BEHAVIORAL AND PHYSIOLOGICAL PHENOTYPE OF THE VIABLE YELLOW MOUSE (A^{VY}/a)

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by
DENISE WARZAK

Dr. R. Michael Roberts and Dr. Cheryl S. Rosenfeld, Thesis Supervisors

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

BEHAVIORAL AND PHYSIOLOGICAL PHENOTYPE OF THE VIABLE YELLOW MOUSE (A^{VY}/a)

Presented by Denise Warzak,

A candidate for the degree of Master of Science

And hereby certify that in their opinion it is worthy of acceptance.

_____

Professor R. Michael Roberts

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Professor Cheryl S. Rosenfeld

_____

Professor Jonathan Green

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LIST OF ABBREVIATIONS

A	*Agouti* gene
a/a	Non-agouti mice
A^{vy}/-; A^{vy}/*a*	Viable Yellow Mouse
ACTH	Adrenocorticotropic Hormone
AMP	Adenosine Mono-Phosphate
ASIP/AGRP	Agouti Signaling/Related Protein
ATP	Adenosine Triphosphate
BPA	Bisphenol A
cm	Centimeter
CpG	Cytosine-Guanine
CST	Central Standard Time
DNMT	Deoxyribose Nucleic Acid Methyltransferase
DOHaD	Developmental Origin of Health and Disease
EDC	Endocrine Disrupting Compound
EPM	Elevated Plus Maze
EtOH	Ethanol
Fig.	Figure
h	Hour
HAT	Histone Acetylatransferase
HDAC	Histone Deacetylase
IAP	Intracisternal A Particle
IGF	Insulin-like Growth Factor

I/R	Infrared
IRT	Inter-response Time
LTR	Long Terminal Repeat
LSD	Least Significant Difference
MCR	Melanocortin Receptor
min	Minute
miRNA	Micro Ribose Nucleic Acid
MSH	Melanocyte Stimulating Hormone
PS1A	Pseudoexon 1A
RMR	Reference Memory Ratio
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
s/sec	Second
SEM	Standard Error of the Mean
siRNA	Small Interfering Ribose Nucleic Acid
SUMO	Small Ubiquitin-like Modifier
vs.	Versus

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Denise Warzak

Dr. R. Michael Roberts and Dr. Cheryl S. Rosenfeld, Thesis Supervisors

ABSTRACT

Epigenetics is the study of how heritable phenotypes are transmitted without changes being made to the DNA sequence. One of the best characterized animal models for epigenetics is the viable yellow (A^{vy}) mouse. These animals exhibit a range of coat colors that reflect the degree of methylation of an intracisternal A particle (IAP) inserted in the *agouti* gene. Yellow coat color A^{vy} mice, with an under-methylated IAP, exhibit altered expression of the *agouti* gene, which leads to obesity and diabetes; in contrast, brown A^{vy} siblings, with a highly methylated IAP, and black (*a/a*) pelage siblings, lacking the IAP, remain healthy. Along with overt phenotypic differences, epigenetic modifications are also thought to change normal behaviors. Here, we examined whether behavioral and body weight differences exist between brown and yellow morph (A^{vy}/a) and black pelage (*a/a*) mice as they age. In another experiment, a diet high in methyl donors was fed to the A^{vy}/a mice to determine if it would have an effect on coat color, weight, and/or diabetic status. We demonstrated that there are body weight and behavioral differences between *non-agouti* and *agouti* mice. Yellow mice were more lethargic and less motivated to complete the mazes compared to darker furred mice. Brown and black mice outperformed their yellow counterparts in almost all mazes and weighed less by puberty. Caloric differences in the diets explain most phenotypic differences seen in the mice but not in the increase in serum glucose concentrations in mice on the methyl-donor diet.

CHAPTER 1

LITERATURE REVIEW

The viable yellow (A^{vy}/a) mouse model is one of the earliest and best known epigenetic mouse models available for studying various diseases (Rosenfeld, 2010). As it ages, this model is predisposed to hyperglycemia and obesity, and so has been used to examine how environment and diet might be contributing to the mounting obesity/Type II diabetes epidemic that plagues the US and is becoming a global concern (Wolff et al., 1986 and 1999; Weaver, 2009). The less methylated the intracisternal particle (IAP) region of the *agouti* gene (*A*) (Fig. 1) on these mice, the more yellow their coat color becomes, with increased tendency to become obese and diabetic. These unhealthy characteristics are most pronounced within the yellow coat color A^{vy}/a mice; while their browner siblings remain lean and less prone to diabetes (Duhl et al., 1994). The coat color of offspring can be shifted towards a greater percentage with brown pelages and healthy phenotype by feeding *a/a* (black, non-agouti) dams harboring A^{vy}/a offspring various methyl-supplemented diets. These diet-induced responses have generated a great deal of interest in this animal model (Cooney et al., 2002; Wolff et al., 1998). In particular, these findings may have implications for pregnant women, who may inadvertently be increasing or decreasing the risk for these adult-onset diseases in their offspring based on the diet they consume while they are pregnant and nursing (Lamers, 2011). In this section, I will delve into how these curious mice were originally discovered and their risk for various diseases, including obesity, diabetes, and cancer. As the coat color and accompanying disease spectra of these mice are impacted by epigenetic mechanisms, I shall consider the various epigenetic mechanisms that appear to be

operating on the *A* gene and how they cause variation in coat color. The potential for various maternal supplemented diets to alter their offspring's coat color and the corresponding risk for diseases will be considered. Finally, I shall then examine the general concept of developmental origin of health and disease (DOHaD) as it relates to humans.

