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Genetic Background Influences Behavior and Responses to Epigenetic Changes Induced by a Methyl-Donor Diet

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Genetic Background Influences Behavior and Responses to Epigenetic Changes
Induced by a Methyl-Donor Diet

By

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Dedication

To both of my granddads: for their friendship, love, and dedication to making sure I received an education.

Acknowledgements

I would like to thank the following undergraduates at the University of South Carolina for their assistance with many aspects of this work: Amy Owen, Trish Cakora, Vanessa Anderson, Jasmine Allen, Eileen Wang, Julie Jacobs, Nam Phan, and Krupesh Dave. I would like to recognize April C.H. South for performing statistical analyses for much of this work. I appreciate conversations and assistance with behavior projects from Dr. Sandra Kelly, Amy Finch, and Dr. Lawrence at the University of South Carolina. I would like to thank the following persons from the University of Missouri: Sarah Johnson for analyzing Barnes Maze videos, Angela Javurek and Michelle Painter for analyzing Elevated Plus Maze Videos, Dr. Ellersieck for performing some of the statistics, and Dr. Cheryl Rosenfeld for overseeing the activities there. I thank Dr. Michael Felder and Dr. Paul Vrana for assisting me in the lab on multiple occasions. I would like to acknowledge my family for their support. I wish to acknowledge and thank my husband, Joseph Lara, for his support, care, and love through the past five years. Finally, I wish to acknowledge that I would not have survived graduate school without a lot of help from God.

Abstract

With recent strides in epigenetics, mainstream media informs the public that we can “beat our genes” by, for instance, changing our diet. Genetics, however, still plays a role in phenotype. Folate and other methyl-donor pathway components are widely supplemented due to their ability to prevent neural tube defects during prenatal development. In addition to vitamins, these compounds are also added to commercial flour, energy drinks, and other supplements. Several lines of evidence suggest that these supplements act through epigenetic mechanisms, including altering DNA methylation. Increasing evidence suggests potential deleterious effects of excessive folate. Given the benefits of these compounds, risk statements must be made with caution.

We hypothesized that excess dietary methyl donors during development might contribute to the apparent rise in neurobehavioral disorders such as attention-deficit disorder (ADD), obsessive compulsive disorder (OCD) and autism spectrum disorders (ASD). To test these hypotheses, we used wild-derived *Peromyscus* (deer mice) stocks. *Peromyscus* are common native North American mammals and exhibit great natural variation. We used two species that are known to differ in physiology, epigenetic control, and behavior. Specifically, *P. maniculatus* (BW stock) are susceptible to repetitive behaviors and are more aggressive in a neutral space. *P. polionotus* (PO stock) exhibit greater sociality and less repetitive behavior and are better able to buffer stress.

In addition the two species can form fertile hybrids in BW female x PO male crosses thus enabling genetic basis of such phenotypes to be determined.

Here we have determined genetic mechanisms by which behaviors differ between BW and PO. Additionally, we discovered behavioral differences in a naturally occurring wide band agouti (A^{Nb}) deer mouse (on a BW background) when compared to BW. Using the same methyl-donor diet used in the classic mouse agouti viable yellow allele (A^{vy}), we demonstrated that the effects of the diet are different across three genotypes (while two genotypes, BW and A^{Nb} , are very similar). These effects included various adult defects, mortality, and behavioral changes. Here we also present data from additional behavioral parameters in both PO and BW animals developmentally exposed to the methyl-donor diet. We also present data showing paternal genotype affects DNA methylation status at the imprinting control region of the *Peg10/Sgce* locus.

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Chapter 1

Introduction to Epigenetics and Peromyscus

Epigenetics

The term epigenetics was first conceived by British embryologist and geneticist Conrad Waddington in 1942. According to Waddington, epigenetics could be defined as “causal interactions between genes and their products which bring the phenotype into being.” [1]. Medawar and Medawar took a much broader approach to epigenetics in 1983 when they defined epigenetics as “‘Epigenesis’ stands for all the processes that go into implementation of the genetic instructions contained within the fertilized egg. Genetics proposes, epigenetics disposes.” [1]. Today, epigenetics is often defined simply as a change in phenotype without a change in genotype. This effect is seen since epigenetic mechanisms affect gene transcription and therefore an organism’s phenotype.

Epigenetic Mechanisms

Two of the most well understood mechanisms of how the epigenome controls the genome are DNA methylation and histone modifications. Other components of the epigenome include different types of RNAs. None of these mechanisms is thought to act alone. Often, DNA methylation may play a role in histone

conformation or in transcription of the RNAs that can affect epigenetic regulation. Differential DNA methylation typically takes place at CpG dinucleotide residues in animals (Figure 1.1). In particular, methylation occurs at CpG islands where the G:C content is 55% or higher within 500 base pair sequence [2]. Enzymes that aid in methylation are deoxynucleotide methyltransferases, or DNMT's. These DNMT's include DNMT1 (the maintenance methyltransferase which acts after cell division) and DNMT3a, DNMT3b, and DNMT3L which are *de novo* methyltransferases. Methylation at CpG islands stops transcription factors from binding to recognition elements. Methylation of CpG islands recruits methyl DNA binding proteins (MBD's) such as MECP2. Recruitment of MBD's activates enzymes that modify chromatin structure such as histone deacetylases [3].

Core histones can undergo many types of post-translational modifications. Most of the modifications are reversible although they cause structural changes in chromatin. Histone modifications can include methylation, acetylation, phosphorylation, sumoylation, ubiquitylation, and biotinylation [4]. The primary histone modifications are on histone tails (N-terminus) that extend outward from the nucleosome [5]. Histone proteins are arranged as octamers within nucleosomes. Nucleosomes then comprise chromatin [6]. Histone modifications therefore can either aid or stop the association of chromatin with DNA repair proteins and transcription factors (Figure 1.2). Euchromatin refers to the "open" state of chromatin that is less tightly packed, and therefore is transcribed. Heterochromatin refers to the more tightly packed state in which transcription factors cannot access the chromatin (Table 1.1).

Several RNA species are known to regulate gene expression. Small interfering RNAs (siRNAs) perform regulatory functions by associating with chromatin or by direct antisense RNA interference [7]. siRNAs, therefore, can repress translation without DNA methylation. Silencing of heterochromatin is performed by this mechanism [8]. Larger non-coding RNAs (lncRNAs) act in a similar manner to siRNAs and are associated with mechanisms such as X inactivation and other imprinted domains. Micro RNAs (miRNAs), which are small, noncoding RNAs, can suppress translation by binding to a partially completed messenger RNA (mRNA) [9,10]. miRNAs are critical to normal cellular processes including development, differentiation, and death [11]. Recent studies indicate miRNAs show tissue- and disease- specific effects.

Epigenetic Heritability

Factors that can affect epigenetic regulation include nutrition or diet, environmental agents or toxins, stress, radiation exposure, infectious agents, and immunological factors [12]. Epigenetic status has been shown to be transmitted from generation to generation. This includes transmission of DNA methylation marks. Dietary folate leads to DNA methylation in a one-carbon metabolic pathway that leads to the generation of S-adenosylmethionine (the methyl donor molecule), which donates a methyl group to DNA.

DNA methylation, in particular, has been shown to be sensitive to methyl donors in the diet such as folic acid. Folate is metabolized in the one-carbon metabolism pathway which, by using vitamin B₁₂, produces methionine. Methionine is converted to S-adenosylmethionine (SAM). SAM is the methyl

donor molecule (Figure 1.3). DNA methyltransferases then catalyze the enzymatic addition of the methyl group from SAM to DNA.

Perhaps one of the most well-known studies on the effects of a methyl donor diet on DNA methylation are the *Agouti* or *A^{vy}* locus studies that have been done using *Mus* or house mouse. The intracisternal A particle (IAP) retroelement insertion at the 5' end of the *Agouti* promoter (*A^{vy}* allele) drives expression of AGOUTI in *Mus* to give a phenotype yellow coat color, obesity, and diabetes. It was noted that when AGOUTI overexpressing were provided a methyl donor chow rather than normal lab chow, offspring showed a heterogeneous reduction in expression of AGOUTI. That is, coat colors varied from still being yellow to being dark [13-16].

A recent increase in the amount of folic acid in the human diet correlates with a rise in the frequency of various diseases including cancers, neurological disorders, growth syndromes, respiratory disorders, and multiple sclerosis [18-21]. Notably Autism Spectrum Disorders (ASD) have increased in frequency as well with a 78% increase in diagnoses since 2000 [20]. Women who are planning to become pregnant or who are already pregnant are prescribed 800 to 1000 micrograms of folic acid supplementation to their diet. Women with a mutation in *MTHFR* (which codes for methylenetetrahydrofolate reductase) are often prescribed up to 4 milligrams of folic acid during pregnancy. Additionally, the FDA began fortification of grains with folic acid in the 1990's. The prescribed folic acid and fortification of grains served to increase prenatal folic acid consumption, which has been correlated with a decrease in neural tube defects which cause

spina bifida. The mechanisms by which folate affects the closing of neural tubes are unknown but are thought to be epigenetic in origin [21].

Heritable epigenetic changes can be persistent over multiple generations. The process by which epigenetic status is transmitted transgenerationally involves several mechanisms. For one, the presence of certain alleles in a parent can influence the offspring's phenotype. This can occur by simple direct transmission of factors via gametes. Transmission via gametes occurs due to interaction with other alleles present in the offspring or through changes in parent behavior [22]. Other transgenerational effects result from parental exposure to any environmental factors that alter the parental epigenome.

Transmission of an altered epigenome to later generations can result in altered disease risk in offspring [22]. First discovered by Sonneborn in the late 1930s, cortical inheritance is a mechanism by which transgenerational effects are established early in embryonic development. Cortical inheritance results from the transmission of information through organelles that exist in the cortical cytoplasm (superficial cytoplasm of a cell) [23]. Early embryonic development is therefore primarily controlled by products of maternal genes obtained by eggs during oogenesis [23]. This was discovered during Sonneborn's study on *Paramecium aurelia*. This research demonstrated that pre-existing structures on the cell surface are passed to offspring for many generations [24] (Figure 1.4).

Parent of Origin Effects

Mendelian traits involve one locus and the transmission of an allele from both the mother and father to a diploid offspring. This is the most basic mode of

inheritance. It is applicable to many diseases and disorders, but sometimes the Mendelian rules of inheritance are not followed. One such non-Mendelian phenomenon is collectively referred to as “parent of origin effects.” Parent of origin phenotypic effects occur in such a way that is dependent upon the gender of the parent from which the effect originated. Effects may only be visible if inherited from the male or the female parent but are not observable if inherited from the other gender. Such effects have caused hardship in genome-wide association studies when trying to explain the heritable component of complex diseases. There are four types of parent-of-origin effects including oocyte derived maternal effects, mitochondrial maternal effects, sex chromosome effects, and genomic imprinting (Figure 1.5).

Oocyte-Derived Maternal Effects

Oocyte-derived maternal effects are observed during early embryogenesis. Oocytes store many necessary factors that are sufficient for the embryo to develop without a contribution from the paternal genome [25]. In general, sperm do not contribute many factors to early embryonic development. Rather, mutations in early maternal factors determine the phenotype of the offspring. This phenomenon can be seen in parthenogenesis, where embryos develop for a long time without a male genetic component [26]. These *in utero* effects are observed solely in the offspring as the phenotypic change is not seen in the mother. Environmental factors can give rise to additional oocyte-derived maternal effects including the previously mentioned change in expression of A^{ν} in *Mus* due to consumption of a diet high in methyl donors [22].

Mitochondrial Maternal Effects

While nuclear DNA is transmitted by both parents, mitochondrial DNA is exclusively maternally inherited. This is due to the fact that mitochondria contributed to an embryo by sperm are marked for degradation by ubiquitylation at fertilization [22]. Several diseases are associated with this parent-of-origin effect including Leber's hereditary optic neuropathy, maternal inheritance Leigh's Syndrome, and Kearns-Sayre syndrome [27]. Mutations in maternally-inherited mitochondrial DNA result in polysystemic degeneration of certain tissues in these syndromes.

Sex Chromosome Effects

Sex chromosome effects are tied to the sex chromosomes, X and Y, in mammals. Humans, most mammals, and many vertebrates are of the male gender if they possess a Y chromosome. The Y chromosome has traditionally been thought to be gene poor. Several genes have been mapped to the Y chromosome and some male-specific effects have been found [28-29]. As males only possess one X chromosome, X-linked effects are often more common in males and are considered to be a maternal effect. An example of an X-linked effect is color-blindness.

The Y chromosome has, in some instances, been linked to behavioral effects and brain functions. The non-pseudoautosomal region of the Y chromosome (YNPARG) is exclusively transmitted paternally to male offspring. This region has been studied extensively in mice and rats where lines have been created that differ only in the YNPARG region [22]. These studies showed that

aggressive behaviors and the morphology of the hippocampus of the brain seem to be associated with this Y chromosome region [22].

The X chromosome, however, has been implicated in many more sex-chromosome effects. The presence of multiple X chromosomes leads to X-inactivation of at least one of the X chromosomes in many species. This is necessary for proper dosage compensation. Some genes, however do escape X-inactivation. Interestingly, many of the genes that escape silencing are map within the YNPARG [30] (Figure 1.6). Individuals with sex chromosome aneuploidies such as Turner's Syndrome (45,X) and Klinefelter's Syndrome (47,XXY) display abnormal behavioral phenotypes that vary in a parent of origin manner. That is, the abnormal behavioral phenotypes differ depending on which parent donated the only or additional X chromosome, respectively [22]. This is likely due to the fact that much of one of the X chromosomes is subject to X inactivation in mammalian females.

X inactivation is mediated by a lncRNA called *Xist*. *Xist* is actively transcribed from the X chromosome that is inactivated. *Xist* binds the X chromosome from which it was transcribed to inactivate gene on the X chromosome that are subject to X inactivation. Both X chromosomes express *Xist* in small amounts, but during X inactivation, the X chromosome that is to remain active ceases to express *Xist* [31-33]. A transcript antisense to *Xist*, *Tsix*, overlaps the *Xist* gene. *Tsix* is another lncRNA. Expression of *Tsix* leads to *Xist* silencing and an active X chromosome [31-33]. The inactive X chromosome has

high levels of methylated DNA and histone 3 lysine 9 (H3K9) methylation which are associated with gene silencing [31-33] (Figure 1.7).

Genomic Imprinting

One parent of origin effect that is highly associated with DNA methylation is known as genomic imprinting. Imprinting is a mechanism by which one allele (inherited from one parent) is silenced while the other allele (inherited from the other parent) is expressed. Silencing of one allele versus the other is based upon the gender of the parent from which the offspring inherited the allele. Maternal imprinting is when the maternally inherited allele is silenced; paternal imprinting is when the paternally inherited allele is silenced (Figure 1.8).

Genomic Imprinting in Mammals

Genomic imprinting has been observed in eutherian [34] and marsupial mammals [35]; however, it is most often studied in mice and humans. Many imprinted genes are neither imprinted in all tissues nor are not imprinted at all times in mammals. This has complicated the identification of imprinted genes. Imprinted genes in mice and humans code for proteins involved in several different cell processes such as embryonic growth and development and post-natal development, as they are involved in placental development and in metabolism [36]. Many genes imprinted in humans are also imprinted in mice, but there are some differences. Some genes imprinted in either mouse or human have no ortholog in the other organism [36]. Therefore, although imprinting is conserved, many species-specific differences exist in genes that are imprinted.

Imprinted Domains and the Role of Methylation

Imprinted genes often are found in clusters that include combinations of maternally and paternally imprinted genes. These clusters are typically regulated by one imprinting control region (ICR) that has at least one CpG island. These CpG islands are methylated in a parent-of-origin specific manner during gametogenesis. ICRs that arise during gametogenesis (also known as intergenic germ line differentially methylated regions, or IgDMRs) are the primary epigenetic marks of imprinted genes. Secondary DMRs arise during embryonic development [37]. DNA methylation, therefore, plays a significant role in the establishment of genomic imprinting. Organization of imprinted domains can vary. Maternally-methylated IgDMRs tend to encompass the promoter region of one or multiple imprinted genes. Paternally-methylated IgDMRs are in intergenic regions and do not directly associate with a promoter region [38].

The simplest of imprinting mechanisms involves direct methylation of the promoter of an imprinted gene. This often occurs in imprinted domains that contain multiple genes but can also function in the independent regulation of a single gene. One example of the latter is in the regulation of murine *Nap1L5*. *Nap1L5*, a maternally imprinted gene, has a methylated IgDMR in the promoter of the maternally inherited allele and an unmethylated IgDMR in the paternally inherited allele near the promoter. The methylated IgDMR on the maternal allele silences *Nap1L5* transcription from this allele in tissues where imprinting regulates the expression. *Nap1L5* is, however, expressed from both parental alleles in other murine tissues [39] (Figure 1.9).

The *Snurf-Snrpn* imprinted domain, which is also in humans known as the Prader-Willi Syndrome Imprinting Control Region (PWS-IC), contains multiple genes that are affected by the *Snurf-Snrpn* IgDMR. This region is similarly regulated in mouse and human. This IgDMR silences the promoters of *Snurf-Snrpn* but also performs long-range silencing of several transcripts that are in this imprinted region. Genes in this region include *Ube3a*, *Gabrb3*, and several snoRNAs, each of which is critical for neurological function [40-43]. PWS-IC is in a bipartite imprinting center that includes the Angelman Syndrome-IC (AS-IC). The PWS-IC bi-directionally activates paternally expressed genes [44]. The AS-IC suppresses the PWS-IC on the maternal chromosome through methylation [44] (Figure 1.10).

Maternally-methylated IgDMRs can also be associated with bidirectional promoters in which two paternally-expressed genes are transcribed in opposite directions. Two well-known examples of this are the cases of the *Peg3-Usp29* and *Peg10-Sgce* domains, which are regulated similarly between mouse and human. For these domains, the transcription start sites are within 500 bases of each other. The IgDMR starts in the intergenic space, spans the first exon of *Peg3* or *Peg10*, and continues into the first intron [45,46]. *Peg3* plays roles in both behavior and in apoptosis during early neonatal brain development [47-49] while *Peg10* has been identified as an ASD locus and is overexpressed in cancers such as leukemia [21,50-52] (Figure 1.11).

Maternally-methylated IgDMRs can also regulate large imprinted domains using lncRNAs. LncRNAs are typically longer than 50 kilobases in length and

have a promoter that is in the IgDMR. Methylation directly silences the transcription of lncRNAs. The lncRNAs are responsible for silencing the rest of the imprinted domain. An example of this mechanism is seen in the case of *Kcnq1ot1*. *Kcnq1ot1* is a lncRNA that is paternally expressed due to a maternally-methylated DMR known as the KvDMR. Therefore, the other genes in this imprinted domain are maternally expressed. This regulation is conserved between mouse and human. *Kcnq1ot1* performs bidirectional silencing of genes in the *Kcnq1* domain by establishing a repressive chromatin structure through the recruitment of chromatin- and DNA- modifying proteins [53]. A truncated transcript of *Kcnq1ot1* leads to reactivation of all paternal transcription in this imprinted region [54-56]. Deletion of the IgDMR also reactivates paternal transcription of all genes in this imprinted region [54,55] (Figure 1.12).

Unlike maternal methylation marks, paternal methylation marks are found in the intergenic regions of imprinted genes. The intergenic region, which is about 90 kb, often divides a paternally-expressed gene (such as *Igf2*) from a maternally expressed non-coding RNA (such as *H19*) [57]. The imprinting control region, or ICR, is about 2 kb upstream of the *H19* transcription start site. Deletion of the *H19* ICR results in a loss of imprinting of both *H19* and *Igf2* [58]. The *H19* ICR has multiple binding sites for an insulator protein known as CCCTC-binding factor (which is encoded by the *CTCF* gene). CTCF can only bind the specific binding sites if they are unmethylated [59,60]. CTCF binding to an unmethylated ICR prevents downstream enhancers from activating *Igf2* (Figure 1.13).

Igf2 and *H19* each have secondary DMRs within their promoter regions. Methylation of these secondary DMRs is associated with silencing of the *cis* allele of that gene. Expression of *Igf2* and *H19* requires several tissue-specific enhancers that span 3 regions 10-120 kb downstream of *H19*. CTCF an enhancer-blocking protein. It binds the methylated *H19* ICR and prevents access of *Igf2* to downstream transcription enhancers by stopping chromatin looping. Notably, the DMRs (DMR1 and DMR2) within *Igf2* affect chromatin looping [61,62]. On the maternal chromosome, the *H19* ICR interacts with DMR1 and a 3' region of the *Igf2* gene called *Mar3*. On the paternal chromosome, the *H19* ICR interacts with DMR2 [61,62]. CTCF binding to the *H19* ICR then mediates higher order chromatin structure on the maternal allele [61] (Figure 1.14).

Histone modifications also factor in the expression of *Igf2*. *Igf2* is silenced on the maternal chromosome, where the *Igf2* region has repressive methylation at H3K9 and H3K27 [63,64]. Activating histone marks (specifically H3K4 methylation and histone acetylation) are found predominantly on the maternal chromosome near the *H19* ICR, the *H19* promoter-gene region, and on the paternal chromosome at the *Igf2* promoter-gene region [64]. Notably, it has become more evident that *H19/Igf2* imprinting is much more complex than previously thought. DNA methylation, histone modifications, and higher order chromatin structure all play a role in the imprinting of the *H19/Igf2* region.

While the data above are from mouse experiments, human regulation of all imprinted gene examples shown appears to be the same as in mouse [65].

Peromyscus: a Model for Studying Biomedical Science

Peromyscus, or deer mice, are among the most common native North American mammals [66] (Figure 1.15). Many species and subspecies have adapted to areas from Alaska to Central America. Deer mice can be found in a range of habitats, from sea-level wetlands and beaches to forests, prairies, deserts, and on mountains at elevations of up to 14,000 feet [66]. There are significant differences among these species. Some species are much larger in size compared to others, and many naturally occurring coat color mutations exist. Naturally occurring behavioral differences are prevalent and are of interest to scientists as well. There exist certain advantages to using *Peromyscus* in the lab over standard *Mus* or *Rattus* lines. *Peromyscus* lab strains are derived from natural populations that remain outbred over the generations, which allows the animals to remain more like the wild population from which they were derived.

Because deer mice are abundant, they have been used in studies in physiology, endocrinology, parasitology, epidemiology, evolution, toxicology, ecology, genetics, behavior, and epigenetics [65]. More recently, small rodents such as *Peromyscus* have become a model organism for Hepatitis C research as they can become infected with a similar virus [67]. Hantavirus (Sin Nombre Virus) [68] and Lyme disease [69] have already been studied in *Peromyscus* as deer mice are carriers of each of these. Other studies include the effects of prenatal BPA exposure on behavior in progeny [70], alcohol consumption studies [71], hybrid growth disorder studies [72-73], and behavior studies to show aggression

[74], monogamy [75], and other phenotypes in *Peromyscus* stocks. The studies mentioned are only a few for which *Peromyscus* have been used in the lab.

Current data from labs using *Peromyscus* along with the development of a genetic map, available genome sequences for several species (<http://www.ncbi.nlm.nih.gov/assembly/84591/>), and interspecies transcriptome data are assisting in the further development of this novel model organism.

Great potential exists for utilization of this novel model organism. For instance, models for diseases can be found in different stocks. One such example is the possibility of using *P. maniculatus bairdii* (BW) as a model for Autism Spectrum Disorders (ASD). High quantities of repetitive behaviors, or stereotypies, have been documented in this species whereas *P. polionotus* (PO) are less inclined to repetitive behavior [76,77]. Stereotypic behavior, or repetitive behaviors that are performed without function or purpose, is one of the diagnostic criteria for ASD. Genetic and behavioral differences between BW and PO have proven useful in developing the genetic map among other studies (Table 1.2). One such study is interspecies hybrid growth disorders in *Peromyscus*. When a PO female is crossed with a BW male, a subsequent loss of imprinting at several genes leads to aberrant phenotypes, including overgrowth, that often results in death for the mother and offspring. Meanwhile, the opposite cross of BW female with PO male leads to undergrowth in the offspring [72,73].

Specific Aims

The central aim of this work is to further develop *Peromyscus* as a biomedical model. The specific aims are (1) to assess the extent of behavioral differences

between BW and PO and to determine genetic mechanisms responsible for the behavioral differences (2) investigate the effects of a diet high in methyl donors on phenotypes including coat color and behavior in a naturally occurring *Agouti* variant of *P. maniculatus* (termed “wide band Agouti”, or A^{Nb}), (3) investigate the effects of a diet high in methyl donors on behavior in PO and BW and determine if genetic background influences the effects of a methyl donor diet, and (4) to determine the effects of a methyl donor diet on the epigenetic status of selected genes, including some imprinted genes and genes on the X chromosome. Here we show that some complex genetic mechanisms underlie differences in behavior between *Peromyscus* species, and that the effects of a methyl donor diet differ between species indicating genetic background influences epigenetic responses to such a diet.

Table 1.1: Epigenetic Marks in Euchromatin and Heterochromatin.

Chromatin Feature		Euchromatin	Heterochromatin
	Structure:	Less Condensed; Open/Accessible	Condensed; Closed/Inaccessible
	DNA Sequence:	Gene Rich	Repetitive Elements
	Activity:	Expressed	Repressed/Silenced
Epigenetic Marks	DNA Methylation	Hypomethylation	Hypermethylation
	Histone Acetylation	Hyperacetylation of Histone H3, H4	Hypoacetylation of Histone H3, H4
	Histone Methylation	H3K4me2 H3K4me3 H3K9me1	H3K27me2 H3K27me3 H3K9me2 H3K9me3

Table 1.2: Relevant Known Differences Between BW and PO

	BW	PO
Glucose Tolerance	Poor	Good
Stress Buffering	Poor	Good
Social Behavior	Low	High
Repetitive Behavior	High	Low
Alcohol Consumption	Low	High
Parenting	Poor	Good
Monogamy?	No	Yes

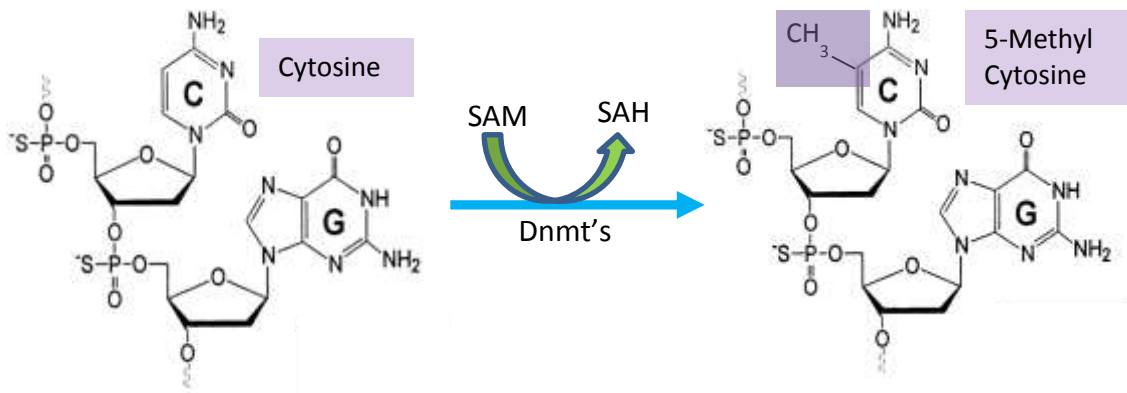


Figure 1.1: DNA methylation at a CpG dinucleotide. The methyl group is added to the cytosine residue at the 5 position (5-methylcytosine). DNA methyltransferases catalyze the transfer of the methyl group from S-adenosylmethionine (SAM) to DNA.

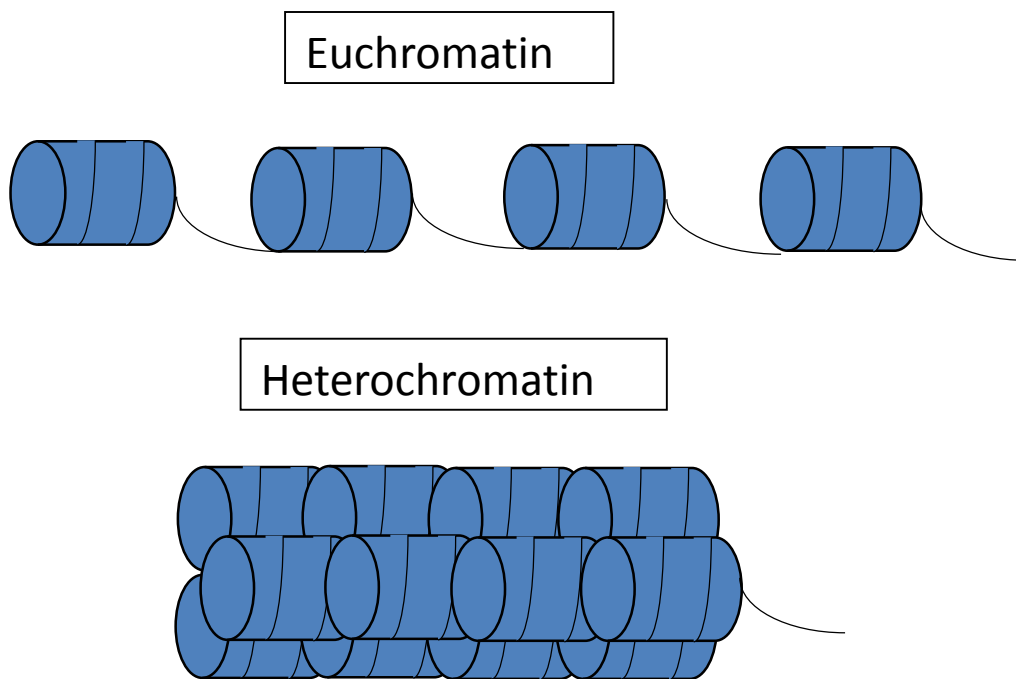


Figure 1.2: Euchromatin vs. heterochromatin. Euchromatin is transcribed due to less compaction whereas heterochromatin is compacted which inhibits transcription factors from accessing chromatin. Histone acetylation is more often associated with euchromatin while histone methylation is more often associated with heterochromatin.

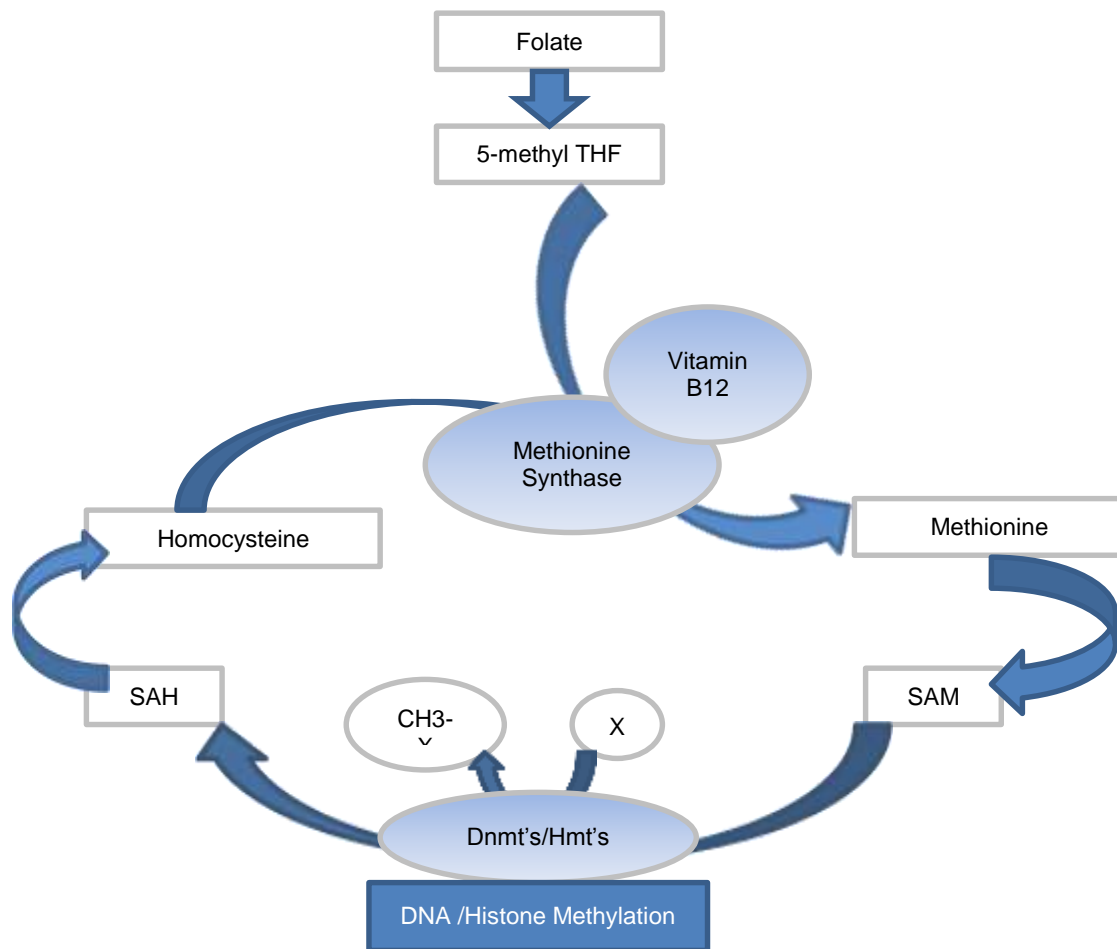


Figure 1.3: The methyl donor pathway. The methyl donor pathway begins with folate (folic acid) in the diet. Folate is eventually converted to methionine using Vitamin B12 as a cofactor. Methionine is converted to S-adenosylmethionine (SAM) using adenosine triphosphate (ATP) as a cofactor. SAM is the methyl donor molecule that donates a methyl group in DNA methylation reactions that use DNA methyltransferases to methylate DNA, RNA, histones, lipids, and proteins.

