of the designed primers. Primers were optimized further for number of denaturing/annealing cycles in a MJ Research PTC-100 Thermo Cycler (Bio-Rad Laboratories, Hercules, CA) using the previously optimized conditions. These bands were then assessed for brightness: bands too dark for proper exposure could not be analyzed; bands too bright are too overexposed for proper analysis. This step is particularly important in that if too many cycles are used in the PCR, differences in brightness between samples that may be detectable between samples at 28 cycles may not be detectable after 36 cycles – for example – due to redundant denaturing and annealing in one sample, while another sample may still engage in annealing of cDNA not previously bound to primers. This would result in an inability to detect a difference in mRNA expression between samples where a difference may exist. Finally, with conditions optimized, the same PCR reaction was executed with varying amounts of cDNA (0.5, 1.0 and 2.0 μL) added to ensure fidelity of the

reaction process. If the bands increased in brightness correlated with increases in cDNA used in the PCR reaction, then the primer optimization process was complete.

Analyses of PCR products were carried out semi-quantitatively using NIH Image/J software. Brightness peak values of each PCR product of a gene variant were normalized to the peak values of a housekeeping gene – here, *Gapdh* – executed on each sample.

Statistical Power and Analysis

Pairwise comparisons between individual generational, age and treatment cohorts were carried out using two-way Student's T-tests. A three-way analysis of variance (ANOVA) was used to determine the effect of intervention (EX vs. SED), age and generation on global DNA methylation levels and post-hoc analyses completed by pairwise comparisons (T-test). Both of the aforementioned were carried out using SAS statistical analysis software (SAS Institute Inc., Cary, NC). The threshold for which statistical significance was accepted was as $p<0.05$.

Chapter 3: Review of Literature

Overview

In this review, I discuss a few essential concepts of epigenetics in the context of how they affect the present study. More specifically, the content includes a brief overview on some of the genes and proteins that affect *de novo* methylation and demethylation of DNA, how they are affected by environmental factors such as physical activity, and how the effects of these genes and proteins can propagate themselves across generations.

Epigenetics

One of the core tenants that scientists have established in defining a living organism is whether or not it contains its own set of DNA that it may propagate to offspring to ensure survival of the species. Since the mid-20th century, DNA has been identified as a sort of blueprint that dictates the organizational and developmental processes in every living organism. From this remarkable substance readily available substrates are converted from simple organic compounds to the complex array of multi-dimensional proteins all organisms require to survive and perpetuate their species' existence.

When the Human Genome Project was first proposed, the general scientific consensus was that by decoding the human genome, humans would at last establish dominion over this mysterious material and the mysteries that enshrouded it.

However, as the project drew to a conclusion, the seemingly providential endeavor generated far more questions than answers.

As a result, it became increasingly apparent that DNA as a substance must be viewed in a different light. Like the proteins derived from it, the primary structure of the DNA – the recently decoded nucleotide sequence – explained only a fraction of how cellular machinery and DNA interacted with each other to foster physiological changes. Other features inherent to the DNA itself, such as the presence of organic compounds bound to its nucleotides and its affinity to the histones it is typically bound to, were now emerging as primary suspects in elucidating the way in which intracellular signals interact with its own chromatin. This field, that which presides over how structural traits of DNA affects its place in the central dogma of biology, is epigenetics.

Though as a field epigenetics is arguably in its most high-profile state ever, it has existed since at least the 1960s and has been the focus of seminal publications for decades. For example, in 1975, Holliday and Pugh published a review – inspired largely by Holliday's observations in previous years regarding 5-methyl cytosine – speculating on the mechanisms by which DNA methylation could affect transcriptional and therefore downstream developmental pathways.[1]

Since, DNA methylation has been the focus of much of the study in the field of epigenetics, though that is not to say that it is the only epigenetic trait that affects transcription levels. A review by Kouzarides in 2007 outlines a large number of other epigenetic mechanisms based largely on histone modification that affect the binding affinity of transcription factors to given genes and their proximity to said modified

histones.[29] In brief, it is outlined how in the default packaging of cellular chromatin in the form of nucleosomes, 147 bp long segments of DNA are wrapped around histones, which themselves are comprised of an octamer formed by four core residues. Modifications of these histone segments via eight known processes – acetylation, methylation, sumoylation, ubiquitination, phosphorylation, ADP ribosylation, deamination or proline isomerization – can result in alteration of chromatin structure and interactivity including transcription, DNA repair, chromatin condensation and DNA replication.[29]

DNA Methylation and Gene Transcription

Overview

DNA methylation involves the addition of a methyl group to a cytosine nucleotide that is adjacent in sequence to a guanine residue in the DNA sequence. These sites for methylation are referred to as CpG sites due to the phosphate bond that connects the two nucleotides and occur frequently throughout the genome. In addition, CpG sites are most commonly found in regions of the genome densely saturated with CpG sites, called CpG islands –or CGI's - which exist most frequently in the upstream, non-coding promoter regions of genes.[1,30] Addition of methyl groups to CpG sites, and more frequently to CGIs on a given gene has been shown to be correlated with lower levels of transcription and thus mRNA transcripts and resultant protein from the hypermethylated gene.[3,29] The results of differing levels of methylation can be gene specific, where hypo- or hypermethylation of CpGs on a CGI in a gene promoter stimulates or inhibits transcription, respectively. Conversely,

when methylation levels are altered on a global level – on a series of sequences with CpG islands that are representative of the whole genome, such as LINE-1 segments – chromatin instability and other carcinogenic phenotypes have been observed.[2]

Dnmt1 and DNA Methylation

DNA methyltransferases exist to regulate the addition of methyl groups onto CpG sites throughout the genome. Within the DNMT family, all proteins share a common trait in that they cause the transfer of a methyl group from a methyl donor S-adenosyl methionine (SAM) to the 5-carbon of a cytosine bound to a guanine.[3,29,31] However, the vast majority of the DNMT member proteins execute the roll of adding methyl groups to unmethylated CpG sites in a permanent or transient fashion. Meanwhile DNMT1 functions primarily by recapitulating the methylated cytosines that remain on a hemimethylated strand of post-replicated DNA onto the newly-polymerized strand.[31]. As a result, DNMT1 activity provides valuable insight into assessing how environmental conditions affect epigenetic homeostasis, as lower levels of functioning DNMT1 in most cases should lead to lower levels of global DNA methylation over time.

In the context of the present study, Dnmt1 is synthesized and active in the testes as well as the spermatocytes it produces; however, mRNA expression of the *Dnmt1* gene tends to decrease as the nascent spermatogonium-cum-spermatocyte develops.[32] This suggests that maintenance of DNA methylation markings from the father organism to the sperm it passes on to its progeny occurs very early in the development of the gamete, and prior to its release from the testis. As a result, one

could assert that much of a father's epigenetic legacy on his progeny is decided in the pre-pachytene stage of spermatocyte development.

Dnmt3a and Dnmt3b Engage in *de novo* DNA Methylation

The addition of novel methyl groups to CpG sites around the genome, known as *de novo* methylation, occurs throughout the development and lifespan of most organisms. In mammals, these *de novo* methylation groups are considered to be added to unmethylated cytosine residues primarily through two proteins: DNMT3a and DNMT3b[33]. DNMT3a and DNMT3b have been shown to orchestrate *de novo* methylation in a number of different tissues across different points of mammalian development, and at certain points – such as in embryonic development – where their action is critical for survival[34] including the germ line. Studies have shown the methylating influence of these proteins is especially marked in the male germ line, such as in the formation of primordial germ cells [35] and pre-meiotic spermatogonia. [36]

Though DNMT3a and DNMT3b have the ability to methylate previously unmethylated CpG sites autonomously, they are also capable of interacting with a DNA methyltransferase-like protein - DNMT3l - to facilitate the process. In fact, early study of the protein – which has no enzymatic methylation capacity of its own – showed increased levels of *de novo* methylation in mice when Dnmt3a and Dnmt3b liaised with Dnmt3l.[37] This effect has also been shown in humans, with DNMT3A showing a three-fold increase in methyltransferase activity when combined with DNMT3l. [37] In addition, DNMT3l showed its worth as a vital component to *de novo* methylation-based mechanisms for development in a study involving Dnmt3l

knockout mice. In this study, Dnmt3l -/- mothers were unable to produce viable offspring, with all fetuses undeveloped and incapable of maturation. [38] *DNMT3L* is expressed in almost all mammalian tissues, though in different tissues, the gene is expressed in different, tissue-specific isoforms. In the context of the current study, for example, *Dnmt3l* is expressed in three different splice variants endemic to round spermatids and pachytene spermatocytes in the post-natal testis.[26] Thus, it can be conjectured that these proteins likely play an integral role in inducing the methylation-based epigenetic changes required for successful post-zygotic development, but also in influencing the epigenetic legacy left by the environmental exposures of the father.

Demethylation

The crux of the present study is heavily focused on the creation of *de novo* methylation markings to induce an epigenetic change. Conversely, methylation-based epigenetic changes occur just as frequently through the transient removal of methyl groups from CpG sites. In spite of the in-depth research being conducted on the subject, the act of both active and passive DNA demethylation is not yet well understood. Current studies best describe active DNA demethylation as a process primarily executed by means of base excision repair (BER). The first proposed method of BER-based demethylation is thought to occur using activation-induced cytosine deaminase (AID), which functions by deaminasing methylated cytosines, converting them to thymines.[39] From here, it is conjectured that a T-G mismatch glycosylase, such as methyl-CpG-binding domain protein 4 (MBD4), would engage in BER mechanisms removing the aberrant thymine residue from the DNA strand.