Historical Background of the A^{vy}/- Mouse

The viable yellow (A^{vy}/-) mouse was originally reported over 50 years ago (Dickie, 1962). The mutation was identified in a C3H/HeJ female mouse, and to propagate the line, this female was bred for multiple parities to her brother, producing 10 of 18 offspring that were yellow and the remainder agouti or brown in color. Prior to this finding, there were only two yellow mutations that had been characterized: Cuénot's/lethal yellow and the recessive yellow (Dickie, 1962). The former is denoted as A^{y}/- and is embryonic lethal when the offspring are homozygous for the lethal yellow allele. When these conceptuses were implanted into the uterus, they underwent resorption by day four of gestation. As the heterozygous (A^{y}/a) animals aged, they developed obesity and diabetes. The genetic change in the lethal yellow mouse was shown to be due to mutations at two loci, such that the dual homozygous recessive (*a/a* and *e/e*) led to heterozygous yellow coat color mice (Dickie, 1962). Recessive yellow mice (*a/a* or *e/e*), on the other hand, become obese and diabetic with the proportion of the percentage of recessive offspring born for both genes matching expected Mendelian ratios (Dickie, 1962).

Viable yellow mice demonstrate altered *agouti* expression and coat color patterns, just as the lethal and recessive yellow mice do. The underpinning molecular changes in the A^{vy}/a mice differ dramatically from lethal and recessive yellow mice. After several back-crossings of the now so-called viable yellow mice into the C57BL/6J strain, researchers were able to conclude that the viable yellow mouse was able to breed successful homozygotes that looked phenotypically the same as their heterozygote counterparts (Dickie, 1962). The investigators concluded that A^{vy} is of the agouti series of mutations based on the absence of non-agouti offspring in the back-crossing experiments. In contrast to the lethal yellow mouse model, A^{vy} mice had a mottled pattern that varied from large patches of brown to only small, brown areas against the yellow fur (Dickie, 1962).

As the strain was propagated, there was mounting interest in the unusual phenotypes that developed in these animals. Dr. George Wolff has been credited with pioneering many of these early studies. In his 1965 initial paper, Wolff described the various mottled appearances of the A^{vy} mouse, but also noted that approximately 10% of mice possessed an "agouti" or brown coat color (Wolff, 1965). When bred with *a/a* (non-agouti) female mice, partial litters of mottled colored pups along with *a/a* siblings were generated and a greater percentage of yellow in the pelage positively correlated with increased risk for obesity (Wolff, 1965). To confirm this observation the mice were grouped into the following categories: "clear" (all yellow), "lightly spotted," "heavily spotted," and "agouti." Both the males and females of the "clear" and "lightly spotted" groups gained considerably more weight than their "agouti" counterparts. The increased weight gain in the "clear" and "lightly spotted" groups was due, in part to "a larger fat

and water content of the carcass and liver" (Wolff, 1965). Examples of the various coat colors in A^{vy} mice are illustrated in Figure 1. The obesity phenotype is discussed further below.

By the late 1970s, the genetics of the viable yellow mouse were becoming clearer. The *agouti* locus for *Mus musculus* was already known to be present on Chromosome 2 of *Mus musculus* (Wolff, 1978), a region that regulates the amount of eumelanin and/or phaeomelanin laid down in each hair follicle (Wolff, 1978). When brown coat color (agouti males) were bred with an *a/a* female, the resulting progeny unexpectedly were of variable coat colors, ranging from black (*a/a*) and A^{vy}/a (brown to yellow; Fig. 2). The former A^{vy}/a mice were termed "pseudoagouti." In order to study the frequency of the various *agouti* phenotypes described previously in the viable yellow mouse, Wolff backcrossed the C57BL/6J viable yellow male mice to three YS/chWf-*a/a* female sisters. When the dam was yellow in color, no pseudoagouti offspring would arise. In contrast, when *a/a* (black coat color) dams were instead paired with yellow sires, 11 to 12% of offspring had the pseudoagouti phenotype (Wolff, 1978). Based on these breeding experiments, the conclusion was that in order to obtain the full range of phenotypes from the A^{vy}/a mice, an *a/a* dam would need to be paired with a A^{vy}/a sire. Additionally, the above studies and those employing homozygous (A^{y}/A^{y}) lethal yellow mice, led to the conclusion that the *agouti* locus must be activated at an early stage of embryonic development, since the lethal yellow mouse was known to develop further when the mother was of an agouti phenotype rather than a yellow color (Wolff, 1978).

Molecular Biology of the *Agouti* Gene:

It was not until the early 1990s that the various *agouti* mutations were fully characterized. By this time several labs had cloned the *agouti* full length cDNA product that is expressed in a wide-range of species, including humans, and encodes for a small protein, agouti signaling protein (ASIP) that causes melanocytes in hair follicles to switch from deposition of black/brown (eumelanin) to yellow pigment (phaeomelanin) (Duhl et al., 1994). The ASIP of the mouse is 131 amino acids length protein, with its most interesting feature being its carboxy-terminus, where the 10 cysteine residues are spaced similarly to those found in the neurotoxins of some hunting spiders and cone snails (Miltenberger et al., 1997). Under normal transcriptional regulation, expression of ASIP is confined to the mid-phase of the hair cycle, and the protein product, ASIP, binds to the melanocortin-1 receptor, MC1R. This binding prevents alpha-MSH signaling leading to down-regulation of brown/black (eumelanin) granules and increased synthesis of yellow/red (phaeomelanin) pigments, as depicted in Figure 3 (Cone et al., 1996). Northern blot analysis revealed that the *agouti* transcript is also robustly detected in the brain, spleen, kidney, liver, lung, and skin of mottled viable yellow mice; whereas it is weakly detected by using RT-PCR analysis in the brain, spleen, kidney, liver, and lung in the pseudoagouti mice (Duhl et al., 1994). As detailed below, there are additional MCR in the above organs, and the differential and systemic expression of the A^{vy} gene product may account for the greater incidence of metabolic diseases in yellow coat color compared to brown coat color A^{vy} offspring.