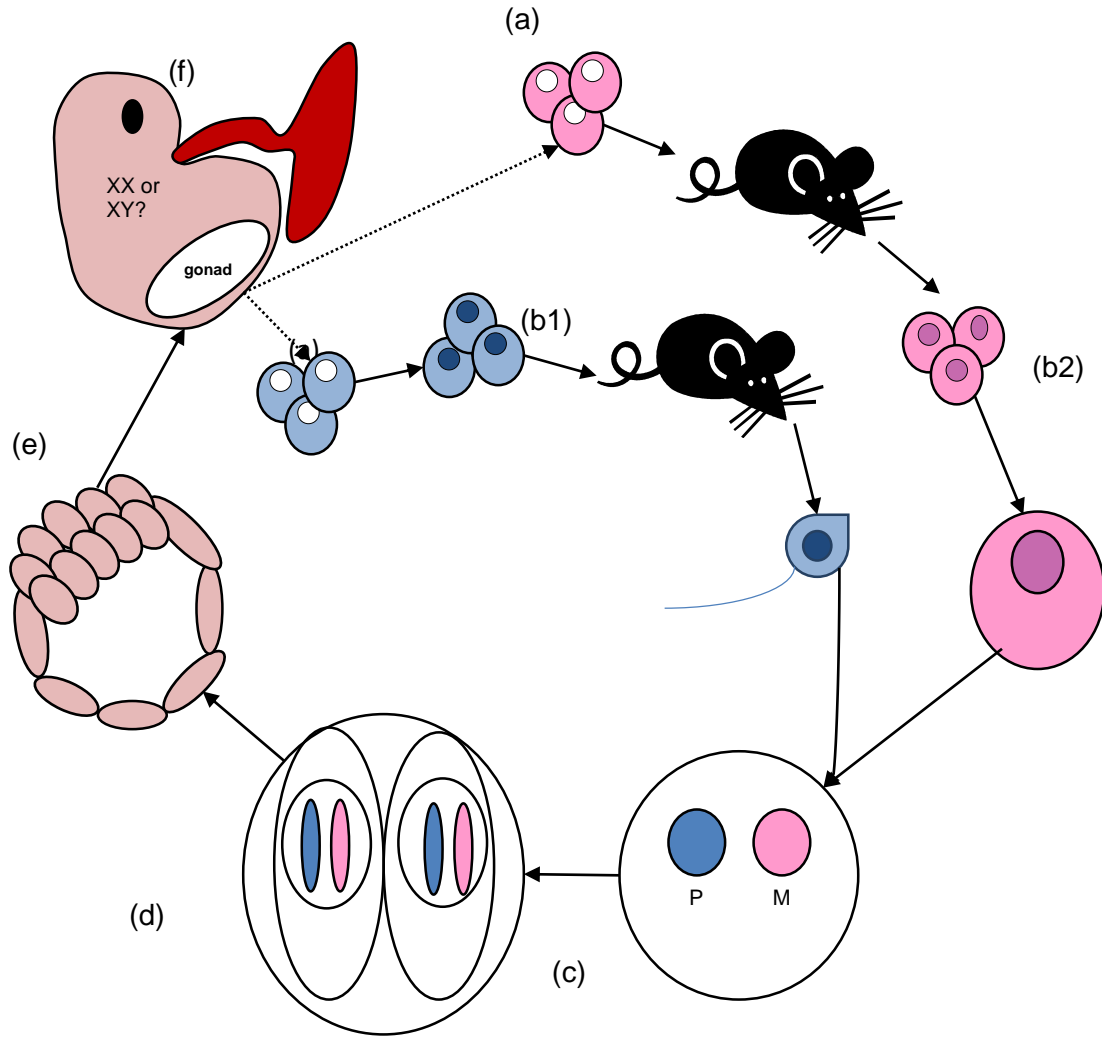


Figure 1.4: Establishment of genomic imprinting. (a) Imprinting is erased in primordial germ cells. (b1) The establishment of genomic imprinting takes place in prenatal male germ cells by *de novo* methylation of imprinted genes. (b2) Female germ cell lines acquire imprinting patterns by *de novo* methylation of imprinted genes in the postnatal stage. (c) Once fertilization of an egg has occurred, the embryo's paternally inherited genome is actively demethylated whereas germline imprints are resistant to demethylation in early embryonic stages. (d) The embryo's maternally inherited genome is passively demethylated. Germline imprints are resistant to active and passive demethylation at the early embryonic stage. (e) *De novo* genomic methylation occurs at the blastocyst stage. (f) Imprinting is maintained in somatic and extra embryonic tissues. Figure adapted from Bartolomei and Ferguson-Smith, Cold Spring Harbor Perspect. Biol. 2011; 3:a002592.

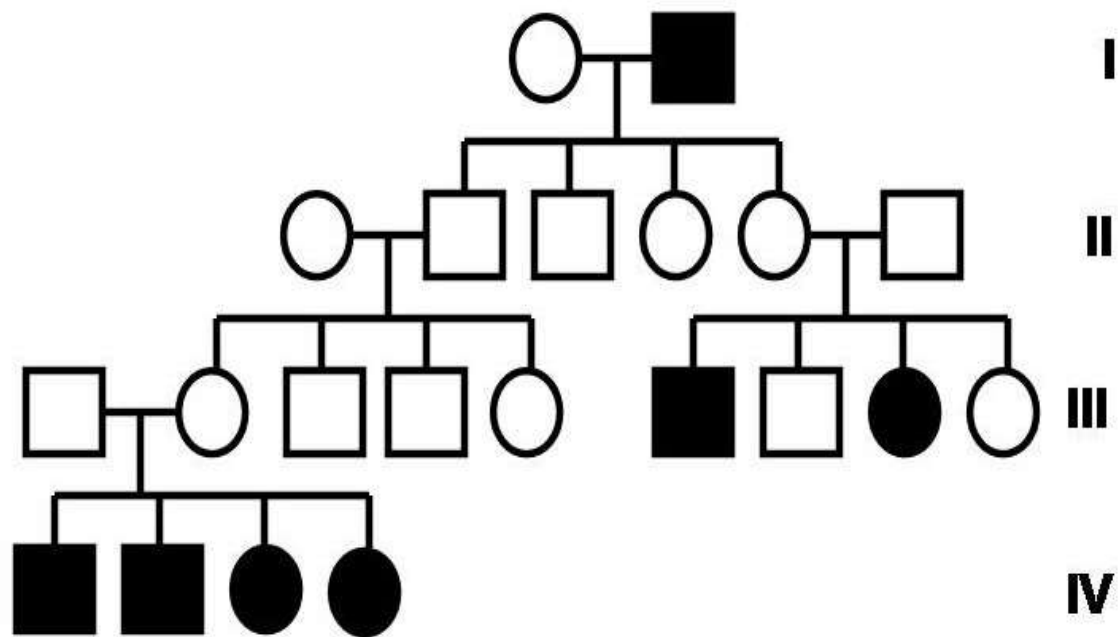


Figure 1.5: Parent of origin pedigree. Parent of origin effects often complicate pedigrees. In the pedigree shown above, the affected phenotype skips generations and only manifests when passed through a female. Therefore, this affected phenotype is likely inherited through an imprinted gene that is maternally expressed (a paternally imprinted gene).

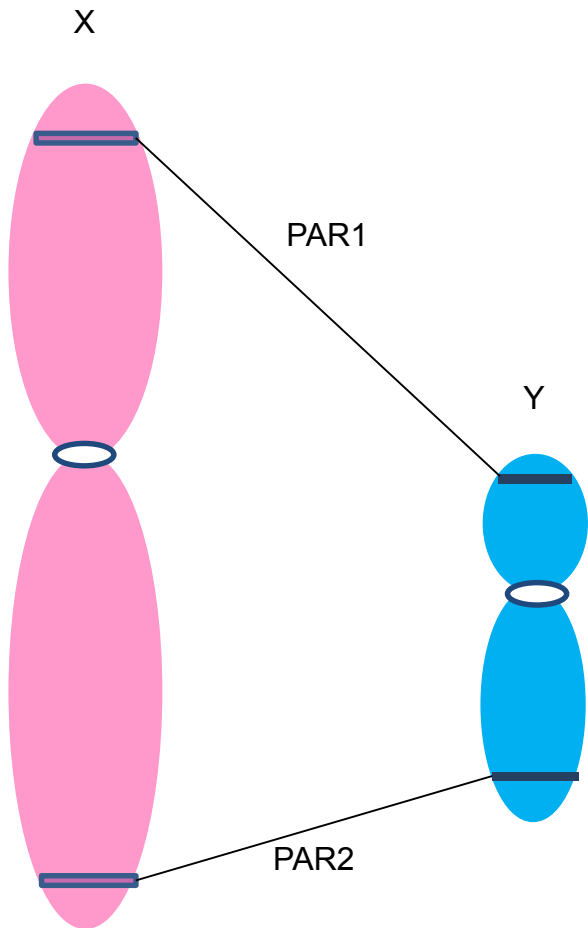
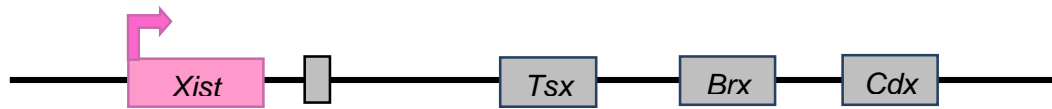


Figure 1.6: X chromosome homology with the Y chromosome. Although the X chromosome is subject to inactivation, some X-linked genes escape inactivation and are silenced. Many genes that escape silencing on the X chromosome are also present on the Y chromosome in the Pseudoautosomal Regions 1 and 2 (PAR 1 and 2).

Inactivated X



Active X

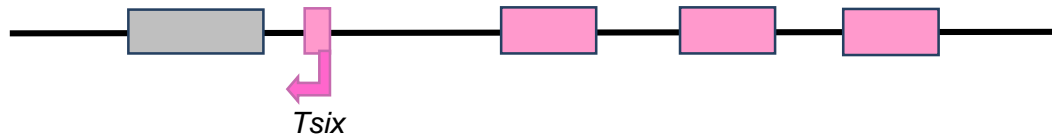


Figure 1.7: X inactivation mechanism. X inactivation depends on transcription of a long-noncoding RNA called *Xist*. *Xist* is transcribed from the inactivated X chromosome where the transcription product of *Xist* binds the X chromosome to inactivate those genes subject to X inactivation. *Tsix* is an antisense transcript that overlaps *Xist*. *Tsix* is transcribed from the active X chromosome (i.e., it is silent on the inactive X). Transcription of *Tsix* leads to silencing of *Xist* (and activation of the X chromosome).

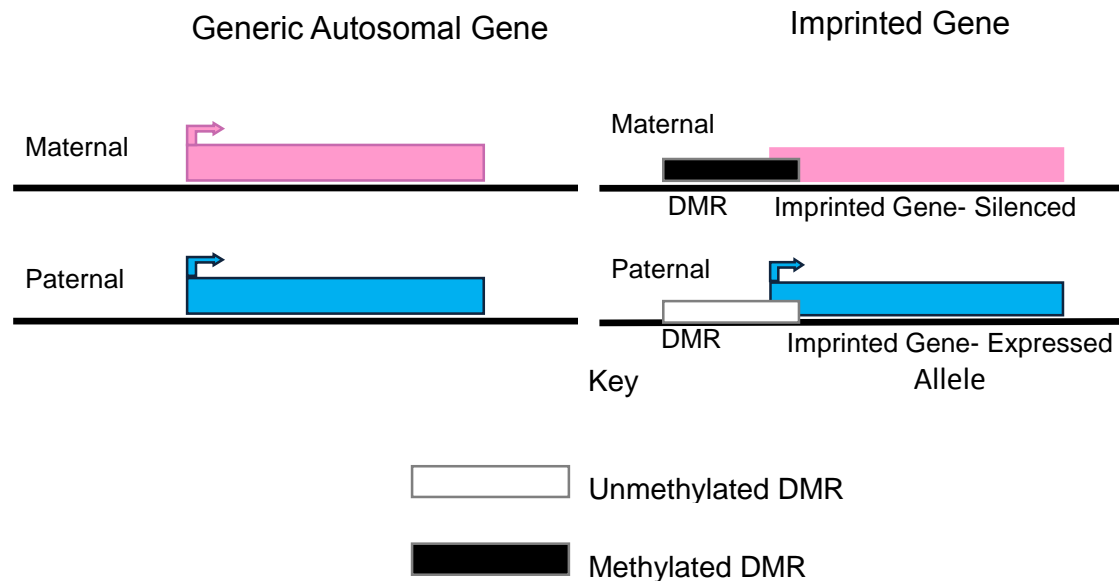


Figure 1.8: Typical Autosomal vs. Imprinted gene. A generic autosomal gene (left) is expressed from both alleles, maternal (pink) and paternal (blue). An imprinted gene (right), however, is expressed in a parent-of-origin specific manner. One allele is expressed and the other is silent due to a methylated DMR or differentially methylated region.

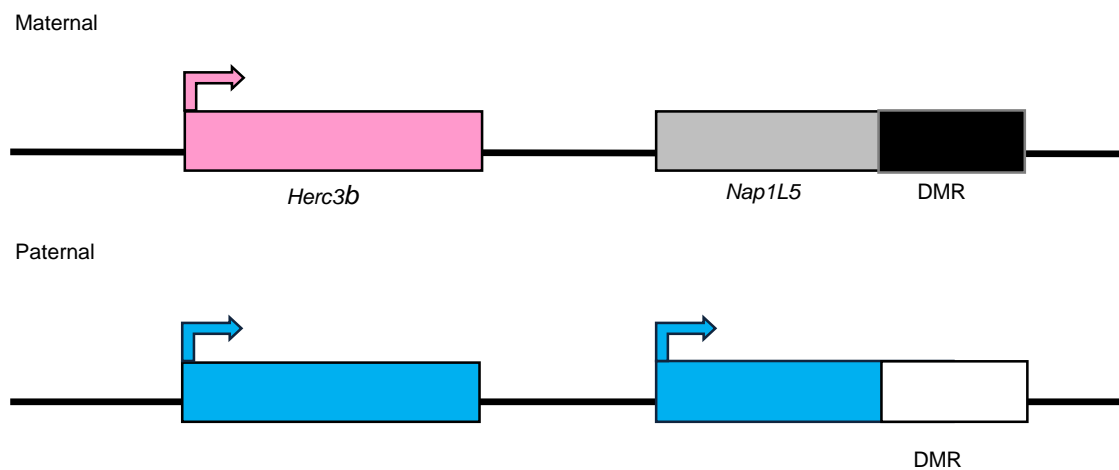


Figure 1.9: *Nap1L5* imprinting. *Nap1L5* imprinting is an example of the simplest imprinting mechanism. This mechanism involves methylation of the promoter of the imprinted gene (*Nap1L5*) that only regulates the single gene. *Nap1L5* is methylated at the IgDMR on the maternal allele and is therefore silenced from the maternal allele.

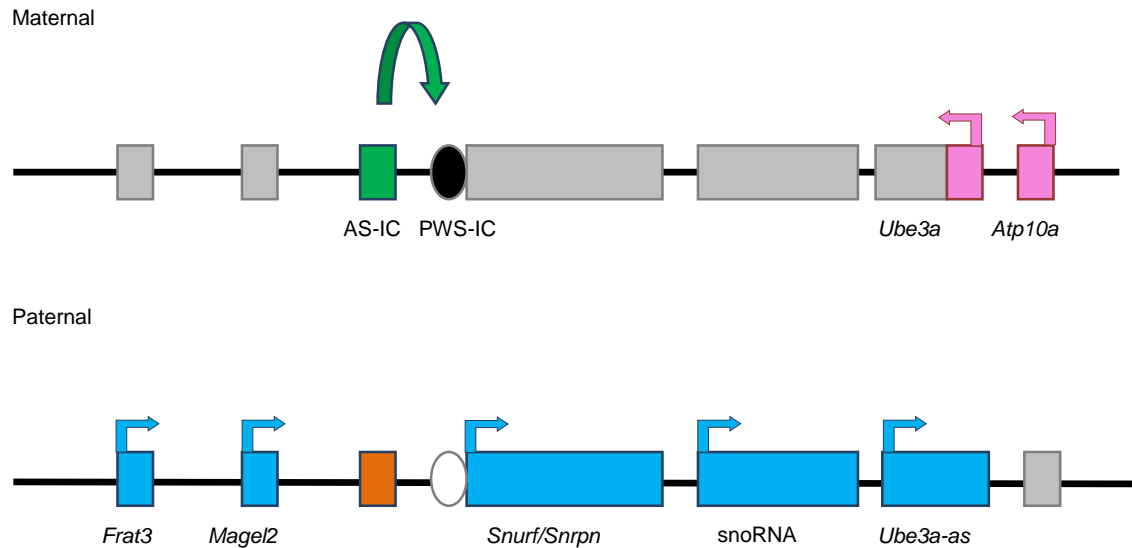


Figure 1.10: The *Snurf-Snrpn* or Prader-Willi Imprinting Center. The Prader-Willi Imprinting Center (PWS-IC) is in a bipartite imprinting center that includes the Angelman Syndrome Imprinting Center (AS-IC). The PWS-IC bidirectionally activates paternally expressed genes. The AS-IC suppresses PWS-IC on the maternal chromosome using DNA methylation. The Prader-Willi Imprinting Center (PWS-IC) contains the promoter, first exon, and part of the first intron of *Snrpn*. This imprinting center regulates neuron-specific expression of a large cluster of genes including *Snrpn*, *Ube3a*, and *Gabrb3*. Each of these is critical for neurological function. Many snoRNAs (non-coding RNAs of the nucleolus that guide rRNA modifications) are also controlled by this IC. Chromatin decondensation occurs specifically at these snoRNA clusters. This region is regulated similarly in mouse and humans.

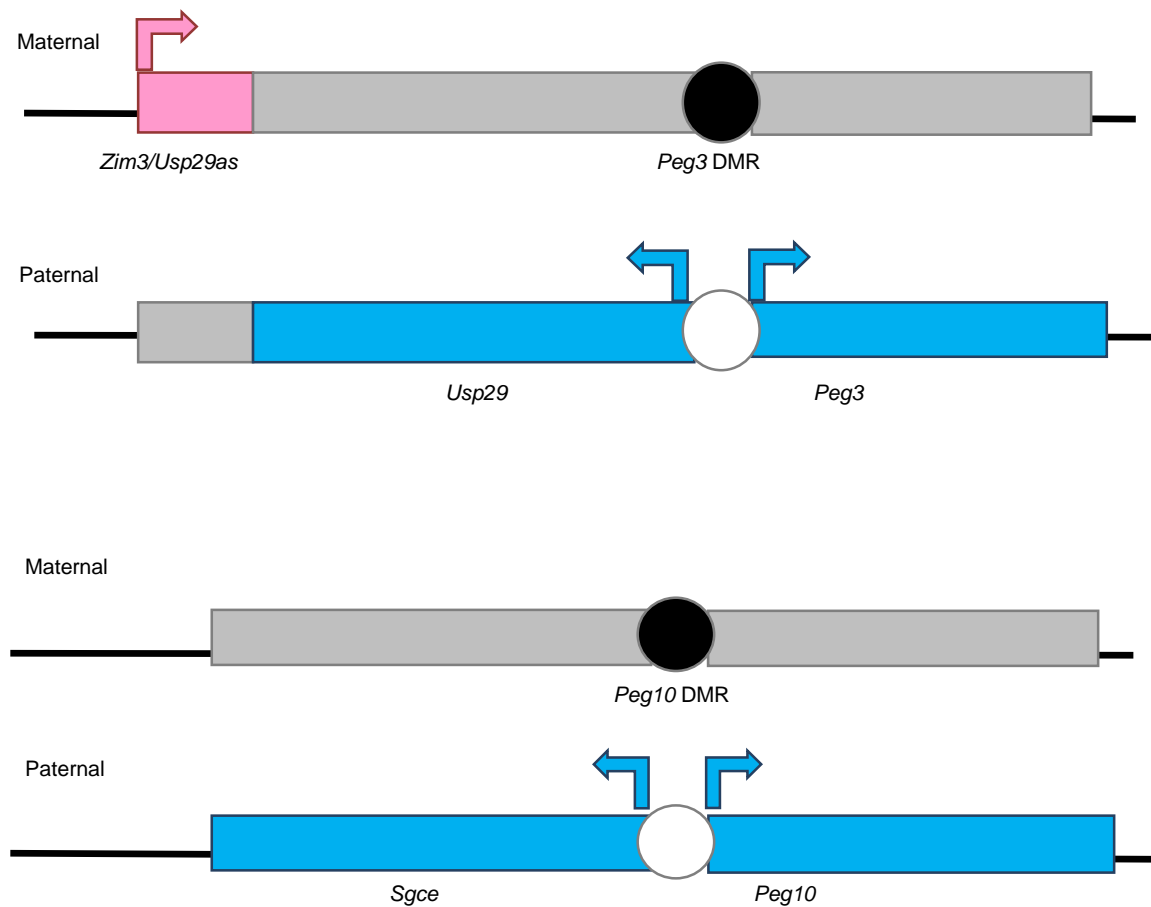


Figure 1.11: The *Peg3* and *Peg10* Imprinting Domains. The *Peg3-Usp29* and *Peg10-Sgce* domains are examples of maternally methylated IgDMRs that are associated with bidirectional promoters. The paternally expressed genes are transcribed in opposite directions with transcription start sites within 500 base pairs of each other. The IgDMR in both cases starts in the intergenic space and spans the first exon of *Peg3* or *Peg10* and continues into the first intron. These two imprinted loci are regulated similarly in mouse and human.

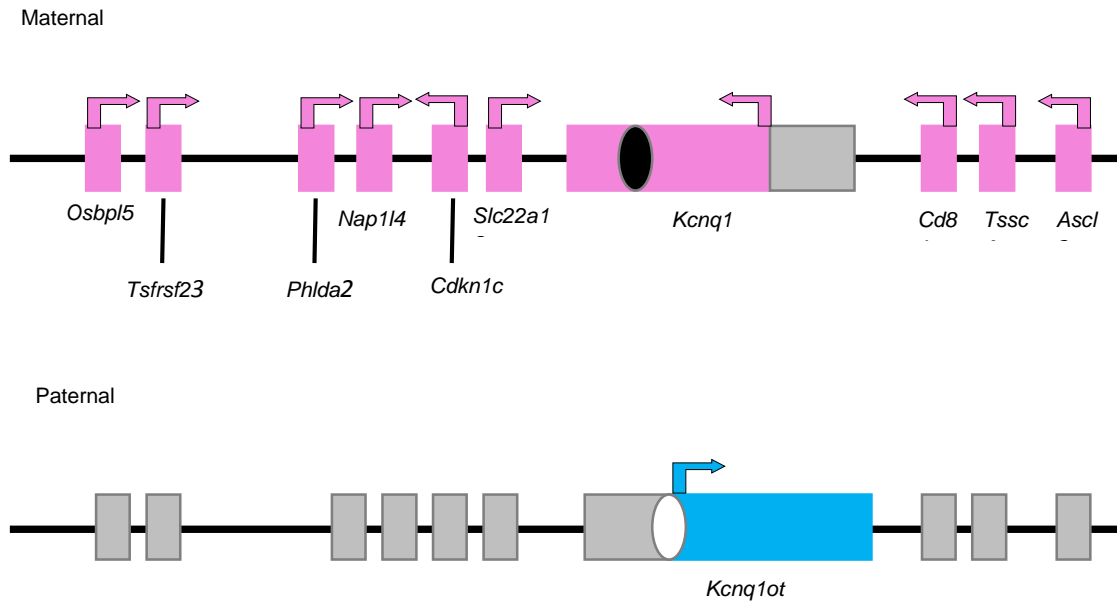


Figure 1.12: The *Kcnq1ot1* Imprinting Center. *Kcnq1ot1* performs bidirectional silencing of the genes in the *Kcnq1* domain by establishing repressive chromatin structure by recruiting chromatin and DNA modifying proteins. The mode by which *Kcnq1ot1* works to control imprinting may be similar to that done by *Xist*. The DMR of *Kcnq1ot1*, a long-noncoding RNA, is responsible for regulating the expression of genes in an imprinting region that includes *Kcnq1* and *Cdkn1c*. This region is regulated similarly in mouse and human.

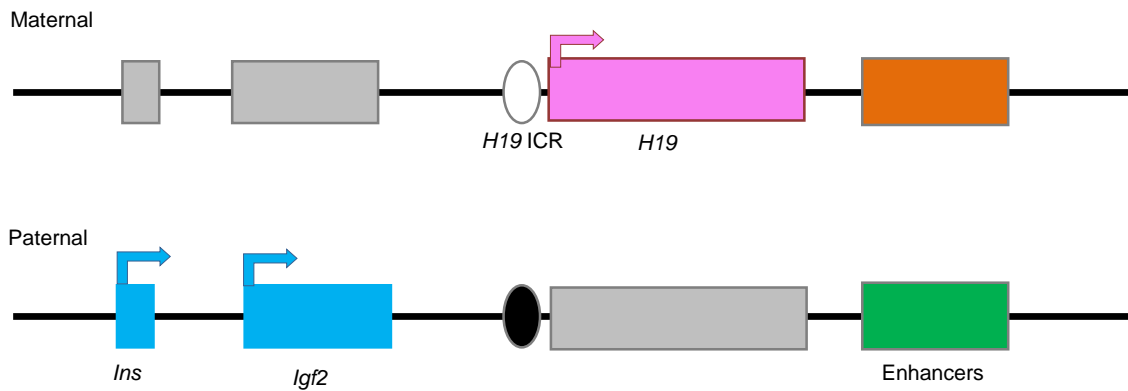


Figure 1.13: The *H19/Igf2* Imprinting Center. Paternal methylation marks are found in intergenic regions of imprinted genes. Such is the case with *H19 ICR*. This ICR is paternally methylated and divides the paternally expressed gene *Igf2* and the maternally expressed gene *H19*. The *H19 ICR* has multiple binding sites for the CCCTC-binding factor (CTCF). CTCF binds the unmethylated ICR and not the methylated ICR. Binding by CTCF prevents *Igf2* access to transcriptional enhancers by suppression of chromatin looping. Regulation of this imprinted region is similar in mouse and human.

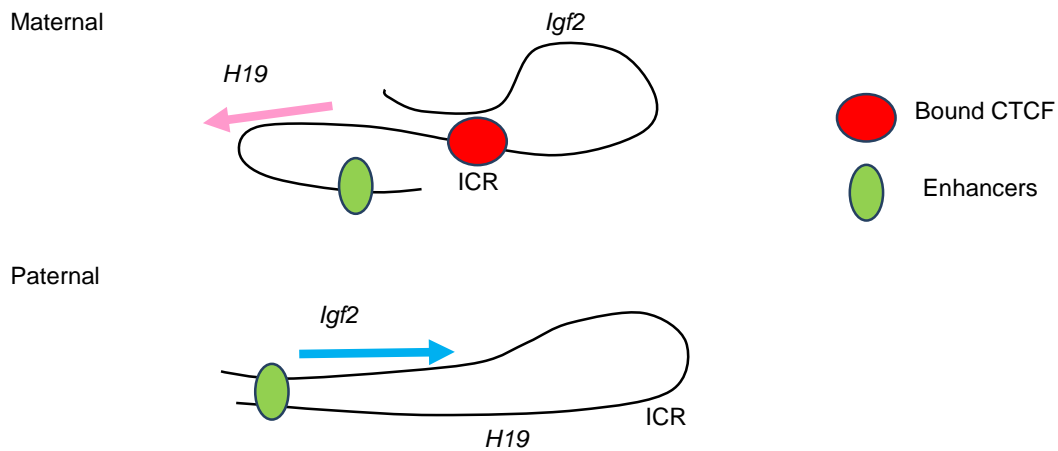


Figure 1.14: The *H19/Igf2* Chromatin Looping Mechanism. When CTCF is bound to the *H19* ICR, chromatin configuration is so that *H19* is closer to enhancers and is transcribed while *Igf2* is further away from enhancers and is silent. On the paternal allele, the *H19* ICR is not bound by CTCF due to methylation. Therefore, on the paternal allele, chromatin configuration is such that *Igf2* has access to transcription enhancers and is transcribed while *H19* is further from the enhancers and is silent.

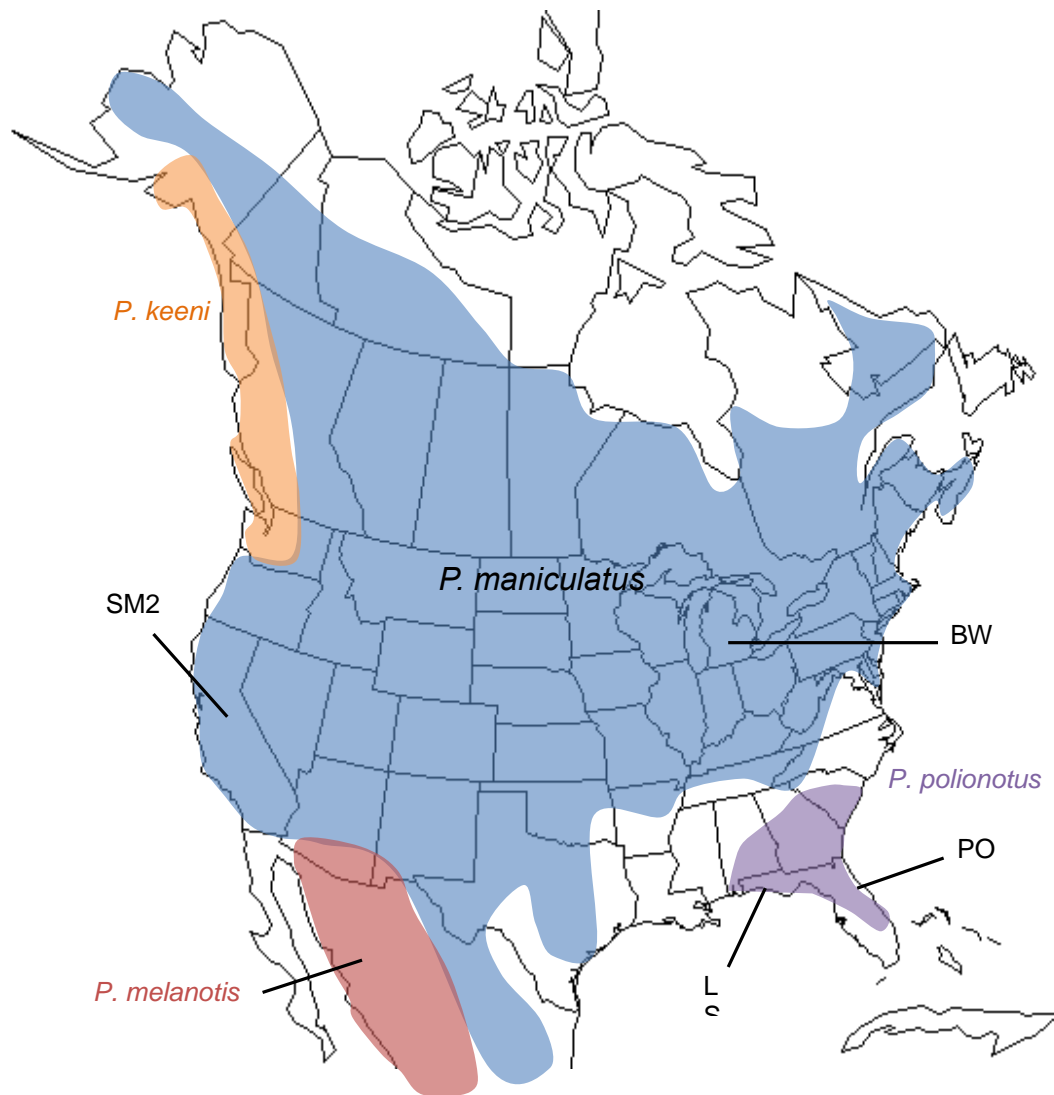


Figure 1.15: Map of *Peromyscus maniculatus* species complex in North America. *Peromyscus* are one of the most common native North American mammals. *P. maniculatus* species are found in most of North America while *P. polionotus* species are found in the Southeastern United States. Founder BW animals at the *Peromyscus* Genetic Stock Center were caught in Michigan. Founder PO animals were captured in Florida.

Chapter 2

Natural Genetic Variation Underlying Differences in *Peromyscus* Repetitive and Social/Aggressive Behaviors ¹

Introduction

Peromyscus (deer and white-footed mice) offer rare opportunities to identify alleles underlying natural variation in biomedically relevant behaviors. The *P. maniculatus* species complex is particularly widespread, variable, and amenable to genetic analyses. Wild-derived stocks of a number of species and populations are maintained at the *Peromyscus* Genetic Stock Center (<http://stkctr.biol.sc.edu/index.html>). These stocks differ from most other commonly used rodent strains in having truly wild-type genomes and not having been deliberately subjected to artificial selection in captivity.

Several *Peromyscus* species have been used extensively in behavioral research, largely with a focus on the effects of environmental/hormonal variables [70, 74, 78]. However, there has been relatively little investigation into the genetic basis of *Peromyscus* behaviors. The BW stock of *P. maniculatus bairdii*

¹Kimberly R. Shorter, Amy Owen, Vanessa Anderson, April C. Hall-South, Samantha Hayford, Patricia Cakora, Janet P. Crossland, Velina R.M. Georgi, Amy Perkins, Sandra J. Kelly, Michael R. Felder, and Paul B. Vrana. 2014. *Behavior Genetics* 44:126-135. Reprinted here with permission of publisher.

(tall grass prairie subspecies, derived from 40 wild caught ancestors in Washtenaw Co MI) and the PO stock of *P. polionotus subgriseus* (derived from 21 animals caught in Ocala National Forest, FL) have proven fruitful in genetic analyses and differ in a number of biomedically and evolutionarily relevant traits.

These two species have been shown to differ in numerous behavioral and physiological characteristics. Notable among these are social behaviors: *P. polionotus* is among the few monogamous mammalian species, and exhibits pair bonding [79,80], while multiple paternity has been demonstrated within wild BW litters [81]. *We hypothesize that many of the interspecific differences may be linked to the differing social behaviors of the two species.*

For example, PO and BW have been shown to differ in aggressiveness towards conspecifics in the resident intruder test, with PO males consistently exhibiting more aggressive behaviors [74]. Glucose homeostasis is much more stable in PO animals of both sexes relative to BWs, although the effect is more pronounced in males. The difference in males appears to be due to PO Y chromosome sequences [82]. This hypothesis was tested via a consomic animal line that has a BW genome except for the Y chromosome (BW Y^{PO}). Several lines of evidence suggest that these differences in regulating blood sugar levels are due to a superior ability of the PO animals to buffer stress.

Importantly, BW animals have also been well studied for their tendency to engage in repetitive behaviors (jumps, backflips, etc.) [77, 83-86]. They are therefore potential models for behavioral/neurological disorders characterized by stereotypies (repetitive behaviors that lack function or purpose), e.g. Autism

Spectrum Disorders (ASD) and Obsessive Compulsive Disorder (OCD) [74]. BW animals are variable in their repetitive behavior at a frequency suggestive of a genetic polymorphism within the stock. Anecdotal observations suggest that PO animals engage in far less such behavior. As PO animals exhibit much less sexual dimorphism in body size and parental behavior (PO males aid in pup-rearing), we hypothesized that BW animals would also be more dimorphic in other measures.

Recent sequencing of both the BW and PO genomes makes identification of the polymorphisms underlying these behavioral differences feasible. Thus, genetic studies of mammalian systems that naturally exhibit variations in social and repetitive behaviors could lead to discovery of causative alleles and subsequent development of natural disease models (e.g. ASD, OCD, ADHD). Simple assessment of whether there are shared genetic components between these characteristics may be relevant to understanding disease etiology. We therefore tested BW, PO, (BW x PO) F₁ hybrids and BW Y^{PO} consomic animals as an initial assessment of the genetic underpinnings of the interspecific behavioral differences.