Though proposed, MBD4 has not yet been shown to be physiologically necessary for survival and normal development in mammals, but has been shown to demonstrate a markedly higher glycosylase activity when phosphorylated. [40]

Active demethylation is also thought to exist through another major pathway involving hydroxymethylation of cytosine residues. Though long evidenced in mammalian chromatin, only recently have any connections been made between hydroxymethyl cytosines (5hmC) and levels of cytosine methylation. However, a role for 5hmc has been shown in maintaining hypomethylated gene promoters.[40,41] A family of proteins known as TET have demonstrated the capacity to convert 5mC to 5hmC in vitro.[41] From there, 5hmC is conjectured to convert to a demethylated cytosine residue – thus illustrating the likely role of TET in DNA demethylation - although no mechanism for this process has been confirmed. Nevertheless, in studies involving young calves 5hmC's, such as those produced by TET, were shown to have glycosylase activity, and thus perceived to lead to DNA repair mechanisms yielding demethylated cytosines.[42]

Passive demethylation on the other hand, as the name suggests, is thought to happen in a less enzymatically-involved manner. The proposed mechanism driving this is via the down-regulation of DNMT1. By inhibiting DNMT1's methylation maintenance function, previously-methylated CpG sites may not be remethylated, such as in hemimethylated strands after DNA replication, or after global methylation erasure post-fertilization in the paternal pronucleus.[43]

Environmental Effects on DNA Methylation

The sequence of DNA, barring any traumatic event to induce mutation, does not change in the lifetime of an organism. As this sequence remains static, it remains the dominion of epigenetics to affect the availability of genes to be accessed for transcription and subsequent translation. The machinery that executes epigenetic effects is therefore completely reliant on environmental stimuli to kickstart the physiological mechanisms that mandate altered levels of transcription of certain genes across the genome. Said differently, epigenetic alterations – either transient or permanent - such as DNA methylation, are one of the most fundamental changes that an organism makes in response to changes in its living environment.

Such environmental stimuli have been the subject of study for epigeneticists for decades and a wide variety of sources of stimuli can change methylation patterns across the genome. Intermittent exposure to doxorubicin, for example, was shown to result in transient fluxes in methylation levels across the proximal *pS2* promoter region of MCF-7 cells. This change coincided with altered levels of DNMT family and demethylation genes.[44]

The maternal environment in which an organism develops in-utero brings with it a host of opportunities for epigenetic influence. A landmark study involving genistein supplementation in pregnant heterozygous viable yellow agouti mouse dams provided direct evidence of this. In this study, levels of genistein supplementation – which simulated varying levels of soy intake in humans – caused phenotypic variations in coat color in offspring towards pseudoagouti. Furthermore, these

phenotypes coincided with increases in methylation in upstream promoters of the *Agouti* gene.[5]

Other studies, involving post-natal interaction of rat and mouse dams with their offspring have yielded comparable results. For example, a study involving rats looked at the nurturing patterns of dams upon their young in a post-natal environment, including grooming and nursing. The study concluded that offspring exposed to more lick grooming and arched-back nursing had differing levels of methylation at the glucocorticoid receptor gene in the hippocampus, resulting in altered responses to stress.[6]

To drive home the importance of both in-utero and post-natal environments, a study was carried out on rats where offspring of dams fed a high fat or control diet were cross-fostered with dams of the opposite cohort. The findings showed that if offspring were exposed to a high fat diet in-utero or while nursing, they would develop a hypertensive phenotype as adults.[7]

In summary, studies have illustrated that environmental stimuli applied to pregnant mammals - primarily those evoking disruptions to metabolic homeostasis – have the ability to affect the epigenetic trajectory of their offspring. In the context of this study, such a stimulus would come in the form of voluntary wheel running – physical activity - as the intervention.

Exercise Alters Methylation Patterns in Humans

In a 2009 study in which young (aged 18-22) and older (aged 40-87) Japanese adults either engaged in brief exercise bouts of moderate intensity every six months

or remained sedentary and without daily exercise, significant epigenetic effects developed between groups. More specifically, methylation analysis at different promoter loci at the *ASC* gene – responsible for expressing pro-inflammatory cytokines – showed that methylation levels were significantly higher in exercising groups in both the young and older groups than in age-matched sedentary cohorts.[45]

Additionally, a landmark study by Zhang et al. in 2010[13] assessed global methylation levels by measuring methylation in LINE-1 inserts across the genomes of men and women of different ages and races who engaged in different levels of physical activity. Though results differed by race, age and sex, one uniform characteristic of the data collected was that subjects who participated in higher quantities of physical activity had lower levels of global methylation.[13]

Further evidence of the role of physical activity in influencing epigenetic states is portrayed in the results of a 2011 study involving patients receiving treatment for schizophrenia.[46] In that study, it was found that increased levels of physical activity had the ability to modulate methylation levels in the promoters of the *COMT* gene in schizophrenia patients.[46] Though not physiologically impactful on most of the human population, this study elegantly illustrates the capability that physical activity has to precipitate patent epigenetic change. This effect was further illustrated by measuring global and gene promoter site-specific methylation in DNA samples derived from skeletal muscle biopsies after acute exercise.[14] In that study, the data showed that exercise bouts of increasing intensities have a dose-dependent effect on methylation of gene promoter sites in metabolic regulatory genes. The resultant decreases in promoter methylation coincided with increased levels of mRNA

transcripts from said genes.[14] Furthermore, a previous study[47] had also demonstrated an exercise-based result in histones: during exercise, histone deacetylases HDAC4 and HDAC5 that serve as transcriptional repressors, were selectively exported from the nucleus on a global scale.[47] And, in a recent study of older females found that those who exercised daily by practicing tai chi had significantly different levels of DNA methylation, including decreases methylated sites associated with aging. [48]

In sum, evidence exists to support the notion that exercise can induce a significant change in DNA methylation. However, what is yet unknown is how the methylation patterns are affected on the level of individual gene sites.

Paternal Influence and Transmittability of Epigenetics

In mammalian reproduction, in order for a zygote to form and begin development, the union of a haploid sperm and egg must occur. As a result, the developing offspring is formed with a genetic sequence of randomly assorted genes from both maternal and paternal lines. Thus, it stands to reason that the offspring would also be influenced by the epigenetic information that its parents' DNA has brought in tow. It is primarily for this reason that scientists have begun studying not only how external stimuli affect the maternal environment, but the environment of the developing gamete – particularly in males. This is because in the fertilization process the father's contribution to the genetic and epigenetic state ends at fertilization, but is an undeniably important contribution nonetheless.

Much study has thusly been carried out to investigate the role of common environmental stimuli in the modern world and their effects on testicular physiology,

as this is the site of synthesis and development of the paternal line's sole genetic and epigenetic contribution to future generations: sperm. Resultant study has identified a number of stimuli that have shown to have direct, impactful effects on testicular function and morphology, as well as altering androgen levels. Androgens play a large role in the production of healthy, viable sperm in males, so it stands to reason that their inhibition would likely affect massive inroads in the spermatogenic process.[49] One of these impactful stimuli discovered is exposure to the herbicide vinclozolin. A study exposing male rats to vinclozolin resulted in significant differences in testicular morphology, increases in spermatocyte apoptosis, global gene expression patterns and levels of expression of genes in the *Dnmt* family. What is more, these phenotypes were perpetuated in F1 through F3 generations – not just the exposed F0 male generation - without changes in the organism's DNA sequence.[12] This provided early evidence of an environmental stimulus that could induce a physiological insult not only to the male germ line in one generation, but also conserved epigenetically across generations.[8]

Nevertheless it is not merely vinclozolin that can affect testicular health in males. Indeed, exposure to other chemicals such as glycol, as well as lifestyle-related outcomes including obesity or use of alcohol, tobacco or drugs can play a massive role in testicular health and resultant spermatogenesis.[50] In an instance in close proximity to the crux of the current study, lifelong chronic physical activity was shown in one study to have beneficial effects on the health and function of the male testis and seminal vesicle. More specifically, C57BL/6 mice that exercised using wheel running over 20 months were better protected from the andropause-like effects

of aging as caused by reactive oxygen species than sedentary matched cohorts. This resulted in mice with higher levels of serum testosterone, larger seminiferous tubule lumens and increased numbers of Leydig cells.[51] Thus, it is logical that wheel running as an environmental stimulus may be sufficient to induce epigenetic change in the male germ line.

Summary

In conclusion, there is a plethora of evidence supporting the assertion that the environmental conditions to which an organism is exposed have the ability to engender epigenetic alterations to the chromatin of the organism. These alterations are executed through *de novo* methylation by genes in the *Dnmt3* family or demethylation-based mechanisms. Said exposures have been demonstrated to be effective in inducing such changes to the organism directly exposed as well as any developing organisms that are confronted with these stimuli in-utero. What is more, literature exists to suggest that an organism's epigenetic legacy can be bequeathed to subsequent generations of progeny via that paternal germ line. Finally, studies in humans have demonstrated the ability that physical activity has to induce epigenetic change in an exercising cohort.

What is not yet known is whether all of the aforementioned factors interact in concert. As yet, no studies have shown the effects of physical exercise in the male germ line or in multiple generations. As such, no mechanism has been suggested for how a metabolic-based intervention such as habitual physical activity could affect the exerciser's offspring via epigenetic alterations of the paternal germ line. Therefore,

the present study endeavored to shed light on the aforementioned by measuring levels of DNA methylation in sperm and mRNA expression of *Dnmt3* family genes in testes of exercising and sedentary mice, as well as in their two subsequent generations of progeny. We hypothesized that we would see significantly higher levels of DNA methylation in the sperm as well as higher *Dnmt3* mRNA expression in EX (exerciser or of exercise ancestry) versus SED (sedentary and of sedentary ancestry) cohorts.

Chapter 4: Results

Anatomical Characteristics of EX and SED cohorts

No significant differences were detected in body weight between EX and SED cohorts of any generation (Table 1). Visceral fat mass tended to be higher in sedentary F0 cohorts than exercisers, but the difference was not statistically significant ($p=0.07$). Testicular mass measured relative to body weight was significantly higher in the EX groups of the F0 and F1 generations ($p<0.05$).

Litter sizes and sex splits

There were no significant differences in litter sizes in the F1 and F2 cohorts (offspring of intervention-matched F0 and F1 cohorts, respectively). However, F1 EX litters had a significantly higher percentage of male offspring than did their SED counterparts ($p<0.01$; Table 2).