In the A^{vy} mouse, a pseudo-exon upstream of the 5' ATG codon contains an intracisternal particle long terminal repeat (IAP LTR) that is responsible for the various

coat color patterns and accompanying risk for the above diseases. The mutation in the agouti gene in A^{vy} mice, originated from a duplication and then inversion of exon 2 leading to pseudoexon 1a (Wolff, 1996). After this gene duplication event, a retrotransposon, termed an intracisternal A particle (IAP), possessing its own cryptic promoter site became embedded in pseudoexon 1a (Fig. 4). The range of colors in the A^{vy}/a mice stems from differences in transcription from this cryptic promoter, which is not under tissue specific control, relative to the normal promoter, which is under strict tissue specific control (Cooney et al., 2002; Morgan et al., 1999; Rosenfeld, 2010; Waterland and Jirtle, 2003; Wolff et al., 1998).

Effects of ASIP in A^{vy}/a Mice on other MCR and Disease Association:

Melanocortin receptors (MCR) are expressed in other cells and tissues besides hair follicle cells. As mentioned previously, MC1R is the one associated with determining the coat color of animals, and ASIP binding to this receptor governs the change in coat color in A^{vy} mice (Abdel-Malek, 2001). Melanocortin-2 receptor (MC2R) is specific to adrenocorticotropic hormone (ACTH) and expressed in the adrenal cortex where hormone binding to the receptor leads to an increase in cortisol production (Abdel-Malek, 2001; Yang, 2011). There is a paucity of information about the roles of MC3R and MC5R in mediating normal physiological processes. In mice that lack MC3R, it has been postulated that this MCR controls energy homeostasis based on the observed obesity in the mutant mice (Yang, 2011). In mice that lack MC5R, it appeared to decrease activity of exocrine glands, particularly sebaceous glands in the skin (Yang, 2011). The

MC4R can be found in the hypothalamus and other regions of the brain and may be the receptor responsible for "genetic obesity" (Abdel-Malek, 2001).

There are two proposed models as to why ASIP binding to the MC4R increases the risk for disease, including obesity within the mottled to yellow coat color A^{vy}/a mice. The first is that the ectopic expression of ASIP may allow its binding to other MCR, such as MC4R, in addition to the MC1R found in the hair follicle. In this manner, ASIP might antagonize the binding of alpha-melanocyte stimulating hormone (α-MSH) to its cognate receptor in a range of tissues not normally influenced by ASIP. Another potential mechanism is that ASIP binds to its own, as yet, unidentified receptor and activates a pathway that interferes with the action of α-MSH on its own receptor, MC4R, in the brain and elsewhere (Duhl et al., 1994). However, there has been little evidence for an ASIP receptor, to support this latter hypothesis. Most of the current models therefore are based on the former hypothesis.

The expression of the *agouti* gene in A^{vy} mice directly led to the observed disease phenotypes (Klebig et al., 1995). For example, when introduced as a transgene into wild type strains of mice, some of the transgenic mice expressed the gene in diverse organ systems and the level of expression was directly related to the level of obesity and severity of hyperglycemia and insulemia (Klebig et al., 1995). However, this study conflicts with past studies in A^{vy} mice, where no relationship between the total percentage of yellow pelage and obesity has been identified (Yen et al., 1994). The researchers in the former study concluded that the results may be partially due to decreased lipolytic rates in the adipocytes in the transgenic mice that, for unclear reasons, are not observed in A^{vy} mice. The authors also attributed the obesity to ASIP binding in antagonistic manner to

MC4R in the hypothalamus that may have led to hyperphagia in the animals, but this notion remains to be rigorously tested (Klebig et al., 1995).

Diseases Described in the A^{vy}/- Mouse Model

Obesity and Diabetes:

As viable yellow mice age, the more yellow coat colored mice have a higher propensity for weight gain and diabetes than their darker coat colored counterparts. This statement is especially true for male mice as they exhibit a greater lipogenesis rate, i.e. accumulate greater fat deposits, and triacylglycerol content that increases with maturity (Wolff, 1965). This finding is more characteristic of Type-II diabetes, the most prevalent form of diabetes in (Yen et al., 1976), arguably making A^{vy}/a mice a useful animal model for this form of human diabetes.

A range of treatments has been employed in order to alleviate the diabetes and obesity observed in the viable yellow mice. This section presents a sampling to provide some representative idea of said designed treatments. Caloric restriction at 70% of the *ad libitum* diet from weaning onwards was observed to prevent extreme obesity in female viable yellow mice of the C3H background (Wolff et al., 1999). Interestingly, only the brain of the calorie restricted viable yellow mice was significantly heavier than those of their congenic *A/a* counterparts (Wolff et al., 1999). This result was consistent with their hypothesis that the *agouti* gene affects somatic growth in some manner, possibly by increasing circulating concentrations of insulin-like growth factor- I (IGF-1) levels (Wolff et al., 1999).