We also tested animals heterozygous for the wide-band *Agouti* allele (*A^{Nb}*). The *A^{Nb}* allele is a natural variant of the *Agouti* (*a*) locus that has been bred onto a BW genetic background [87]. This allele overexpresses the *Agouti* gene, resulting in a more yellow coat color. This allele is thought to be adaptive, as animals carrying *A^{Nb}* live in a sandy habitat [88]. We are also using *A^{Nb}* as a biomarker for epigenetic effects, similar to the lab mouse viable yellow allele of

Agouti (A^{vy}) [89]. *Peromyscus* lacking AGOUTI expression (black or non-agouti) have been shown to be less aggressive and groom more than their wild-type AGOUTI counterparts [90]; these differences are thought to be due to the AGOUTI protein's function as a melanocortin receptor antagonist [91]. We therefore expected the opposite trend from A^{Nb} animals (i.e. more aggressive, less grooming). Moreover, as PO animals are lighter colored than BW, we hypothesized that A^{Nb} behaviors might be more similar to PO animals in some aspects of social behavior.

As an initial step towards these goals, we employed a simple behavioral test battery that can be employed on hundreds of back- or inter- cross animals as initial assessment of these species differences. Thus, we used an open field test and a novel individual/social interaction test in this study. Major goals of this study were to 1) quantitate basic interspecific differences; 2) assess whether these simple tests would uncover sufficient variation to undertake back and/or intercross tests and 3) assess basic inheritance patterns of the interspecific differences.

Materials and Methods

Animals

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina. Animals were taken from the stocks maintained at the *Peromyscus* Genetic Stock Center. Animals were kept on a 16:8 hour light-dark cycle and were given food and water *ad libitum*. All animals tested were 4-6 month old (young adult; both species live 4+ years)

virgins. All animals had been housed with other same-sex animals post-weaning, and were tested in the middle of the light period (>4 hours from both lights on/off). We bred BW females to PO males to obtain F₁ hybrids. We bred BW females to homozygous A^{Nb} males to generate A^{Nb} heterozygotes. Apart from breeding records and coat-color, A^{Nb} genotype was also determined by several SNPs [88]. PCR primers to generate a ~200 bp amplicon for sequencing were: Agouti F gggattcgttttccagggtt and Agouti R aacgctgtgggttcagactc. These A^{Nb} heterozygotes, BW, PO, (BW x PO) F₁ hybrids BW Y^{PO} consomic (15th generation backcross, as previously described [82] were all tested.

Behavioral Testing

We tested twelve males and twelve females of BW, PO, F₁, and A^{Nb} stock and twelve males from the Y consomic stock (which are only male). Each open field test consisted of first placing a single animal into a standard rat (10.25"W x 19"L x 8"H) opaque polycarbonate cage with ~ 0.75 inches of aspen shavings and a ventilated transparent cover. After five minutes of observation, we introduced a novel animal of the same sex and species. The subsequent five minute period was the social interaction test. The novel animal's tail was marked with a non-toxic marker to distinguish it from the animal being tested. The cage was cleaned between each animal tested (including replacement of bedding).

Video Analysis

All behaviors were recorded with a digital camcorder. We used the Noldus Observer XT software (<http://www.noldus.com/>) to score behaviors from the video data. For the open field test, we scored the following behaviors: burrowing,

freezing, jumping, back-flipping, running in circles, and grooming. Based on these videos, we considered straight vertical jumping, back-flipping, and running circles as repetitive behaviors. We also scored exploratory behaviors (e.g. walking the cage perimeter) and instances where the animal remained stationary, but these were not included in the analyses as they did not appear informative.

For the social interaction test videos, we scored the same behaviors as in the open field test with the addition of social and aggressive behaviors. General social behaviors included sniffing, following, and allogrooming. Aggressive behaviors included biting, chasing, boxing, and mounting. Many of these had been described by Eisenberg in the “Behavior Patterns” chapter of the first comprehensive *Peromyscus* compilation [92].

All behaviors were scored by incidence; we assessed behavior type at five second intervals throughout the video. Two people scored each video; overall inter-rater reliability was at least ninety-five percent. At least one scorer was blind to the genotype of the animals being scored. When specific behavioral assessments disagreed, we alternated accepting the assessment of scorer 1 vs. scorer 2. The data collected by scoring videos were graphed with Microsoft Excel. Behaviors are reported as percentage of incidence of behavior. Statistics were calculated using the Minitab and SPSS software packages. Note that we used Kruskal–Wallis one-way analysis of variance in cases where there was clearly a non-normal distribution in one or more of the groups being compared, and ANOVA in other instances.

Results

Differences in Repetitive Jumping Behavior between Stocks and Sexes.

Because the data did not meet the assumption of normality for analyses of variance, the data were analyzed using the non-parametric Kruskal-Wallis test. As predicted, BW animals engaged in more repetitive behavior than other stocks in combined sex analyses (Figure 2.1). BW animals exhibited significantly higher amounts of repetitive behaviors when compared to PO, F₁, A^{Nb} and BW Y^{PO} animals ($p \leq 0.008$, Kruskal-Wallis test). The difference with the latter two categories is most surprising as both stocks have a genetic make-up that is almost entirely BW. The differences between BW and the (BW x PO) hybrids also suggest dominant PO sequences in suppressing such behavior. We also assessed sexual dimorphism of repetitive behaviors within each stock (Figure 2.2). While males of each stock had higher levels of repetitive behavior, the difference was only significant in the A^{Nb} stock ($p = 0.049$, Kruskal-Wallis test).

As noted, previous studies have shown that BW animals fall into at least two groups based on jumping frequency (i.e. high-frequency vs. low-frequency jumpers). Such a pattern is evident in males of the BW, PO, and Y consomic stocks (Figure 2.3). Significance could not be calculated for A^{Nb} males as only one high jumper was recorded. Surprisingly, the BW x PO hybrids did not have two apparent groups; this may be due to the limited number of parents we employed to generate F₁ animals used in this study.

A bimodal jumping distribution is also evident in BW female animals, but not females of other stocks (Figure 2.4). This finding may be attributed to the low

average amount of jumps in females within stocks other than BW, at least during the short interval we observed.

Differences in Burrowing between Stocks and Sexes

The open field tests yielded only one significant difference between stocks in digging/burrowing behavior: A^{Nb} animals dug more than BW animals ($p=0.017$, Kruskal-Wallis test; Figure 2.5). In social interaction tests, however, digging is significantly higher in PO, F_1 , and A^{Nb} animals as compared to BW animals ($p\leq 0.014$, Kruskal-Wallis test). This suggests that PO alleles are dominant in inducing a predisposition to digging, and that variation at the *Agouti* locus may be a major contributor to these differences. Consistent with this hypothesis, BW Y^{PO} consomic males are similar to BW males in digging incidence (Figure 2.5).

Sex differences in digging incidence were apparent across all groups, with females always having a greater propensity to dig/burrow. However, only the difference between female and male F_1 animals was found to be significant ($p=0.026$, Kruskal-Wallis test; Figure 2.6).

Grooming Differences between Stocks and Sexes

BW animals (combined sexes) self-groom significantly less than PO and F_1 animals ($p\leq 0.043$, Kruskal-Wallis test; Figure 2.7). This again suggests dominant PO alleles that mediate such behavior. These inter-stock differences are more pronounced in males: BW males groom significantly less than PO males and Y consomics ($p\leq 0.019$, Kruskal-Wallis test) (Figure 2.8).

In contrast, females of each stock tested perform self-grooming behaviors in similar amounts (Figure 2.9). Sexual dimorphism in grooming behaviors was

most noticeable in PO animals: Male PO animals groom significantly more than female PO animals ($p=0.025$, data not shown). This sexual dimorphism is not evident in any of the other stocks tested.

Surprisingly, PO, F_1 , and BW Y^{PO} males exhibit an apparent bimodal distribution for grooming behavior. This pattern is not evident in BW or A^{Nb} males (Figure 2.10), and thus consistent with being influenced by PO alleles of Y chromosome sequences. Similar to jumping, there appear to be high grooming, low grooming, and no grooming categories. The differences between high versus low/no grooming groups in males of stocks noted above were confirmed as significant using t-tests.

Comparisons of Social Behaviors between Stocks and Sexes

BW animals engaged in significantly less general social behavior (as noted- allogrooming, sniffing, following) than animals of the PO, F_1 , and A^{Nb} stocks ($p \leq 0.002$, Kruskal-Wallis test; Figure 2.11). Only BW Y^{PO} consomic males registered levels of social behavior similar to BW males (i.e. alluding to the fact that the Y chromosome plays no significant role in these species differences). PO animals also exhibited more social behavior than both A^{Nb} and F_1 animals ($p \leq 0.001$, Kruskal-Wallis test). Thus the higher levels of PO social behavior are consistent with a single semi-dominant locus or perhaps several loci (e.g. one dominant, one recessive). The A^{Nb} stock animals exhibit these behaviors at the same levels as the F_1 animals, suggesting a role for the *Agouti* gene in mediating such behaviors.

Intra-stock sexual dimorphism in these general social interactions is evident in several stocks. Male F_1 animals are more social than female F_1 animals ($p=0.024$, Kruskal-Wallis test) and male A^{Nb} animals are more social than female A^{Nb} animals ($p=0.006$, Kruskal-Wallis test; Figure 2.12).

Differences in Aggressive Behaviors between Stocks

The general social behaviors observed in A^{Nb} animals appeared to frequently lead to aggressive encounters. This hypothesis is supported by data showing that the incidence of aggressive behaviors (biting, boxing, chasing, mounting) was significantly higher in A^{Nb} animals than any other stock ($p\leq 0.022$, Kruskal-Wallis test; Figure 2.13).

The greatest contrast was with the PO animals, for which we did not record any aggressive behaviors. However, the BW, F_1 and Y consomic lines were intermediate between the PO and A^{Nb} lines (though the BW animals had much less variability than the latter two lines). Thus these data suggest a combination of BW and the A^{Nb} (or a tightly linked) alleles results in the most aggressive behavior. In this case, the BW alleles appear to be dominant to those of PO, and the PO Y chromosome does not appear to play a role.

Discussion

These data indicate the great potential of using this *Peromyscus* species group to elucidate the genetic (& epigenetic) basis of mammalian behaviors. The data presented here show that multiple genetic modules underlie the complex behavioral differences between the monogamous species *P. polionotus* and the polygamous *P. maniculatus* as well as their variants (e.g. the wide band agouti

stock, A^{Nb}). In combination with the nascent resources (genome sequences and a genetic map of the BW and PO stocks), back- or intercrosses may be used to discover the genetic architecture underlying several important traits [93].

The pathways underlying BW repetitive behaviors (jumps flips, circle running) appear to be affected by variation at multiple loci. First, we hypothesize that an ancestral polymorphism underlies the bimodal distribution observed within both the BW and PO stocks (i.e. a single locus with two additive alleles; for example, $HH > Hh > hh$). An additional locus or loci must therefore underlie the significant differences in repetitive behaviors between the two populations. In males, the Y chromosome must play a role, as the BW Y^{PO} consomic animals are not distinguishable from their PO male ancestors in the incidence of repetitive behavior. It is possible that epigenetic variation also plays a role in etiology of these stereotypies, as environmental factors reduce the incidence later in life [83]. Definitive genetic tests must be performed to determine the genetic vs. epigenetic contribution to the BW distribution (e.g. mating high incidence animals and assessing repetitive behaviors in the offspring).

We suggest that the *Agouti* gene (*a*) may be also involved, given the reduced jumping in the A^{Nb} animals and potential pleiotropic effects of this hormone pathway. However, while the A^{Nb} has been bred onto the BW background for decades, it is possible that genes tightly linked to *Agouti* have not recombined. If so, these animals may have non-BW alleles which are the source of differences in the A^{Nb} line. There are several loci (largely with unknown

function) that overlap the large *Agouti* locus and thus necessarily cannot recombine when selecting for the A^{Nb} allele.

While the PO allele(s) of the loci affecting the intra-specific differences in repetitive behavior must be dominant, it is not necessarily clear which is the derived (vs. ancestral) condition. There is variation even within *P. maniculatus* in such behaviors: a forest subspecies, *P.m. gracilis*, jumps and freezes less than *P.m. bairdii* (e.g. BW) [94].

The deeper, more elaborate burrows built by PO animals are influenced by a major and several minor autosomal loci [95,96]. The distinct nesting styles may be indicated by the differences in digging activity we observed even in these short duration tests. In this case, the PO alleles underlying this difference appear clearly dominant, as shown by the burrowing activity of the hybrids. The PO Y chromosome clearly does not play a role, as evidenced by the similar profiles of BW and Y consomic animals. However, the *Agouti* locus again is a suspect in these differences, as the A^{Nb} animals are similar in profile to the PO stock. This raises the possibility that the A^{Nb} or a tightly-linked allele was selected for behavior in addition to the cryptic coloration.

There is some indication of an ancestral sexual dimorphism in burrowing, as females in every stock had a higher percentage of burrowing activity. While this difference only achieved statistical significance in the BW x PO hybrids, we suggest that testing additional animals may resolve this issue. It seems possible that the differential burrowing activity is related to greater nest-building by females.

Self-grooming behaviors are more complicated. Females of each stock self-groom in near-equal amounts, but males differ significantly with PO males grooming much more than BW males. Again the PO alleles are at least semi-dominant, as reflected by increased (relative to BW) self-grooming in both the hybrid and Y consomic lines. However, PO males also have an apparent bimodal distribution in terms of self-grooming levels; the apparent presence of two such groups in both the F₁ and Y consomic lines is consistent with an effect of Y chromosome sequences. How the PO Y chromosome would induce such a distribution in a line (lacking a bimodal distribution) is less clear. Our hypothesis that *A^{Nb}* animals would groom less was clearly contradicted, nor is there convincing evidence from these studies that this locus is involved in the interspecific differences in self-grooming.

In *Mus*, self-grooming is considered an anxiety behavior [97,98]. This interpretation is intriguing given that PO animals have significantly higher levels of the stress hormone corticosterone than BW animals, but appear able to buffer its effects better as reflected by their ability to regulate blood glucose levels [81]. Interestingly, the Y consomic animals exhibited significantly lower corticosterone levels than either stock, and had blood glucose drop to very low levels when challenged [82]. The hypothesis that PO Y chromosome sequences affect self-grooming is also supported by PO males grooming significantly more than PO females. Thus it is possible that the PO Y chromosome is the sole determinant of the inter-specific and male intra-specific differences, but interactions with autosomal loci seems likely.

Apart from the susceptibility of BW to stereotypies, perhaps the most intriguing differences between these two species are those involving social behaviors. Indeed we hypothesize that the greater social interactions frequently seen in monogamous species requires greater stress buffering in order to engage in these behaviors (as observed in PO). As hypothesized, PO animals engage in such behaviors significantly more than BW animals. The intermediate status of the F₁ animals suggests the PO trait is semi-dominant, or affected by multiple loci. The presence of significant sexual dimorphism in the F₁ hybrids (but not in PO) is more consistent with the latter.

Despite greater amount of these interactions in male hybrids, the Y chromosome appears to play no role in these behaviors: BW Y^{PO} males were indistinguishable from standard BW animals. The *Agouti* locus, however, is again a candidate, as the A^{Nb} animals exhibit comparable levels of social interaction to the F₁ hybrids and have a similar sexual dimorphism in those behaviors (with males engaging in more interactions).

A major difference in A^{Nb} social encounters is that they led to aggressive behaviors at twice the frequency of any other stock; note that this supports the hypothesis that levels of the AGOUTI protein are causal to aggressiveness [90]. The multiple behavioral effects (burrowing and aggression) of A^{Nb} raises the question of whether the lighter color it confers (i.e. cryptic coloration) is the only cause for selection of this allele [88,99].

The A^{Nb} aggression frequency is most divergent from the PO animals, for which we did not record any aggressive behaviors. While PO males have been

documented as being more aggressive than BW, this was in a resident intruder test wherein the first male had been housed alone for several weeks before introduction of the second male (i.e. allowing establishment of a territory [74]). Also, animals in the present study were housed under long day (16 hrs light) conditions, and aggression is maximized under short days [74,100] as well as using unfamiliar animals [101]. For aggressive behaviors under these conditions (meeting of an unfamiliar animal in an open neutral space), the BW alleles appear dominant, as the F1 (and Y consomic) exhibit similar frequencies. The latter is surprising, as the Y chromosome has extensive documentation as contributing to differential aggression in (inbred) *Mus* lines [102-104]. However, the Y chromosome and testosterone are generally considered to be more involved in territorial aggression while the current study would likely measure what would be considered defensive aggression [105].

Unlike other more commonly used mammalian models, *Peromyscus* offer the opportunity to assess the effects of natural genetic variation on disease/disorder predisposition. Moreover, their behavioral repertoire offers opportunities not present in laboratory mice or rats. These initial studies suggest that a number of important characteristics (e.g. repetitive behavior susceptibility, social interaction tendencies) are tractable through genetic studies via these simple behavioral assays. In addition to straightforward back or intercrosses, these analyses show that consomic or variants at individual loci may also be informative. For example, assessing the male offspring of homozygous A^{Nb}

females bred to BW Y^{PO} males may yield further insights into the genetic basis of the behaviors described here.

Thus, further behavioral genetic studies of these *Peromyscus* stocks may lead to novel and more natural biomedical models for conditions such as ASD, anxiety-related disorders, and those related to impaired social interactions. For example, a number of *Mus* inbred strains have been extensively characterized for social and repetitive behaviors [106-107]. Of these, the C58 strain has evolved as an ASD model [108-109]. Behavioral variation in these *Peromyscus* lines appears to compare favorably to the *Mus* lines; more extensive testing (e.g. elevated plus maze, Barnes Maze) will aid further comparisons. While these animals do not yet have the molecular tools available in *Mus*, the *Peromyscus* lines offer several advantages. These include their wild-derived genomes, outbred status (e.g. natural heterogeneity in repetitive behavior exhibited by the BW animals) and social behaviors not seen in *Mus* (pair-bonding). Additionally, this system has a unique potential for understanding the evolution of monogamy and co-selected traits.

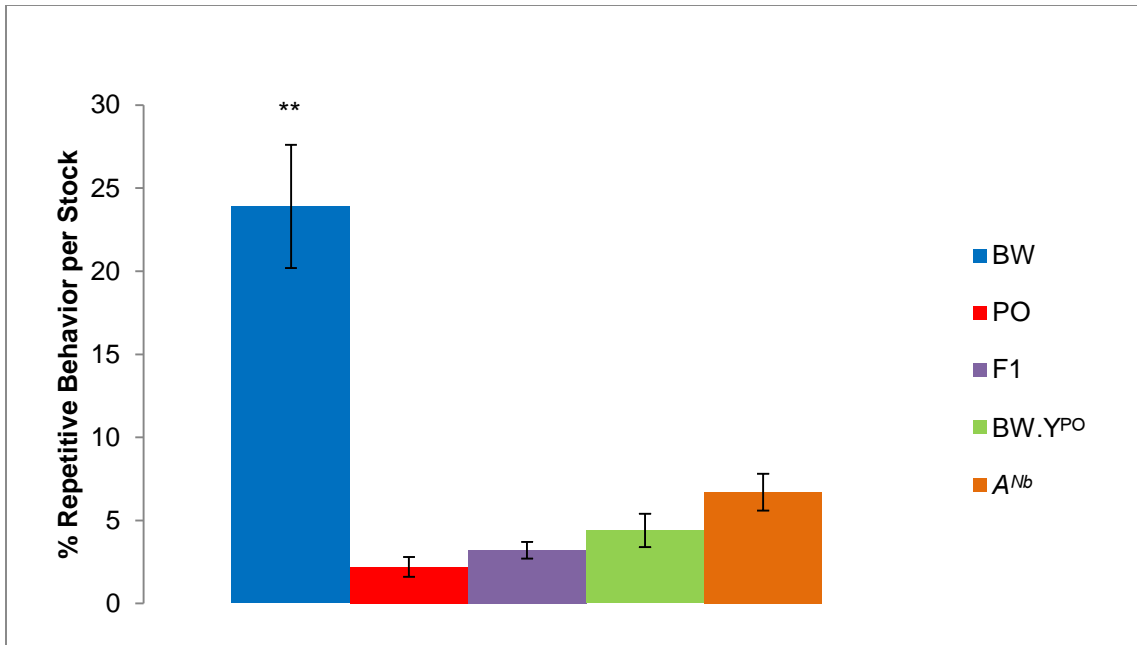


Figure 2.1: Frequency of repetitive behaviors. Frequency of repetitive behaviors (various kinds of jumps, circle running) in each stock were tested as a percentage of total behaviors. Mean values with standard error (bars) are shown. BW values are significantly different when compared to each of the other stocks ($p \leq 0.008$, Kruskal-Wallis test). Other stocks show no significant differences in pair-wise comparisons using Kruskal-Wallis. Double asterisk indicates $p \leq 0.01$ comparing BW to other stocks.

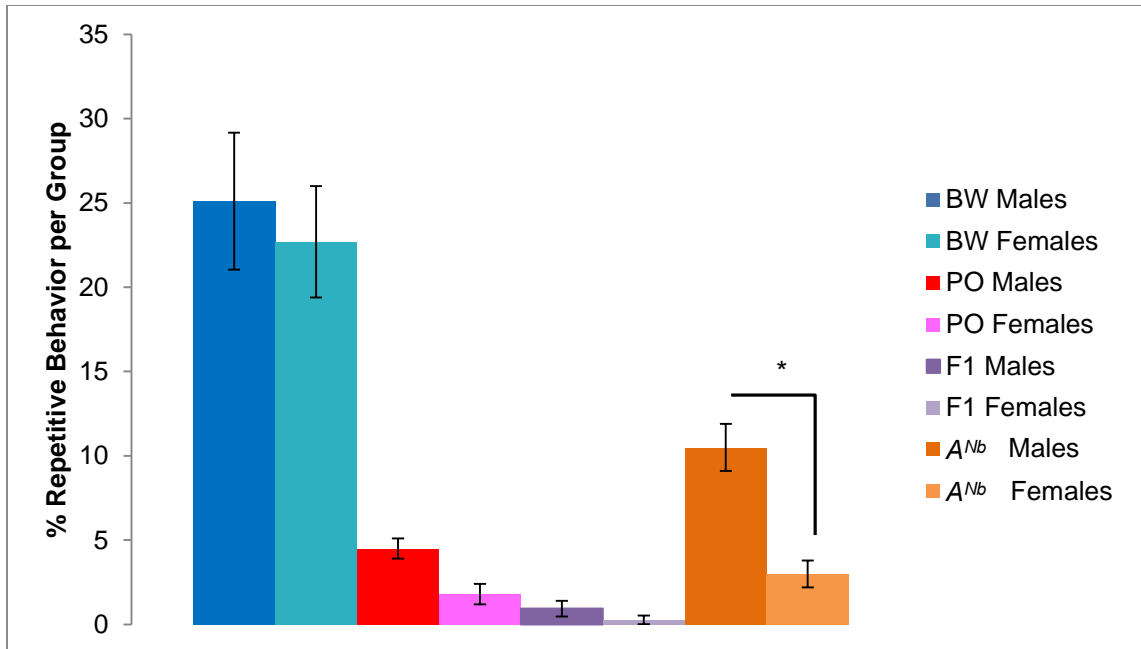


Figure 2.2: Repetitive behavior differences between sexes. Mean values with standard error (bars) are shown. A^{Nb} males perform repetitive behaviors significantly more than A^{Nb} females ($p=0.049$, Kruskal-Wallis test; $p=0.041$, 1-way ANOVA). BW and PO males perform repetitive behaviors more than the females of their respective stocks, but these differences are not statistically significant according to a 1-way ANOVA. A single asterisk indicates $p<0.05$ between the sexes of a given stock.

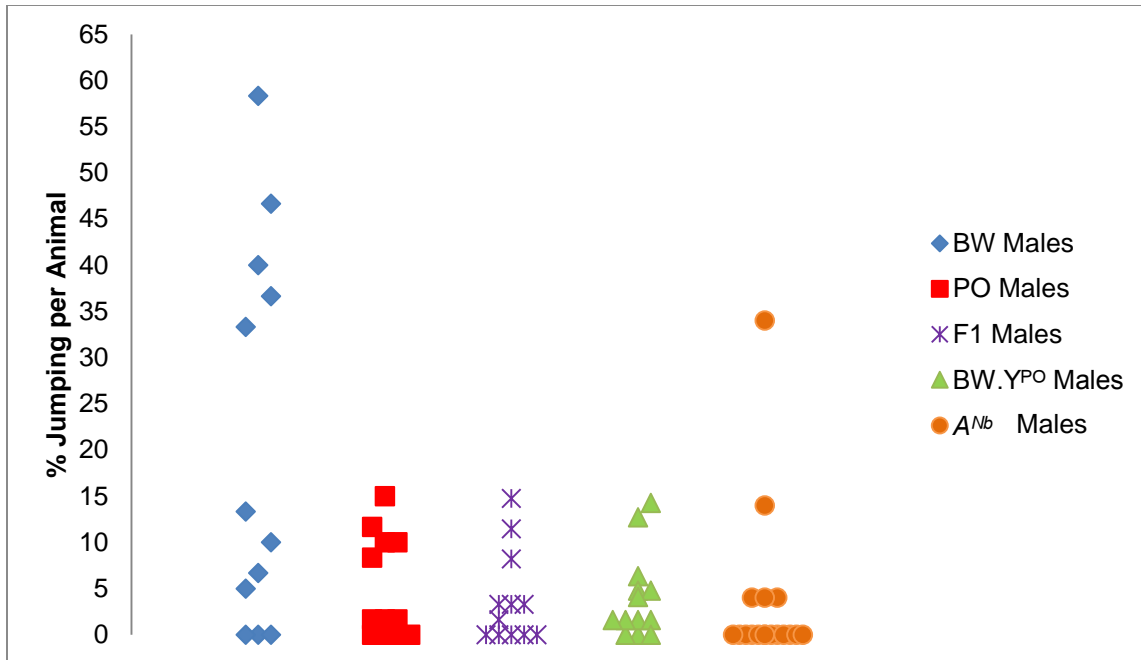


Figure 2.3: Potential bimodal distribution of jumping in males. High jumping groups were compared to low jumping groups in the same stock using a 2-tailed t-test: Male BWs (test high, N=5, vs. low jumper, N=7) $t=7.87$, $p=0.001$, $DF=5$; t test PO males (high, N=5, vs. low jumper, N=7) $t=8.11$, $p=0.001$, $DF=4$; Y consomics (high, N=2 vs. low jumper, N=9) $t=12.87$, $p<0.001$, $DF=6$. Differences were significant for the BW ($p=0.001$), PO ($p=0.001$), and BW YPO ($p<0.001$). Differences between high and low jumpers were not significant for ANb and F1 males although for these groups, too few high jumpers were recorded.

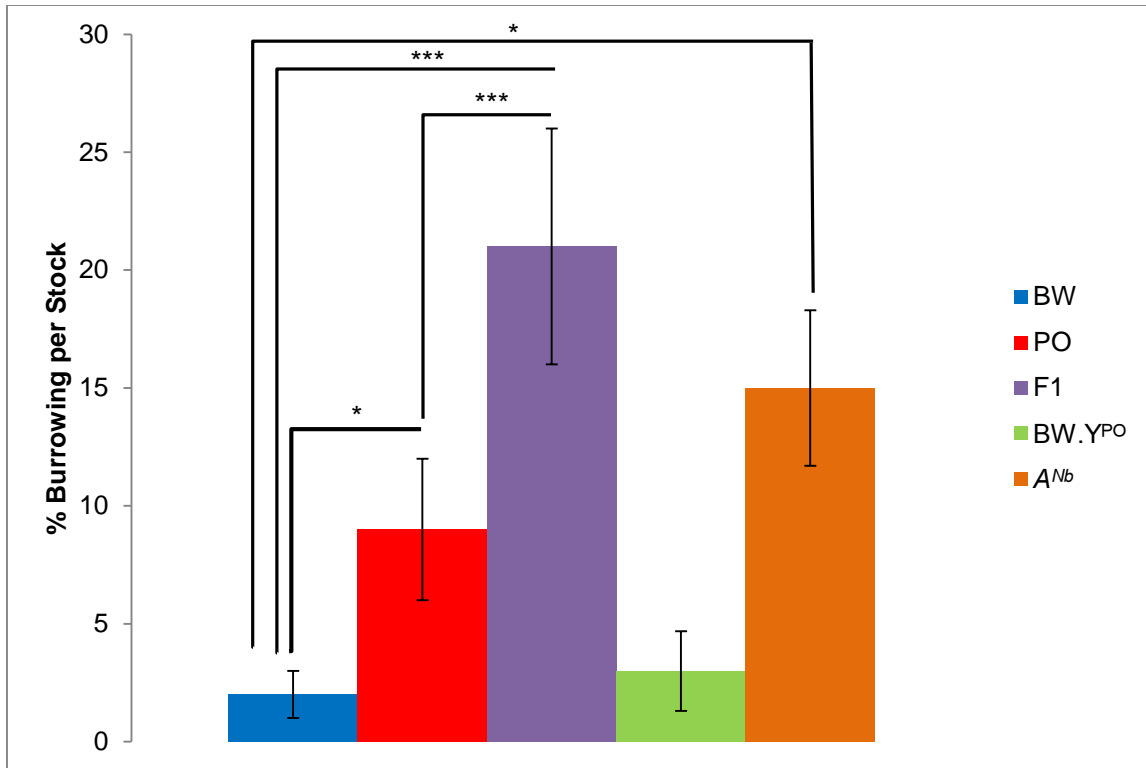


Figure 2.5: Frequency of digging/burrowing behaviors. These are from social interaction tests. Mean values with standard error (bars) are shown. Burrowing is significantly higher in PO, F₁, and A^{Nb} animals than in BW and BW Y^{PO} animals ($p \leq 0.014$, Kruskal-Wallis test). F₁ animals burrow significantly more than PO animals as well ($p = 0.013$, Kruskal-Wallis test). Asterisks indicate significance compared to BW (* indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$).

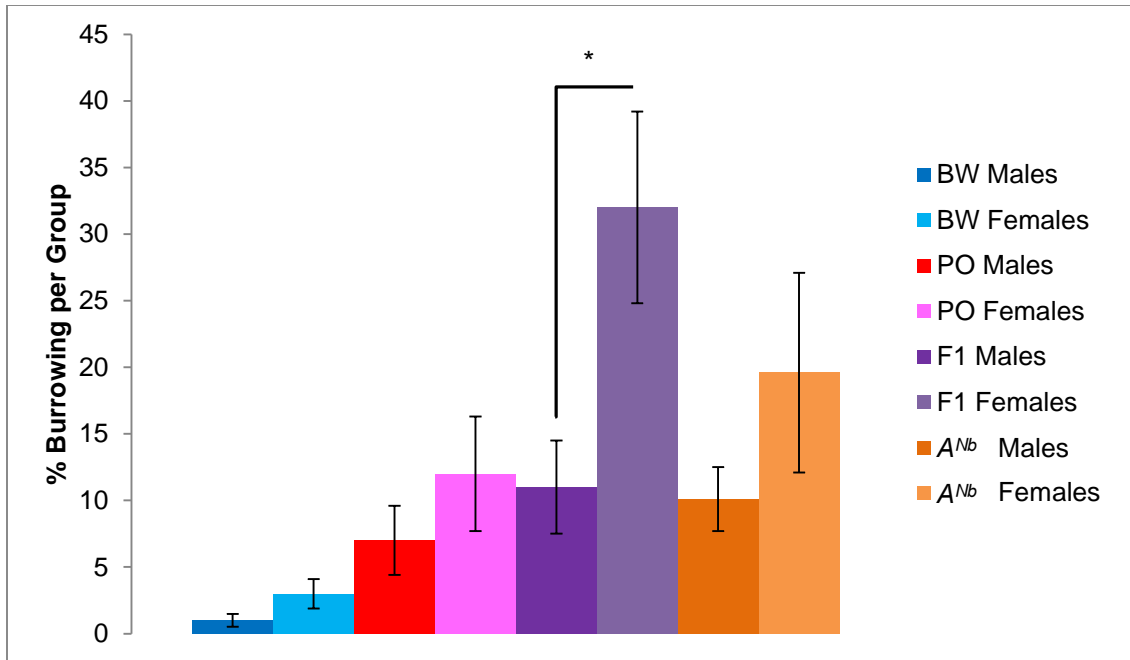


Figure 2.6: Sexual dimorphism in digging/burrowing. The difference observed between F₁ males and females was statistically significant ($p=0.026$, Kruskal-Wallis test; $p=0.013$, 1-way ANOVA). Females of each stock burrow more than males but are not statistically significant by 1-way ANOVA. A single asterisk indicates $p<0.05$ between sexes of a given stock.

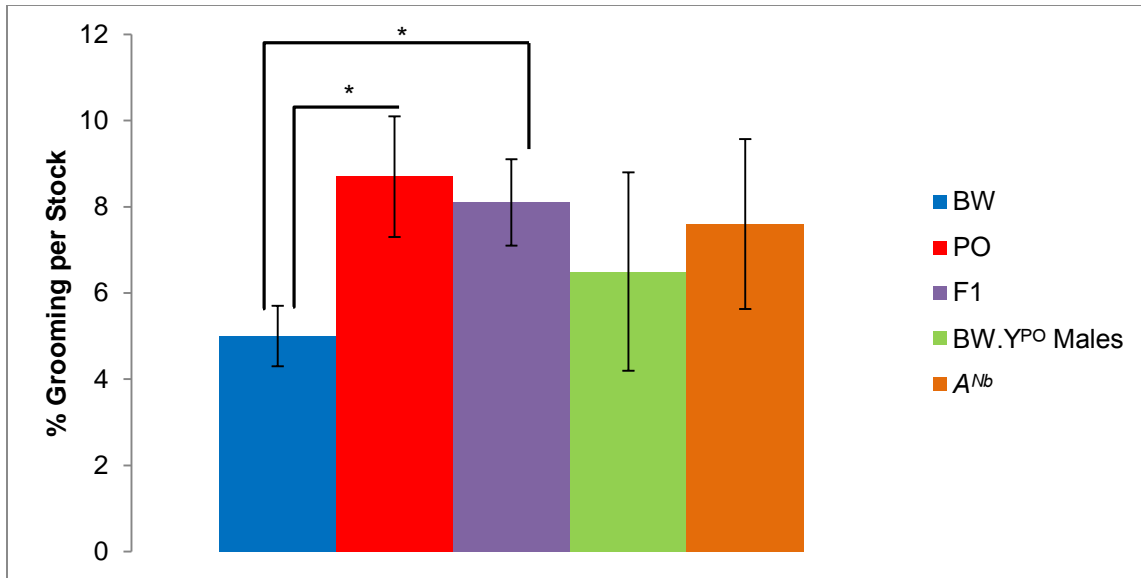


Figure 2.7: Self-grooming frequency in each stock. Mean values with standard error (bars) are shown. Self-grooming is higher in PO and F1 animals than in BW animals ($p \leq 0.043$, Kruskal-Wallis test). A single asterisk indicates $p < 0.05$ in comparison to BW.