Testicular Dnmt Gene Expression

In F0 28 week mice, no significant differences in mRNA expression for transcripts of the *Dnmt3* family were observed between EX and SED cohorts (Figure 1). However this was not the case in subsequent generations as F1 28 week mice of EX ancestry expressed significantly higher levels of mRNA for *Dnmt3a1, Dnmt3a2, and Dnmt3lf1* gene transcripts versus their SED ancestry counterparts (all $p<0.05$; Figure 2). Levels of these same genes transcripts as well as for *Dnmt3b2/3* were significantly higher in the subsequent F2 28 week EX ancestry cohort compared to mice of SED ancestry (all $p<0.05$; Figure 3). *Dnmt3a1, Dnmt3lf1* and *Dnmt3lf3*

mRNA transcripts were significantly higher (all $p<0.05$), and *Dnmt3b2/3* tended to be higher ($p=0.08$) in F1 8 week old EX cohorts than SED cohorts (Figure 4). In contrast mRNA expression of *Dnmt3b2/3* was significantly lower in F1 8 week EX versus SED ancestry males ($p<0.005$), but *Dnmt3lf3* tended to be higher in EX versus SED ancestry males ($p=0.07$; Figure 5).

Global Methylation Levels of Sperm DNA

The results of a three-way ANOVA (shown in Appendix C) showed a significant main effect for age ($p<0.001$) and generation ($p=0.007$) on the global methylation levels of sperm samples across all cohorts. Post-hoc analyses showed that 8-week male had significantly higher levels of DNA methylation than 28-week males ($p<0.001$; see Figure 9), and F0 males had significantly higher levels of DNA methylation than F1 and F2 males ($p=0.007$). There was no significant main effect for condition (e.g. EX versus SED). There were also significant interaction effects for condition X generation ($p=0.026$) and condition X generation X age ($p=0.039$).

Based on follow-up pairwise comparisons, there were no significant differences were observed in global methylation levels of sperm DNA between EX and SED cohorts in F0 28 week, F1 28 week, or F2 8 week-old male cohorts. However, EX males had significantly higher levels of global DNA methylation than SED cohorts in F2 28 week-old males ($p<0.011$), whereas SED males had significantly higher levels of global DNA methylation than EX males in the F1 8-week cohort ($p=0.026$; Figure 6). Within just the EX groups, F0 EX males had significantly higher levels of global DNA methylation than F1 28 wk EX males ($p<0.012$) and F2

28 wk EX males ($p=0.024$) but not F1 8 week-old EX males (Figure 7). Similarly, F1 8-week old EX males had significantly higher levels of global DNA methylation than F1 28 week-old EX males ($p=0.004$) and F2 28 week-old EX males ($p=0.029$; Figure 7). F0 SED males were also found to have higher levels of global DNA methylation than age and intervention-matched cohorts in subsequent generations: significantly in F2 28 wk SED males ($p=0.009$), and with a tendency in F1 8 wk SED males ($p<0.055$; Figure 8). F1 8 week-old SED males had significantly higher levels of DNA methylation than F0 SED males ($p=0.018$), F1 28 wk SED males ($p<0.001$), and F2 28 wk SED males ($p<0.001$; Figure 8). The average global DNA methylation level of all 8 week-old males was found to be significantly higher than the average global DNA methylation level of all 28 week-old males ($p<0.001$; Figure 9).

Table 1: Anatomical characteristics of EX and SED cohorts across generations.

F0		Ex n=8	Sed n=10	
	Body Weight (g)	30.401±1.505	31.283±0.906	p=0.606
	Fat (g)	0.989±0.209	0.894±0.123	p=0.687
	Testicular mass (mg/g)	3.353±0.084	3.423±0.096	p=0.595
F1		Ex n=10	n=10	
	Body Weight (g)	28.625±0.338	29.461±0.519	p=0.194
	Fat (g)	0.501±0.031	0.585±0.075	p=0.317
	Testicular mass (mg/g)	3.501±0.099	3.161±0.164	***p=0.035***
F2		Ex n=10	Sed n=10	
	Body Weight (g)	28.188±0.584	28.586±0.596	p=0.43
	Fat (g)	0.544±0.057	0.518±0.043	p=0.742
	Testicular mass (g/g)	3.381±0.096	3.221±0.164	p=0.388
F0 Control		Ex n=9	Sed n=10	
	Body Weight (g)	28.385±0.497	30.051±0.739	p=0.072
	Fat (g)	0.428±0.055	0.757±0.096	***p=0.007***
	Testicular mass (mg/g)	3.600±0.069	3.251±0.096	***p=0.0048***

Values presented as means ± SEM***. Bold italics*** are significant with p-value < 0.05. Testicular mass measured in grams and normalized to body weight (g/g).

Table 2: Litter sizes and sex splits.

F1	Males/litter	4.750±0.796	2.600±0.400	
	Females/litter	1.630±0.42	3.700±0.473	
	Litter Size	5.101±0.473	6.310±0.597	p=0.927
	% Males	71.090±8.77	40.910±5.10	***p<0.007***
F2	Males/litter	2.857±0.670	2.000±0.723	
	Females/litter	2.480±0.612	3.000±0.535	
	Litter Size	5.286±0.819	5.000±0.483	p=0.820
	% Males	50.172±11.910	31.630±10.961	p=0.274

Values presented as means ± SEM***. Bold italics*** are significant with p-value < 0.05.

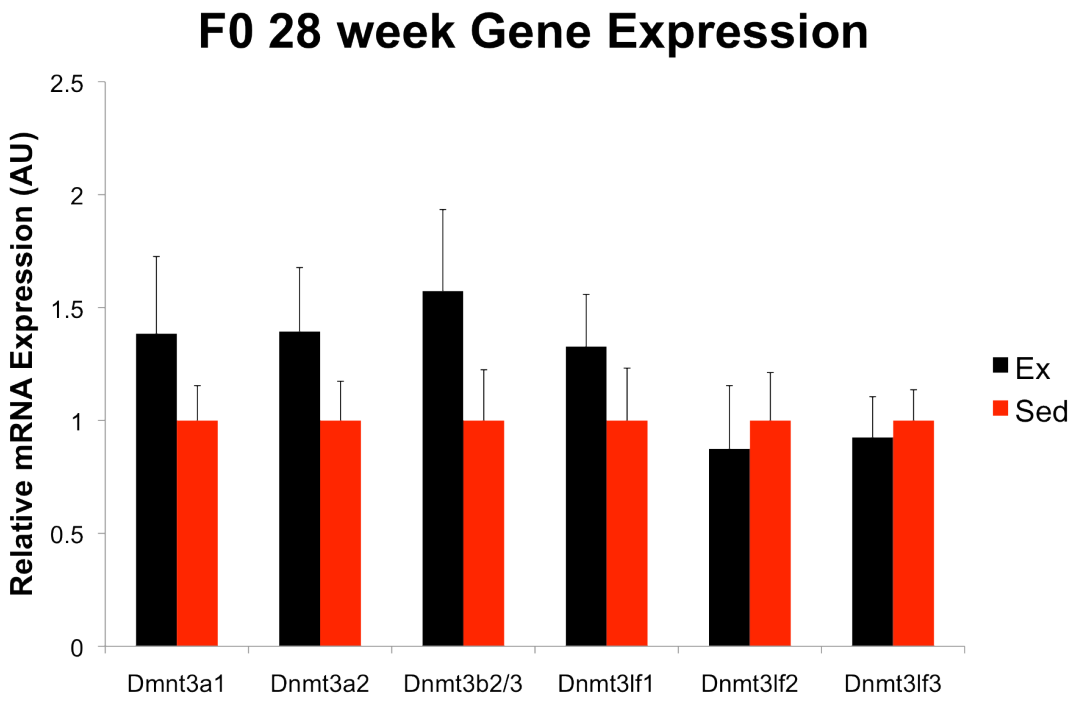


Figure 1 Gene expression data from testes of F0 Exercise and Sedentary 28 week-old cohorts relative to Gapdh. No statistically significant differences were seen through semi-quantitative PCR and gel electrophoresis.

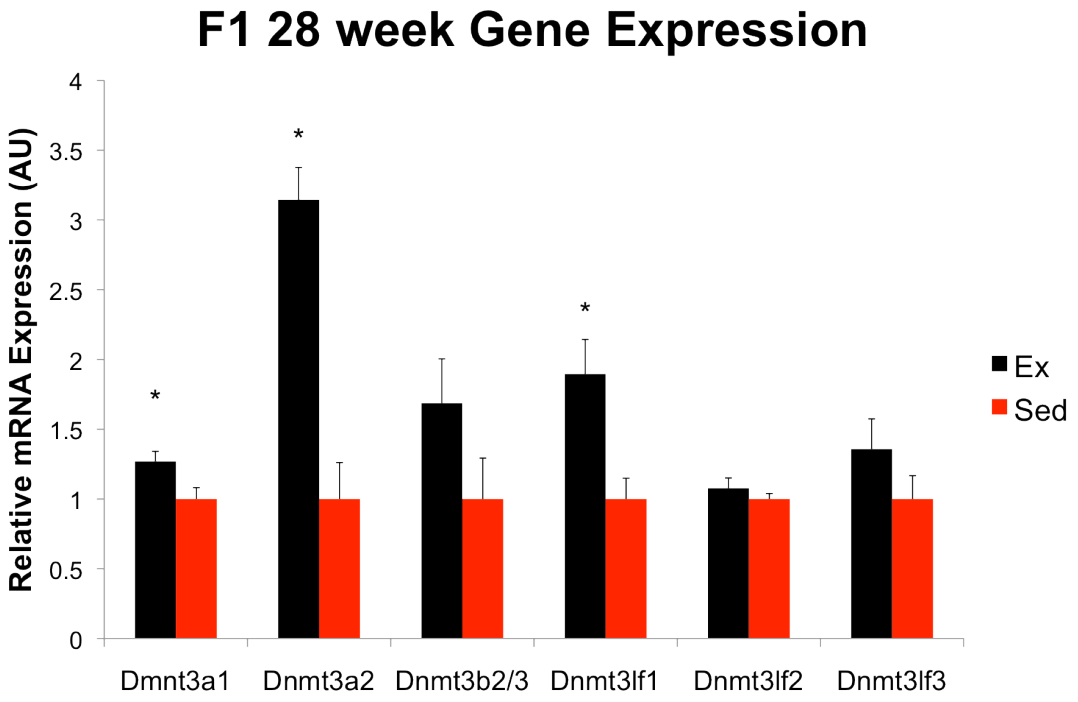


Figure 2. Gene expression data from testes of F1 Exercise and Sedentary 28 week cohorts relative to Gapdh. Significantly higher levels of expression were seen in males of exercise versus sedentary ancestry in Dnmt3a1 ($p=0.02$), Dnmt3a2 ($p<0.01$) and Dnmt3lf1 ($p<0.01$) genes as determined through semi-quantitative PCR and gel electrophoresis.
*Significantly different than adult male cohort of sedentary ancestry ($p<0.05$).