A drug that has anti-diabetic effects on viable yellow mice is MTP-1307 (Yen, 1987). MTP-1307 works by promoting glucose utilization in adipocytes, inhibiting gluconeogenesis in the liver, and increasing insulin secretion from the pancreas. Animals treated with a low dose (25 mg/kg) of this compound demonstrated improved glucose intolerance, i.e. they were less likely to become hyperglycemic, compared to controls and those animals treated with a high dose (300 mg/kg) of the drug (Yen, 1987). The objective is thus to treat yellow coat color A^{vy} mice with postprandial glucose intolerance without causing hypoglycemia. The drug ephedrine, which has now been restricted due to abuse and causing potential heart attacks, has been another weight loss drug that had been tested on viable yellow mice prior to being sold to the public (Yen et al., 1981). The underpinning mechanism of action for this latter treatment was presumed to increase thermogenesis in brown adipose tissue of mice.

Antiobesity compounds, such as fluoxetine and/or other appetite suppressants, may also be potential treatments to reduce body weight in adult obese A^{vy} mice (Yen et al., 1994). This finding, along with the decreased lipolysis rate in transgenic agouti mice, suggests that there might be a defect in the signaling mechanism for generating and/or maintaining intracellular cyclic AMP, especially in adipocytes (Yen et al., 1994). Hyperinsulinemia and insulin resistance also form as the mice age in non-treated mice. It is not currently known how much or if the diabetes and obesity affect each other due to the fact that β-cell hyperplasia is already evident in viable yellow mice before the onset of obesity (Miltenberger et al., 1997). Estrogen concentrations present in females might protect against the onset of diabetes (Yen et al., 1994). Parabiosis of yellow obese mice, such as the lethal yellow, with normoinsulemic mice fails to induce obesity in the non-

obese recipient. These studies indicate that the obese phenotype is not attributed to circulating steroid hormones or other potential obesogens, compounds that disrupt normal lipid metabolism (Wolff, 1965). Adrenalectomies and hypophysectomies also fail to completely remove the propensity of A^{vy} mice to gain weight and become hyperinsulinemia with age (Miltenberger et al., 1997).

Cancer:

Along with obesity and diabetes, the lethal and viable yellow mouse mutants in certain backgrounds exhibit greater propensity to develop various cancers (Roberts et al., 1984). Heterozygous A^{vy} mice in several genetic backgrounds develop mammary tumors, hepatomas, lung adenomas, and spontaneous cholangiomas in response to chemical or viral treatment that is not observed in their non-agouti counterparts (Roberts et al., 1984). Because the agouti locus appears to have some control over the enzymes and signaling pathways that underpin coat color, it was hypothesized that it might also regulate other obesogens, but these remained to be elucidated (Roberts et al., 1984).

The susceptibility to cancer in A^{vy} mice was also tested in skin-derived fibroblasts (Furst et al., 1991). The growth rate of untreated fibroblasts derived from A^{vy} mice was compared against their black (*a/a*) litter mates and it was found that the colonies from fibroblasts derived from A^{v} mice exhibited greater proliferation rates than those from *a/a* mice.

Epigenetics:

Epigenetics is defined as a mitotically or meiotically heritable change in gene expression that occurs without a concomitant change in the actual DNA sequence.

(Morgan and Whitelaw, 2008; Rosenfeld, 2010). Well known and useful animal models and human studies for understanding epigenetics include the *Axin 1* fused (aka "kinky tail") mice, viable yellow mice (A^{vy}), and monozygotic or identical human twins. Select means by which an epigenetic alteration can occur are attributed to the addition or removal of specific molecular markers such as methyl groups that lead to gene silencing or activation, respectively (Morgan and Whitelaw, 2008). The nucleic acids that comprise the DNA or the surrounding histone proteins can be subject to these chemical modifications. When the epigenetic "marks" involve the germline, transgenerational propagation of the altered gene expression state can occur, such that the epigenetic change effects not just one generation but the descendants of the original individual. Examples of well characterized epigenetic changes are discussed below.

Examples of Epigenetic Changes:

DNA methylation: DNA methylation is an epigenetic change where a methyl group is placed upon a cytosine in CpG islands, particularly within the promoter site of various genes, to silence transcription (Waterland et al., 2003). The DNA methyltransferases (DNMTs) mediate this addition. During early embryogenesis and meiosis of germ cells, the methyl marks are removed by putative demethylase enzymes and then replaced via *de novo* methylation during development of the blastocyst (Wu et al., 2010). The addition of the methyl groups to the DNA requires S-adenosylmethionine (SAM) and a co-factor, zinc. Synthesis of SAM is dependent upon "dietary folates, vitamin B_{12}, methionine, choline, and betaine" (Wolff et al., 1998). The end result of this synthesis, S-adenosylhomocysteine (SAH), can in turn suppress DNMT activity leading to select regions of the DNA remaining in an unmethylated state (Fig. 5), which might be

one means to prevent aberrant DNA methylation (Cooney et al., 2002). DNMT1 is expressed in the oocyte and early zygote stages, and during mitosis, this enzyme ensures that the daughter cells are methylated in the same regions as the parental cell. In contrast, DNMT3A and DNMT3B enzymes are expressed at the blastocyst stage, and in the presence of SAM, novel or *de novo* methylation occurs with methylation occurring within CpG islands of the daughters cells that were not evident in the parental cell (Fig. 6; Wu et al., 2010).