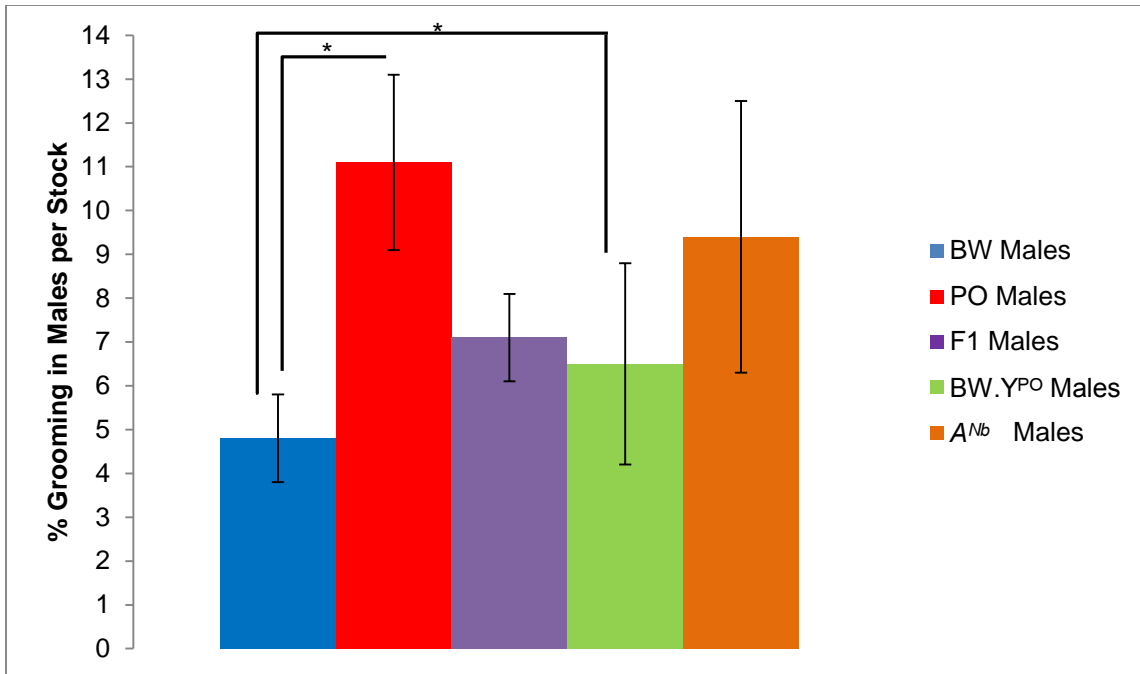


Figure 2.8: Self grooming differs between males of different stocks. This is particularly true for PO males vs. BW males ($p=0.009$, Kruskal-Wallis test). BW Y^{PO} animals groom significantly more than BW males ($p=0.019$, Kruskal-Wallis test). Although grooming may appear to be different when comparing BW vs. F₁ and PO vs. F₁, these differences were not significant using Kruskal-Wallis tests. Asterisks signify significance in comparisons to BW males (* indicates $p<0.05$).

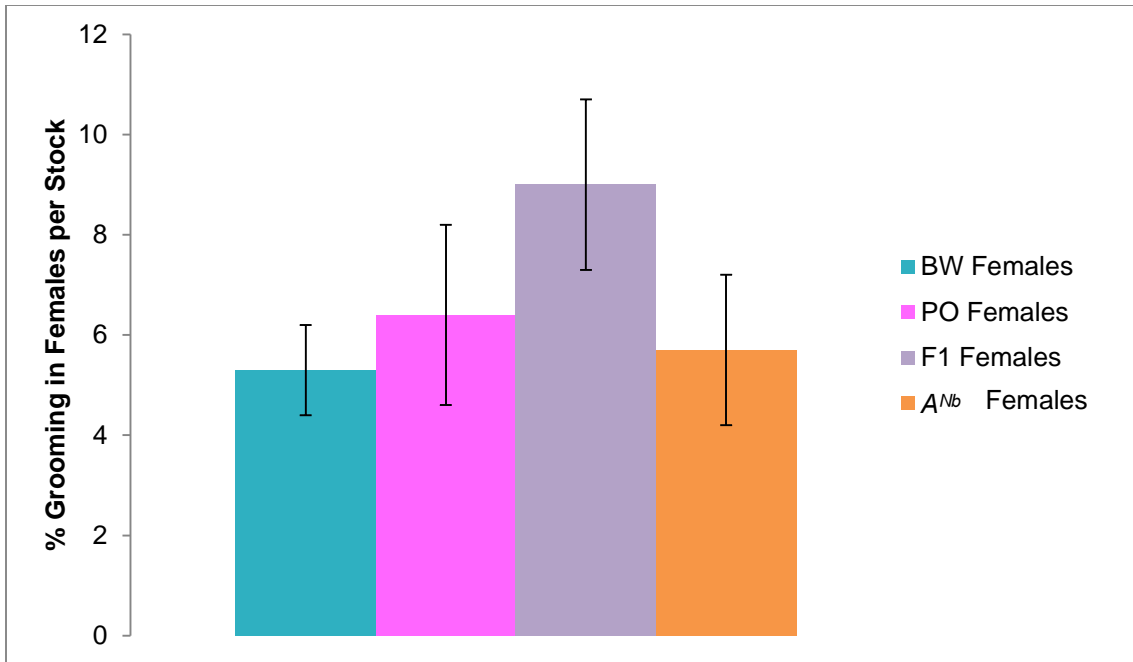


Figure 2.9: Self grooming is similar in females of different stocks. There is no significant difference between females of stocks in self-grooming as determined by Kruskal-Wallis tests.

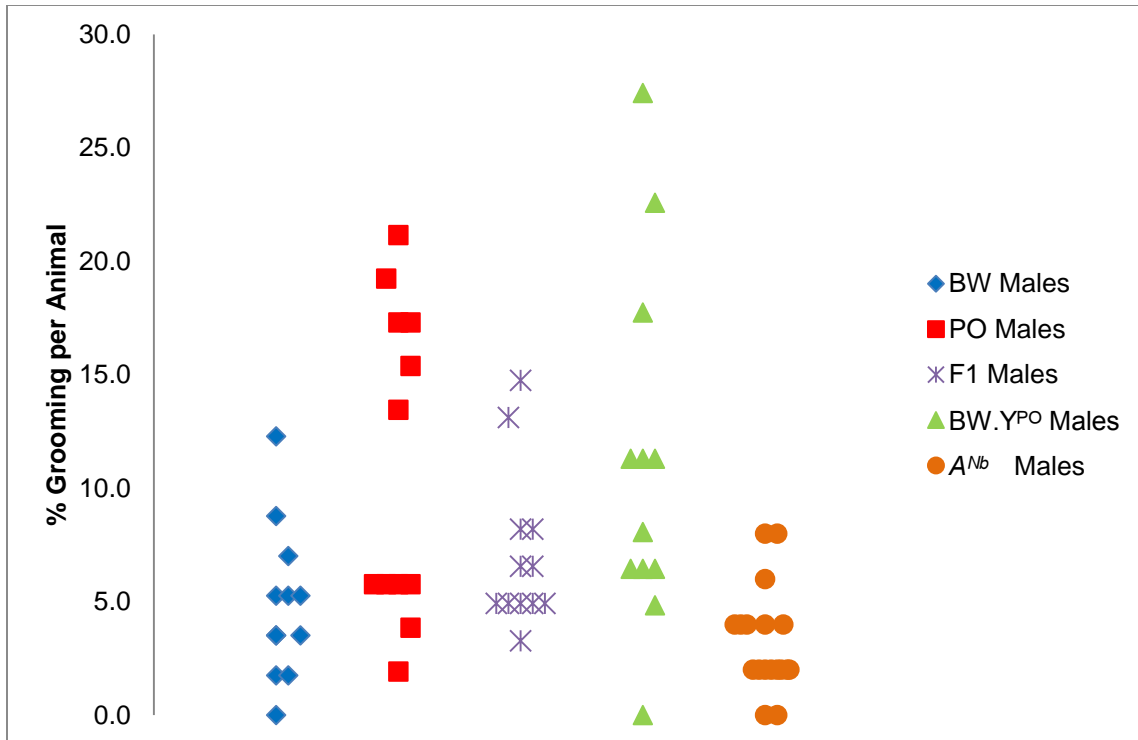


Figure 2.10: Possible bimodal distribution of self-grooming in males. Self-grooming shows a bimodal distribution in PO, F₁, and BW Y^{PO} male animals but not in BW or A^{Nb} animals. High grooming groups were compared to low grooming groups in each stock using a 2-tailed t-test: PO male (high, N=5, vs. low groom, N=7) $t=9.66$, $p<0.001$, $DF=8$. F₁ male (high, N=2, vs. low groom, N=11) $t=3.43$, $p=0.042$, $DF=3$. Y consomic (high, N=4, vs. low groom, N=8) $t=4.82$, $p=0.017$, $DF=3$. There were no high and low groomer groups within BW and A^{Nb}, so statistics between two groups could not be calculated.

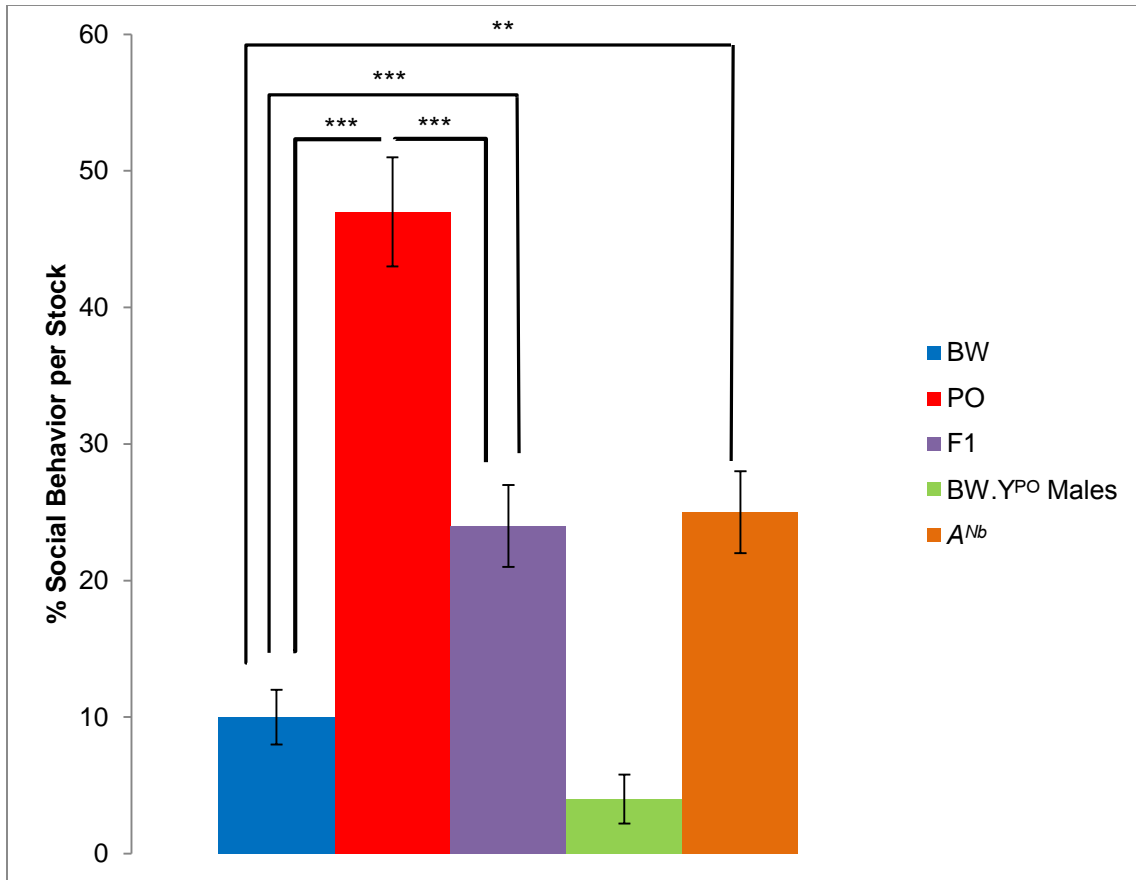


Figure 2.11: Social behavior frequency. Social behaviors occur more frequently in PO animals when compared to BW animals ($p<0.001$, Kruskal-Wallis test). Mean values with standard error (bars) are shown. F₁ animals are significantly different from both BW ($p=0.001$, Kruskal-Wallis test) and PO ($p<0.001$, Kruskal-Wallis test), indicating an incomplete dominance mode of inheritance. A^{Nb} animals are also more social than BW animals ($p=0.002$, Kruskal-Wallis test). Asterisks indicate significance when compared to BW (* indicates $p<0.05$, ** indicates $p<0.01$, and *** indicates $p<0.001$).

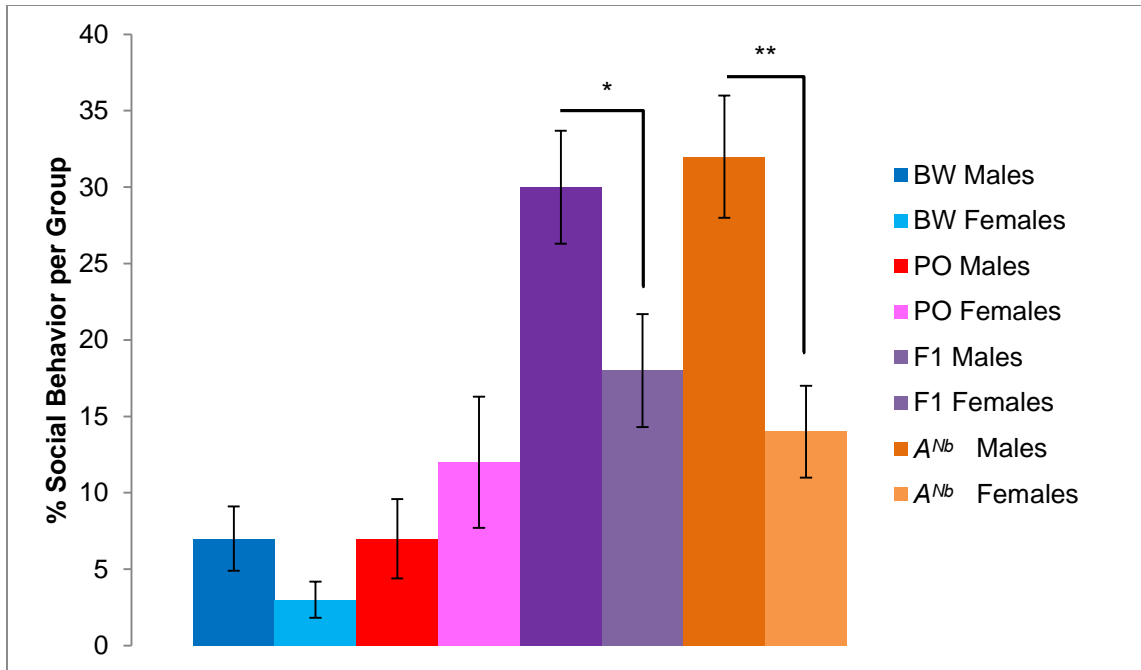


Figure 2.12: Sexual dimorphism in social behaviors. The social behavior difference between male and female is significant only in F₁ animals ($p=0.024$, Kruskal-Wallis test) and A^{Nb} ($p=0.006$, Kruskal-Wallis test) stocks. Asterisks indicate significance differences between the males and females of a given stock (* indicates $p<0.05$ and ** indicates $p<0.01$).

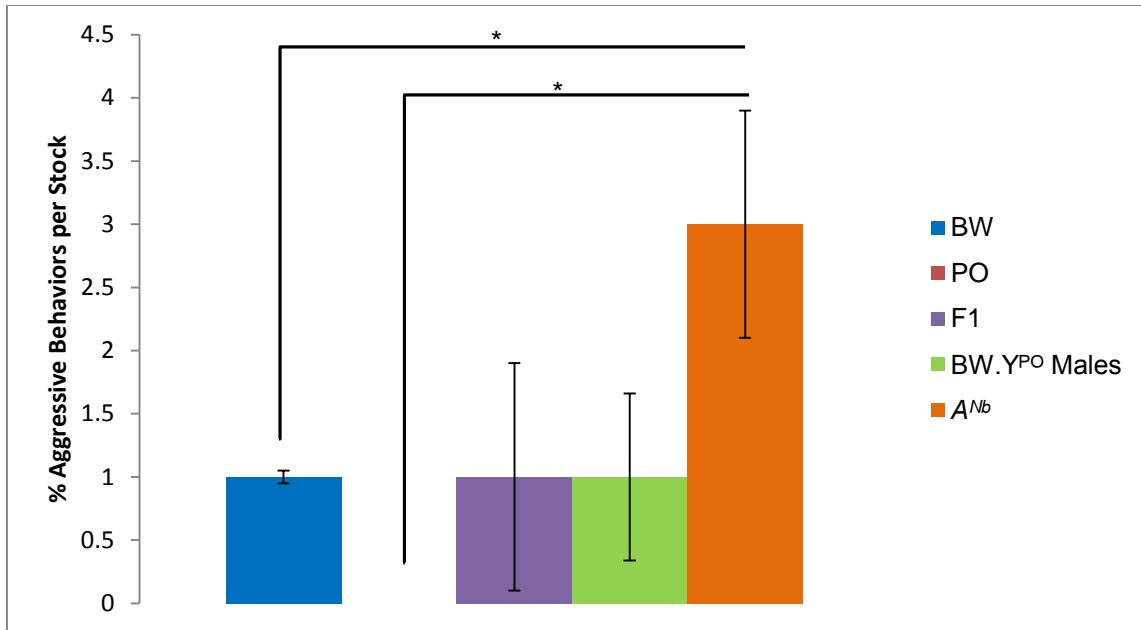


Figure 2.13: Aggressive behavior frequency. A^{Nb} animals exhibit higher amounts of aggressive behaviors than other stocks tested although comparisons of A^{Nb} to F_1 and $BW.Y^{PO}$ are not significant using Kruskal-Wallis. Importantly, A^{Nb} animals are significantly more aggressive than BW animals ($p=0.022$, Kruskal-Wallis test). A^{Nb} also were more aggressive than PO animals ($p=0.014$, Kruskal-Wallis test). A single asterisk indicates $p<0.05$. Note that PO animals performed no aggressive behaviors during the test.

Chapter 3

Pleiotropic effects of a methyl-donor methyl donor diet in a novel animal model¹

Introduction

Folic acid and related B vitamin consumption has increased over the last decade, due not only to direct supplementation (i.e. vitamin tablets/capsules) but also to enrichment of grains [110,111], and addition to other products such as energy drinks (for example, 5-hour energy drinks).

The 1-carbon/methyl donor pathway, to which these molecules contribute, is essential to many biological processes. Since these components are involved in production of SAM (S-Adenosyl Methionine), this and other data suggest that these nutrients act through epigenetic mechanisms, as methylation of DNA and histone amino acid residues are known to mediate epigenetic effects [13,112].

Few studies have been done on natural variants or examination of other potential effects of a methyl-donor diet such as that used in previous *A^{vy} Mus* studies [15,16]. *Peromyscus* are wild-derived North American rodents and thus represent natural populations/genomes in ways that more widely used models do not [113]. We therefore tested the 1x diet originally used in the *A^{vy}* studies on *P*.

¹Kimberly R. Shorter, Vanessa Anderson, Patricia Cakora, Amy Owen, Keswick Lo, Janet Crossland, April C.H. South, Michael R. Felder, and Paul B. Vrana. 2014. Submitted to PLoS ONE.

maniculatus. We employed a naturally occurring variant termed wide-band agouti (A^{Nb}) as a biomarker for the effects of the diet [87,113]. The A^{Nb} allele is otherwise on a BW (http://stkctr.biol.sc.edu/wild-stock/p_manicu_bw.html) genetic background, a *P. maniculatus* stock whose genome has recently been sequenced (<http://www.ncbi.nlm.nih.gov/assembly/84591/>) and mapped [93]. Effects of the diet on the A^{Nb} animals would suggest general effects of the diet, as there is no evidence for a retroelement in this allele [114].

We therefore wished to assess whether the diet overtly affected behavior in addition to potential effects on the A^{Nb} allele. These studies provide novel evidence of deleterious effects of large doses of these compounds typically considered therapeutic or preventive to disease.

Methods

Ethics Statement

All procedures were approved by the University of South Carolina Institutional Animal Care and Use Committee (IACUC; protocol #1809-100340-061011).

Animal Husbandry & Mating Schemes

Animals were taken from the stocks maintained at the *Peromyscus* Genetic Stock Center (<http://stkctr.biol.sc.edu/>). Animals were kept on a 16:8 hour light-dark cycle and were given food and water *ad libitum*. Matings of BW female x A^{Nb} male were established and maintained on either the methyl donor diet (Table 3.1) or normal rodent chow (i.e. controls). Offspring were weaned at approximately 25 days of age and maintained on the methyl donor diet or normal rodent chow until reaching six months of age (to obviate any concerns about

maturity of coat-color; note that these animals live >4 yrs). Additional tissues from both ages are available to interested investigators.

Behavioral Testing

Offspring of the BW female x A^{Nb} male matings were evaluated in open field and social interaction tests at 4-6 months of age, as previously described [20]. These tests were conducted during mid to late light cycle (late morning to early afternoon) and were done during late summer to early fall on 10 separate testing days. We tested 62 experimental animals (39 ♀ & 23 ♂) and 30 controls (12 ♀ & 18 ♂). Briefly, these tests consisted of first placing a single animal into a standard rat (10.25"W x 19"L x 8"H) cage with aspen shavings and ventilated transparent cover. After five minutes of observation, we introduced a novel animal of the same sex and species. The subsequent five minute period constituted the social interaction test. The novel animal's tail was marked with a non-toxic marker to distinguish it from the animal being tested. The cage was cleaned between each animal tested (including replacement of bedding).

All behaviors were recorded with a digital camcorder. We used the Noldus Observer XT software (<http://www.noldus.com/>) to score behaviors from the video data. For the open field test, we scored the following behaviors: burrowing, freezing, jumping, back-flipping, running in circles, and grooming. Based on these videos, we considered straight vertical jumping, back-flipping, and running circles as repetitive behaviors.

For the social interaction test videos, we scored the same behaviors as in the open field test with the addition of social and aggressive behaviors. General

social behaviors included sniffing, following, and allogrooming. Aggressive behaviors included biting, chasing, boxing, and mounting.

All behaviors were scored by incidence; we assessed behavior type at five second intervals throughout the video. Three people scored each video; overall inter-rater reliability was at least 80 percent. At least two scorers were blind to the diet of the animals being scored. When specific behavioral assessments disagreed, we alternated accepting the assessment of the three scorers. The data collected by scoring videos were graphed with Microsoft Excel. Behaviors are reported as percentage of incidence of behavior. Statistics were calculated using the Minitab and SPSS software packages. Note that we used Kruskal–Wallis one-way analysis of variance in cases where there was clearly a non-normal distribution.

Tissue Analyses

After behavioral testing, animals were euthanized via CO₂ chamber. Whole pelts were taken in order to analyze coat color differences. Tissues (skin sample, brain, and liver) were obtained and flash frozen in liquid nitrogen. DNA isolation was done later using the Qiagen DNeasy Blood and Tissue Kit. DNA concentration was analyzed using a Nanodrop 2000 Spectrophotometer from ThermoScientific.

Measurement of Agouti (Yellow) Band Lengths

Hair tufts were pulled from the dorsal midline behind the ears from each pelt. Tufts of hair were placed on a microscope beside a micrometer and pictures were taken using a light microscope/digital camera combination. Agouti (yellow)

band lengths in the hair were measured in millimeters (mm). We assessed 67 experimental animals (40 ♀ & 27 ♂) and 30 controls (12 ♀ & 18 ♂).

DNA Isolation & Bisulfite Analyses

Bisulfite treatment of DNA was performed using the Epitect Bisulfite Kit from Qiagen. Bisulfite primers for the *Agouti* promoter were:

F TTTTAGTGTTGAAAATTGGTAGAAATTT and

R CCTACAATACAAATAATTCAACTCC.

PCR products were produced with Bioline MyTaq HS mix

(<https://www.bioline.com/>) using the following

thermocycler program: 95°C for 10 minutes, [95°C 30", 49°C 20", 72°C 40"] x 30 cycles, followed by 72°C for 8 minutes. PCR products were cloned using

Invitrogen TOPO TA Cloning Kit. Plasmid DNA was isolated using the GeneJet Plasmid Miniprep Kit from ThermoScientific and sequenced at Eton Bioscience Inc. (<http://www.etonbio.com>).

Results

Methyl Diet Affects Coat Color & Body Weight

Matings were established to obtain offspring heterozygous for the dominant A^{Nb} allele. As this allele results in higher expression of *Agouti*, heterozygotes exhibit a longer yellow band of hair and thus overall lighter appearance. A number of animals raised on the methyl-donor diet exhibited visibly darker coats than the controls (Figure 3.1A).

To quantify these changes, we prepared pelts and measured the yellow (agouti) band length on the dorsal midline from 67 methyl diet animals (40♀,

27♂) and 41 controls (18♀, 23♂; Figure 3.1B). These data revealed that while the control A^{Nb} animals had yellow band lengths tightly clustered around 3.1 mm, the treatment group had a broader distribution with an average yellow band length of 2.21 mm (Figure 3.1C). These differences were deemed significant by t-test ($p < 0.005$).

A number of the methyl diet A^{Nb} animals appeared visibly larger than the controls. We therefore weighed the animals at the time of sacrifice (Figure 3.2). Female methyl diet animals averaged 20.2g compared to 18.7g for control females; this shift was significant ($p < 0.05$; t-test). Despite the presence of two much larger animals, the male methyl diet average (22.6g) was essentially the same as the control average (22.0g).

Abnormalities & Mortality

Unexpectedly, we noted that a number of methyl-donor animals died between weaning and adult assessments of coat-color and behavior (4-6 months). While mortality was especially pronounced in males ($p < 0.001$; Table 3.2), it was also significant in females ($p = 0.005$). Note that there was no mortality in control animals over this time period (*P. maniculatus* live 4-5 years in captivity).

When we took tissues from sacrificed animals for nucleic acid analyses, we noted a number of abnormalities in methyl diet animals not present in controls (Table 3.2). Again, the number was higher in methyl diet males (9 of 28 methyl diet males had at least one abnormality; $p < 0.005$), but also significant in females (5 of 40 methyl diet females had at least one abnormality; $p < 0.01$). These apparent defects (Table 3.2) were varied, and showed no effect of litter (i.e. were

randomly distributed between the litters). They included ovarian cysts (Figure 3.3A), size/consistency differences in ribcage, heart, and lungs (Figure 3.3B), cataracts (Figure 3.3C) and asymmetrical testes (Figure 3.3D). In addition, we noted consistency differences in other organs (e.g. brain).

Methyl Diet Affects Behavior

Animals still alive at six months were subjected to a simple open-field test and social interaction test, as described [115]. Major categories scored included repetitive behaviors (jumping, backflips, circle running) and general social behaviors (sniffing, following, allogrooming). We also assessed aggressive behaviors, including biting, boxing, mounting, and chasing.

Female methyl diet animals performed significantly higher numbers of repetitive behaviors than control diet females (Figure 3.4; $p < 0.01$, Kruskal-Wallis test). Examples are shown in the supplementary video. Female methyl diet animals were, on average, more social, but this was not deemed significant (Figure 3.4; $p = 0.064$, Kruskal-Wallis). Similarly, male methyl diet animals trended towards more aggression than control diet males, but this was not statistically significant ($p = 0.069$, Kruskal-Wallis test). *A^{Nb}* animals are more aggressive and exhibit less repetitive behavior than standard BW animals [115]. Thus, it is possible that some of these behavioral effects are due to suppression of the *Agouti* (or a tightly linked) locus itself.

DNA Methylation at the Agouti locus

Prior studies have noted this diet's ability to affect DNA methylation status at the *Agouti* locus in the *A^{vy}* animals (albeit in the IAP element). We therefore used

bisulfite mutagenesis/PCR/sequencing techniques to assess the diet's effects on DNA methylation at the A^{Nb} allele. Results yielded a significant increase in methylation in methyl diet animals when compared to controls (Figure 3.5). The amount of DNA methylation increase was dependent on yellow band length in the hair tufts. A methyl diet female with a yellow band of 1.9mm had 95% methylation ($p < 0.001$, Chi-squared) while a methyl diet male with a yellow band length of 2.5mm had 78% methylation ($p < 0.01$, Chi-squared). The control shown is a combination of a male and a female, each with a yellow band length of 3.1mm, had 57% methylation.

Discussion

We set out to assess whether the methyl-donor diet would affect the *Peromyscus* natural agouti variant A^{Nb} in a similar manner to the *Mus* A^{vy} and whether the behavior of these wild-derived animals was obviously altered by the diet. The data presented here further indicate that these dietary components do indeed affect the A^{Nb} agouti allele, especially with DNA methylation increases at the *Agouti* promoter. The apparent lack of a retroelement in this allele suggests more broad effects than previously reported in the mouse A^{vy} and $Axin^{Fu}$ studies. Further, female repetitive behavior and weights were significantly increased. Unexpectedly, the diet resulted in significant increases in mortality and abnormalities, with a greater effect in males.

The data presented here indicate that dietary intake of methyl-donors may have multiple adverse outcomes in a true wild-type mammalian model. To our

knowledge, this is the first study to associate these particular defects, mortality or altered behavior in wild-type animals with these dietary factors.

We note that increasing evidence points to gene-environment interactions underlying the etiology of many diseases. Folic acid and other methyl-donor pathway components are typically thought of as preventing, rather than being causal to human health issues. Addition of these nutrients to flour appears to have dramatically reduced neural tube defects [116], and deficiencies are also thought to contribute to neuro-cognitive disorders. However, these data add to a growing number of recent studies suggesting deleterious effects of developmental exposure to high doses of these compounds [110, 116-123]. For example, mutations in some loci involved in neural tube development are exacerbated (rather than rescued) by excess folic acid [119], and neurons developmentally exposed to high folic acid may be more susceptible to seizure [121]. Further, studies using these same components have shown increased colitis susceptibility and allergic airway disease (e.g. allergic asthma) in standard laboratory mice (C57BL/6J) [124,125].

Through counting of food pellets consumed, we estimated that these animals took in approximately one food pellet per day. This amount is roughly equivalent to a human consuming around 1750-2000 micrograms of folic acid in a day (based on weight of the animals and 0.0043 grams folate/kg food). We note that such consumption is quite feasible, as many commercial supplements contain 800 micrograms folate (e.g. <http://www.vitaminshoppe.com/p/folic-acid-800-mcg-100-capsules/vs-1148#.UwetE8pWQ7w>), which are taken in addition to

the amounts found in enriched flour and sports drinks. Other Ingredients in this diet are also consumed in copious amounts. For example, the decaffeinated version of the popular 5- hour energy drink contains additional Vitamin B₁₂ and choline in addition to folic acid

(<http://www.5hourenergy.com/healthfacts.asp?Product=decaf>). While rodent and human metabolism differ substantially, it is worth considering whether these dietary components may contribute to human behavioral variation [126].

Clearly, much additional work is required to assess the scope and mechanisms of these adverse effects. For example, we are currently undertaking additional behavioral assays (e.g. Barnes Maze) to ascertain effects on learning and memory. Besides molecular characterization of these changes, we plan to test the dietary effects on an interfertile species (*P. polionotus*), which is more social and less prone to repetitive behaviors [115]. We hypothesize that certain genotypes will be more susceptible to specific epimutations that result in neurological disorders or have other deleterious effects.

That is, we hypothesize that certain genotypes in combination with threshold amounts of these nutrients at specific developmental time points may result in negative effects. As observed in our studies, we predict that such effects will also be highly sexually dimorphic.

Table 3.1: Comparison of differing components in Harlan-Teklad Standard rodent (8604) vs. Methyl-Donor (7517) diet (g/kg of chow).

	Standard (8604)	Methyl Donor (7517)
Betaine	0	5
Choline	2.53	7.97
Folic Acid	0.0027	0.0043
Vitamin B ₁₂	0.051	0.53

Table 3.2: Mortality & abnormalities in methyl vs. control diet animals.

	Methyl Diet						Control Diet		
	♀ %	p value	♂ %	p value	% Litters	p value	♀ %	♂ %	% Litters
Mortality	7.8	p=0.005	22.2	p<0.001	47.1	p<0.001	0	0	0
<u>Abnormalities:</u>	<u>10.6</u>	<u>p<0.0025</u>	<u>32.1</u>	<u>p<0.001</u>	<u>58.8</u>	<u>p<0.001</u>	<u>0</u>	<u>0</u>	<u>0</u>
Ovarian Cyst	6.4	N/A	N/A	N/A	17.6	N/A	0	0	0
Asym. Testes	N/A	N/A	10.7	N/A	17.6	N/A	0	0	0
Cataracts	2.1	N/A	7.1	N/A	11.8	N/A	0	0	0
Enlarged Liver	0	N/A	7.1	N/A	11.8	N/A	0	0	0
Other	2.1	N/A	10.7	N/A	23.5	N/A	0	0	0

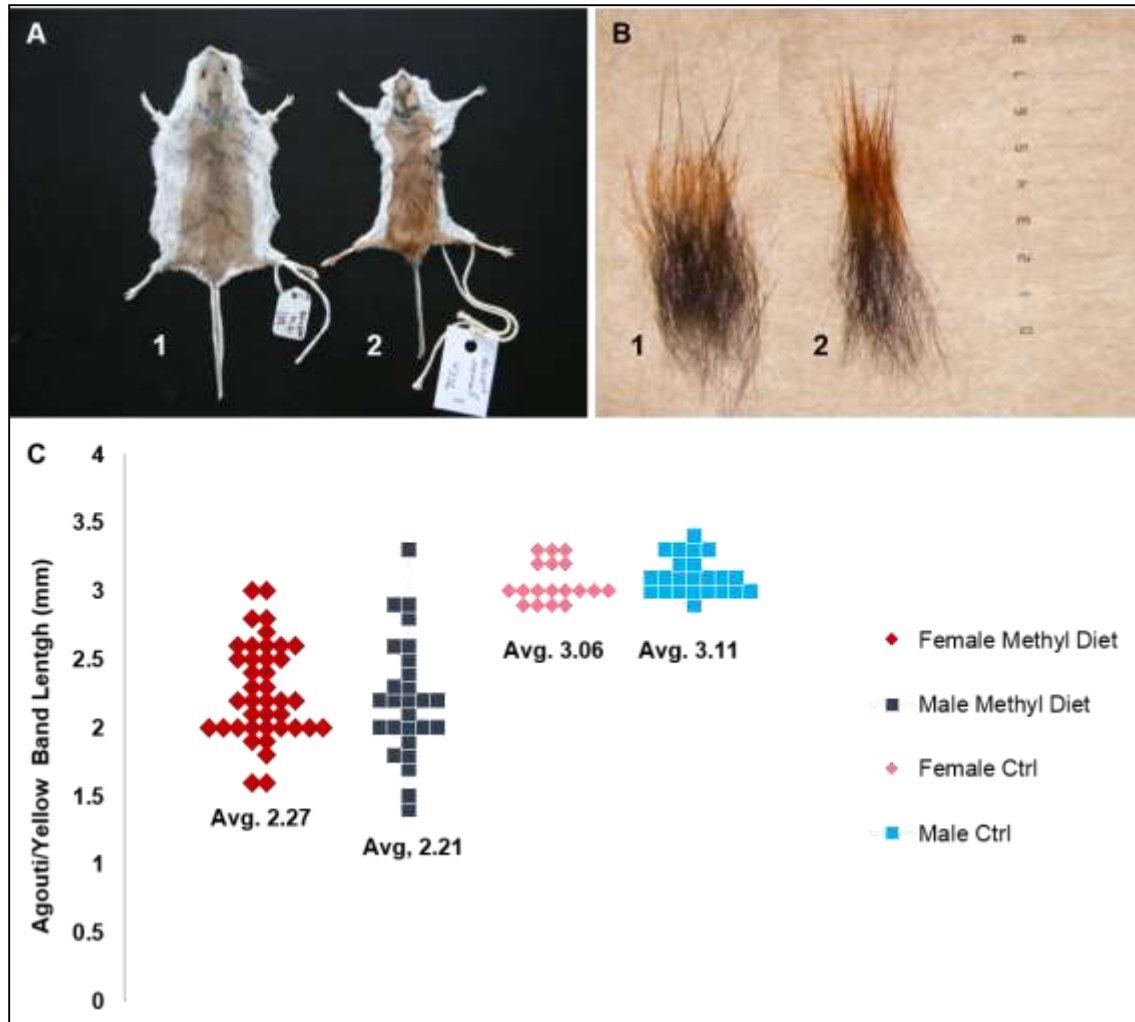


Figure 3.1: Effects of methyl-donor diet on coat-color/pattern. (A) Whole pelts and (B) corresponding hair tufts from representative six-month old female A^{Nb} methyl diet (#1) and control diet (#2) animals. Note the visible differences in yellow band length in hair tufts and size. (C) Distribution of yellow band lengths (in mm) in tufts of hair. A t-test was used to determine significance between methyl diet animals and control animals: $t(107)=15.9$, $p<0.005$, $d=2.2$. The calculated Cohen's D value of 2.2 indicates a large treatment effect.