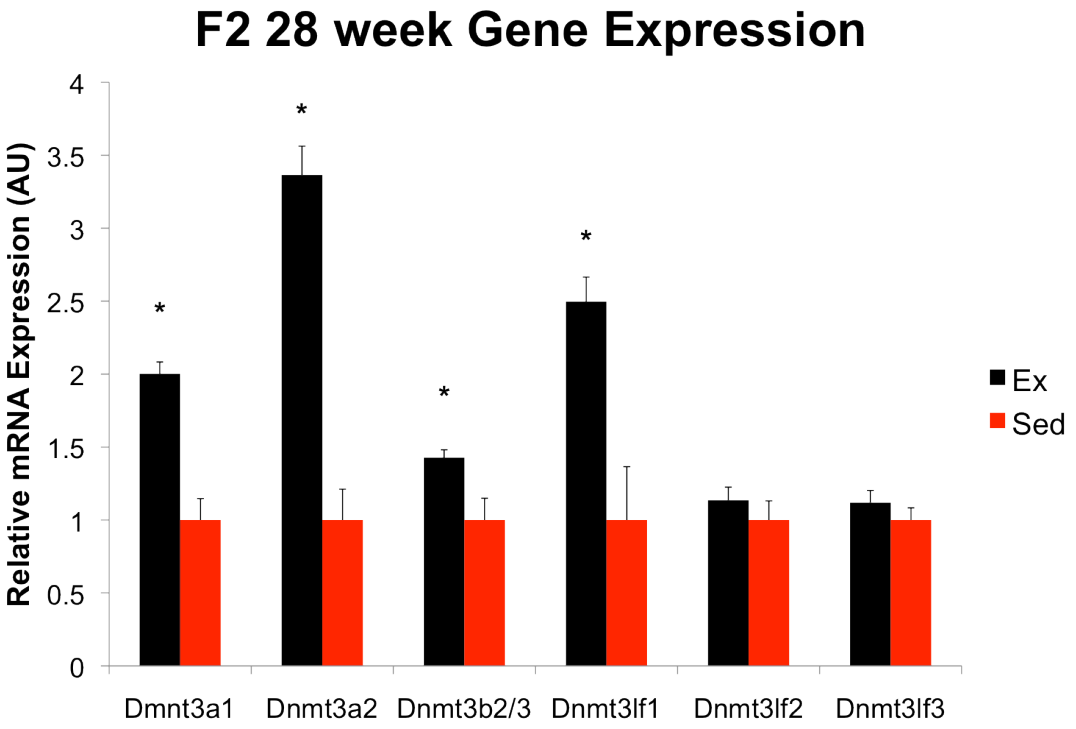


Figure 3. Gene expression data from testes of F2 Exercise and Sedentary 28 week cohorts relative to Gapdh. Significantly higher levels of expression were seen in males of exercise versus sedentary ancestry in Dnmt3a1 ($p<0.01$), Dnmt3a2 ($p<0.01$), Dnmt3b2/3 ($p=0.01$) and Dnmt3lf1 ($p<0.01$) genes as determined through semi-quantitative PCR and gel electrophoresis.
*Significantly different than adult male cohort of sedentary ancestry ($p<0.05$).

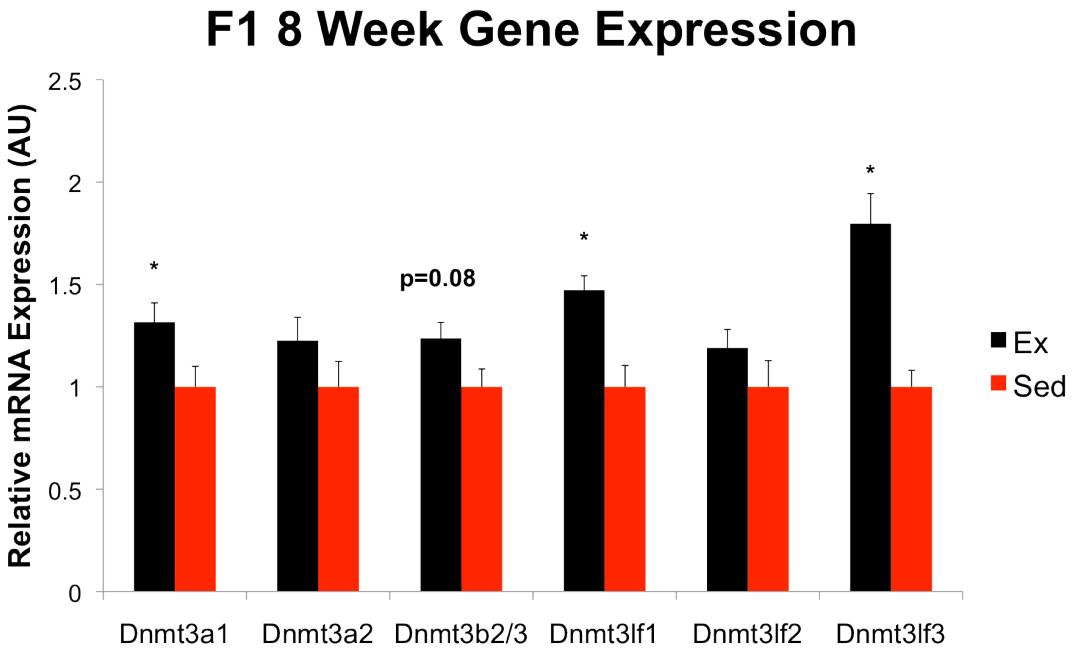


Figure 4. Gene expression data from testes of F1 Exercise and Sedentary 8 week cohorts relative to Gapdh. Significantly higher levels of expression were seen in males of exercise versus sedentary ancestry in Dnmt3a1 ($p=0.04$), Dnmt3lf1 ($p<0.01$), and Dnmt3lf3 ($p<0.01$) genes, and tended to be higher in Dnmt3b2/3 ($p=0.08$) as determined through semi-quantitative PCR and gel electrophoresis. *Significantly different than adult male cohort of sedentary ancestry ($p<0.05$).

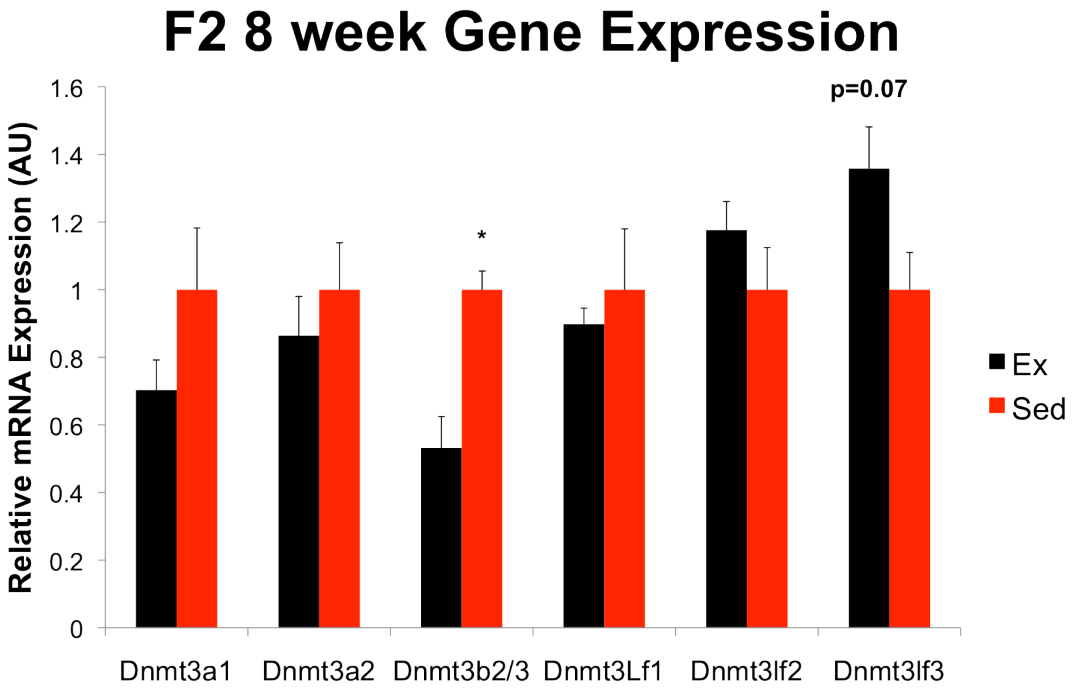


Figure 5. Gene expression data from testes of F2 Exercise and Sedentary 8 week cohorts relative to Gapdh. Levels of expression were seen to be significantly higher in males of sedentary versus exercise ancestry in Dnmt3b2/3 ($p=0.005$), but tended to be lower in Dnmt3lf3 ($p=0.07$) genes as determined through semi-quantitative PCR and gel electrophoresis.
*Significantly different than adult male cohort of sedentary ancestry ($p<0.05$).