Histone modifications: Histones are the primary proteins that the DNA helix surrounds, and the combination of DNA and the surrounding histone proteins is termed a nucleosome (Fig. 7). Histone proteins, including H2A, H2B, H3, and H4, are also subject to chemical modifications. Acetylation of the N-terminal region lysine residues within the histone proteins leads to gene activation, which might be due to neutralization of the positive charge that otherwise exists, although there have been some reports disputing this hypothesis (Wu et al., 2010). In contrast, deacetylation removes the acetyl groups from the lysine residues such that the histone proteins resume complete binding to the DNA and obstructing transcriptional factor access to the promoters. Enzymes that can acetylate and deacetylate the lysine residues are histone acetylatransferases (HATs) and histone deacetylases (HDACs), respectively (Wu et al., 2010). Methylation of the lysine and arginine residues in the various histone proteins can lead to gene activation or silencing depending on which amino acids and histone proteins are modified (Wu et al., 2010). Other modifications, such as phosphorylation, SUMOylation, and ubiquitination, can also alter gene expression, but the net effect of these changes can be variable. Histone protein alterations can also alter how DNA is repaired and how the chromosomes

condense during mitosis (Wu et al., 2010). Variable histone protein patterns have been documented in the 5' long terminal repeat of the viable yellow (A^{vy}) allele, suggesting that this epigenetic change may also contribute to the "yellow obese syndrome" observed in these mice (Dolinoy et al., 2010).

microRNA (miRNA): MicroRNA (miRNA) are small RNA molecules that bind to mRNA and in general lead to their destruction by the DICER enzyme (an endoribonuclease), although there has been some recent suggestion that select miRNA may activate gene transcription by uncertain mechanisms (Chuang et al., 2007). There are sub-categories of miRNA, one being small interfering RNA (siRNA) which is the predominant miRNA that can modulate DNMT and histone methylases. It has also been shown that many of these siRNA are involved in up-regulating tumor suppressor mRNA that silence oncogenes, that may have been previously down-regulated due to aberrant methylation, though the exact mechanisms are uncertain. Anti-tumorigenic drugs may be designed to mimic the action of these siRNA that effectively lead to increase tumor suppressor gene activation and thereby decrease the spread of diverse cancers, such as breast, liver, lung, and colon (Chuang et al., 2007).

Chromatin arrangement: Chromatin is tightly packed DNA and proteins that make up the nucleus of a cell (Pray, 2008). The DNA weaves around histone proteins to pack it into the tight confines of the cell's nucleus, with non-histone proteins assisting in this condensation. Chromatin is generally considered in either a silenced (heterochromatin) or active (euchromatin) state within a cell (Pray, 2008). If a gene is located along heterochromatin, it is most likely in a silenced state with the opposite being true for a gene on euchromatin. As mentioned previously, the type of histones and DNA marks

may alter the chromatin state. A chromosome has the two main parts: the centromere, which is the region where the chromosome divides from to form two sister chromatids during mitosis, and the telomeres, which are at the ends of the chromosome and are responsible for its stability (Pray, 2008; Fig. 8). The likelihood that select genes are transcribed can depend on their location within the chromosome. However, if a gene is translocated from a euchromatic to a heterochromatic region, otherwise termed position effect variegation, the potential for gene activation is decreased, as shown in Figure 8 (Pray, 2008).

Epigenetics and the A^{vy}/a Mouse Model

As the field of epigenetics has gained currency in explaining various disease aspects, it is now accepted that the varying degrees of mottled coat color patterns in viable yellow mice is attributed to epigenetic inheritance (Wolff et al., 1998). In these animals, the greater number of methyl "marks" placed on the proximal IAP LTR of the viable yellow's agouti gene, the more likely it is that the mouse will become lean and develop a more agouti-type coat color. The fewer the number of CpG sites methylated in the IAP cryptic promoter site, the more likely the mouse will have this gene constitutively and ubiquitously "turned on," and correspondingly these offspring will possess a yellow coat color and become obese in time. Moreover, these epigenetic marks are transmitted from the mother to the offspring without changing the actual genotype of her progeny (Wolff et al., 1998). There is also transgenerational inheritance of the methylation status through the maternal but presumably not the paternal germline, as evidenced by the fact that mothers and grandmothers that possess a yellow coat color are

more likely to give rise to greater percentage of yellow coat color offspring and grand-offspring, respectively (Wolf et al., 1998).

Since Wolff and his colleagues suspected that epigenetics underpinned the various coat color patterns in these mice, they sought to determine if they could change the outcome of the pup's coat color by feeding *a/a* (non-agouti, black coat color) dams two weeks prior to breeding and throughout gestation a methyl-supplemented diet (Wolf et al., 1998). The females were then bred to A^{vy}/a males, which resulted in them harboring A^{vy}/a and *a/a* offspring. Notably, this pioneering study revealed that dams provisioned with a diet high in methyl donors and cofactors birthed pups that had a higher ratio of pseudoagouti and darker mottled pups compared to control dams.

As a follow-up to the aforementioned study, female C57blJ6 (*a/a*) mice were placed on diets with varying levels of methyl supplementation and bred to viable yellow males to compare the DNA methylation changes in offspring derived from dams that had been on the higher to lower end of methyl supplementation (Cooney et al., 2002). This research group also adapted the "Y coat color" scoring system with 'Y_0' standing for pseudoagouti mice, 'Y_{1-2}' for heavily mottled mice, 'Y_3' for mottled mice, 'Y_4' for slightly mottled mice, and 'Y_5' for all yellow mice. By this time, it was also realized that in order to obtain the complete "Y color spectrum", an *a/a* mouse had to be bred with A^{vy}/a mice and not with A^{vy}/A^{vy} mice, as the latter produced greater number of yellow coat color offspring (Cooney et al., 2002). In order to determine the methylation status of viable yellow pups, liver and/or kidney samples were collected from pups as soon as their coat colors emerged (approximately 7 days after birth) and DNA was PCR amplified. A restriction digest with methyl specific enzyme (HaeII) was then performed on the

amplified bands to determine methylation patterns for each mouse. The researchers were able to determine that along with coat color, the dams that had been fed the higher methyl supplemented diets produced more pups with greater number of methylated regions in the IAP site within the A^{vy} locus. This study was essentially confirmed by a second independent research group (Waterland et al., 2003), who instead employed bisulfite sequencing to screen the methylation status of the various CpG islands within the IAP cryptic promoter site embedded in the A^{vy} gene.