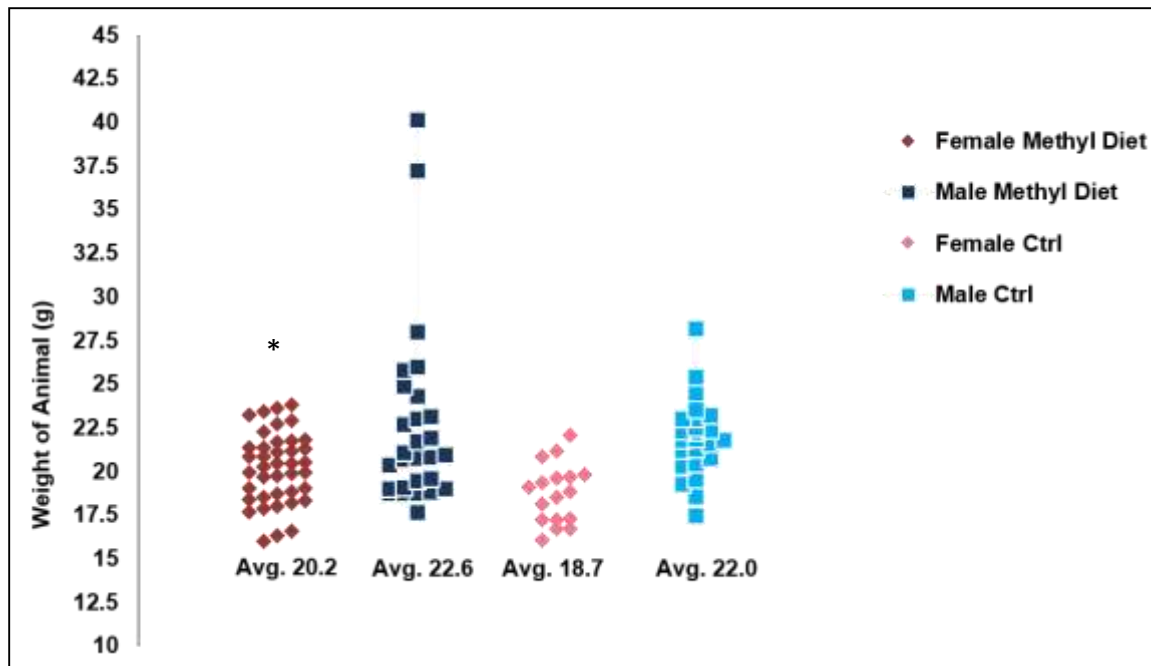


Figure 3.2: Weight distributions of methyl-diet vs control diet A^{Nb} animals. We weighed 68 experimental animals (40 ♀ & 28 ♂) and 40 controls (12 ♀ & 18 ♂) at six months of age. The difference between female experimental & female control (ctrl) was significant ($p < 0.05$; t-test), male averages were not significant. However, there were two methyl-diet males that were much larger than the control population.

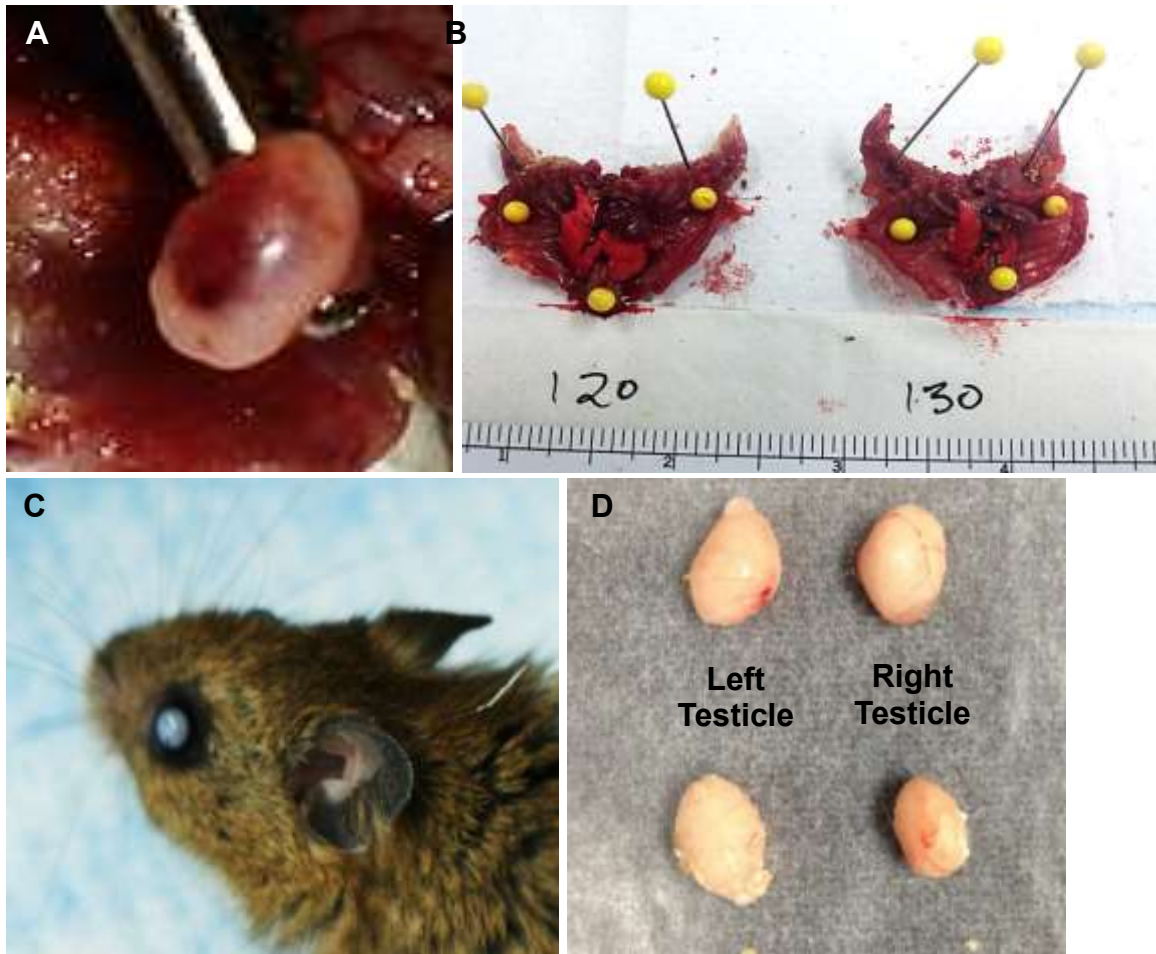


Figure 3.3: Representative abnormalities in methyl diet A^{Nb} animals. (A) Hemorrhagic ovarian cyst in a methyl diet female. (B) Normal diet animal's ribcage, heart, and lungs (left) compared to one methyl diet animal's ribcage, heart and lungs; note abnormalities in size and shape of lungs and heart. (C) Cataracts were visible in the left eye of some animals. (D) Left and right testes from a control diet male (top) and a methyl diet male (bottom). Chi squared tests suggest significant size differences between right and left testes in these three methyl diet males.

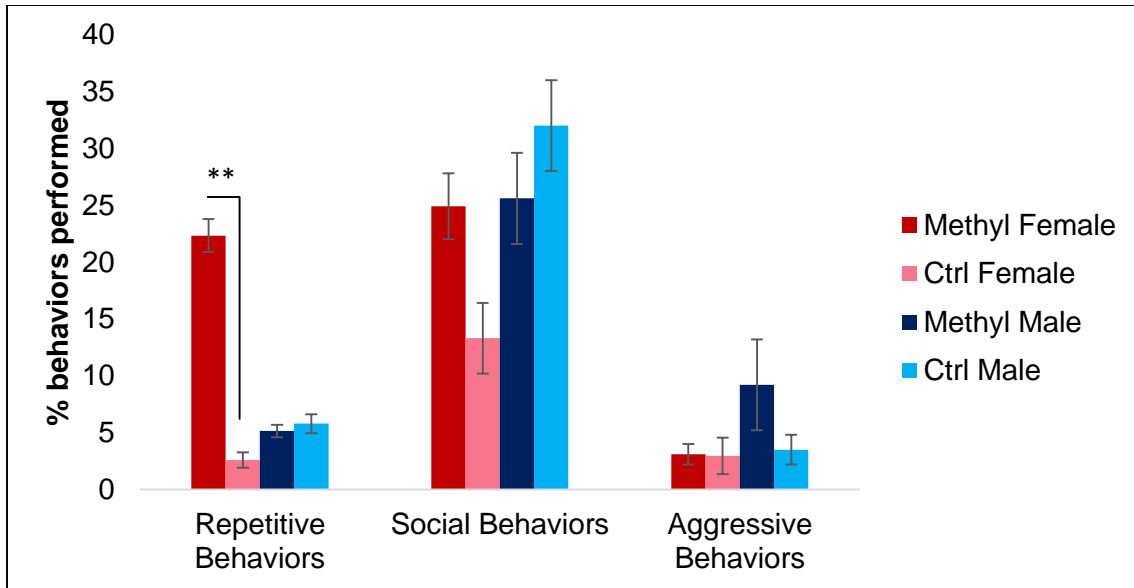


Figure 3.4: Effects of methyl-donor diet on behavior in A^{Nb} animals. Repetitive behaviors included jumping, back-flipping, and running in circles. Female methyl diet animals performed significantly higher numbers of repetitive behaviors than control diet females ($p < 0.01$, Kruskal-Wallis test). Social behaviors included sniffing, following, and allogrooming. Female methyl diet animals were, on average, more social, but this was statistically insignificant ($p = 0.064$, Kruskal-Wallis). Aggressive behaviors included biting, boxing, mounting, and chasing. Male methyl diet animals were, on average, more aggressive than control diet males, but this was statistically insignificant ($p = 0.069$). Bars represent standard error.

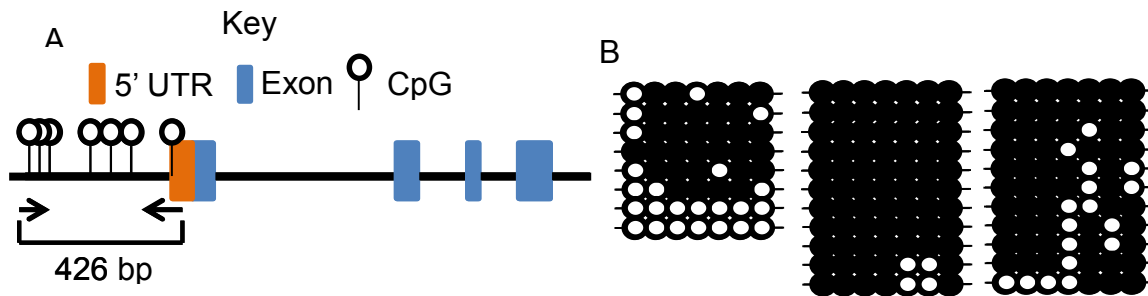


Figure 3.5: *Agouti* bisulfite sequencing in A^{Nb} animals. A schematic of the *Agouti* locus in *Peromyscus* is shown with the location of forward and reverse primers as arrows. Bisulfite sequencing results are shown in B. Each line in B represents a clone that was sequenced that contained a copy of the PCR product. Each circle represents a CpG dinucleotide. Filled-in circles represent methylated CpGs while open circles represent unmethylated CpGs. The controls (1 male and 1 female combined) are shown (left) with 57% methylation. One methyl diet female (middle) with an agouti band length of 1.9mm had 95% methylation ($p < 0.001$) while a methyl diet male (right) with an agouti band length of 2.5mm had 78% methylation ($p < 0.01$). Chi-squared statistics tests were used.

Chapter 4

BW-PO and Gender Differences in Barnes and Elevated Plus Mazes

Introduction

As seen in Chapter 2, many behavior differences exist between BW and PO *Peromyscus*. Therefore, we tested BW and PO in additional behavior tests to gain insight into further differences between these two species and to determine if there differences between the genders.

The Barnes Maze can be used to test learning and memory while the elevated plus maze (EPM) is used to assess anxiety-like behaviors [70]. During the Barnes Maze, animals were subjected to testing for 7 days, with 2 trials per day. Latencies were recorded during testing. Cleversys was used to analyze additional parameters such as sniffing correct versus incorrect holes as well as search strategy.

EPM videos were analyzed for time spent in closed versus open arms as well as exploratory behaviors such as head-dipping and rearing. These Barnes Maze and EPM studies suggest that PO animals are much less anxious than BW animals. Additionally, these studies indicate that the Barnes Maze is useful for studying memory and learning in PO animals, but not in BW animals.

Materials and Methods

Barnes Maze Testing

We utilized a modified Barnes Maze for use with *Peromyscus* as previously described [70]. The maze consisted of a white polypropylene platform 99 cm in diameter that was 70 cm above the floor. A schematic of the Barnes Maze is shown in Figure 4.1. A digital camcorder was centered 1.5 m above the platform. The platform was enclosed by an aluminum wall 50 cm high around the maze to prevent animals from jumping out of the maze. A cue made of black cardboard construction paper was placed every 90 degrees along the sides of the maze; each of the 4 cues was a different shape (triangle, star, square, and circle). Near the base of the aluminum wall, there were twelve evenly spaced black 2 inch diameter escape holes leading to black polypropylene elbows (90 degrees).

Each animal tested was assigned an escape hole. All holes except for the escape hole remained plugged during testing. Exit holes were alternated 90 degrees to eliminate odor cues. The maze was cleaned with 70% ethanol after each individual trial, also to eliminate odor cues. The escape hole location and visible cues within the maze remained constant for each individual. The escape hole led to a typical *Peromyscus* housing cage that contained clean aspen wood-chip bedding. Barnes Maze tests were conducted during the late light phase. At the beginning of each test day, animals were transferred to the testing room 30 minutes prior to testing to reduce any additional stressing.

We tested 36 experimental PO animals (18 ♀ & 18 ♂) and 24 control PO animals (13 ♀ & 11 ♂) while we tested 20 experimental BW animals (11 ♀ & 9 ♂)

and 24 control BW animals (12 ♀ & 12 ♂) of 3-6 months of age in the Barnes Maze. The animals were tested in the Barnes Maze twice per day for 7 days in a row, with a 90 second trial on day 10. The two tests per day for each animal (on days 1-7) were separated by 30 minutes. During testing, a stimulatory light shined onto the platform. Before the first test on day 1, animals were placed in the center of the maze and were guided to their escape hole that led to a clean cage. This was due to the observation that PO animals would not search for their escape hole, but would rather enter a random hole and stay there (if not previously shown their escape hole).

During actual testing, animals were placed into an open ended cylinder in the center of the maze in order for the Cleversys software to begin tracking the animal. The cylinder was lifted after 2-3 seconds and the latency (time it took for the animal to go into their home cage) was recorded using a stopwatch. If the animal did not find and enter their escape hole within 5 minutes, they were carefully and gently guided to their escape hole. Cleversys was used to track the animal, verify latency, and determine the number of correct versus incorrect holes each animal sniffed.

Barnes Maze Data Analyses

A repeated measures ANOVA was used to analyze the Barnes Maze latencies. All possible interactions with species, gender, and day were tested. For these tests we utilized the SPSS software package.

A repeated measures ANOVA was utilized to determine if there existed significant differences in search strategies between species, genders, and days.

Again, all possible interactions with species, gender, and day were tested. The three discrete search strategies for the escape hole (serial, random, and direct) were defined as described previously [70]. The serial search strategy involves the animal searching each hole in a pattern (usually traveling in one direction while searching each hole). The random search strategy entails searching each hole, but not in a pattern. The direct search strategy refers to when the animal goes directly to the correct hole, searches the correct hole, and exits the maze.

Sniffing the correct versus incorrect hole, as well as total holes sniffed during trials, were other parameters we tested. Testing these parameters could potentially provide more information regarding animals' exploratory behaviors in the maze. We tested this parameter due to the fact that some PO animals, particularly in earlier trials, would not search for the exit hole, while BW animals would stop entering the correct exit hole in later trials. A repeated measures ANOVA was once again employed to test for significance between species, gender, and day.

Elevated Plus Maze Testing

The EPM was used as described previously (Chapter 4). Testing for the EPM took place during mid light phase (3 hours of testing on a given test day).

We tested 24 PO animals (13 ♀ & 11 ♂; from 6 different litters) and 24 BW animals (12 ♀ & 12 ♂; from 9 different litters) of 3-6 months of age in the EPM. EPM testing occurred one week before Barnes Maze tests began. Animals were placed into the center of the EPM and were recorded for 5 minutes. If animals

jumped or fell off of an open arm, they were quickly and gently placed back into the center of the maze within 10 seconds.

Elevated Plus Maze (EPM) Data Analyses:

The proportion of total EPM time spent in open and closed arms, head dipping, and immobile, as well as total number of arm entries, average velocity, total distance travelled, and number of times rearing were analyzed by ANOVA, which included the effects of gender and species, as well as gender x species. SAS version 9.2 Software (SAS Institute, Cary, NC) was also employed for these analyses.

Results

Barnes Maze Latency Differences

It is clear that PO animals show a pattern consistent with learning and memory while the BW animals do not show this pattern (Figure 4.2). This difference is clear upon viewing graphs for both PO females vs. BW females and PO males vs. BW males. BW males and females do not differ significantly from each other, but as stated before, neither shows a pattern consistent with learning in the Barnes Maze. PO males and females do not differ significantly in latencies in the Barnes Maze.

Barnes Maze Sniffing Correct Hole Differences

PO males, in general, sniffed the correct hole more than PO females. On day 2, PO females did sniff the correct hole more than males ($p=0.049$; repeated measures ANOVA). On day 6, PO males sniffed the correct hole significantly more than PO females ($p=0.038$; repeated measures ANOVA). BW males, in

general, sniffed the correct hole more than BW females. The only day this was significant was day 5 ($p=0.043$; repeated measures ANOVA). It appears that there may not be a difference between PO females and BW females. The BW females sniffed the correct hole more than PO males on day 6 ($p=0.008$; repeated measures ANOVA), but the lines for the two groups over 7 days intersect several times. In other words, BW females sniffed the correct hole more on some days, while PO females sniffed the correct hole more on other days. PO males; however, sniffed the correct hole more than BW males (in general). PO males sniffed the correct hole significantly more than BW males on days 1 ($p=0.05$; repeated measures ANOVA) and 7 ($p=0.015$; repeated measures ANOVA).

When comparing total holes sniffed between genders and species, it was evident that PO males and females did not differ in how many total holes they sniffed (Figure 4.4). The same was true for BW males and females as the BW males did not sniff significantly more than BW females. Using repeated measures ANOVA, it was determined that BW females sniffed more holes than PO males during days 3 ($p=0.019$), 4 ($p=0.001$), 5 ($p=0.005$), and 6 ($p=0.01$). This difference; however, may simply reflect the fact that PO females learned to enter the correct exit hole while BW females did not. Using repeated measures ANOVA, it was determined that BW males also sniffed more holes than PO males on days 3 ($p=0.005$), 4 ($p=0.004$), 5 ($p<0.001$), 6 ($p=0.002$), and 7 ($p=0.003$). This, again, may simply reflect the fact that BW males did not learn to

enter the correct exit hole while PO males did learn to enter and exit through the correct hole.

Barnes Maze Search Strategies

BW and PO animals differ in search strategy only on day 7 when PO animals use the direct search strategy significantly more than BW animals ($p=0.005$; repeated measures ANOVA; Figure 4.5). PO females appear to use the direct search strategy more than BW females (especially during days 2, 5, 6, and 7), but this was not significant (Figure 4.6). PO females do not differ in search strategy from PO males. PO males do differ from BW males in search strategy as PO males use the direct strategy significantly more on day 3 ($p=0.05$; repeated measures ANOVA; Figure 4.6) and on day 7 ($p=0.003$; repeated measures ANOVA). BW males differ from BW females as BW females use the random strategy more than serial on day 3 ($p=0.041$; repeated measures ANOVA) and on day 7 ($p=0.047$; repeated measures ANOVA).

Elevated Plus Maze

Time spent in the open arms of the maze were significantly different between PO males and females ($p=0.003$; ANOVA) as PO females spent less time in open arms than PO males (Figure 4.7). The time spent in the open arms was also significantly different between BW and PO male animals ($p=0.03$; ANOVA) as BW males spent less time in open arms than PO males. PO females spend less time head-dipping than PO males ($p=0.03$; ANOVA; Figure 4.8). BW males spend less time head-dipping than PO males ($p=0.006$; ANOVA). The behavior termed “rearing” was also significantly changed between some groups, as

frequency of rearing was significantly higher in BW females than PO females ($p=0.001$; ANOVA; Figure 4.9). Finally, BW females reared more than BW males while in the EPM ($p=0.0009$; ANOVA). No other data was indicated as significant using ANOVA.

Discussion

Comparing the BW and PO species in Barnes Maze and EPM reveals more behavioral differences between the two species that may be useful in further studies to determine genetic basis of behavioral traits in *Peromyscus*. The sexual dimorphism apparent in some cases should be further studied to determine if there is a link to sex chromosome for these differences.

One apparent difference during the Barnes Maze is BW animals do not appear to learn during Barnes Maze testing. This was indicated by a lack of pattern in BW male and female latencies, while PO males and females had a clear trajectory that indicates learning and memory. This was also evident upon reviewing how many holes total were sniffed by each group. BW animals sniffed more than PO animals for the duration of the Barnes testing after day 3. PO males seemed to sniff the correct hole more than PO females (although this is mostly not significant). This may indicate better memory with a desire to explore the maze. The same may have been true for BW females as they sniff the correct hole more than PO females, even though BW females did not follow a learning trajectory when viewing their latencies.

EPM data revealed that PO males were less anxious than PO females, and PO males are less anxious than BW males as PO males spent significantly

more time in the open arms of the EPM. BW females appeared to be less anxious and more exploratory than BW males and PO females since BW females reared more than both BW males and PO females.

BW males show signs of heightened anxiety (when compared to other groups) during the EPM in three parameters tested. This could account for their learning and memory deficits in the Barnes Maze, as learning/memory and anxiety may be linked since hippocampal dysfunction is apparent in anxiety disorders while the hippocampus is involved in memory formation [127]. This hypothesis is uncertain. An additional hypothesis is that the Barnes Maze acts as environmental enrichment for BW animals, so they would rather explore the maze than to exit the maze. This is somewhat evident when comparing the number of holes sniffed by both BW and PO animals, as in both genders, BW animals sniff more holes than PO animals, suggesting BW are exploring the maze more than PO animals (instead of learning the location of the exit hole). A final hypothesis is that the difference between BW and PO in burrowing behaviors alters how well BW and PO perform in the Barnes Maze. BW animals burrow significantly less than PO animals (Chapter 2). Therefore, BW animals may lack motivation in this test since the “reward” for exiting the Barnes Maze through the correct hole was a cage with clean bedding.

The sexual dimorphism in anxiety and exploratory behaviors is evident when comparing EPM results of BW and PO. BW females are more exploratory than PO females, but the reverse was true in males as PO males were more exploratory than BW males.

Testing these additional behavioral differences in the future, in combination with behavioral differences in Chapter 2, may assist in locating a locus or multiple loci responsible for certain behaviors in *Peromyscus*. It is hypothesized some behaviors tested previously in Chapter 2 may be linked to the same gene/gene region as genes that affect learning and memory, particularly in the Barnes Maze, between these two species. This also provided insight into the fact that the Barnes Maze is not an appropriate memory and learning test for BW animals.

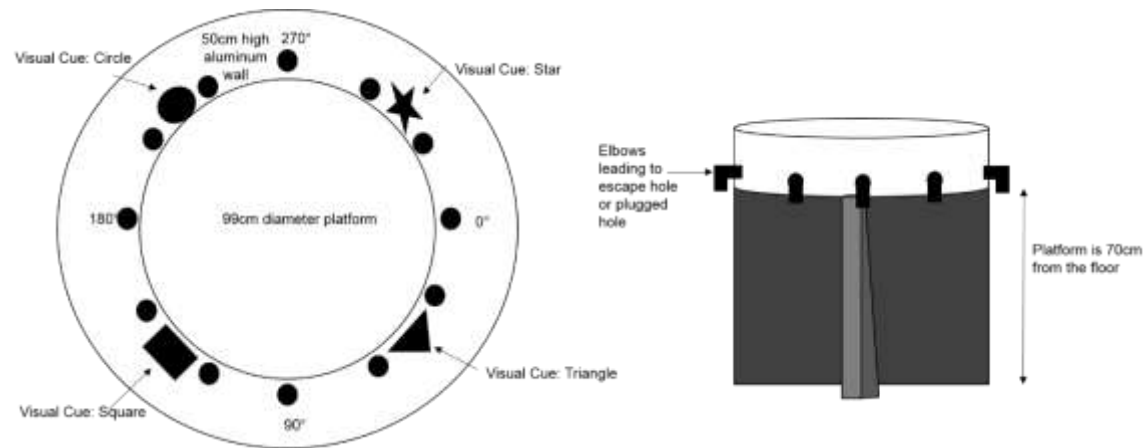


Figure 4.1: Barnes Maze schematic. Left: a 99cm diameter platform is surrounded by a 50cm high aluminum wall. Twelve evenly spaced pipes that lead to plugged holes (except for the hole to the escape cage) surround the maze 2 inches above the platform. Animals were assigned an exit hole (0, 90, 180, or 270 degrees) and had only visual cues (star, triangle, square, and circle) to assist with learning where their escape hole was. Right: the Barnes Maze platform is 70cm from the floor.

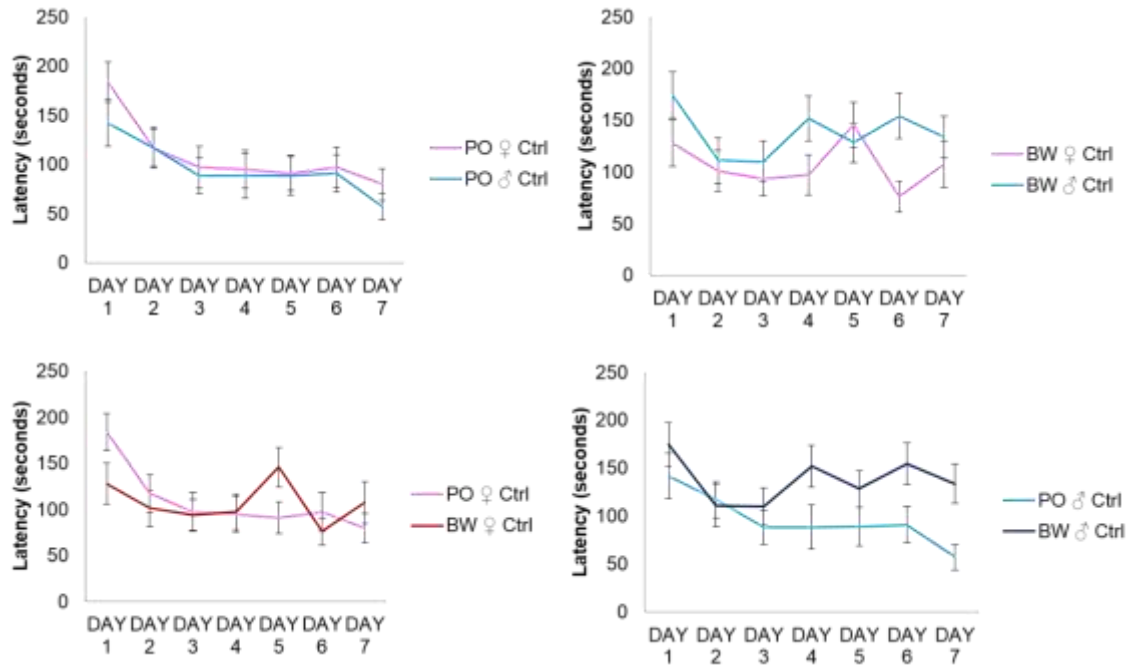


Figure 4.2: Latency for 7 day Barnes Maze trials for BW and PO. It is clear that PO animals show a pattern consistent with learning and memory while the BW animals do not show this pattern. This difference is clear upon viewing graphs for both PO females vs. BW females and PO males vs. BW males. BW males and females do not differ significantly from each other, but as stated before, neither shows a pattern consistent with learning in the Barnes Maze. PO males and females do not differ in latencies in the Barnes Maze. Error bars are standard error.

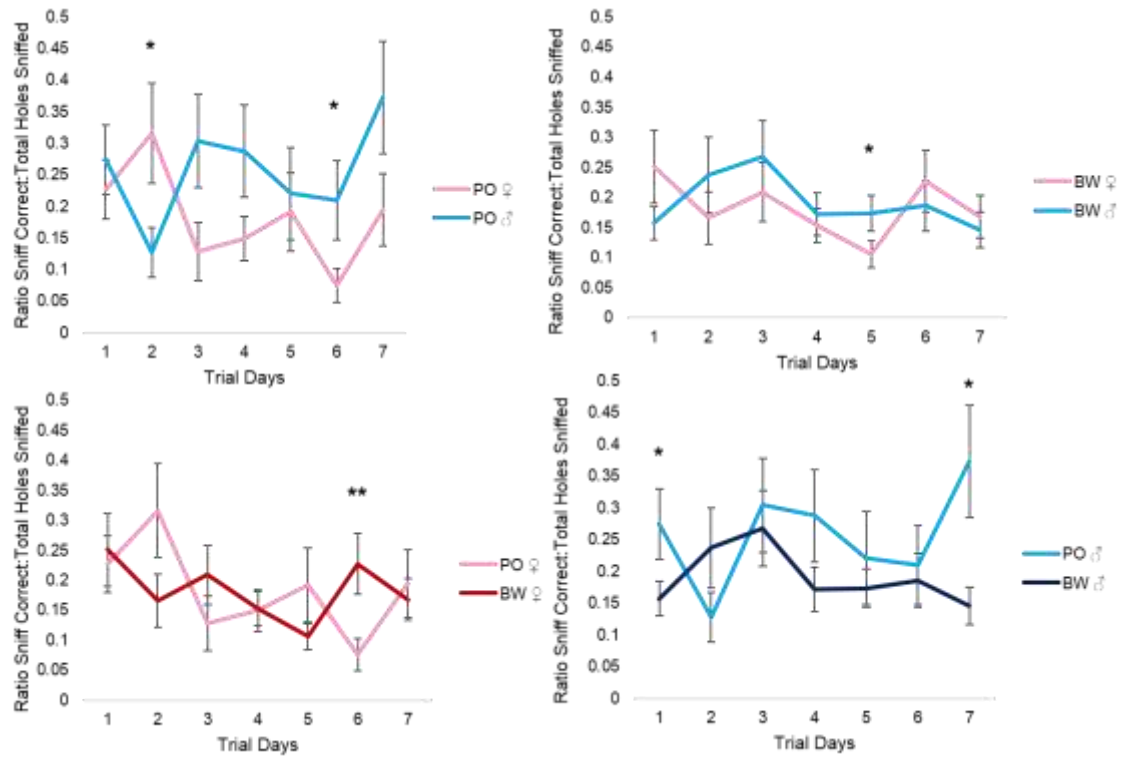


Figure 4.3: Ratio of Correct Holes Sniffed in BW and PO in Barnes Maze. PO males, in general, sniffed the correct hole more than PO females. On day 2, PO females did sniff the correct hole more than males ($p=0.049$, repeated measures ANOVA). On day 6, PO males sniffed the correct hole significantly more than PO females ($p=0.038$, repeated measures ANOVA). BW males, in general, sniffed the correct hole more than BW females. The only day this was significant was day 5 ($p=0.043$, repeated measures ANOVA). It appears that there may not be a difference between PO females and BW females although BW females sniffed the correct hole more than PO males on day 6 ($p=0.008$). PO males sniffed the correct hole more than BW males (in general). On days 1 ($p=0.05$, repeated measures ANOVA) and 7 ($p=0.015$, repeated measures ANOVA), PO males sniffed the correct hole significantly more than BW males. Error bars are standard error.