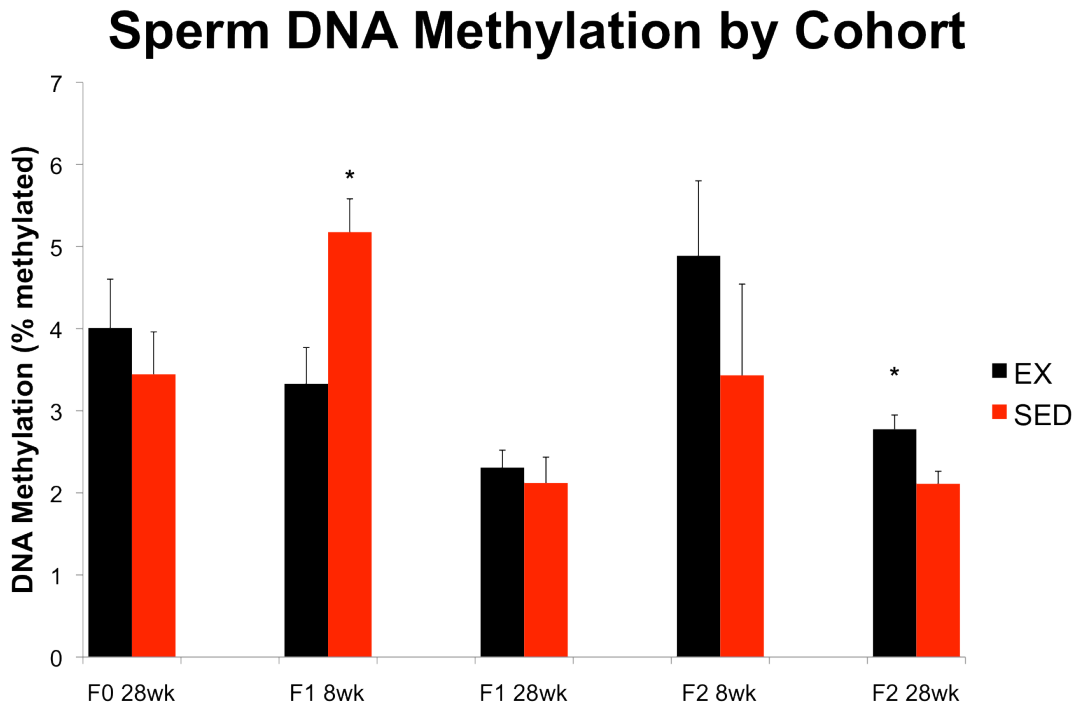


Figure 6. Pairwise comparisons of global DNA methylation levels in sperm samples of all EX versus SED cohorts within each generation. Global methylation levels were significantly higher in the EX cohort than the SED cohort in the F2 generation ($p=0.034$), but higher in the SED cohort than the EX cohort in F1 8 week-old mice ($p<0.001$).
*Significantly different between EX and SED cohorts of the same age and generation cohort ($p<0.05$).

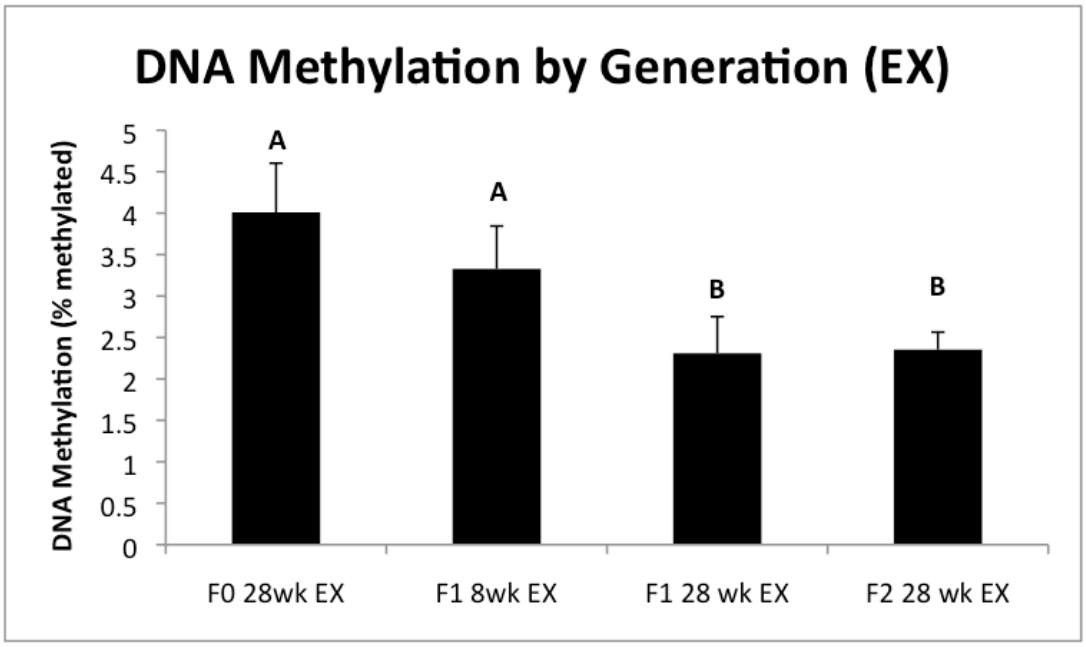


Figure 7. Comparisons of global DNA methylation of sperm samples in EX cohorts across generations. F0 28 wk EX males had significantly higher levels of DNA methylation than F1 28wk EX males ($p=0.012$) and F2 28wk EX males ($p=0.024$); F1 8wk males had significantly higher levels of DNA methylation than F1 28 wk EX males ($p=0.004$) and F2 28 wk EX males ($p=0.029$). Sample sizes in F2 8wk males were too small to provide reliable comparisons.
Letter differences indicate significant differences between cohorts ($p<0.05$).

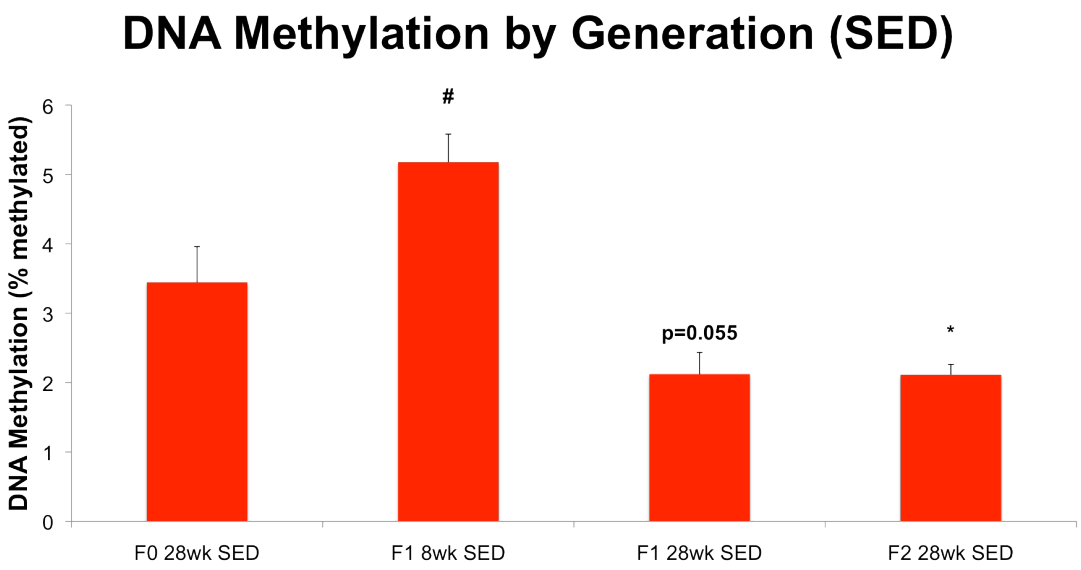


Figure 8. Comparisons of global DNA methylation of sperm samples in SED cohorts across generations. Males had significantly lower levels of DNA methylation than F0 SED males in the F2 SED cohort ($p=0.009$) and tended to be lower in the F1 SED cohort ($p=0.055$). F1 8 week-old males had significantly higher levels of DNA methylation than F1 SED males ($p<0.001$) and F2 SED males ($p<0.001$). Sample sizes in F2 8wk males were too small to provide reliable comparisons.
*Significantly different from F0 28wk SED cohort ($p<0.05$).
Significantly different from all 28wk SED cohorts ($p<0.05$).

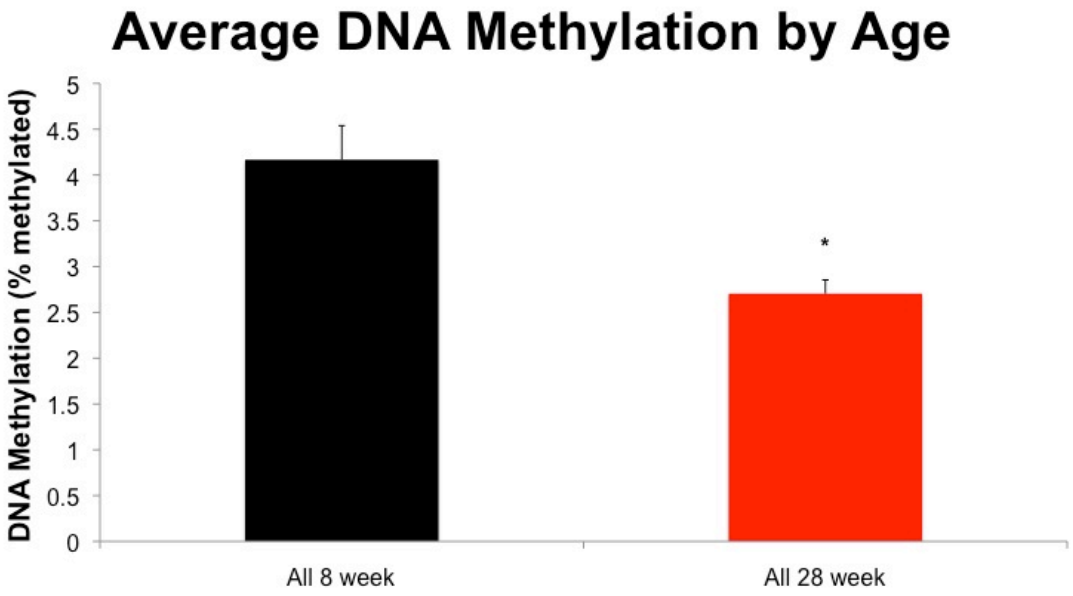


Figure 9. Comparisons of global DNA methylation sperm samples averaged across EX and SED cohorts of all generations, sorted by age. Males aged 28 weeks had significantly lower levels of DNA methylation than 8 week-old males ($p<0.001$). *Significantly different between 8 and 28 week cohorts ($p<0.05$).

Chapter 5: Discussion

This study is the first to assess the effects of chronic exercise on epigenetic factors of an organism in a heritable fashion across multiple generations. Furthermore, it is the first study to assess the effects of chronic exercise on portions of the epigenetic machinery of the male mammalian testis. Our data indicate that while chronic exercise may not ostensibly cause significant epigenetic changes in the mammalian testis in the exposed generation, chronic exercise does appear to significantly alter gene expression of *de novo* methylation genes in subsequent generations (F1 and F2) of mice offspring. What is more, we found that this significant difference in mRNA levels of *Dnmt3* family genes tied to exercise versus sedentary ancestry correlated with the significant differences in global DNA methylation levels of the sperm. Our results indicate that given where these significant differences appear in subsequent generations, that the effect of chronic exercise on multigenerational epigenetic factors is maternal environment-based, as discussed below.