Developmental Origin of Health and Disease (DOHaD)

The notion of developmental origin of health and disease (DOHaD) first emerged in the late 1980s, after Dr. David Barker published a trio of articles describing the correlation found between low birth weight and death due to coronary disease in people living in England (Barker et al, 1986, 1989, and 1993). This led to an interest in what is also now known as the "Barker hypothesis", whereby fetuses that are exposed to poor *in utero* conditions will develop into unhealthy adults or vice versa, the *in utero* environment might confer protection against the progeny succumbing to later adult-onset diseases (Wadhwa, 2009). Epigenetics might be a primary means by which environmental cues can lead to developmental changes that alter the risk for disease. For instance, in the viable yellow mouse model, dams carrying viable yellow pups that are provisioned with methyl supplements are more likely to birth brown as opposed to yellow coat color offspring (Cooney et al., 2002; Waterland et al., 2007; Wolff et al., 1998). One of the first retrospective and becoming increasingly characterized cases of DOHaD in humans is the Dutch Hunger Winter Period (1944-1945). During this time, malnourished

pregnant women gave birth to underweight children who were at greater risk for obesity, hyperlipidemia (abnormally high amounts of lipid in the blood), and coronary heart disease later in life (Weaver, 2009). Those children born to famished mothers also demonstrated epigenetic changes, including hypomethylation of insulin growth factor- 2 (IGF-2), which is an essential gene for regulating growth and metabolism (Weaver, 2009). Therefore, the presumption is that the up-regulation of IGF2 expression might have led to the observed later metabolic disturbances in these offspring. It is likely that the *in utero* environment might lead to an imprint on other candidate genes and subsequently adult diseases. Once we understand how the pre- and post-natal environment affects later disease risk physicians can better advise pregnant women and treatments can be designed to alleviate these diseases in at-risk children.

It is becoming increasing clear that animals and humans are the product of both their genes and their environment with epigenetics being the primary means by which environmental factors can impact gene expression. However, several questions remain, including whether enviromental factors , such as diet, later in life can reverse harmful epigenetic marks and thereby alleviate the risk for associated diseases. Additionally, we are just beginning to understand how epigenetics can impact various complex responses, such as cognition and learning and memory. The rest of the thesis will summarize my graduate studies that have attempted to address these key issues.

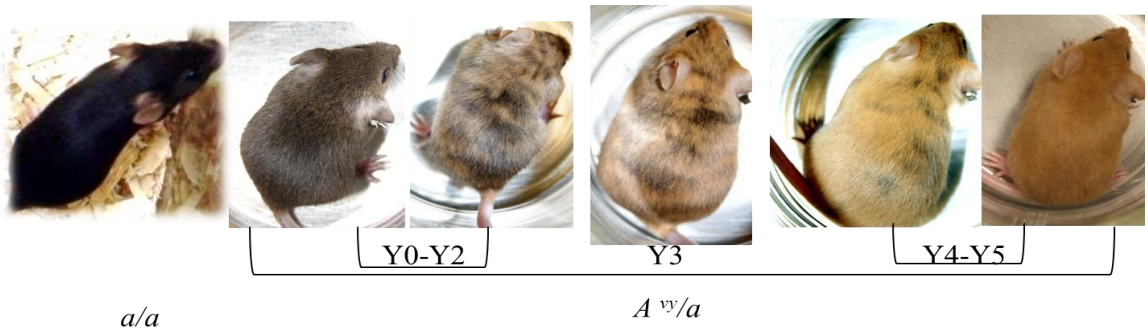


Figure 1: Y Coat Color Scoring System- Mice were analyzed based on coat color with *a/a* (black), Y_0-Y_2 (brown), and Y_4-Y_5 (yellow) being the three groups. (Photos taken by Paizlee Sieli)