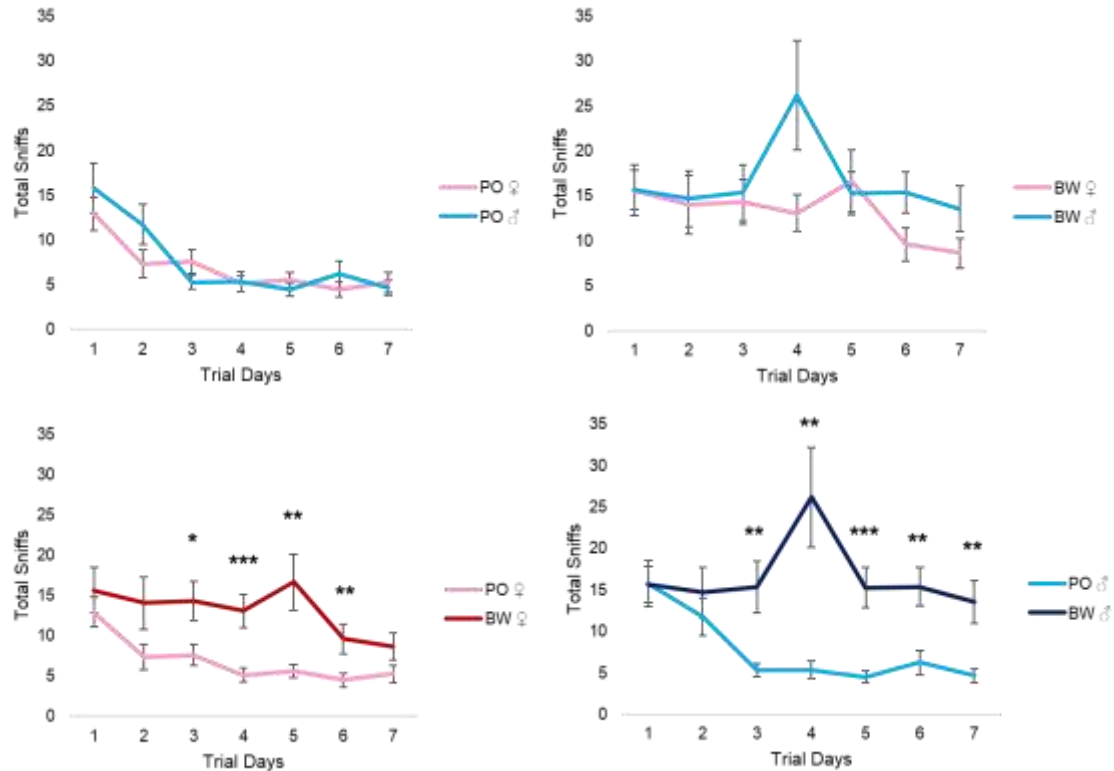


Figure 4.4: Total holes sniffed by BW and PO. PO males and females did not differ in how many total holes they sniffed. The same was true for BW males and females as the BW males did not sniff significantly more than BW females. Using repeated measures ANOVA, it was determined that BW females sniffed more holes than PO males during days 3 ($p=0.019$), 4 ($p=0.001$), 5 ($p=0.005$), and 6 ($p=0.01$). This difference; however, may simply reflect the fact that PO females learned to enter the correct exit hole while BW females did not. Using repeated measures ANOVA, it was determined that BW males also sniffed more holes than PO males on days 3 ($p=0.005$), 4 ($p=0.004$), 5 ($p<0.001$), 6 ($p=0.002$), and 7 ($p=0.003$). This, again, may simply reflect the fact that BW males did not learn to enter the correct exit hole while PO males did learn to enter and exit through the correct hole. Error bars are standard error.

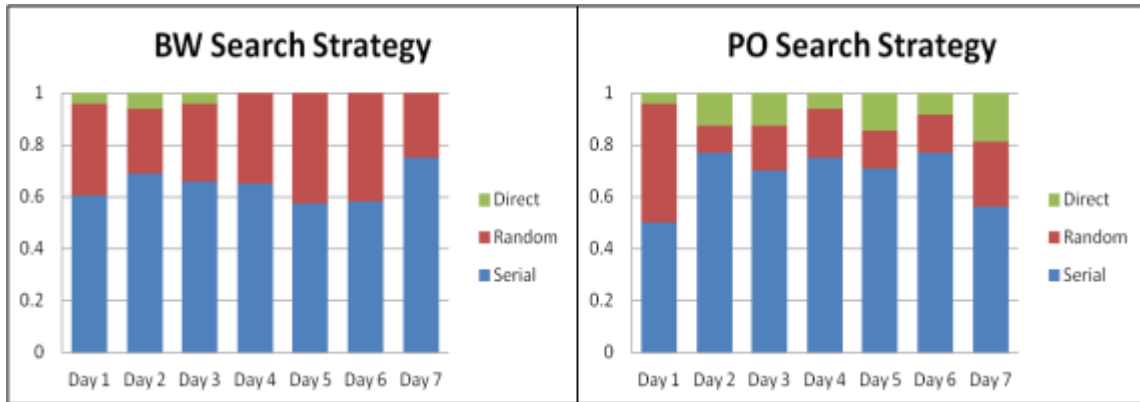


Figure 4.5: BW versus PO search strategies in Barnes Maze. The search strategy is significantly different between BW and PO only on day 7 ($p=0.005$; repeated measures ANOVA). On day 7, it is clear PO animals use the direct search strategy significantly more than BW animals as BW animals do not use the direct search strategy on day 7. It appears PO animals use the direct search strategy more than BW animals on other days as well, although for days other than day 7, the differences were not significant.

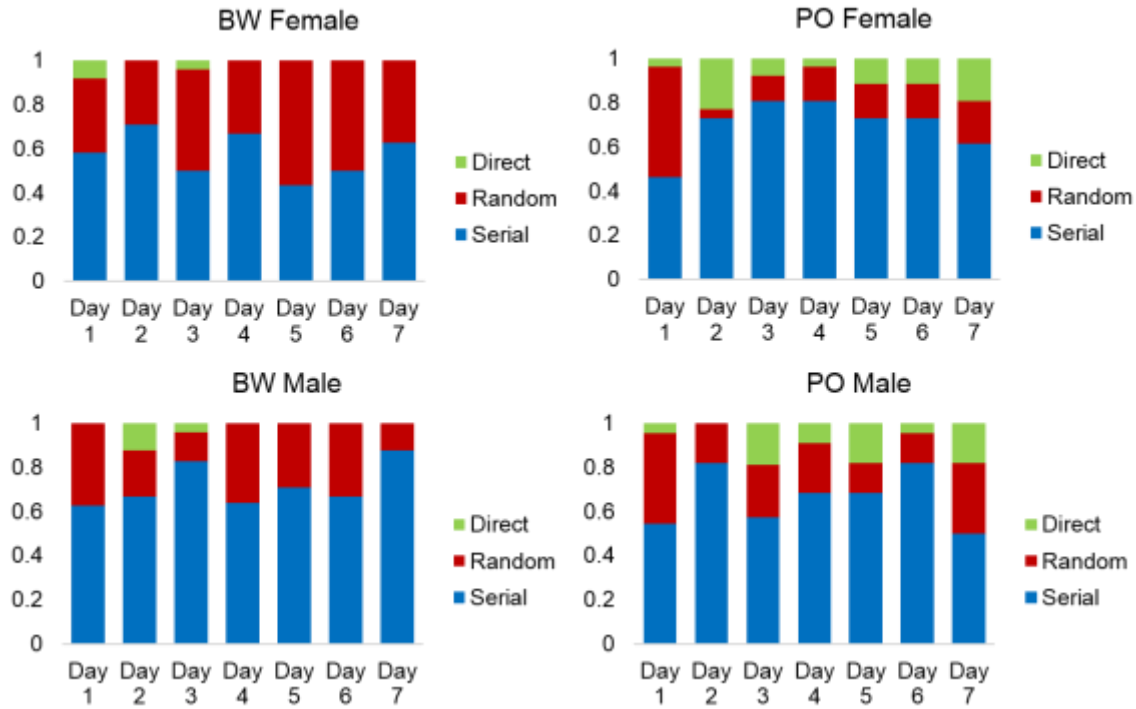


Figure 4.6: Search strategy in PO and BW males and females. PO females appear to use the direct search strategy more than BW females (especially during days 2, 5, 6, and 7), but this was not significant. PO females do not differ in search strategy from PO males. PO males do differ from BW males in search strategy as PO males use the direct strategy significantly more on day 3 ($p=0.05$, repeated measures ANOVA) and on day 7 ($p=0.003$, repeated measures ANOVA). BW males differ from BW females as BW females use the random strategy more than serial on day 3 ($p=0.041$, repeated measures ANOVA) and on day 7 ($p=0.047$, repeated measures ANOVA).

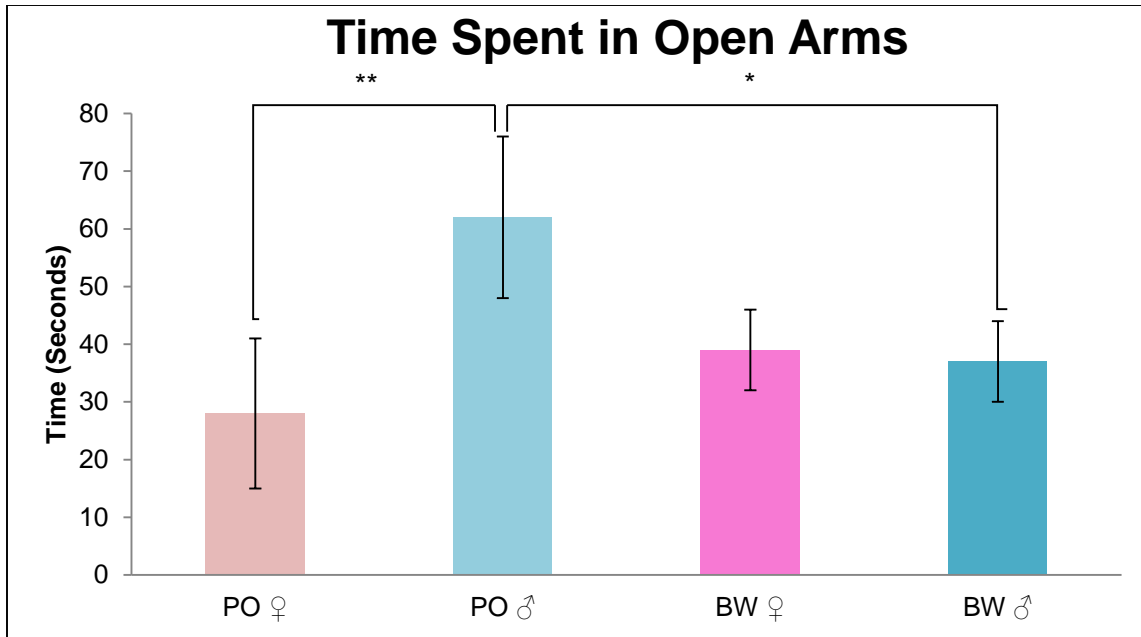


Figure 4.7: Time Spent in Open Arms for BW and PO. PO females spent less time in open arms than PO males ($p=0.003$; ANOVA). BW males spent less time in open arms than PO males ($p=0.03$; ANOVA). Error bars are standard error.

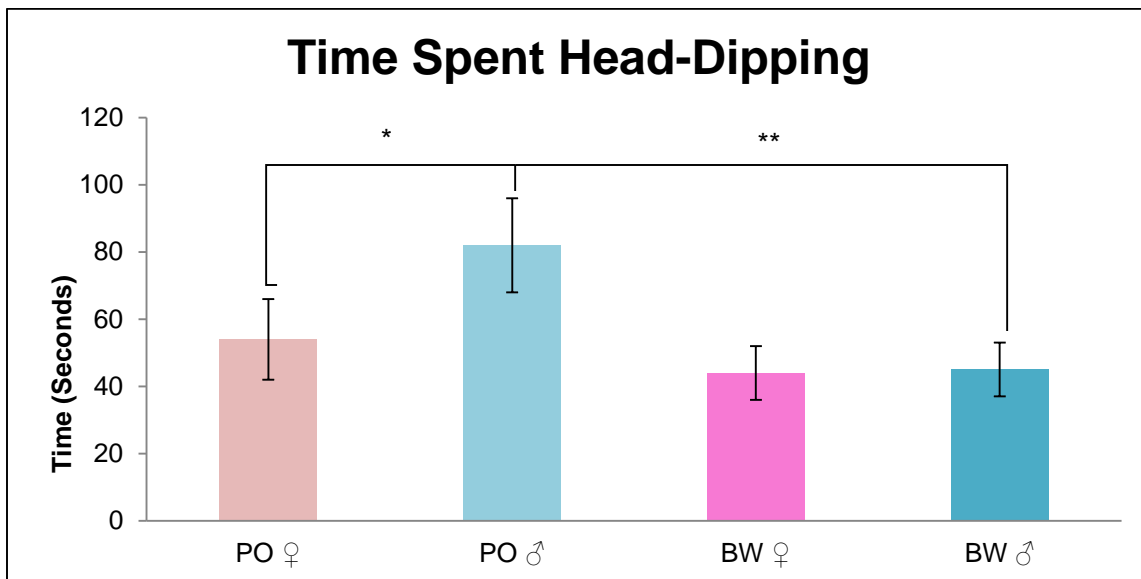


Figure 4.8: Time Spent Head-Dipping for BW and PO. PO females spend less time head-dipping than PO males ($p=0.03$; ANOVA). BW males spend less time head-dipping than PO males ($p=0.006$; ANOVA). Error bars are standard error.

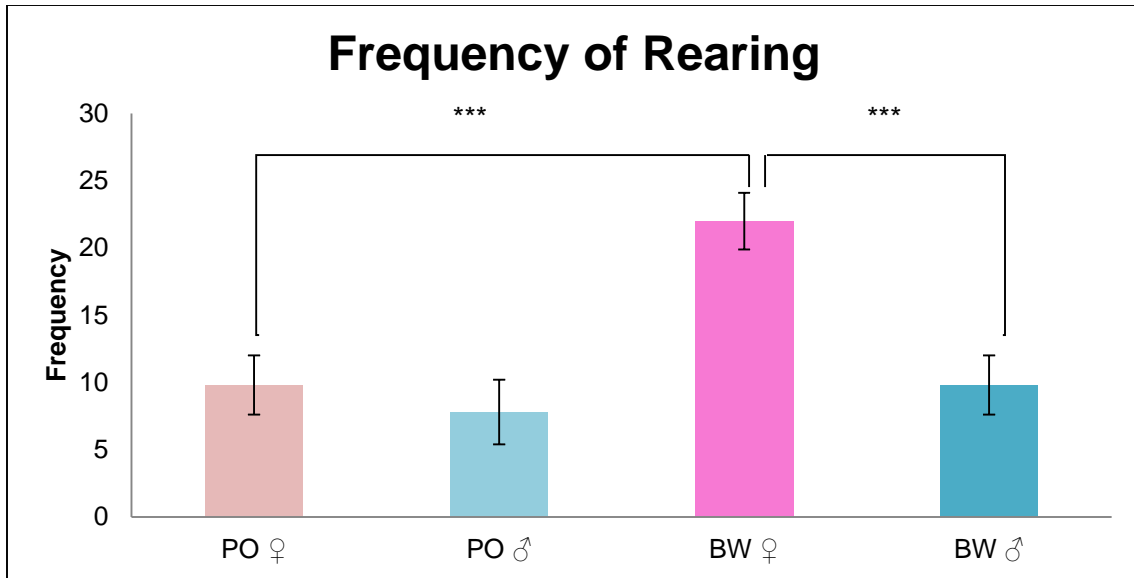


Figure 4.9: Frequency of Rearing in BW and PO. Frequency of rearing was significantly higher in BW females than PO females ($p=0.001$; ANOVA). BW females reared more than BW males ($p=0.0009$; ANOVA). Error bars are standard error.

Chapter 5

Genetic Background Influences Effects of the Methyl-Donor Diet

Introduction

Epigenetic changes have been found to alter behavior, learning and memory, and anxiety in humans and mice. For instance, prenatal maternal mood in humans has been shown to alter DNA methylation at the glucocorticoid receptor (*NR3C1*) in offspring, which is associated with stress response [128]. It is also well recognized that stressful life events in mice can alter gene expression [129, 130]. DNA methylation at promoters of some genes and changes in how GABA regulates epigenetic changes and gene transcription are all associated with anxiety in mice [129]. Age-associated decline in memory has been attributed to loss of DNA methylation in mice and is thought to be similar in humans [131,132]. Memory-related epigenetic changes in the mouse amygdala are associated with DNA Methyltransferase activity [133]. Additionally, in *Mus*, rescued expression of *Dnmt3a2* (a *de novo* DNA Methyltransferase) in hippocampus of aged mice resulted in recovered cognitive abilities when compared to control aged mice [132].

In our *A^{Nb}* methyl diet studies, the methyl-donor diet has been shown to affect various phenotypes in *A^{Nb} Peromyscus*. As expected, supplementation with folic acid induced increased DNA methylation. Increased DNA methylation is

associated, in general, with gene silencing. The data then allowed us to correlate yellow band length in hair tufts with amounts of DNA methylation increase. Due to large variance in effects on coat color, abnormalities, weight, and behavior, it is likely that multiple loci and/or pathways (not just *Agouti*), are affected by the methyl donor diet.

Utilizing the same methyl donor diet described previously (Chapter 3), we characterized and compared the behavioral effects of this diet in BW and PO deer mice stocks. Therefore, we sought to determine whether genetic background influences the effects of the methyl donor diet in BW and PO. Our previous work showed that A^{Nb} *Peromyscus* behavior was altered in offspring of parents that were on the methyl donor diet. It is plausible to hypothesize that effects on BW may differ despite the fact this allele has been bred onto a BW background for 16 generations since the A^{Nb} allele affects social and aggressive behaviors [115]. We also hypothesized effects on PO would differ from both BW and A^{Nb} considering the significant genetic differences between BW and PO.

We assessed the effects of the methyl-donor diet on behaviors in open field and social interaction tests as previously described (Chapters 2 and 3). Additionally, potential effects of the methyl-donor diet on memory were assessed using the Barnes Maze. These studies were conducted at the same time as the studies in Chapter 4. Memory has been associated with DNA methylation changes [131-132]. Therefore, it is reasonable to hypothesize that since DNA methylation affects memory, then the methyl-donor diet may induce changes in memory.

The elevated plus maze (EPM) was used to test levels of anxiety-like behaviors as epigenetic factors can be associated with anxiety in offspring [134]. Since anxiety has a clear link to epigenetics, particularly DNA methylation [134-136], it is plausible to hypothesize that the methyl-donor diet may have effects on anxiety levels.

In DNA methylation analyses, we used neonatal whole brains to determine methylation changes at an imprinted gene (*Peg10*) that is known to be associated with autism [21]. An additional subspecies of *Peromyscus*, SM₂, or *P. maniculatus sonoriensis* (a different subspecies of *P. maniculatus*), were used in a cross with BW in order to have single nucleotide polymorphisms (SNPs) for distinguishing maternal and paternal alleles.

We hypothesized that changes seen in each species of BW and PO deer mice will differ both between species and between sexes, which would indicate that genetic background does, in fact, influence the effects of the methyl-donor diet.

Materials and Methods

Animal Husbandry & Mating Schemes

Animals were taken from the stocks maintained at the *Peromyscus* Genetic Stock Center (<http://stkctr.biol.sc.edu/>). Animals were kept on a 16:8 hour light-dark cycle and were given food and water *ad libitum*. Matings of BW female x BW male and PO female x PO male were established and maintained on either the methyl donor diet (as previously described) or normal rodent chow (i.e. controls). There were 11 POxPO methyl diet matings, 6 POxPO control matings,

4 BWxBW methyl diet matings, and 6 BWxBW control matings. Offspring were weaned at approximately 25 days of age and maintained on the methyl donor diet or normal rodent chow until sacrifice. The control BW and PO offspring obtained are the same animals that were used in the studies in Chapter 4.

Behavioral Testing- Open Field and Social Interaction Tests

Offspring of BW and PO crosses (methyl and control groups for each cross type) were evaluated in open field and social interaction tests at 3-6 months of age, as previously described [115]. These two tests were always conducted during mid-light phase (3 hours of testing during this light phase). Open field and social interaction tests took place one week before EPM and two weeks before Barnes Maze for a given animal. We tested 69 experimental PO animals (37 ♀ & 32 ♂) and 26 control PO (14 ♀ & 12 ♂), and 21 experimental BW animals (12 ♀ & 9 ♂) and 24 control BW (12 ♀ & 12 ♂). Briefly, these tests consisted of first placing a single animal into a standard rat (10.25"W x 19"L x 8"H) cage with aspen shavings and ventilated transparent cover. After five minutes of observation, we introduced a novel animal of the same sex and species. The subsequent five minute period constituted the social interaction test. The novel animal's tail was marked with a non-toxic marker to distinguish it from the animal being tested. The cage was cleaned between each animal tested (including replacement of bedding).

All behaviors were recorded with a digital camcorder. We used the Noldus Observer XT software (<http://www.noldus.com/>) to score behaviors from the video data. For the open field test, we scored the following behaviors:

burrowing, freezing, jumping, back-flipping, running in circles, and grooming. Based on these videos, we considered straight vertical jumping, back-flipping, and running circles as repetitive behaviors.

For the social interaction test videos, we scored social and aggressive behaviors as well as the open field test behaviors mentioned above. General social behaviors included sniffing, following, huddling, and allogrooming. Aggressive behaviors included biting, chasing, boxing, and mounting. All behaviors were scored by incidence; we assessed behavior type at five second intervals throughout the video. Two people scored each video; overall inter-rater reliability was at least 95 percent. One scorer was blind to the diet of the animals being scored. When specific behavioral assessments disagreed, we alternated accepting the assessment of the two scorers. The data collected by scoring videos were graphed with Microsoft Excel. Behaviors are reported as percentage of incidence of behavior. Statistics were calculated using the Minitab and SPSS software packages. Note that we used Kruskal–Wallis one-way analysis of variance in cases where there was clearly a non-normal distribution.

Barnes Maze Testing

We utilized a modified Barnes Maze for use with *Peromyscus* as previously described [70, Chapter 4]. Methyl diet Barnes Maze tests were conducted at the same time as experiments that were conducted on controls in Chapter 4. Each animal tested was assigned an escape hole. All holes except for the escape hole remained plugged during testing. Exit holes were alternated 90 degrees to eliminate odor cues. The maze was cleaned with 70% ethanol after each

individual trial, also to eliminate odor cues. The escape hole location and visible cues within the maze remained constant for each individual. The escape hole led to a typical *Peromyscus* housing cage that contained clean aspen wood-chip bedding. Barnes Maze tests were conducted during the late light phase. At the beginning of each test day, animals were transferred to the testing room 30 minutes prior to testing to reduce any additional stressing.

We tested 36 experimental PO animals (18 ♀ & 18 ♂) and 24 control PO animals (13 ♀ & 11 ♂; Chapter 4) while we tested 20 experimental BW animals (11 ♀ & 9 ♂) and 24 control BW animals (12 ♀ & 12 ♂; Chapter 4) of 3-6 months of age in the Barnes Maze. The animals were tested in the Barnes Maze twice per day for 7 days in a row, with a 90 second trial on day 10. The two tests per day for each animal (on days 1-7) were separated by 30 minutes. During testing, a stimulatory light shined onto the platform. Before the first test on day 1, animals were placed in the center of the maze and were guided to their escape hole that led to a clean cage. This was due to the observation that PO animals would not search for their escape hole, but would rather enter a random hole and stay there (if not previously shown their escape hole).

During actual testing, animals were placed into an open ended cylinder in the center of the maze in order for the Cleversys software to begin tracking the animal. The cylinder was lifted after 2-3 seconds and the latency (time it took for the animal to go into their home cage) was recorded using a stopwatch. If the animal did not find and enter their escape hole within 5 minutes, they were carefully and gently guided to their escape hole. Cleversys was used to track the

animal, verify latency, and determine the number of correct versus incorrect holes each animal sniffed.

Barnes Maze Data Analyses

A repeated measures ANOVA was used to analyze the Barnes Maze latencies. All possible interactions with species, gender, diet, and day were tested. For these tests we utilized the SPSS software package.

A repeated measures ANOVA was utilized to determine if there existed significant differences in search strategies between species, genders, diet, and days. Again, all possible interactions with species, gender, diet, and day were tested. The three discrete search strategies for the escape hole (serial, random, and direct) were defined as described previously [70, Chapter 4].

Sniffing the correct versus incorrect hole, as well as total holes sniffed during trials, were other parameters we tested. Testing these parameters could potentially provide more information regarding animals' exploratory behaviors in the maze. We tested this parameter due to the fact that some PO animals, particularly in earlier trials, would not search for the exit hole, while BW animals would stop entering the correct exit hole in later trials. A repeated measures ANOVA was once again employed to test for significance between species, genders, diet, and day.

Elevated Plus Maze Testing

The EPM had two open arms and two closed arms. The maze was made entirely of polypropylene. Each arm was 46.5 cm in length and 5 cm in width with the white floor of the maze being 46.5 cm above the floor. Walls of the enclosed

arms were black polypropylene and measured 46.5 cm in height each. During testing, an aluminum wall 30 cm in height encircled the maze in order to contain the animal if they fell or jumped off of the maze. Testing for the EPM took place during mid-light phase (3 hours of testing in a given day).

We tested 36 experimental PO animals (18 ♀ & 18 ♂) and 24 control PO animals (13 ♀ & 11 ♂) while we tested 21 experimental BW animals (12 ♀ & 9 ♂) and 24 control BW animals (12 ♀ & 12 ♂) of 3-6 months of age in the EPM.

EPM testing occurred one week before Barnes Maze tests began. Animals were placed into the center of the EPM and were recorded for 5 minutes. If animals jumped or fell off of an open arm, they were quickly and gently placed back into the center of the maze within 10 seconds.

Elevated Plus Maze (EPM) Data Analyses:

The proportion of total EPM time spent in open and closed arms, head dipping, and immobile, as well as total number of arm entries, average velocity, total distance travelled, and number of times rearing were analyzed by ANOVA, which included the effects of sex, diet, and sex x diet. SAS version 9.2 Software (SAS Institute, Cary, NC) was also employed for these analyses.

DEXA Scans

DEXA scans were performed on 6 month old experimental PO animals (18 ♀ & 14 ♂) and control PO (8 ♀ & 9 ♂). Animals were anesthetized with 2% isofluorane and remained under anesthesia during the DEXA scan. After the scan, animals were returned to their home cage and remained under surveillance until they were awake.

Tissue Harvesting

At 6 months of age, animals were anesthetized with 2% isofluorane and blood was collected by retro-orbital bleed in order to obtain serum for later studies. Animals were then euthanized and tissues were harvested: hippocampus, hypothalamus, brainstem, liver. Ovaries and uteri were additionally collected from females while testes or sperm were collected/ isolated from males. All tissues were flash frozen with liquid nitrogen. DNA isolation was performed using the Qiagen DNeasy Blood and Tissue Kit. DNA concentrations were analyzed using a Nanodrop 2000 Spectrophotometer from ThermoScientific.

Bisulfite Sequencing

For bisulfite sequencing of imprinted genes, we used whole brain from neonatal offspring of control and methyl-donor diet crosses between a BW female and an SM2 male as well as BW female by PO male crosses. Neonatal brains were used in order to analyze (as close as possible) *in utero* effects without sacrificing parents in order to obtain additional litters. Crosses used were to ensure the presence of sufficient SNPs in order to distinguish the maternal from paternal allele. Bisulfite treatment of DNA was performed using the Epiect Bisulfite Kit from Qiagen. The *Peg10/Sgce* ICR PCR was amplified with the following primers: F TGTAGGAGAGTAATTAAATGTAAAAG and R ATCTAATACCACCATCATACAATAA.

A gene on the X chromosome that is subject to X-inactivation was studied for promoter methylation. *Mecp2*, which has been shown to be aberrantly methylated in some autism patients, was amplified using the following primers:

F GGGTATAGATGGTTAGTAGTTTATTAA and
R TAAACACCTAACTACTACATAATCAAATC.

An autosomal gene was sequenced from PO homozygous offspring as there is no need to distinguish parental alleles. The glucocorticoid receptor (*Gcr*) was amplified in hypothalamus and brain stem tissues of PO methyl and control male offspring using the following primers:

F TTAGAGTTTTTAAGGGTGATAGGTAGT and
R CCCCCAACTAAACTCACAATAC. PO methyl male samples were chosen based on having high amounts of time spent in closed versus open arms of the elevated plus maze.

PCR products were produced with Bioline MyTaq HS mix (<https://www.bioline.com/>) using the following thermocycler program for *Peg10/Sgce* and for *MeCP2*: 95°C for 10 minutes, [95°C 30", 49°C 20", 72°C 40"] x 30 cycles, followed by 72°C for 8 minutes. *Gcr* was amplified with the program 95°C for 10 minutes, [95°C 30", 55°C 20", 72°C 40"] x 35 cycles, followed by 72°C for 8 minutes. PCR products were cloned using Invitrogen TOPO TA Cloning Kit. Plasmid DNA was isolated using the GeneJet Plasmid Miniprep Kit from ThermoScientific and sequenced at Eton Bioscience Inc. (<http://www.etonbio.com>).

PCR using *Sry* and *M33* primers was done to determine sex of offspring that were tested for changes in DNA methylation. *Sry* is a gene specific to the Y chromosome and *M33* is specific to the X chromosome. The *Sry* primers were:

F TCAAGCGMCCCATGAAYGCATT and

R ATATTTATAGTTYGGGTATTTCTC. *Sry* was amplified using the following program: 95°C for 10 minutes, [95°C 30", 52°C 20", 72°C 40"] x 35 cycles, followed by 72°C for 8 minutes. *M33* was amplified using the following primers: F GCTCCCGTGTCAATTTCTTTCAC and R AGACAAGAGCAGTCATTCTGTCACC. The same program for amplifying *Sry* was used to amplify *M33*.

Results

Abnormalities and Mortality in BW and PO

No mortality was seen in PO methyl diet liveborn offspring. Only 2 male adult animals (6 months of age) had any possible abnormal phenotype as their livers were discolored and spotted in appearance, possibly indicating fatty liver (Figure 5.1). This awaits further confirmation by histology. To determine if any death/abnormalities were occurring before birth, we harvested embryos from 3 methyl donor PO crosses and 2 control PO crosses. Of a total of 16 unborn methyl diet embryos, 3 had some abnormality or aberrant morphology (Figure 5.2). In contrast, many offspring from the methyl diet BW crosses died before weaning age (24 days old), with most of the deaths occurring soon after birth ($p < 0.001$; Chi-squared; Table 5.1). However, mortality seemed to be limited to the early postnatal period as no death was observed in animals between the age of weaning and 6 months of age. Further, physical abnormalities were not readily visible in BW offspring of parents on the methyl donor diet (e.g. cataracts). Dissections of BW animals were performed and there was no significance in abnormalities seen in offspring of parents on the methyl donor diet.

Weight and DEXA Scans

At 6 months of age, PO and BW controls and methyl diet offspring were weighed. A total of 92 experimental (47 ♀ & 45 ♂) and 41 control (21 ♀ & 20 ♂) PO animals were weighed while a total of 21 experimental (12 ♀ & 9 ♂) and 24 control (12 ♀ & 12 ♂) BW animals were weighed. There was no significant difference in PO weight for males or females, although the weight ranges in methyl diet offspring were much larger than controls (Figure 5.3). In BW, however, weight was significantly decreased in methyl diet offspring when compared to controls (Figure 5.4).

DEXA scans were performed to determine if bone mineral content (BMC) or percent body fat were altered in the methyl diet animals. DEXA scans on PO animals revealed there were no changes in BMC, however, the PO methyl diet offspring had a significantly higher percent body fat than controls (Figure 5.5).

Open Field and Social Interaction Tests

In open field tests using PO, repetitive behavior was significantly increased in methyl diet females ($p < 0.05$; Kruskal-Wallis; Figure 5.6). There were no other changes in other behaviors for PO. Open field tests using BW, however, showed a significant increase in grooming in both methyl diet males ($p < 0.05$; Kruskal-Wallis) and females ($p < 0.01$; Kruskal-Wallis; Figure 5.7)

Social interaction tests using PO revealed many changes in behavior in methyl diet offspring. Repetitive behaviors were significantly increased ($p < 0.01$; Kruskal-Wallis) and social behaviors were significantly decreased in methyl diet males ($p < 0.001$; Kruskal-Wallis) and females ($p < 0.001$; Kruskal-Wallis; Figure

5.8). A significant increase in aggressive behaviors was seen in methyl diet males ($p < 0.001$; Kruskal-Wallis).

In BW methyl diet offspring, there was a significant decrease in repetitive behaviors in females ($p < 0.05$; Kruskal-Wallis) while the females also had a significant increase in aggressive behaviors ($p < 0.05$; Kruskal-Wallis; Figure 5.9). Social behavior was not significantly changed, although BW methyl diet males were somewhat more social than controls.

Elevated Plus Maze

Females of both species tested (BW and PO) had no significant change in anxiety-like behaviors during the EPM. Time spent in open vs. closed arms, as well as head-dipping behaviors (exploratory behaviors) were assessed. BW methyl diet males spent more time in closed arms ($p < 0.05$; ANOVA) and less time head dipping ($p < 0.01$; ANOVA) than control BW males (Figure 5.10). The same is true for PO methyl diet males as they spent more time in closed arms ($p < 0.05$; ANOVA) and less time head dipping ($p < 0.05$; ANOVA) than control PO males (Figure 5.11). No other parameters tested were significant (data not shown).

Barnes Maze

PO methyl diet females had significantly higher latencies compared to PO control females only on days 1 ($p = 0.05$; repeated measures ANOVA; Figure 5.12) and 2 ($p = 0.044$; repeated measures ANOVA). PO methyl diet males had significantly higher latencies compared to PO control males only on days 1 ($p = 0.006$; repeated measures ANOVA) and 7 ($p = 0.042$; repeated measures ANOVA). The

methyl diet did not improve the performance of BW animals in the Barnes Maze, as a learning trajectory was not seen in methyl diet males or females. In fact, the trajectories of each are similar to their control counterparts that were seen in Chapter 4.

PO methyl females, after day 2, sniffed the correct hole more than control females, particularly during day 4 ($p=0.05$; repeated measures ANOVA; Figure 5.13) and day 6 ($p=0.045$; repeated measures ANOVA). Control PO males sniffed the correct hole more than methyl diet PO males. This effect was only significant on day 7 ($p=0.014$; repeated measures ANOVA). BW methyl diet females sniffed the correct hole more than control BW females on day 2 ($p=0.042$; repeated measures ANOVA) and day 4 ($p=0.043$; repeated measures ANOVA). There was no significant difference in sniffing the correct hole for BW males. When comparing total holes sniffed between groups, it became apparent there was a significant difference between BW methyl diet females and control BW females. Methyl diet BW females sniff more holes total than control BW females on day 6 ($p=0.043$; repeated measures ANOVA; Figure 5.14) and day 7 ($p=0.005$; repeated measures ANOVA), which could indicate more exploratory behavior in methyl diet females.

It is important to note that there are no significant changes in search strategy between methyl and control animals of each stock and sex (data not shown).

Bisulfite Sequencing

Bisulfite sequencing of whole neonate brains from BW female x SM2 male and BW female x PO male crosses (methyl diet and control) reveal different effects on DNA methylation at the DMR of the *Peg10/Sgce* imprinted domain. BW female x SM2 male offspring from methyl diet crosses gained methylation on the paternal allele in 1 out of 4 methyl diet offspring tested. The affected offspring was a male while the others were 2 females and 1 male. BW female x PO male offspring, however, lost methylation at the maternal allele in 3 out of 8 methyl diet offspring tested (2 representative methyl samples, one male and one female, were chosen for the figure) (Figure 5.15).

Currently, 4 clones have successfully been sequenced for *Mecp2*. Two of these clones are from a control female while the other 2 are from a methyl diet male. We currently have a 50% methylation pattern in the control female (expected) while there is a gain of aberrant methylation in the methyl diet male (data not shown).

Bisulfite sequencing of the glucocorticoid receptor (*Gcr*) promoter in hypothalamus and brainstem tissues from brains of 6 month old PO to date reveal significant increases in methylation of both hypothalamus and brainstem *Gcr* in methyl diet PO males ($p < 0.001$; Chi-squared; Figure 5.16). DNA methylation changes in *Gcr* in hypothalamus are also significant although it is unknown how DNA methylation changes seen in both brainstem and hypothalamus might affect mRNA levels.