A primary focus of this study was that of the epigenetic effect that chronic voluntary exercise has upon an organism and its offspring. Naturally, in order to study the effect on offspring, it is imperative to assess the effect of the environmental intervention not only on the in-utero environment of the developing organism, but also on the gamete-producing regions that spawned it. Furthermore, it is crucial to understand how the intervention – here, chronic exercise – affects the development of the gamete-producing organs in the pre-natal, neonatal and pre-pubertal life stages, as this undoubtedly affects their function at the time of maturation. While the present

study was not able to precisely address these questions, previous studies that have can play a valuable role in contextualizing the results of the present study.

The planned outcomes of this study were two-fold: 1) to gauge how chronic exercise can affect DNA methylation in multiple generations on a whole genomic – or global – scale; 2) to assess how exercise and sedentary activity or ancestry affected the expression of key genes involved in the *de novo* addition of DNA methylation markings. For the DNA methylation aspect, our data show a significantly higher level of global DNA methylation levels in the sperm of 28 week-old male mice of exercise ancestry than counterparts of sedentary ancestry in the F2 generation – two generations removed from the original exercising cohort. This seems to suggest an effect of exercise that was especially prominent in the ova of F1 females of exercise ancestry - as all ova were all formed in the *in utero* environment of an exercising F0 mother, and when copulated with the F0 male offspring of exercise ancestry (who through upregulation of various *Dnmt3* mRNA transcripts) were ostensibly affected by their exercise stimulus may have been sufficient to foster a significant difference in the global DNA methylation of their offspring versus their sedentary counterparts. Additionally, our results show that 8 week-old males of sedentary ancestry in the F1 generation had significantly higher levels of global DNA methylation than males of exercise ancestry. Though no literature exists supporting a physiological rationale behind this finding, it certainly merits further study. However, this fact coupled with the fact that this was not observed in age-matched males in the F2 generation and that by 28 weeks, these same cohorts showed no significant differences in global methylation suggests the possibility that this finding was an artifact attributable to a

sample size smaller than other cohorts measured in this facet of the study. This small sample size in F2 mice was likely due to the comparatively small number of F1 EX females born to F0 EX mothers, which in turn led to an overall smaller F2 EX cohort. Finally, our results indicated a significant effect of age and generation on methylation wherein 8-week old males had significantly higher levels of global methylation than 28 week-old males, and that 28 week-old males in generations following the F0 generation – suggestion a potential demethylating stress-based effect of the altered pre- and or post-natal environment to which mice from both EX and SED cohorts bred in generations after F0 were exposed. These practices were likely different than the husbandry practices of Jackson Laboratories, where the mice in the present study were purchased, and due to the consistency of our husbandry practices between cohorts, may explain the consistently lower global DNA methylation levels in both EX and SED 28-week cohorts in F1 and F2 generations versus their F0 counterparts.

With regard to our analysis of key methylation-related genes, our results show a significant upregulation in mRNA transcript expression across a number of genes governing de novo methylation of chromatin in the testes of EX versus SED cohorts of 28 week old mice of the F1 and F2 generations: *Dnmt3a1*, *Dnmt3a2* and *Dnmt3lf1* higher in the EX F1 cohort; *Dnmt3a1*, *Dnmt3a2*, *Dnmt3b2/3* and *Dnmt3lf1* higher in the EX F2 cohort. This effect was also seen in the F1 8 week old animals: *Dnmt3a1*, *Dnmt3lf1* and *Dnmt3lf3* higher in the EX F1 8 week group (with *Dnmt3b2/3* approaching significance – $p=0.08$); and *Dnmt3b2/3* higher in the EX F2 8 week group (with *Dnmt3lf3* approaching significance – $p=0.07$). In contrast, the F0 generation – which was directly exposed to the chronic exercise wheel running –

exhibited no significant differences in testicular mRNA expression of *Dnmt3* family genes. As a result, this suggests one of two possibilities: first, that the effect of voluntary wheel running as recorded was unable to induce an epigenetic impact on male mice that resulted in *de novo* epigenetic changes in the testis. The second, and less likely possibility, is that the wheel running exposure indeed was able to cause such a methylation-based effect in the male testis, but the effect was so ephemeral it was not detectable at the time of sacrifice – approximately 48 hours after losing access to a running wheel. This possibility is less likely, since significant epigenetic differences were found to be detectable in subsequent generations – including *Dnmt3* mRNA expression (F1 & F2) and DNA methylation (F2) - that were not ever exposed to running wheels. Therefore, this suggests any epigenetic effects that came as a result of running wheel exposure were not paternally derived in the F0 generation, and thus, came from the maternal line.

A large number of studies have provided evidence of a marked effect of exercise during pregnancy on the maternal environment of prenatal offspring and the phenotypes that these effects yield postnatally. For example, one study has shown that when diabetic mice exercise during pregnancy, they produce more viable offspring with higher insulin sensitivity than diabetic mice that do not exercise while pregnant.[15] Additionally, mice who engage in voluntary wheel running – the same exercise mode used in the present study – when pregnant, have been shown to produce offspring with enhanced spatial memory capabilities.[52] Our data – particularly the sex ratios and significant mRNA expression of *Dnmt3* genes in F1 -

demonstrate yet another example by which the *in utero* effect of exercise can impose a significant phenotypic change in offspring.

The scope of the present study aimed to examine any potential paternal-line contributions to the propagation of exercise-induced epigenetic changes to an organism's progeny. This becomes relevant to the maternal environment in the way that the maternal environment affects the developmental trajectory of the nascent organism – and more specifically, its gonadal tissues. In mammalian development, the testes and ovaries develop from a group of non-sex specific pluripotent cells that form the genital ridge. The development into testes or ovaries hinges on the expression of the *Sry* gene prior to 11.5 days post-coitum. In its presence, it sets off a biomolecular cascade, resulting in the differentiation of these proto-gonadal cells into Sertoli cells and later fully-formed testes; in its absence they develop into ovaries.[53] These Sertoli cells are then arranged in tubular arrays surrounding newly-formed seminiferous tubules, a process that occurs early in embryonic development.[54] It has also been demonstrated that Sertoli cells proliferate during fetal and neonatal development stages as well as just prior to puberty[49], illustrating the impact the maternal environment has upon its progeny and paternal links to future generations. Applied to the context of the present study, exercising versus sedentary F0 mothers likely provided drastically different environments for development in the fetal and neonatal life periods of their progeny. This factor alone may help account for the many differences viewed in testicular *Dnmt3* mRNA gene expression and global DNA methylation levels between exercising and sedentary ancestry cohorts, most markedly in the F2 generation. The differences would arguably be particularly

profound in the F2 generation, as they were the byproduct of sperm formed by Sertoli cells fostered under drastically different metabolic in-utero conditions (i.e. F0, in which mothers exercised throughout pregnancy).

This possibility, in short, suggests that epigenetic effects in multiple generations such as those indicated in this study are a spermatogenesis-derived issue moderated by the state of the Sertoli cells, as dictated by the in-utero environment in which they developed. The in-utero environment thusly causes a change that is inherent to the offspring and may not be rescueable by lifestyle interventions.[49,50] One example of this involves in-utero exposure of nascent offspring to testosterone, which has been shown in previous studies to be one of the most crucial factors in mediating Sertoli cell proliferation in the fetal and neonatal periods.[49] This possibility becomes all the more relevant when considering that studies in which rats who engaged in a forced swim test tended to have higher levels of endogenous testosterone compared to sedentary rats.[55] Additionally, further evidence exists in humans showing that both high intensity exercise[56] and endurance-type exercise [57] increases serum testosterone, even in females. Therefore, it is possible that F0 EX females had different levels of endogenous testosterone resulting in significant differences in Sertoli cell development in F1 males *in utero*, resulting in changes in the F2 generation. Though this conclusion is impossible to directly address given the constraints of the present study, a future study with the same parameters that compared endogenous androgen levels in EX versus SED F0 females when paralleled with qualitative and quantitative assessments of the cross-sections of F1 testes might help corroborate such a hypothesis. Such a physiological perturbation may have also played a role in the

significantly higher ratio of males to females in the F1 generation. Evidence has previously shown that environmental stressors can significantly affect sex ratios of offspring in mammals.[58] In particular, significantly higher proportions of male offspring were produced when circulating levels of serum testosterone were elevated in females during the preovulatory and perifertilization periods of bovine mammals.[59] If this same effect is conserved across other mammalian species, this effect – in addition to explaining differences in F1 Sertoli cell differences - would explain the significantly higher number of males in the F1 generation from EX cohort parents versus SED counterparts. Due to the likelihood that exercise-induced increases in serum testosterone in F0 EX dams would not be present in the sedentary F1 EX dams, a significantly higher ratio of male to female offspring would be expected in the F1 but not in the F2 generation. Furthermore, evidence exists showing the role of environmental conditions that induce emotional stress such as overcrowding[60] in the periconceptional period can affect sex ratios of offspring, preferentially favoring female offspring.[61] Our results, showing significantly more males per litter than females born to mothers who exercised in the periconceptional period, suggests that pregnant dams were not as emotionally stressed as the mothers who were confined to cages not equipped with running wheels. This, in turn, may explain the more equal ratios of males to females observed in the subsequent (F2) generation. We are aware of no literature examining the role of exercise on sex ratios at birth in multiple generations.

The direct exposure effect to which the developing F1 generation offspring were exposed via different *in utero* metabolic exposures may have resulted in

significant differences in *Dnmt3* family expression transmittable to the F2 generation – but it did not appear to affect global methylation significantly between intervention cohorts. This fact was demonstrated in that no significant differences were observed in global DNA methylation in sperm between EX and SED cohorts in the F0 or F1 generation at the 28-week post-copulation time point. Significant differences at this time point were observed, however, between EX and SED cohorts in the F2 generation – wherein the EX cohort was observed to have a higher percentage of methylated DNA. Additionally, while F2 EX males had expressed significantly higher levels of mRNA transcripts from four different *Dnmt3* family genes than SED counterparts, F0 EX males had none. In contrast, three different *Dnmt3* family genes were expressed at significantly higher levels by F1 EX versus SED males, however between them there was no significant difference in global DNA methylation. This result seems to imply that though there were no differences in global methylation at 28 weeks seen in F0 caused by the wheel running exposure, it may have been able to induce significant change on the male germ line in exercisers through mechanisms other than those moderated by *Dnmt3* gene expression - though not males exposed *in utero* (e.g. F1 males). As for the methylation differences in F2 male sperm the interpretation becomes more difficult. One possible interpretation is that the epigenetic mechanism driving the increase in global methylation in F2 EX males is derived from epigenetic alterations to the germ line of the F1 EX females, who themselves were directly exposed *in utero* to the wheel running intervention and due to the focus on males and male tissues of the present study, remain uninvestigated.