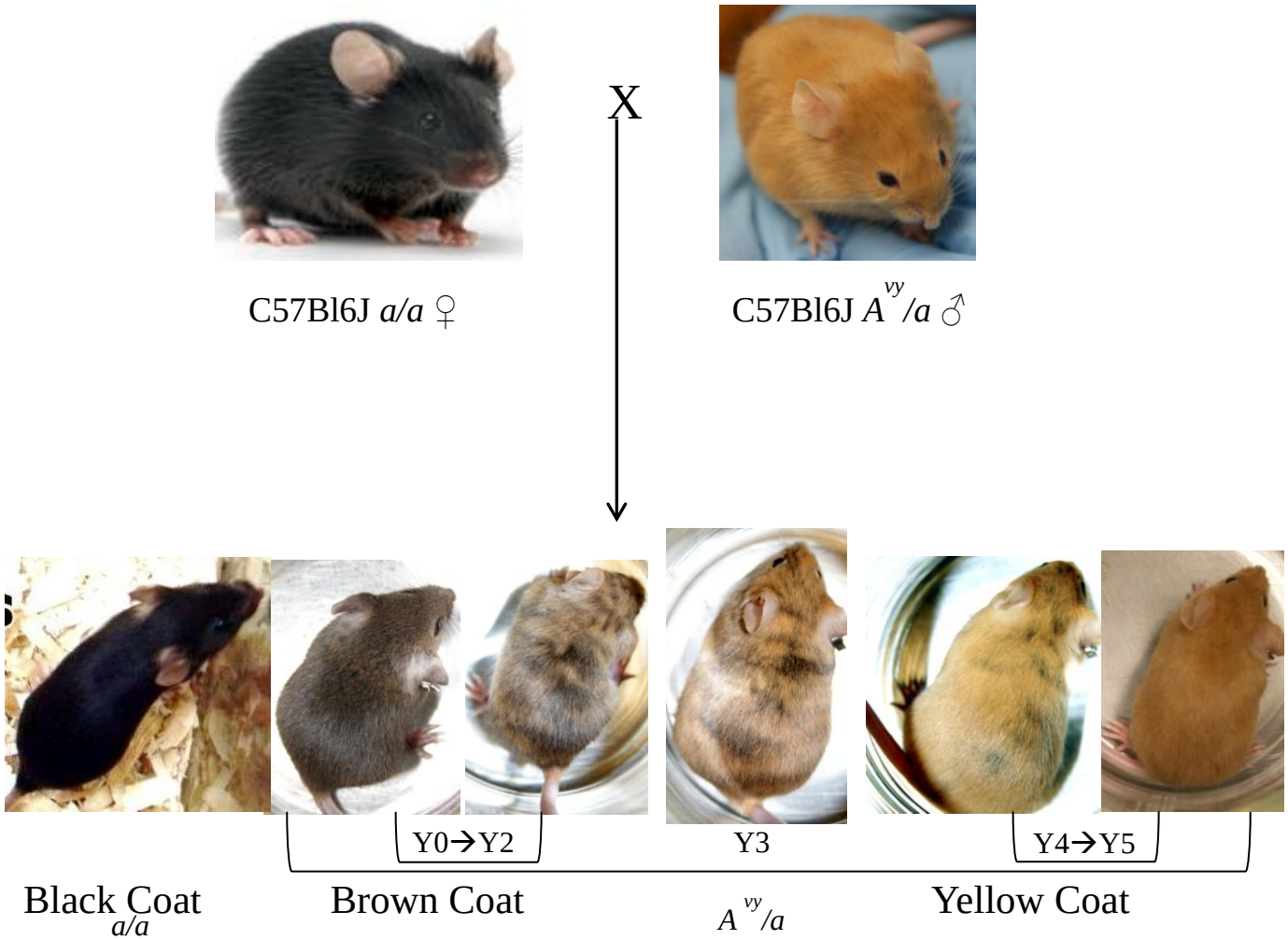


Figure 2: Viable Yellow mating scheme with possible outcomes. When a *a/a* female is mated with a A^{vy}/a male, approximately ½ of the offspring are *a/a* with the other half being a variation of A^{vy}/a.