Discussion

We set out to assess whether or not genetic background influenced epigenetic response to the methyl-donor diet. The data presented here indicate that this is the case, although mechanism(s) by which this happens remain elusive. This is, in part, due to the fact that the effects are pleiotropic and there could be many genes involved in the different effects seen. Prenatal abnormalities and death in PO as well as juvenile deaths in BW indicate the diet has the potential to induce negative physiological effects by an epigenetic mechanism that has not yet been determined.

This could be the consequence of DNA or histone methylation, although this assumption may not necessarily be the case as we saw loss of methylation with a change of the offspring's paternal inheritance. Other factors affect DNA methylation, such as *Tet3*, which codes for ten-eleven translocation 3-mediated hydroxylase, which converts 5-methylcytosine to 5-hydroxymethylcytosine (5-hmc) which can then be converted to unmethylated cytosine [137]. A role for *Tet3* has more recently been discovered to play a critical role in prefrontal cortex for mediation of rapid behavior adaptation and establishment of epigenetic marks (demethylation of cytosine) that promote gene expression [137].

Weight and percent body fat changes indicate a possible link between high levels of methyl donors in the diet and obesity, depending on genotype. Metabolism may be affected due to changes in DNA methylation in the liver, as maternal and post-weaning folic acid supplementation has been shown to affect DNA methylation at specific genes in rat liver [138]. Also, high prenatal folic acid

use in humans (>5mg/day) has been reported to be associated with higher birth weight [139].

Additionally, the behavioral changes seen in PO are likened to that seen in patients with Autism Spectrum Disorders (ASD), yet some changes seen in BW behavior are positive (i.e. less repetitive behavior in females, more social behavior in males). This difference in behavioral changes could be attributed, in part, to the differential changes in DNA methylation at the *Peg10/Sgce* promoter, since *Peg10/Sgce* is located in a region is associated with autism [21]. It is impossible to determine if *Mecp2* methylation is significantly affected due to too few clones that have been sequenced. Further directions include obtaining sequencing for many more clones and individuals.

Severity of anxiety-like behaviors in the EPM seems to be linked to genetic background as well, as BW males on the methyl diet present more anxiety-like behaviors, and to a greater degree, than PO males. Increased anxiety was somewhat unexpected, as anxiety has been attributed to higher homocysteine levels (brought on by a lack of B vitamins) which in turn has been thought to interfere with neurotransmitter levels [140]. Therefore, DNA methylation of genes involved in anxiety-like behaviors such as *Gcr* may be perturbed.

We tested DNA methylation at the CG rich promoter of *Gcr* in hypothalamus and brainstem in PO methyl diet males with high amounts of time spent in closed (versus open arms) in the EPM and compared the data to data for control PO males. To date, brainstem and hypothalamus *Gcr* is significantly

more methylated in methyl diet PO males, although how these DNA methylation changes affect mRNA levels has yet to be determined.

This could account some for the changes in anxiety-like behaviors in males on the EPM since the brainstem is part of the noradrenergic system which is linked to anxiety [141], and generalized anxiety disorder is often associated with dysfunction of the hypothalamus-pituitary-adrenal axis [142]. However, methylation may be perturbed at other loci. Further studies, including current RNA-seq, will be useful in determining if other anxiety linked genes have changes in expression levels in methyl diet PO animals.

PO methyl diet females had significantly higher latencies compared to PO control females only on days 1 and 2. This is indicative of heightened anxiety/stress in methyl PO females rather than a difference in learning and memory. PO methyl diet males had significantly higher latencies compared to PO control males only on days 1 and 7. The difference on day 1 is indicative of higher stress in methyl PO males during the first day. Interestingly, it appeared the stress/anxiety effect on early trials was more significant in methyl diet PO males. There is some evidence indicating learning or memory deficit due to the significant difference on day 7. As stated before in Chapter 4, it is understood that stress/anxiety and memory/learning coincide with each other; that is, higher stress/anxiety can lead to poorer memory/learning. The methyl diet did not improve the performance of BW animals in the Barnes Maze, as a learning trajectory was not seen in methyl diet males or females. The methyl diet animals' trajectories, in fact, were very similar to those of BW control animals.

When comparing how often each group sniffs the correct hole versus total holes sniffed, PO methyl females, after day 2, sniffed the correct hole more than control females, particularly during days 4 and 6. This does not provide any insight into exploratory behavior or memory as the significant days appear to be random. Control PO males sniffed the correct hole more than methyl diet PO males. This effect is only significant on day 7 ($p=0.014$, repeated measures ANOVA). This could further indicate a deficit in learning/memory, as a significant difference was seen in latency for day 7 as well for methyl PO males. BW methyl diet females sniffed the correct hole more than control BW females on day 2 ($p=0.042$, repeated measures ANOVA) and day 4 ($p=0.043$, repeated measures ANOVA). Again, due to the randomness of the days in which there was significance, this likely does not provide any insight into exploratory behaviors or learning/memory.

There was no significant difference in sniffing the correct hole for BW males. Results for latencies and correct versus incorrect hole sniffing indicated there was no effect of the methyl diet in BW animals in learning/memory. Willingness to explore; however, may have been affected in methyl diet BW females as they sniffed more holes total than control BW females. This, again, further supports the hypothesis that the Barnes Maze acts as environmental enrichment for BW animals and is not a good test for learning/memory.

It is apparent that genotype does in fact affect the response to the methyl donor diet in several different ways, from phenotypic abnormalities and mortalities to behavioral changes and aberrant DNA methylation. Crosses of BW and PO could be utilized to determine which genes may contribute to the

differential effects seen in BW and PO on the methyl diet. Such genes could include the aforementioned *Tet3*, or even *Mthfr* (a reductase enzyme in the pathway that metabolizes folic acid through the 1-Carbon metabolism system to lead to SAM, the ultimate methyl donor) [144].

Recently, mainstream media has brought attention to the concept of epigenetics. In fact, they have begun telling the public, “Why DNA Isn’t Your Destiny” [145], how to “Outsmart Your Genes” [146] and NOVA titles have surfaced such as “Epigenetics: Beating our Genes” [147]. This information, however, is somewhat misleading. Humans carry many genetic mutations in many different genes. Without genetic testing, one may not know that she/he has a mutation in such a gene since phenotypic manifestations can be mild (e.g., the C677T *MTHFR* mutation results in mild hyperhomocysteinemia due to less folic acid metabolism) [148]. Attempting to then alter phenotype by altering epigenetics (e.g., by diet) may prove futile or possibly deleterious without knowing how genetics may still control phenotypic destinies.

Examples of such alterations of phenotype can be seen in mice with different gene knockouts (known to induce neural tube defects, or NTDs) that were given a diet high in methyl donors. Folic acid supplementation led to exacerbated NTDs in two separate mouse models, one with a gene knockout of *L3P* and one with a gene knockout of *Shroom3* [119]. Other mouse models with a gene knockout in either *Zic2* or *Grhl2* had improvements in NTDs with the methyl donor diet [119]. High methyl donor supplementation, therefore, has the potential to be deleterious to a developing fetus, especially since women with a

MTHFR mutation are provided with folic acid supplementation of up to 4 mg per day [149]. Therefore, it is reasonable to argue in favor of individualized periconceptional folic acid supplementation that would be based on genetic testing.

Table 5.1: BW methyl diet offspring mortality.

	Born	Weaned
Control	44	42
Methyl	30	21



Figure 5.1: Spotted liver from PO methyl diet offspring. This offspring and one other methyl diet offspring (PO) had spotted livers, possibly indicating fatty liver.

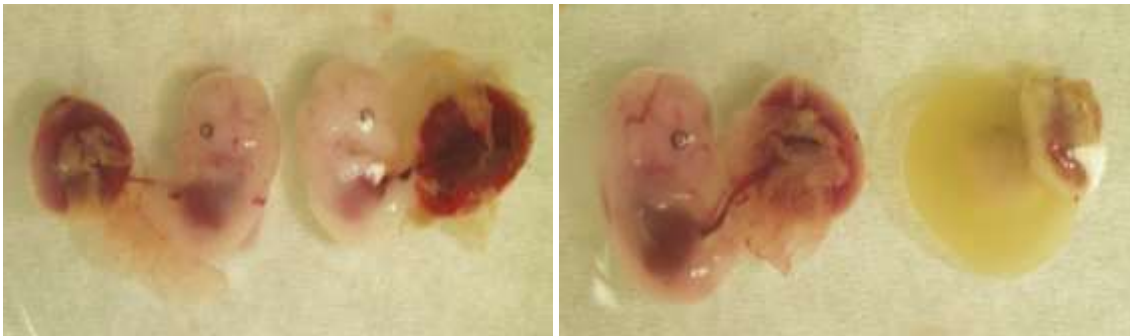


Figure 5.2: Embryos from PO methyl and control crosses. Embryos 1, 2 and 4 are from methyl diet parents while 3 is a control embryo. Embryos 1 and 2 have a notable lack of blood supply when compared to the control. Embryo 2 also has a dysmorphic head and a much larger and redder placenta. Embryo 4 was either being reabsorbed or the embryonic structure failed to develop.

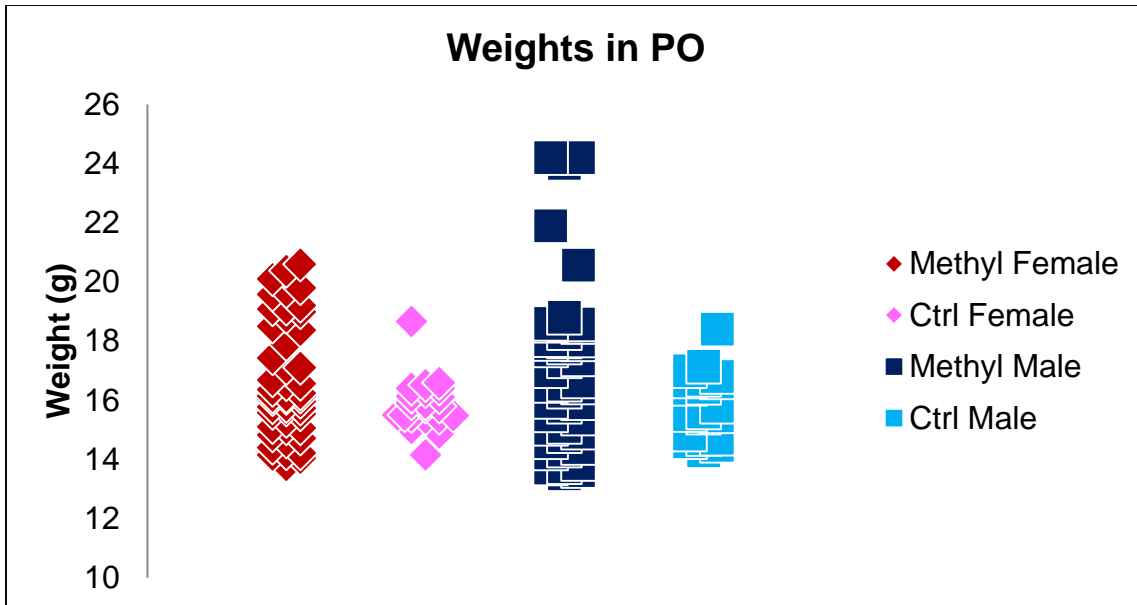


Figure 5.3: Weights in methyl and control PO offspring. There is no significant difference in weight between control and methyl diet groups of each sex. There does, however, appear to be a larger range in weight in methyl diet animals: some methyl diet animals are lighter (both males and females) while some methyl diet animals are heavier (both males and females).

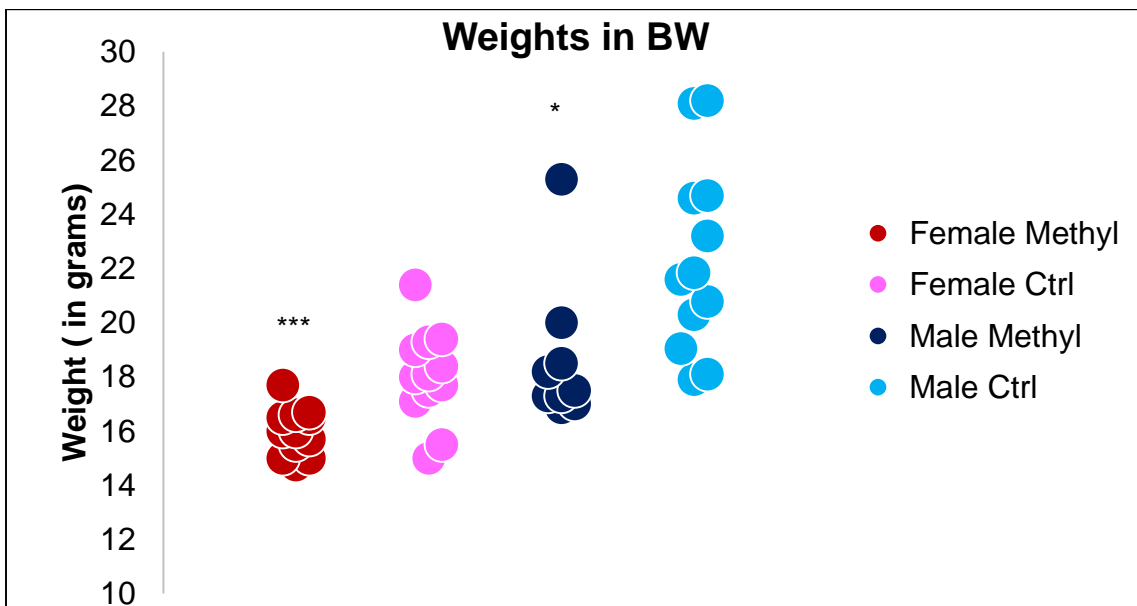


Figure 5.4: Weights in methyl and control BW offspring. There is a significant decrease in weight in methyl diet females ($p < 0.001$, t-test), and in methyl diet males ($p < 0.05$, t-test).

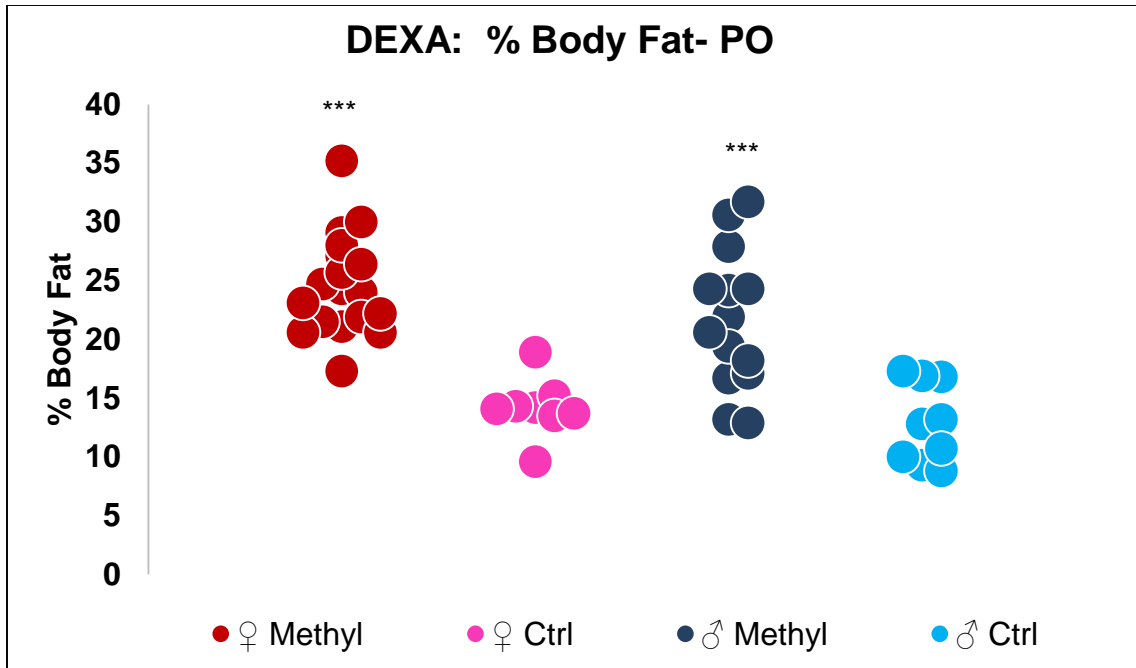


Figure 5.5: Percent body fat in PO methyl and control offspring. There was a significant increase in % body fat in both male and female methyl diet offspring ($p < 0.001$, t-test).

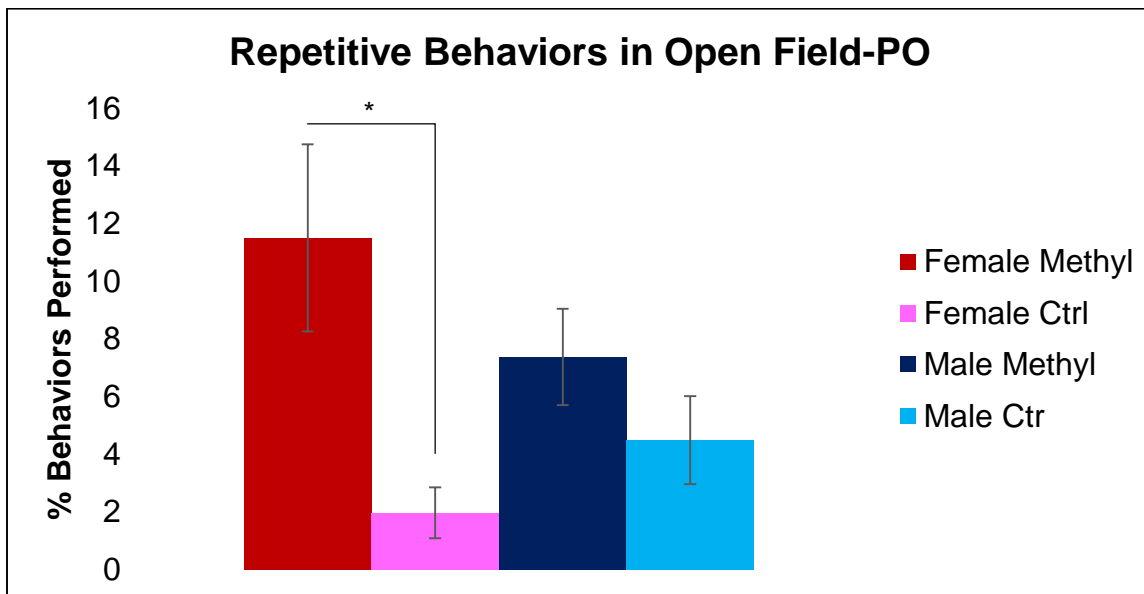


Figure 5.6: Repetitive behaviors in PO in Open Field Tests. PO methyl diet females had a significant increase in repetitive behaviors in Open Field tests where males remained relatively unchanged ($p < 0.05$; Kruskal-Wallis). Error bars are standard error.

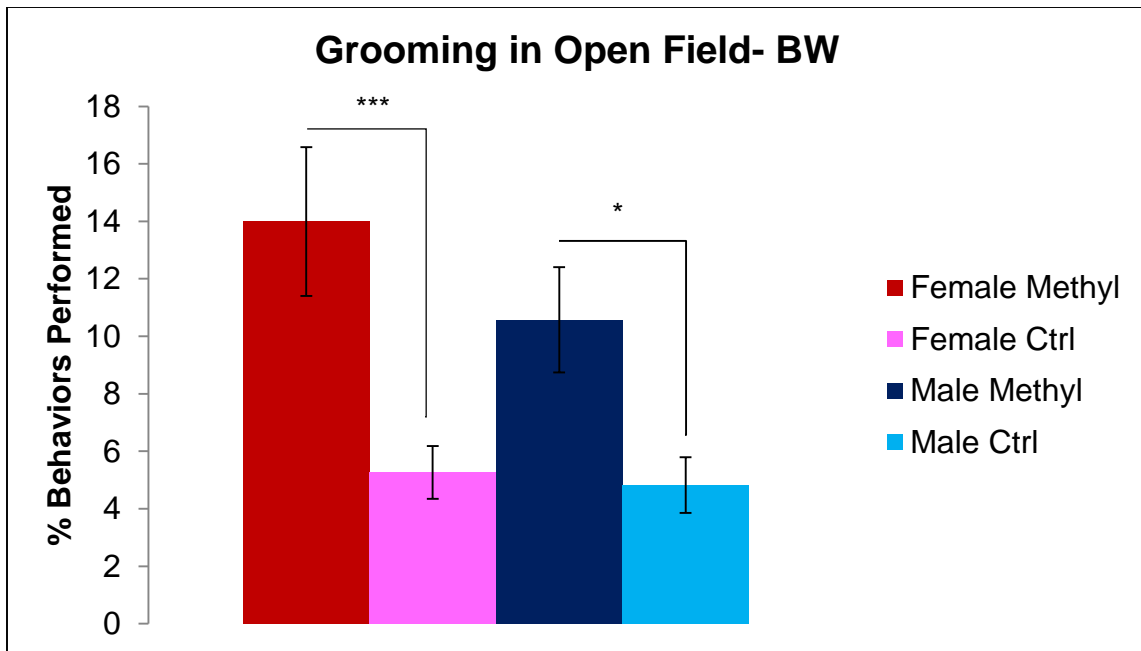


Figure 5.7: Self-grooming in methyl and control BW in Open Field Tests. Methyl diet BW animals groomed significantly more than control BW animals. A larger increase was seen in females ($p < 0.001$; Kruskal-Wallis) than in males ($p < 0.05$; Kruskal-Wallis). Error bars are standard error.

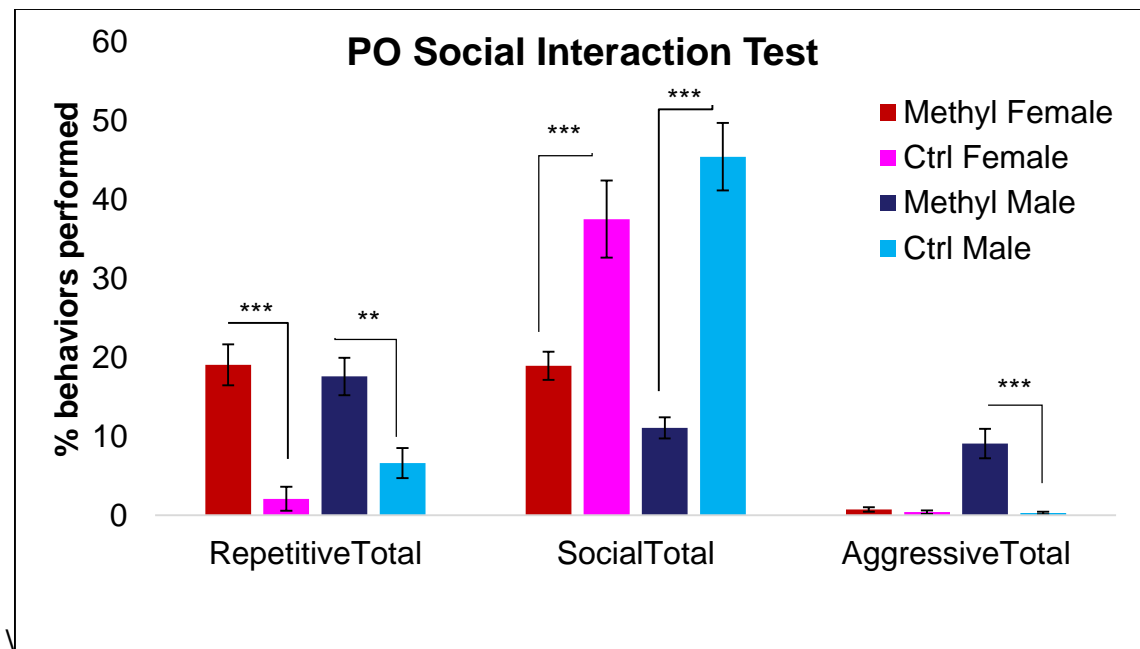


Figure 5.8: Social Interaction Test in methyl and control PO. A significant increase in repetitive behaviors was indicated in both methyl diet males ($p < 0.01$; Kruskal-Wallis) and females ($p < 0.001$; Kruskal-Wallis) while a significant decrease in social behaviors was seen in methyl diet males and females ($p < 0.001$; Kruskal-Wallis). A significant increase in aggressive behaviors was seen only in methyl diet PO males ($p < 0.001$; Kruskal-Wallis). Error bars are standard error.

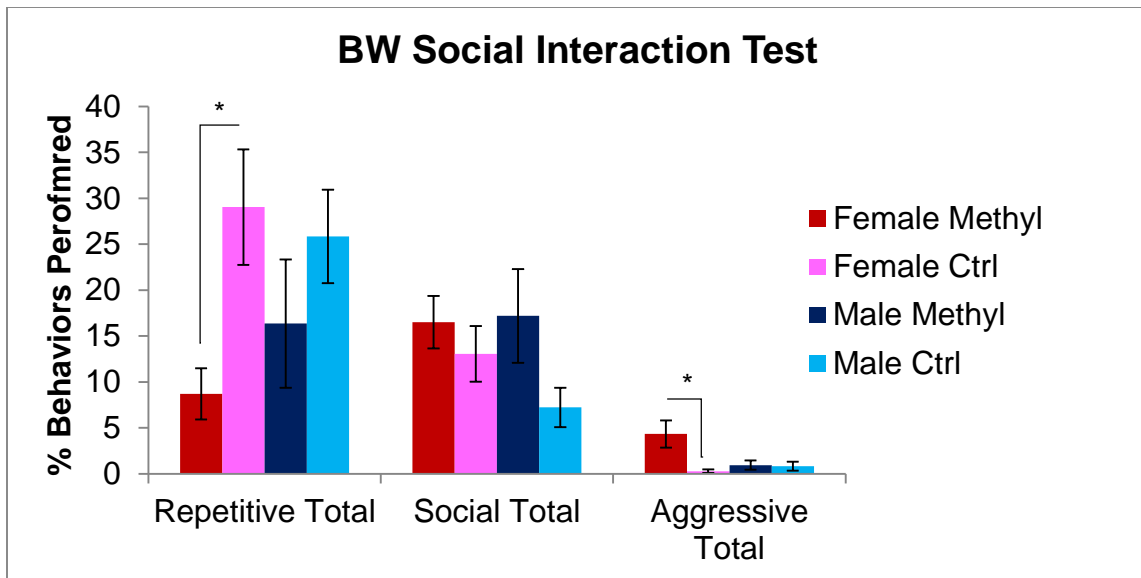


Figure 5.9: Social Interaction Test in methyl and control BW. A significant decrease in repetitive behaviors was evident in methyl diet BW females ($p < 0.05$; Kruskal-Wallis) while there was no change in males. A significant increase in aggressive behaviors was also evident in methyl diet BW females ($p < 0.05$) while there was no change in males. There was a trend toward increased social behavior in methyl diet males although this was not significant ($p = 0.064$; Kruskal-Wallis). Error bars are standard error.

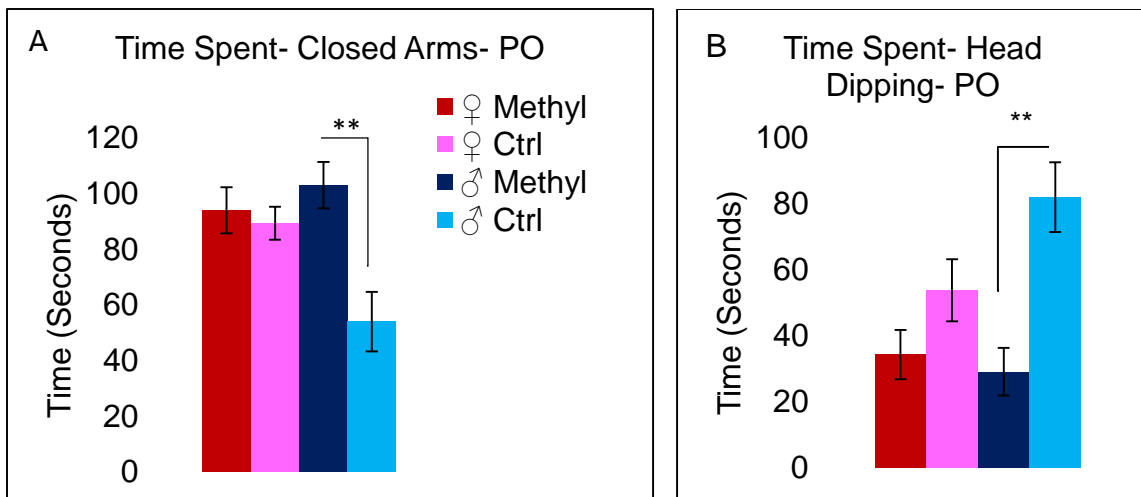


Figure 5.10: EPM results for PO. (A) A significant increase is apparent in the amount of time spent in closed arms for methyl diet male PO ($p < 0.01$; ANOVA). (B) Methyl diet male PO animals also performed significantly less “head dipping” behaviors ($p < 0.05$; ANOVA). There were no changes in PO female methyl diet animals. Error bars are standard error.

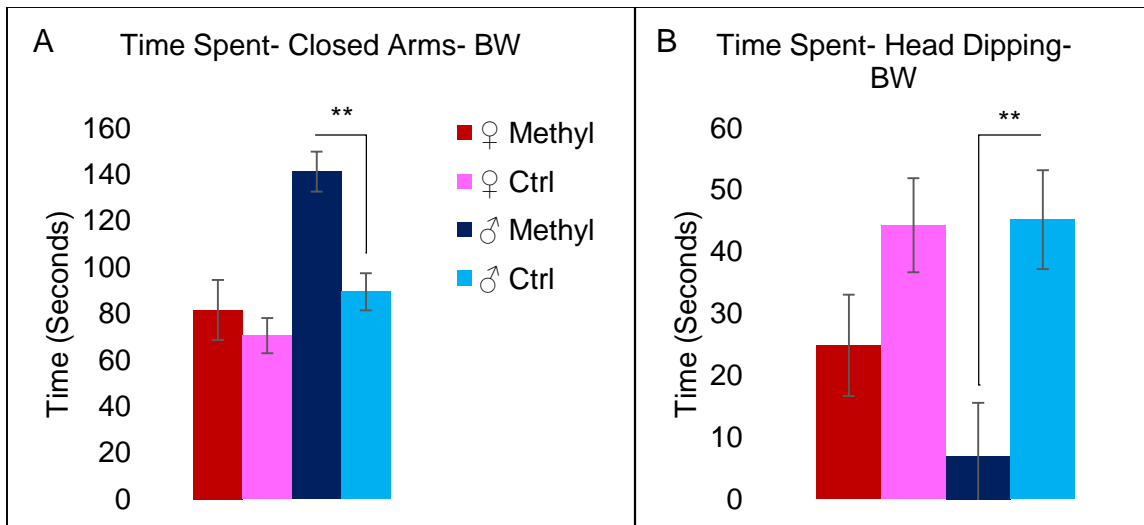


Figure 5.11: EPM results for BW. (A) There was a significant increase in the amount of time spent in closed arms for methyl diet male BW ($p < 0.01$; ANOVA). (B) Methyl diet male BW animals also performed significantly less “head dipping” behaviors ($p < 0.01$; ANOVA). There were no changes in PO female methyl diet animals. Error bars are standard error.

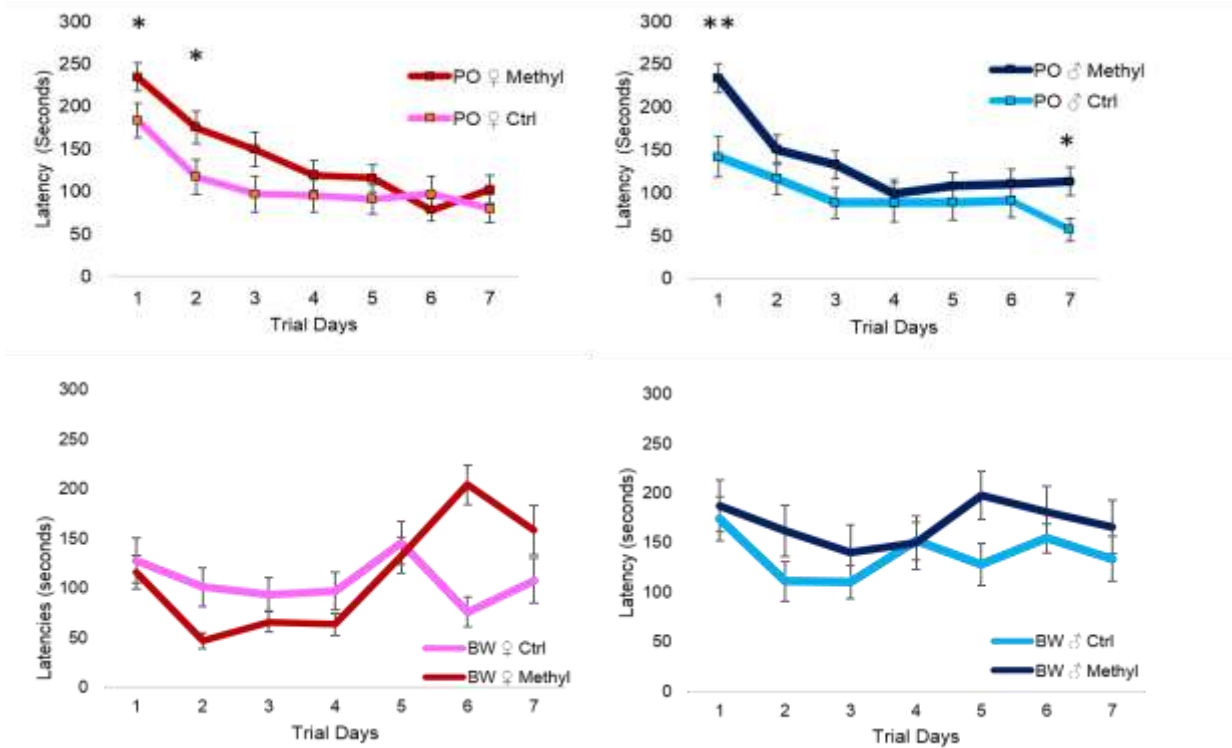


Figure 5.12: Latencies for BW and PO methyl and control animals. PO methyl diet females had significantly higher latencies compared to PO control females only on days 1 ($p=0.05$, repeated measures ANOVA) and 2 ($p=0.044$, repeated measures ANOVA). This indicates stress/anxiety in PO methyl diet females. PO methyl diet males had significantly higher latencies compared to PO control males only on days 1 ($p=0.006$, repeated measures ANOVA) and 7 ($p=0.042$, repeated measures ANOVA). This indicates stress/anxiety but also indicates a possible memory/learning deficit in methyl diet male PO animals. The methyl diet did not improve the performance of BW animals in the Barnes Maze, as a learning trajectory was not seen in methyl diet males or females.