A further aim of the present study was to investigate the heritability of phenotypes to illustrate the legacy of a metabolic intervention such as chronic wheel running or sedentary lifestyle. Our results demonstrated similar significant differences in gene expression in F1 and F2 generations, lending credence to this possibility. However this effect was only partially seen across multiple generations in DNA methylation of sperm, such that only F0 and F2 generations showed higher levels of methylation in EX male sperm. Although a number of previous studies have demonstrated significant methylation-based epigenetic heritability between generations in humans at a gene-specific level[62] and across the genome,[63] little work has been done to suggest consistent patterns of epigenetic heritability on a global scale in animals.

A landmark 1987 study by Wilson et al. demonstrated a significant decrease in percentage of methylated DNA with age across a number of tissues in two different mammalian species - including C57Bl/6 mice.[64] We observed this finding in the present study, as all 8-week-old cohorts save one (F2 8 week SED) showed significantly higher levels of DNA methylation in sperm than their generation-matched 28 week-old counterparts. The small sample size in the F2 8 week SED cohort (n=4) may have contributed to the lack of a significant difference between this cohort and its 28 week-old counterpart, though the data showed the beginnings of a similar trend. This said, Talens et al.[65] showed the temporal stability of DNA methylation markings in identical tissues by showing a high correlation of methylation levels across certain gene promoter sites in samples taken from humans 11 to 20 years apart.[65] Nevertheless, these samples were taken across a more

heterogeneous population – aged 14 to 71, 50% male, most unrelated to one another – and buccal cells were taken. In contrast, our samples were taken from a considerably more homogeneous cohort – all male, same ages – and from spermatocytes. Additionally, the flux in DNA methylation as part of the aging process may differ by species, and it is difficult to compare differences in aging between the 11-20 years in humans from the aforementioned study and the 20 weeks of aging in the present study. Regardless, this is likely the first evidence of physical activity as an environmental stimulus that affects the epigenetic machinery of the male germ line and appears to be transmittable across generations.

Limitations

There exist many current hypotheses with regards to factors that can affect the epigenetic status of offspring. Among these are aforementioned maternal-based influences including maternal *in utero* environment[5], post-natal grooming[6] and lactation[7], which have all been shown to trigger epigenetic differences in rodents. This study aimed to control for these factors, but we cannot assert with supreme confidence that every fetus received the same nutrients *in utero*, suckled identically during lactation, or was groomed equally by their mother.

Additionally, there are a number of factors within the sperm itself that may explain epigenetic contributions to offspring that cannot be accounted for in the present study. Small amounts of RNA are present in the cytoplasm of spermatocytes, and the contribution of this cytoplasm/RNA to the zygote is proposed to have epigenetically-driven phenotypic effects[66,67] that at this juncture are not detectable using the assays proposed – and even more speculative to which to infer causality.

Secondary structure in the chromatin itself via the maintenance or elimination of nucleosomes and the epigenetic alterations of them provide a clear site of interest when looking at factors affecting gene expression both in the spermatocyte itself and in the offspring it forms.[68] This too can neither be accounted for in our experimental methods, nor in existing literature. Finally, there is evidence that different genes are expressed at different levels in spermatocytes during different stages of their progression through the reproductive tract – namely the seminiferous tubules in the testes, the caudal epididymis and ductus deferens[69] and that these are likely caused through methylation differences. As the samples collected contain spermatocytes from both the ductus deferens and the caudal epididymis, there is a possibility that there will be a difference in methylation between the spermatocytes in our heterogeneous sample; however, no literature currently exists to support this possibility.

Lastly, one major limitation of the study lies in the plate-to-plate comparability of the MethylFlash kit utilized to measure global DNA methylation in sperm. The 96-well plates were laid out such that samples from F2 28 week EX and SED as well as 8 week EX and SED cohorts could all be analyzed on the same plate to minimize variability in the comparison of treatment groups and age groups. However, the downside to this layout method is that the variability inherent with different plates being prepared and analyzed on two separate occasions makes it statistically difficult to compare groups from different plates. The normalization of absorbance readings to the standard curve on each plate will normalize each sample to control for possible interplate variability; nevertheless, a more statistically

appropriate way of laying out these plates would have been to assign samples randomly across all plates to spread out the interplate measurement variability across all sample cohorts. Or, conversely, where possible it would have been advantageous to run other plates with the two groups being compared on the same plate. This would be done in lieu making the comparisons across plates as was done in this study.

Chapter 6: Conclusions and Future Directions

Conclusions

The present study was an exploratory study aiming to identify the potential for chronic exercise to alter mouse sperm methylation patterns and determine whether those patterns are transmitted to subsequent generations. In that sense this project was hypothesis-generating and provides information for future well-controlled and mechanistic studies. That said, the results of the present study show for the first time that chronic exercise, as represented by voluntary wheel running, has the ability to significantly affect levels of *de novo Dnmt* genes at the mRNA level in the testes of mice generations after the intervention itself. Furthermore, our novel data show that this effect coincided with significant differences in DNA methylation in sperm in generations following an exercise intervention.

The present study aimed to identify differences in global methylation levels in DNA from sperm obtained from EX and SED ancestry mouse cohorts, and we hypothesized that there would be a significant difference between the cohorts. Based on the significant differences we have observed, most markedly in the F2 28 week old mice where EX cohorts had significantly more methylated DNA than their SED counterparts, we cannot reject our hypothesis and thus infer that chronic exercise via wheel running has the ability to induce epigenetic change in the male germ line, even if not patently detectable in the exercising F0 organism.

Secondly, we hypothesized that between EX and SED cohorts we would detect significant differences in mRNA expression in one or more genes responsible for *de novo* DNA methylation. Having seen significantly higher expression rates in

isoforms of *Dnmt3a* and *Dnmt3l* in EX cohorts of both generations following F0, as well as *Dnmt3b2/3* isoforms significantly higher in F2 and approaching significance in F1 (p=0.13), we cannot reject our hypotheses and thus infer that differences in global DNA methylation (such as those seen in F2) may have been engendered by upregulation of certain *Dnmt3* isoforms.

Lastly, we hypothesized that in the present study we would view a phenotypic difference that would be conserved in multiple generations of offspring as a result of exercise or sedentary ancestry. From the mRNA gene expression measures taken, we have observed significantly higher expression of *Dnmt3a1, Dnmt3a2* and *Dnmt3lf1* in EX versus SED cohorts in F1 that was recapitulated in F2, suggesting an inherited epigenetic phenotype resulting from the running wheel exposure.

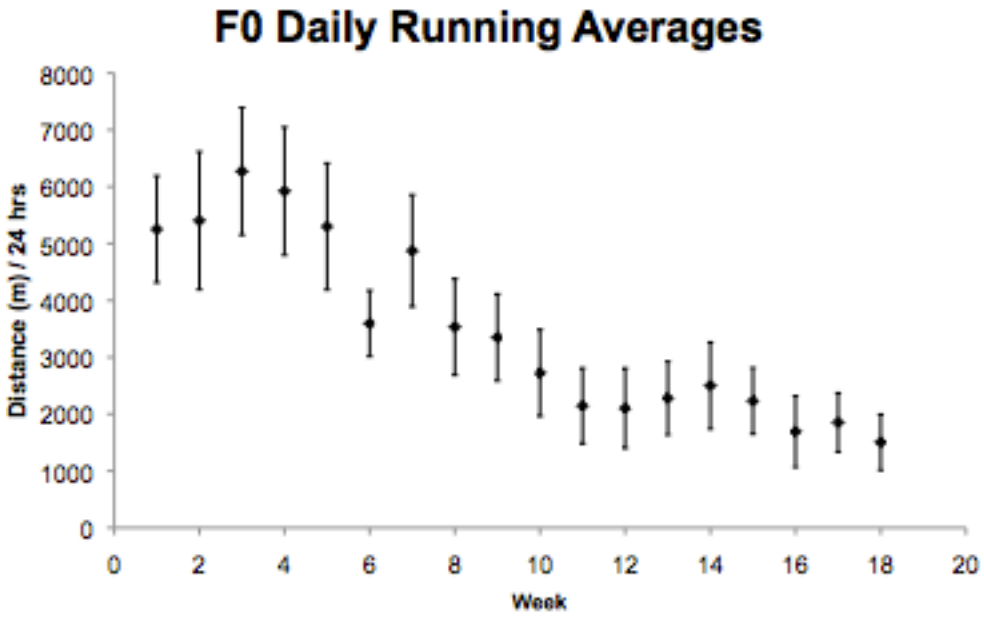
Future Directions

Follow up studies will be required to elucidate possible mechanisms driving these changes in *Dnmt* gene expression and/or DNA methylation patterns. For example, in these prospective studies we would maintain the same breeding and intervention paradigm to replicate those of this present study, however we would add an F3 generation to address unequivocally the role of paternal-line contributions to epigenetic phenotypes. This is possible because in studies that only go as far as the F2 generation – such as the present study – a paternal-line effect is not possible to isolate, as the F2 generation is still affected by the maternal environment (albeit indirectly) in that the F2 generation spawns from F1 ova, which are fully formed in F0 *in utero* conditions. By collecting data from the F3 generation, the paternal line can be explicitly studied by excluding the F0 maternal environmental effect. If

possible we would also measure serum testosterone levels in all breeding females prior to copulation, and in the offspring upon sacrifice we would image stained cross-sections of testes to qualitatively assess Sertoli cell size, number and functionality. Moreover, protein level and protein activity would be measured for *de novo* methylation by Dnmt proteins, as well as potentially in DNA demethylating proteins and proteins involved in maintenance of DNA methylation markings (e.g., TET proteins). When measuring global DNA methylation, we would use a block study-style layout, randomly assigning samples to each plate to mitigate potential interplate variance effects. Finally, we would look to link the methylation differences with *Dnmt* expression by using bisulfite treatment to measure methylation at the upstream promoter sites of genes that affect DNA methylation.