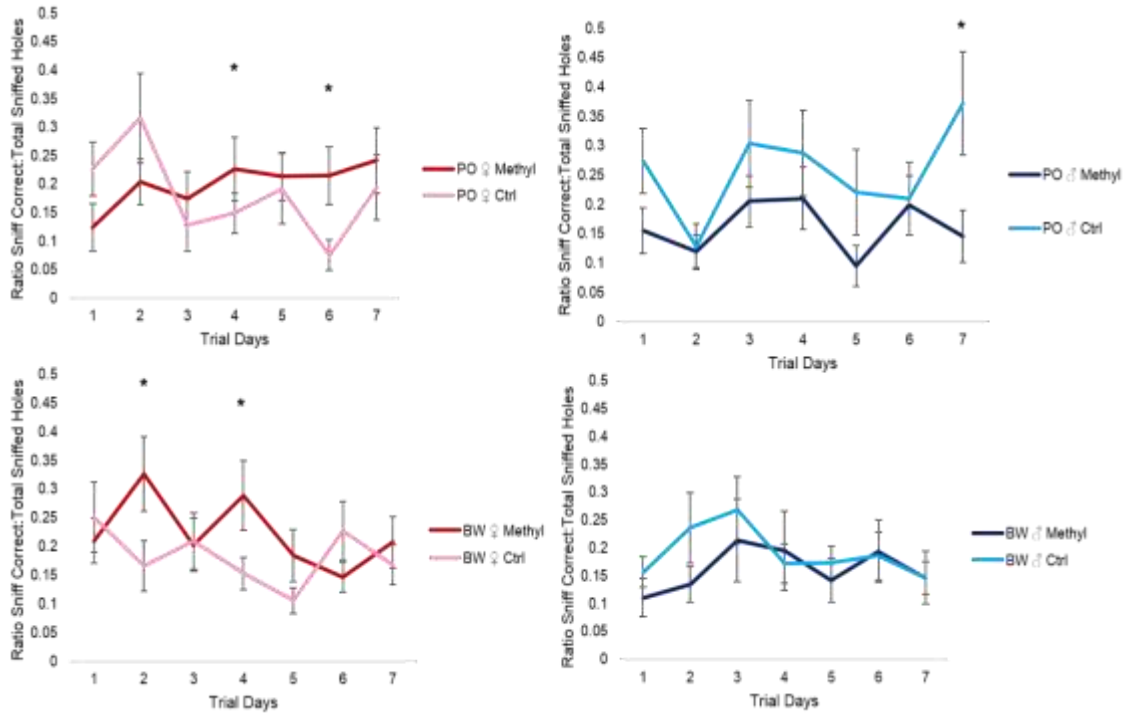


Figure 5.13: Ratio of correct versus total holes sniffed in methyl diet animals. PO methyl females, after day 2, appear to sniff the correct hole more than control females, particularly during day 4 ($p=0.05$, repeated measures ANOVA) and day 6 ($p=0.045$, repeated measures ANOVA). Control PO males sniff the correct hole more than methyl diet PO males. This effect is only significant on day 7 ($p=0.014$, repeated measures ANOVA). BW methyl diet females sniff the correct hole more than control BW females on day 2 ($p=0.042$, repeated measures ANOVA) and day 4 ($p=0.043$, repeated measures ANOVA). There is no significant difference in sniffing the correct hole for BW males.

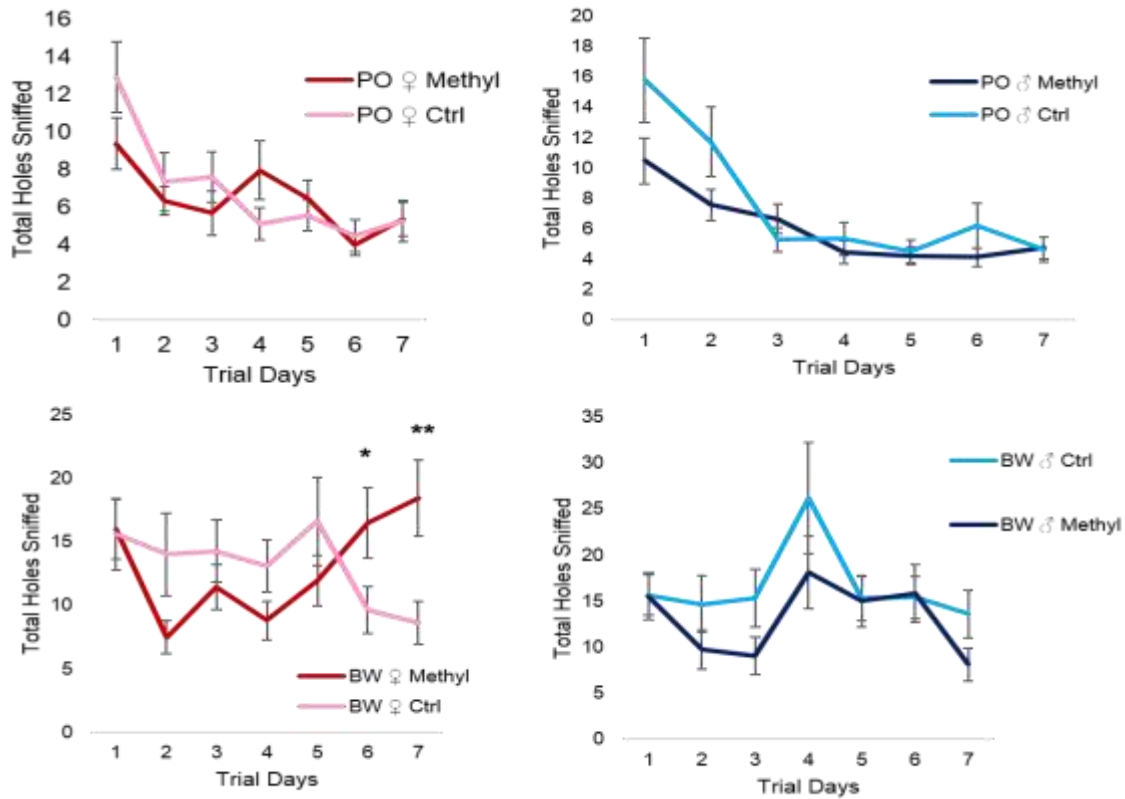


Figure 5.14: Total holes sniffed in methyl diet versus control animals. There was no significant difference in total number of holes sniffed between methyl diet and control PO animals of both genders. The same was true for BW methyl versus control males. BW methyl diet females; however, sniffed significantly more holes than control females during day 6 ($p=0.043$; repeated measures ANOVA) and day 7 ($p=0.005$; repeated measures ANOVA). This may indicate more exploratory behavior in methyl diet BW females compared to controls.

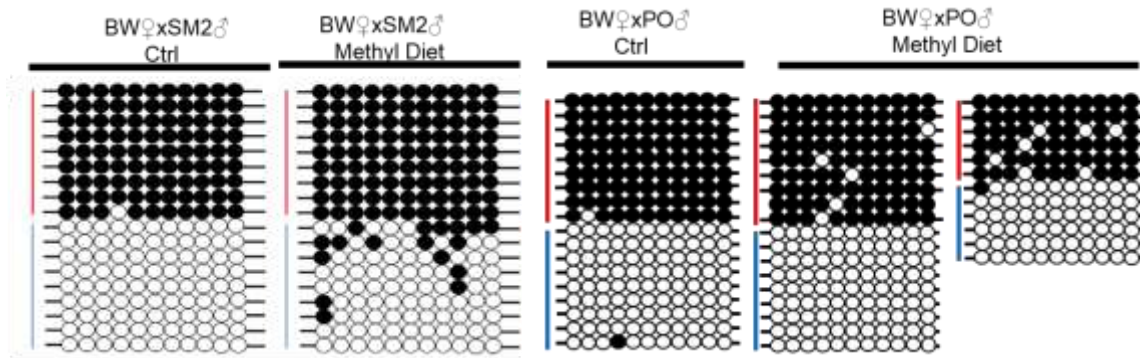


Figure 5.15: Changes in *Peg10/Sgce* Methylation. Each line represents a clone that was sequenced which contained the PCR product. Circles represent CpG dinucleotides. Filled-in circles represent methylated CpGs while open circles represent unmethylated CpGs. Chi-squared analysis reveals a significant increase in DNA methylation in BW female x SM2 male offspring in a 1 in 4 pattern ($p < 0.05$). The affected offspring was a male. BW female x PO male offspring, however, have a significant decrease in methylation ($p < 0.05$; Chi-squared) in 2 of 8 offspring tested. These 2 affected offspring were males. The BW female x PO male control shown is a combination of 2 control offspring, 1 male and 1 female.

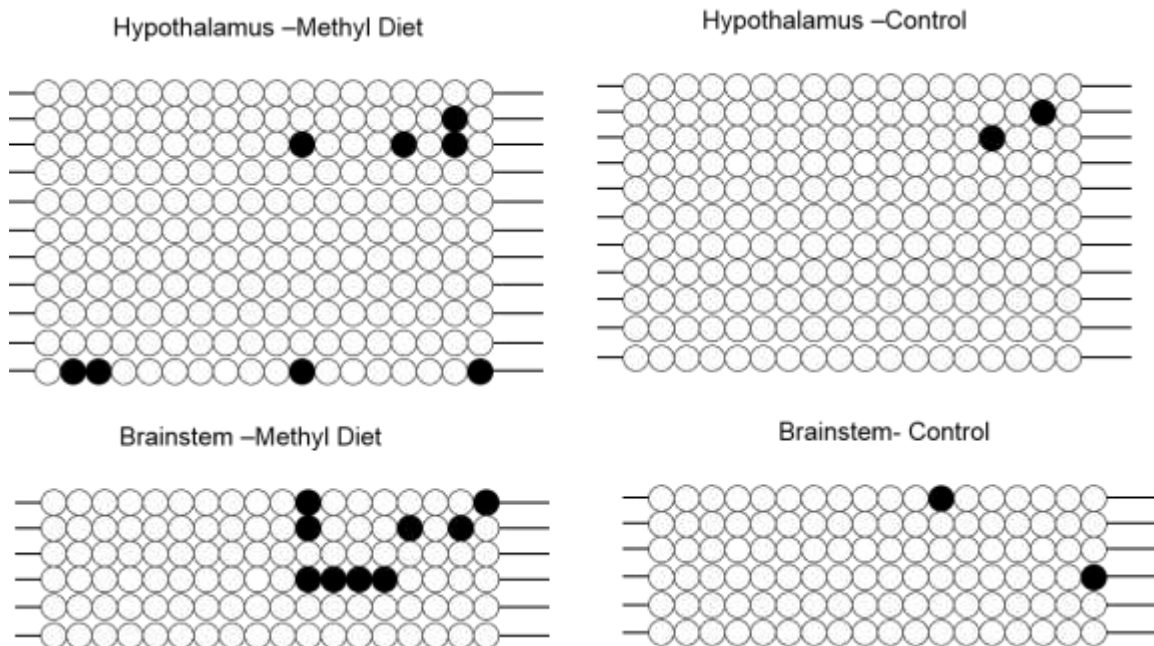


Figure 5.16: Changes in Glucocorticoid Receptor Methylation in PO Males. Each line represents a clone that was sequenced which contained the PCR product. Circles represent CpG dinucleotides. Filled-in circles represent methylated CpGs while open circles represent unmethylated CpGs. Chi-squared analysis indicates a significant increase in hypothalamus ($p < 0.01$) and brainstem ($p < 0.001$) GCR methylation in PO methyl diet males.

Conclusion

Differences in BW and PO behavior are dramatic. This is likely due to genotypic differences which still need to be discovered. Gene discovery for these differences in behavior can be uncovered using backcrosses (i.e. BW female x F1 (BW female x PO male) offspring) of animals of known behavior types. Once these genes are discovered, BW and PO may likely become a much more useful research tool, particularly since BW already serve as a model for certain neurological disorders.

It is likely that the *Agouti* gene is responsible for many behavioral patterns as well. Burrowing and social behavior, in particular, appear to be affected by this gene given that the A^{Nb} stock has been bred onto a BW background for quite some time. The coat color differences between BW and PO suggest it is possible *Agouti* expression may account for certain behavior traits that differ between BW and PO. Further testing would be required to challenge such a hypothesis. The A^{Nb} allele, however, does appear to affect the response to the methyl donor diet, as their phenotypes changed in different ways than in both BW and PO.

As the BW, PO, and A^{Nb} stocks differ widely in response to the methyl donor diet, it is reasonable to assume that genetic background can influence the epigenetic response to the methyl donor diet. Since diet contributes to the

methyl donor pathway, it is likely that epigenetic mechanisms account for the changes seen in methyl donor diet offspring. Support for this hypothesis is reflected in the DNA methylation changes we noted at the *Agouti* locus (Figure 3.5), a locus linked to autism (*Peg10*, Figure 5.15), and one linked to anxiety (*Gcr*, Figure 5.16). The changes seen have sizable implications, especially considering the fact that the methyl donor diet yielded behavior in PO animals that was similar to that of autism patients (e.g., increased repetitive behaviors and decreased social behaviors), while BW and *A^{Nb}* stocks had negative physical attributes (such as cataracts, Figure 3.3C) and mortality (Table 3.2). This leads to the hypothesis that too much periconceptional folate intake in humans (who vary greatly in genetic background) could affect offspring in very different ways. Therefore, folic acid supplementation recommendations for pregnant women may need to be re-evaluated. This would be in addition to providing pregnant women with education regarding folic acid supplementation in foods, drinks, etc.

In conclusion, this research led to more questions. One of which is what genes are involved in the different responses to the diet between species. It is possible that genes coding for enzymes involved in the methyl donor pathway may be linked to these differences. On such gene in humans is *MTHFR*, and it is widely assumed that if a woman has a mutation in *MTHFR*, she should take up to 10 times the FDA recommended intake per day. One possible issue is that it is not known, for instance, how this may affect the developing fetus if the fetus is a heterozygote for the mutation. If it is discovered that genes that code for enzymes involved in the methyl donor pathway are responsible for the differing

effects, then genetic testing for such genes (personalized medicine) may help indicate how much folate women should take during pregnancy.

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Appendix A

Characterization of Dopamine Receptor D4 (*Drd4*) in Species of *Peromyscus*

Introduction

Dopamine receptor D4, or *Drd4*, is a g-coupled protein that plays an important role in the mesocorticolimbic dopaminergic pathway. The mesocorticolimbic dopamine pathway is comprised of several brain structures including the ventral tegmental area, substantia nigra, the nucleus accumbens, and the associated limbic structures [148-152]. The ventral tegmental area and substantia nigra are regions of the midbrain that house cell bodies. Dopamine is synthesized and stored in axon terminals in projection areas. These projection areas are the cortical and limbic areas of the brain. The projection areas include the prefrontal cortex, nucleus accumbens, and dorsal striata [149].

The “rewarding power” of abused drugs such as alcohol is ascribed to the projections of this pathway. *Drd4* is, therefore, partially responsible for mediating the effects of dopamine production in this pathway [149]. Due to the responsibilities of *Drd4* protein in this pathway, it is of little surprise that DRD4 is thought to play a role in some neurological disorders such as schizophrenia and bipolar disorder [149,153]. To date, DNA methylation of *DRD4* has been quantified in monozygotic twins [154] but authors did not note that the 50%

methylation patterns seen at the *DRD4* promoter may indicate an imprinted gene. Therefore, this data raises the question of whether or not *DRD4* is an undiscovered imprinted gene, particularly due to parent of origin effects in diseases associated with *DRD4*.

This question has apparently been debated for some time without any clear answer. This project plans to elucidate whether or not *Drd4* is imprinted in *Peromyscus*. During this project, we additionally uncovered a genomic difference (a deletion in *Drd4* in PO). The deletion may be of importance due to the location within the gene and due to the possible implications this could have in *Peromyscus* behavior differences.

From the data collected for this project, we hypothesize that *Drd4* may be part of a larger and more complicated imprinting scheme. Additionally, we hypothesize that the difference in the gene between the two *Peromyscus* species PO and BW may have a role in behavior differences between the two species.

Materials and Methods

Tissue Harvesting and DNA and RNA Isolation

Brains were harvested from BW, PO, BW female x PO male offspring, and PO female x BW male offspring and were flash frozen in liquid nitrogen. Before isolation of DNA and RNA, brains were ground with mortar and pestel in liquid nitrogen in order to test DNA and RNA from a homogenous mix of brain regions. DNA isolation was performed using the Qiagen DNeasy Blood and Tissue Kit. RNA was isolated using the Qiagen RNeasy Midi Kit. Concentrations of DNA and RNA were read on a Nanodrop Spectrophotometer from ThermoScientific.

Bisulfite Sequencing of DNA

Brains from BW female x PO male offspring were used to detect CG methylation differences in the *Drd4* promoter region. DNA was treated with sodium bisulfite using the Qiagen Epitect Bisulfite Fast Kit. Bisulfite PCR using MyTaq Mix from Bioline was performed with the following primers for *Drd4*:

F TTTATTTAATTTTTTTGTTGAAATTAAGTAT and

R CAAAATTACTAAAAATCCAAAC. These primers extend from slightly upstream of the promoter region into exon 1 (Figure A.1). The PCR program was as follows: 95°C for 10 minutes, [95°C 30", 52°C 20", 72°C 40"] x 30 cycles, followed by 72°C for 8 minutes. PCR products were cloned using Invitrogen TOPO TA Cloning Kit. Sequencing was performed by Eton Bioscience, Inc.

cDNA Synthesis and Reverse Transcriptase PCR

cDNA was synthesized using equal concentrations of RNA from the following brain RNA samples: BW, PO, BW female x PO male, and PO female x BW male. cDNA synthesis was performed using the Tetro cDNA Synthesis Kit from Bioline Taq. Once cDNA was then amplified by PCR using MyTaq Mix from Bioline with the following primers (for exon 1 of *Drd4*): F GCCGGAGCTCATTTAGCTATC and R ATGGCGCACAGATTGAAGAT. The PCR program was as follows: 95°C for 10 minutes, [95°C 30", 52°C 20", 72°C 40"] x 30 cycles, followed by 72°C for 8 minutes. PCR products were then analyzed by acrylamide gel for high resolution of the two band sizes for BW and PO.

Drd4 Genotyping

BW and PO brain samples were used to determine SNPs in *Drd4* between the two species. PCR products were amplified using MyTaq Mix from Bioline. The PCR was performed using the following primers:

F GCCGGAGCTCATTTAGCTATC and R CACGCACACGAGCGAGTT. The PCR program was as follows: 95°C for 10 minutes, [95°C 30", 52°C 20", 72°C 40"] x 30 cycles, followed by 72°C for 8 minutes. PCR products were then cloned using the TOPO TA Cloning Kit from Invitrogen. Sequencing was performed by Eton Bioscience, Inc.

Results

Imprinting in Drd4

No differential CG methylation was apparent between the two alleles (BW and PO) at the *Drd4* promoter (data not shown). Results of previously conducted RT-PCR on the 4th exon of *Drd4* (at U.C. Irvine, by Harry Mutandan) indicate an imprinted pattern (Figure A.2). Results of RT-PCR performed on the 1st exon of *Drd4*, however, do not indicate an imprinted pattern (Figure A.3).

Genotyping of Drd4 in BW and PO

Due to results of bisulfite sequencing, we hypothesized there is a deletion in *Drd4* in the PO sample. Results of *Drd4* genotyping indicate this 57 bp deletion is present in PO. Through determining likely amino acid sequence and comparing to *Mus*, it was determined this deletion in PO is likely in the first extra-cellular domain of the *Drd4* protein and is 19 amino acids.

Discussion

It would at first appear that *Drd4* is not imprinted based on the bisulfite sequencing results and the RT-PCR results of exon 1. I hypothesize, however, that this is misleading. Taking into account the location of *Drd4* (close proximity to the complicated imprinting region of *H19/Igf2*), and the fact exon 4 displays an imprinting pattern, my hypothesis is exon 4 could be imprinted in *Peromyscus* via the complicated chromatin looping mechanism seen in *H19/Igf2*. *Drd4* in *Mus*, in fact, is 1.3 cM away from *H19* on chromosome 7. Further studies would need to be performed in order to determine if this is valid. One possible method would be through CHIP-loop Chromatin Conformation Capture (also known as 3C-CHIP-loop), where it is possible to study two chromosomal region interactions that are mediated by a bound protein (in the case of *H19/Igf2*, there is a bound protein, CTCF, at the methylated ICR) [155].

It does appear that the deletion in PO in the first exon of *Drd4* has the potential to be functional. Further studies to confirm this would be western blotting to determine if the protein is truncated. It seems possible that this deletion could account for one to many of the behavioral differences seen between BW and PO. One possible method to determine this is to make a congenic strain by crossing BW and PO to obtain F1s and backcross F1s with BW for several generations while selecting for the deletion in *Drd4* followed by behavioral testing. This deletion has the potential to clarify, possibly, many questions regarding *Peromyscus* behavior, the most interesting of which is difference in alcohol consumption.

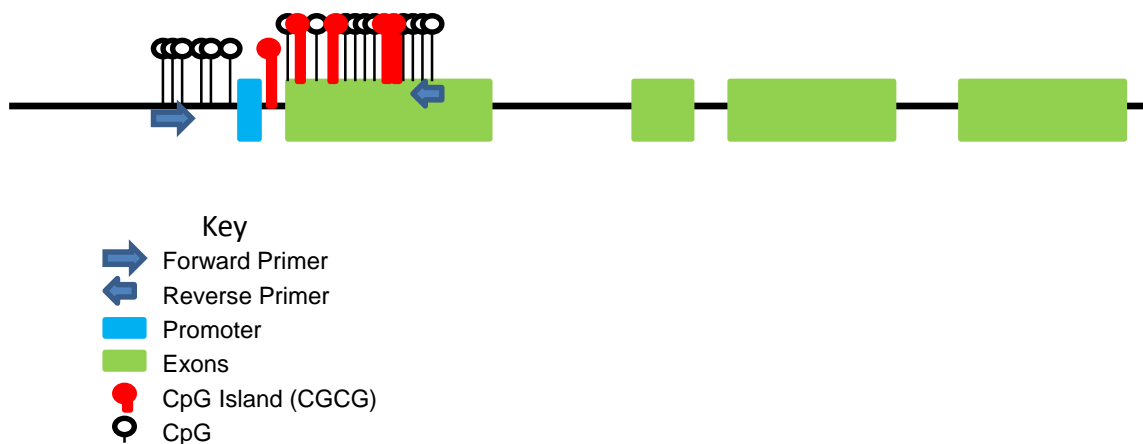


Figure A.1: *Drd4* Diagram with Bisulfite Sequencing Primer Locations. Primers used for bisulfite sequencing of *Drd4* are indicated by the arrows. The primers are 5' to the promoter and extend into exon 1. Several CG islands are located within the amplicon.

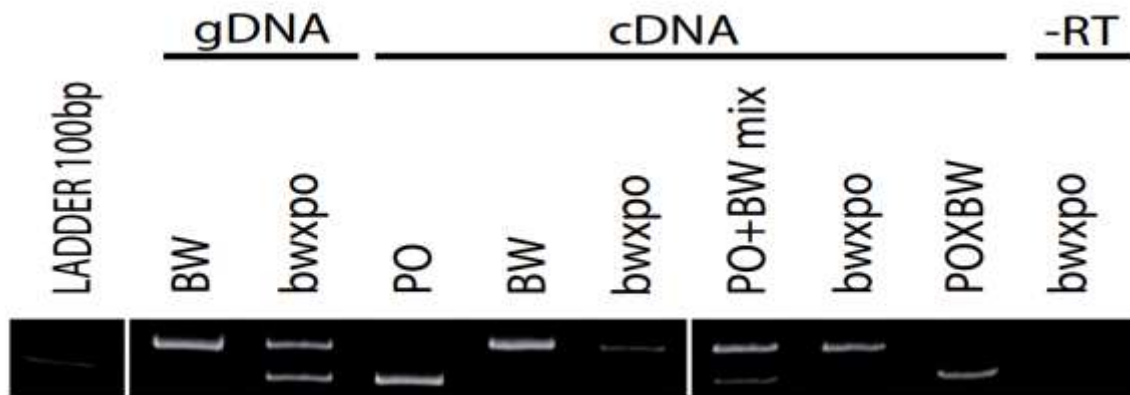


Figure A.2: Reverse Transcriptase Results of *Drd4* Exon 4. From Harry Mutandan, U.C. Irvine, Dr. Vrana's Lab. Genomic DNA (DNA) shows the size difference expected between BW and PO alleles using the same primers used to amplify the cDNA. The Reverse Transcriptase PCR on cDNA indicates genomic imprinting, as the maternal allele is the only one expressed in the bwxpo and POxBW samples. PO+BW mix was used as a control to show both alleles, when together, amplify with the primers. -RT control shows lack of genomic DNA in the cDNA sample.

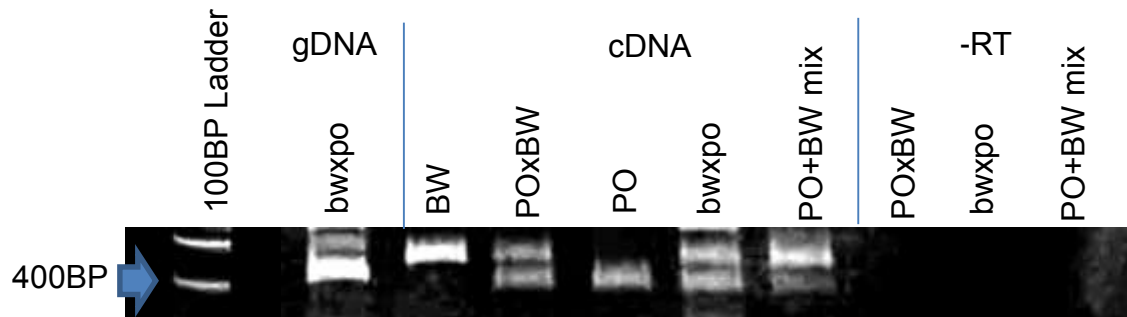


Figure A.3: Reverse Transcriptase PCR of *Drd4* exon 1. gDNA from a heterozygote shows the two bands and their size difference. Reverse Transcriptase on cDNA reveals both PO and BW alleles are expressed from the heterozygous samples used. The –RT controls show no genomic DNA was present in the heterozygous or mixed cDNA sample.

Appendix B

Genetics of Peromyscus Hybrid Post-Natal Growth

Introduction

Overgrown offspring from PO female x BW male crosses are genetically well understood [72,73]. The reciprocal cross of BW female x PO male leads to growth retarded offspring. Through genotyping of several genes (a genome-wide scan), we have attempted to uncover the genetic linkage for the growth retardation phenotype seen in BW female x PO male hybrid offspring. Additionally, we will determine if there is a parent-of-origin effect that can be linked to this phenotype.

Methods

Crosses Used in Analysis

Many markers have been genotyped in offspring from the following crosses: BW female x F1 (BW female x PO male) male, F1 (BW female x PO male) female x PO male, F1 (BW female x PO male) female x F1 (BW female x PO) male, and BW female x F3 (hybrid) male. The F3 hybrid male was obtained by crossing BW female by PO male, then crossing F1 by F1, then F2 by F2 to obtain F3 animals.

DNA Isolation and PCR

DNA was isolated from tail tissue using the Qiagen DNeasy Blood and Tissue Kit. PCR was performed on microsatellites and genes using Bioline MyTaq Mix.

Microsatellites and genes with size polymorphisms between BW and PO were analyzed by acrylamide gel directly after PCR. Other genes analyzed were digested with an appropriate restriction enzyme after PCR products were confirmed on acrylamide gel. Digestion products were then analyzed by acrylamide gel electrophoresis.

Results

Results from BW female x F1 male cross offspring currently indicate linkage for *Sparc*, a gene on *Peromyscus* chromosome 8. All genes and microsatellites genotyped for the BW female x F1 male cross offspring are in Table B.1.

Additionally, results from F1 female x PO male cross offspring indicate X chromosome linkage, particularly to the gene *Mao*. Significance of linkage was determined by Chi-Squared analyses.

Results indicating linkage for *Sparc* were further confirmed using a BW female x F3 (hybrid) male cross. The F1 female x F1 male cross was used to determine if there is a pattern between 45 day weight and genotype for *Sparc*. The pattern was not apparent although more offspring need to be genotyped, and this may indicate a parent of origin effect.

Discussion

Sparc appears to be linked to growth retardation in the *Peromyscus* BW female x PO male hybrids. *Sparc* has been implicated in other organisms, such as *Drosophila*, for growth. *Sparc*, in *Drosophila*, has been identified as an early transcription marker that is upregulated in “outcompeted” suboptimal cells during development to protect these cells by inhibiting caspase activation [156].

Additionally, *Sparc* is required for *Drosophila* embryo and larval development while mutant *Sparc* is associated with growth retardation [157]. The lack of pattern between 45 day weight and *Sparc* genotype in F1 female x F1 male offspring indicates a possible parent-of-origin effect for growth retardation. This would not be surprising since the overgrowth in the reciprocal *Peromyscus* cross can be attributed to parent-of-origin effects. More genes for this cross, however, such as *Peg3* and X chromosome genes, must be genotyped in order to further confirm this hypothesis.

Table B.1: Genotyping Primers and Conditions for Hybrid Growth Genetics

Gene/MS	F Primer	R Primer	Temp	# Cycles	Enzyme
Cd24a	ATGCAAAATCAATCCATAAC	GAGAGCAGAATTACGTTGA	52	35	TaqI
Ednrb	ATGACGCCACCCACTAAGAC	GATGATGCCTAGCACGAACA	62	35	MspI
Frzb	CTATGAAGAyGAGGAACGTTCCAG	CACTTCTCAGCTATAGAGCCTTC	52	35	MnII
Grb10	CATGCCAAATGAGAGTAA	GCAGGCACACATACAG	57	35	Size Poly
Hba	CCCACCACCAAGACCTACTT	CGGTATTTGGAGGTCAGCAC	53	35	MspI
Hmox2	GAAACATTAAGAAGGAGCTA	CTCATTCTGCCCTACATAGT	52	35	BsaHI
Igfbp1	TTTTACCTGCCAAACTGCAAC	GGTAGACGCACCAGCAGAG	59	35	PvuII
Impact	CTGGTATGGAGGGATTCTGC	GCCCATATTTTACCAGTC	49	35	BssSI
Krt17	GGAAGCCGACATCAATGG	TTCTTCAGGTAGGCCAGCTC	52	35	Mbol
Lep	GACCTTAGCCCTGAATGCTG	TGCTTTGCTTCATATCCATCC	52	35	BsrBI
MS-103	TTTCCAGCTTCTGTCAAG	GCAGTTTCTGTTACCCAC	52	30	Size Poly
MS-121	GTCCAAAGTCATACTGG	GAAACGTAACCTTTCTGG	52	30	Size Poly
MS-260	GCAAGGGGAGAAGGCTAG	GAAACCTTCCCATACCAGAG	49	30	Size Poly
MS-397	CTGTGGGTCGTTCTGTCC	TCCAGCAATCAATAAATCTCC	52	30	Size Poly
MS-446	TATCGCTATCCAAATTCTC	CTGTCTCCCTCACTCCATC	49	30	Size Poly
MS-5033	TTAAGGCAGAAATTTGACAG	GTTTATAGTAACACAAGGGAGAA	52	30	Size Poly
MS-5062	TTGTCTCTGAACCTCATACT	GTTTTGGCTTTTCACACATTGTTAT	49	30	Size Poly
MS-5101	GTTAATTTCAGGTAGGAAAGTCTA	GTTCTCTGTCAATGGACTAC	49	30	Size Poly
MS-5107	GAAATTGTCTAACCAGAGA	GTTTCTTCTTTGTGACTTTGTA	49	30	Size Poly
MS-511	ATAAAGAGAGCACCTAGCAAC	AGTCTGAAAGGGGTTAATTC	52	35	Size Poly
MS-5110	GAGTGGCTAAGGAGGACAAT	GTTTAGCAATCAGACCATGTTAG	49	30	Size Poly
MS-5116	CAGTCGGGCGTCATCATTTGCTTCTCTGAAAATCT	GTTTTCCAGCATTCATAATATC	52	30	Size Poly
MS-5151	TCCTACTGTTTTTAAAGCTATC	GTTTGAACATTTGATGCCATAGT	52	30	Size Poly
MS-5163	GTTAGGAAATAGGTCAATAGT	GTTTATGAGATCCTTGGGTCA	52	30	Size Poly
MS-5185	GTTTCTCTTATGTTGCTATATCACT	TTAGGGACAGCAGAAATAT	49	30	Size Poly
MS-5221	GAGATGCCAGCAGATATG	GTTTCATTCTGTGAAACTCTTGTCT	49	30	Size Poly
MS-5254	CCACTTTGCTAAAACCTACA	GTTTGATGGGAGGTCTGAGTA	49	30	Size Poly
MS-5266	GAGGCAGAAAATGAAAGTCTT	GTTTAGACCATCCGAACAGTGTG	52	35	Size Poly
MS-5280	CAGTCGGGCGTCATCAGAACAGCACACCTTAGTC	GTTTCCATAACAGTCAGAGCTACAT	52	30	Size Poly
MS-5297	GTTTCAGAGAACCCAAATTTAGTC	TGAACACCAAGGAATCTCTTAC	52	30	Size Poly
MS-5300	GTTTGGGAATTCGGGCTATG	TCTCAGAGGCATCACTTTACAT	52	30	Size Poly
MS-5310	CAGTCGGGCGTCATCACAATGTAACAGACCCTGAAT	GTTTACACCATCTTATCTCCCTATGT	52	30	Size Poly
MS-5323	ACAGTATACTCAGCTTTTAAG	GTTTCTTACAGGTGATCGTTAA	52	30	Size Poly
MS-540	TAGGCTAACCGTGTCTG	AGGCCAGCTTGACTTAAAG	52	30	Size Poly
MS-5431	CAAACCCAGCTCTTGAAGT	TTTGGGGGCTGACAT	52	30	Size Poly
MS-5455	GTGAGGAATTGCATCTGT	GTTTAATTCAGCAAAGATGAT	52	30	Size Poly
MS-661	CCACCACCAATAACAACTAAC	GAACCTGACTCCACAAACTG	49	30	Size Poly
Peg10	TGGTCAGTAGACGCATGAGC	CGCATGAAGCGCCTATTAGT	59	35	MseI
Peg3	CTGGGTCAGCCATTCGATG	GCTCTGCAGCCTCCACTTC	54	30	MspI
Prdm9	CAAAGAACAAATGAGATCTGA	GTCTTYCTGTAATTGTTGAGATG	52	35	Size Poly
Sparc	CCCCAATGTTTAAATGTTTGG	TTTCGTGTGTCTGTACTTCCCT	54	35	HhaI
Spsb3	CAAGTGCTGCATAACAAG	ACCCACTGACATCATGTA	52	35	BsmAI
Ugp2	TAAGTCTGGCTTGCTGCACACCTT	TCCACCAGTCTCAGTTTGCCTTCA	54	35	AvaII
Xbp1	TGGCCTTGATGATTGAGAACCAGGA	ACAGCGTCAGAATCCATGGGAAGA	55	35	HindIII

Appendix C

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