By doing the aforementioned, we should understand better how the epigenetics of the exercise intervention works from a more statistically sound measurement of global DNA methylation, to the effect of methylation at specific gene loci associated with epigenetic alteration. These results could be paralleled with mRNA expression of those genes, and subsequent protein quantity and activity assays that would help explain phenotypic differences observed between exercising and sedentary mice and their progeny. The final result of such studies would hopefully afford a glimpse at the elegant mechanisms physiologically orchestrated from one generation onto its progeny as a result of chronic physical activity.

APPENDIX A –Running Distances



Supplemental Figure 1. Daily running distance averages in F0 EX adult male C57Bl/6J mice. Week 0 of exposure to running wheel occurred at 8 weeks of age.

APPENDIX B – RT-PCR Primers and Conditions

Primers

All primers listed 5'-3'

Dnmt3a1: Forward – CCCAATGGAGACTTGGAGAA
Reverse - CGTTTCCGTTTGCTGATGTA

Dnmt3a2: Forward – CCAGACGGGCAGCTATTTAC
Reverse - AGAACTTGCCATCTCCGAAC

Dnmt3b2/3: Forward - GGCTTCAAGCCTACTGGGATCGAG
Reverse – CCACAGGACAAACAGCGGTCTTCC

Dnmt3lf1: Forward - TTATACAGACAATTGGGATGTTGG
Reverse – CATAAACAATCCCCACTTAGATCA

Dnmt3lf2: Forward - AGGTGAACATCAGATATCAGGGCT
Reverse – ACCCAGACAGAGCAACTCT

Dnmt3lf3: Forward - ATCCGGTGGAACTGGAACAT
Reverse – GAACATCAGATATCAGGTGGAGAA

Optimized PCR Conditions by Gene

Note: 1 µL cDNA is used per reaction for all 25 µL reactions

Gene	Component	Volume per Sample (µL)
Dnmt3a1	β-mercaptoethanol Buffer	2.5
	dNTP (1.25nM)	4
	Forward Primer (0.01nm/µL)	0.5
	Reverse Primer (0.01nm/µL)	0.5
	Taq Polymerase (5 units/mL)	0.2
	Ultrapure Water	16.3

Annealing Temperature:	52°C
Annealing Time	60 seconds
Cycle Number	29 cycles

Dnmt3a2	β-mercaptoethanol Buffer	2.5
	dNTP (1.25nM)	4
	Forward Primer (0.01nm/μL)	0.5
	Reverse Primer (0.01nm/μL)	0.5
	Taq Polymerase (5 units/mL)	0.2
	Ultrapure Water	16.3

Annealing Temperature:	55°C
Annealing Time	60 seconds
Cycle Number	30 cycles

Dnmt3b2/3	Ammonium Sulfate Buffer	2.5
	dNTP (1.25nM)	4
	Magnesium Chloride (25mM)	1.5
	Forward Primer (0.01nm/μL)	0.4
	Reverse Primer (0.01nm/μL)	0.4
	Taq Polymerase (5 units/mL)	0.2
	Ultrapure Water	15

Annealing Temperature:	60°C
Annealing Time	60 seconds
Cycle Number	30 cycle

Dnmt3lf1	Ammonium Sulfate Buffer	2.5
	dNTP (1.25nM)	4
	Magnesium Chloride (25mM)	1.5
	Forward Primer (0.01nm/μL)	0.5
	Reverse Primer (0.01nm/μL)	0.5
	Taq Polymerase (5 units/mL)	0.2
	Dimethyl Sulfoxide	2.5
	Ultrapure Water	12.3

Annealing Temperature:	51°C
Annealing Time	60 seconds
Cycle Number	29 cycles

Dnmt3lf2	Ammonium Sulfate Buffer	2.5
	dNTP (1.25nM)	4
	Magnesium Chloride (25mM)	1.5
	Forward Primer (0.01nm/µL)	0.5
	Reverse Primer (0.01nm/µL)	0.5
	Taq Polymerase (5 units/mL)	0.2
	Ultrapure Water	14.8

Annealing Temperature:	56°C
Annealing Time	30 seconds
Cycle Number	28 cycles

Dnmt3lf3	β-mercaptoethanol Buffer	2.5
	dNTP (1.25nM)	4
	Forward Primer (0.01nm/µL)	0.5
	Reverse Primer (0.01nm/µL)	0.5
	Taq Polymerase (5 units/mL)	0.2
	Ultrapure Water	16.3

Annealing Temperature:	53.5°C
Annealing Time	30 seconds
Cycle Number	31 cycles

APPENDIX C – ANOVA Results for DNA Methylation

Key

Condition	0 – EX	1 - SED		
Generation:	0 – F0	1 – F1	2 – F2	3 – F0C
Age:	0 – 8 week	1 – 28 week		

The SAS System

The GLM Procedure
Least Squares Means

Condition	Generation	Age	Methylation Prct LSMEAN	LSMEAN Number
0	0	1	3.44210929	1
0	1	0	3.32769374	2
0	1	1	2.50879751	3
0	2	0	4.88944306	4
0	2	1	2.68794378	5
0	3	1	2.92963753	6
1	0	1	4.05906497	7
1	1	0	5.17648620	8
1	1	1	2.49205072	9
1	2	0	3.43169853	10
1	2	1	2.13862607	11
1	3	1	3.02490505	12

Least Squares Means for effect Conditi*Generati*Age
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: MethylationPrct

i/j	1	2	3	4	5	6
1		0.8450	0.0937	0.0348	0.1859	0.4490
2	0.8450		0.1644	0.0288	0.2876	0.5718
3	0.0937	0.1644		0.0007	0.7520	0.5339
4	0.0348	0.0288	0.0007		0.0019	0.0138
5	0.1859	0.2876	0.7520	0.0019		0.7255
6	0.4490	0.5718	0.5339	0.0138	0.7255	
7	0.3118	0.2540	0.0125	0.2524	0.0299	0.1208
8	0.0078	0.0068	<.0001	0.7009	0.0003	0.0035
9	0.0810	0.1477	0.9752	0.0005	0.7240	0.5114
10	0.9886	0.8905	0.2084	0.0812	0.3173	0.5445
11	0.0237	0.0501	0.5143	0.0001	0.3463	0.2524
12	0.5374	0.6670	0.4458	0.0189	0.6246	0.9028

Least Squares Means for effect Conditi*Generati*Age
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: MethylationPrct

i/j	7	8	9	10	11	12
1	0.3118	0.0078	0.0810	0.9886	0.0237	0.5374
2	0.2540	0.0068	0.1477	0.8905	0.0501	0.6670
3	0.0125	<.0001	0.9752	0.2084	0.5143	0.4458
4	0.2524	0.7009	0.0005	0.0812	0.0001	0.0189
5	0.0299	0.0003	0.7240	0.3173	0.3463	0.6246

The SAS System
The GLM Procedure

Class Level Information

Class	Levels	Values
Condition	2	0 1
Generation	4	0 1 2 3
Age	2	0 1

Number of Observations Read 89
Number of Observations Used 89

The SAS System

The GLM Procedure

Dependent Variable: MethylationPrct MethylationPrct

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	67.1135291	6.1012299	4.04	0.0001
Error	77	116.4277816	1.5120491		
Corrected Total	88	183.5413107			

R-Square	Coeff Var	Root MSE	MethylationPrct Mean
0.365659	38.48055	1.229654	3.195521

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Condition	1	0.02686976	0.02686976	0.02	0.8943
Generation	3	5.68573897	1.89524632	1.25	0.2963
Age	1	42.36748632	42.36748632	28.02	<.0001
Condition*Generation	3	10.56430557	3.52143519	2.33	0.0810
Condition*Age	1	1.77443403	1.77443403	1.17	0.2821
Generation*Age	1	0.03128843	0.03128843	0.02	0.8860
Conditi*Generati*Age	1	6.66340598	6.66340598	4.41	0.0391

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Condition	1	0.48854893	0.48854893	0.32	0.5714
Generation	3	19.54218528	6.51406176	4.31	0.0073
Age	1	42.40625065	42.40625065	28.05	<.0001
Condition*Generation	3	14.85298864	4.95099621	3.27	0.0255
Condition*Age	1	0.79326794	0.79326794	0.52	0.4711
Generation*Age	1	0.00006645	0.00006645	0.00	0.9947
Conditi*Generati*Age	1	6.66340598	6.66340598	4.41	0.0391

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September 27, 2011

Dr. Stephen Roth
Kinesiology
University of Maryland
sroth1@umd.edu

Dr. Roth,

This letter is to inform you that on **September 22, 2011** the members of the Institutional Animal Care & Use Committee (IACUC) reviewed and approved the annual review for the protocol:

Role of Maternal Exercise Environment on Transgenerational Offspring Health

R-10-93

Please note that an approved protocol is valid for three (3) years unless there is a change in the protocol. Thus, this protocol is valid until **November 18, 2013**. Federal laws indicate that protocols must be reviewed yearly. You must submit your next annual review in November, 2012. All work extending beyond the approval date of the protocol must be submitted to the IACUC as a new protocol.

Sincerely,

W. Ray Stricklin
IACUC Chair

CC: Doug Powell, Amanda Underwood

Glossary

DNMT	DNA Methyltransferase protein (human)
DNMT	DNA Methyltransferase gene (human)
Dnmt	DNA Methyltransferase protein (mouse)
Dnmt	DNA Methyltransferase gene (mouse)
EX	Exercising or Exercise Ancestry cohort
SED	Sedentary or Sedentary Ancestry cohort
F0	First generation of animals exposed to Exercise or Sedentary lifestyle intervention
F1	Second generation; offspring of F0 cohorts
F2	Third generation; offspring of F1 cohorts
F0C	F0 Control; mice purchased and raised in identical conditions to F0 generation but did not breed
AID	Activation-Induced cytosine Deaminase
BER	Base-Excision Repair
PCR	Polymerase Chain Reaction
SAM	S-Adenosyl Methionine, a methyl donor compound
TET	Proteins that hydroxymethylate cytosines; thought to be involved in active demethylation of chromatin

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