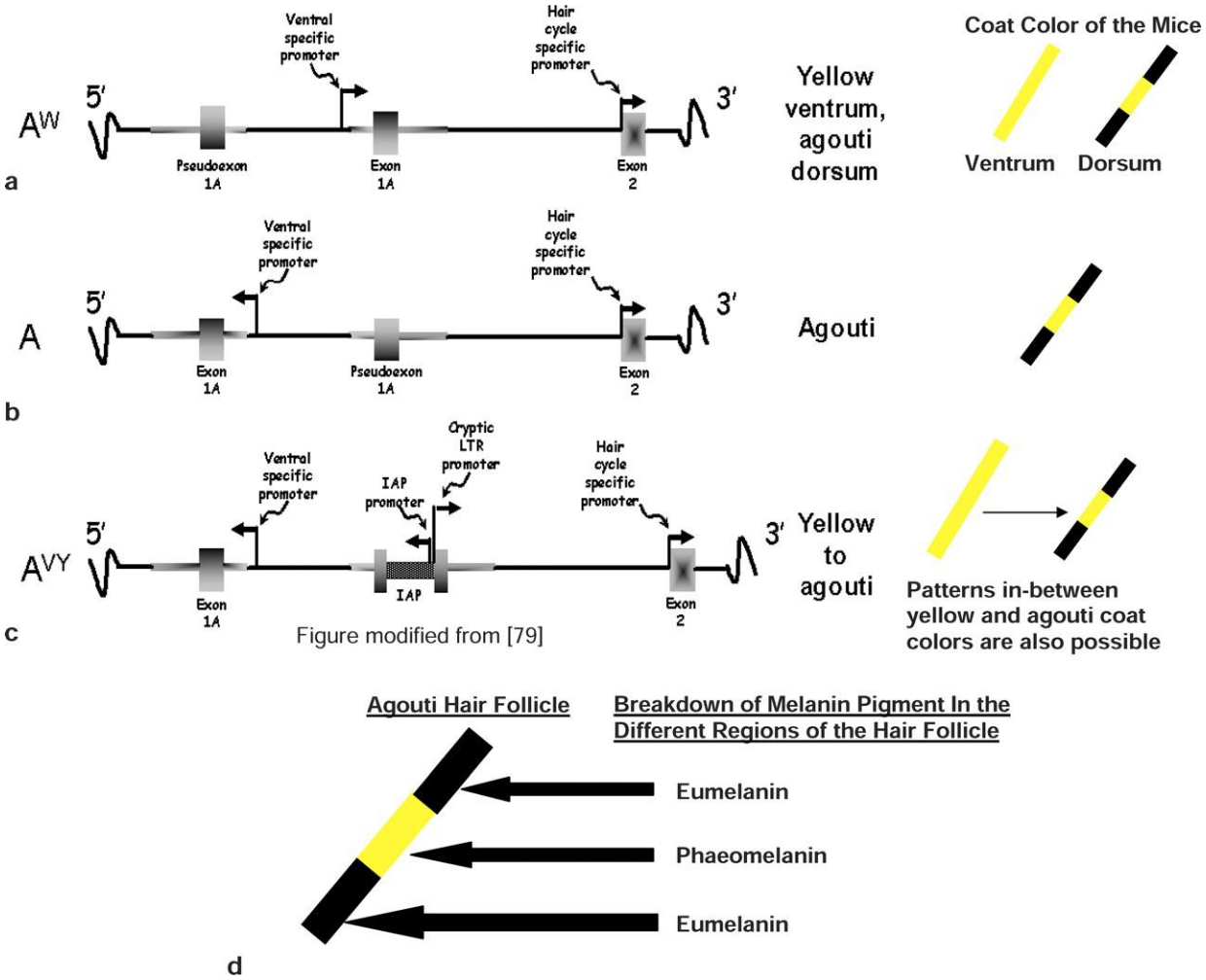


Figure 3: Examples of dominant agouti (*A*) allele forms. a) AW. Correct orientation of the ventral specific promoter results in completely yellow coat color region in the ventrum. In the dorsum region, expression originates from the hair cycle-specific promoter, which results in agouti (brown) patterned hair in the dorsum. b) A. In this allelic form, there is an inversion of the region containing the ventral-specific promoter (exon 1A) and pseudoexon 1A that results in a loss of ventral agouti expression except for that arising from the hair cycle-specific promoter. As a result, the agouti gene is confined in its expression during the hair cycle growth to the mid phase of the hair cycle, with a resulting brown coat color in both the ventrum and dorsum regions. c) A^{vy}. In this allelic form, a retrotransposon, or IAP, has inserted into pseudoexon 1A of the *A* allele. This naturally occurring viral insertion results in agouti expression being driven from a cryptic promoter in the LTR of the IAP. Although endogenous transcription factors cannot suppress the viral promoter within the IAP site, its expression is governed by methylation of cytosines within this sequence. When the LTR is fully demethylated, ubiquitous agouti expression in all organs occurs and yellow obese syndrome result. However, if this promoter site is fully or partially methylated, the mice have brown to in-between coat color patterns, respectively. These latter forms will remain healthy compared with those displaying a yellow coat color. d) The hair follicle and melanin pigment of an agouti coat color hair follicle is displayed here to demonstrate the differences in melanin pigment that are laid down during the hair follicle cycle. The hair follicles of yellow coat color mice contain exclusively pheomelanin. Black coat color mice (*a/a*), in contrast, only contain eumelanin throughout the hair follicle. Modified from Figure 1 in Chen et al., with permission from the Genetics Society of America. (From Rosenfeld, 2010)

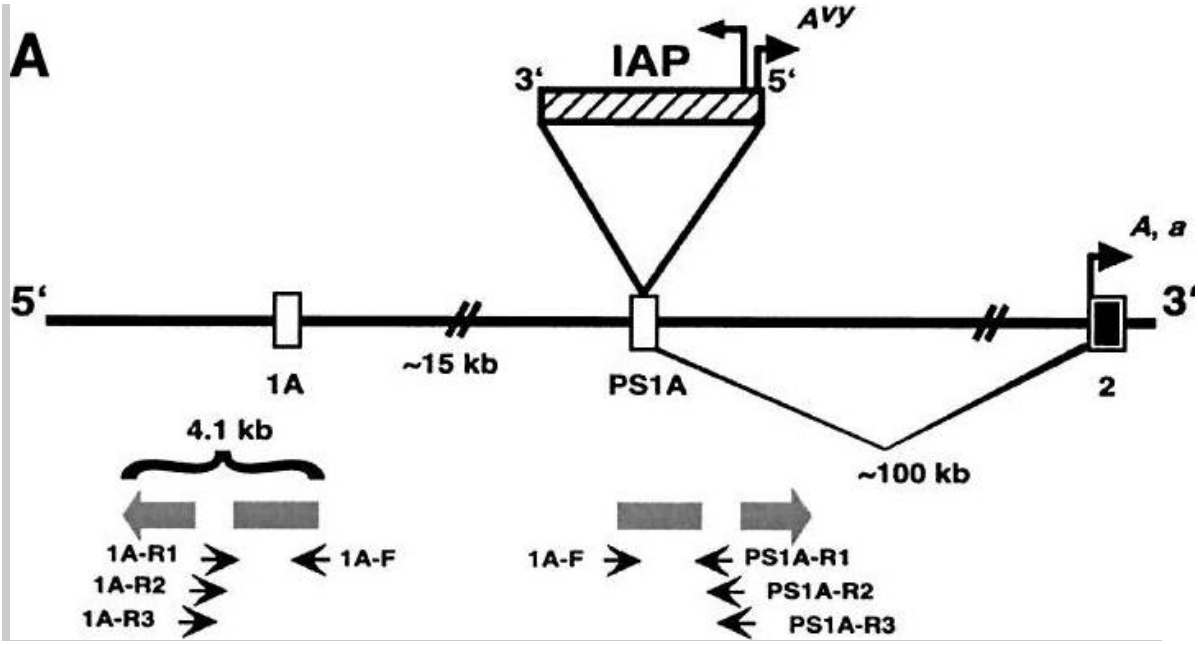


Figure 4: IAP insertion site in A^{vy} allele. (A) Exon 1A of the murine agouti gene lies within an interrupted 4.1-kb inverted duplication (shaded block arrows). The duplication gave rise to pseudoexon 1A (PS1A). On the A allele, PS1A is located ≈100 kb upstream of exon 2 and ≈15 kb downstream of the contraoriented exon 1A (6). The A^{vy} mutation was caused by a contraoriented IAP insertion (striped bar; tall arrowhead shows direction of IAP transcription). A cryptic promoter within the long terminal repeat proximal to the agouti gene (short arrowhead labeled A^{vy}) drives ectopic agouti expression in A^{vy} animals. In *A* and *a* animals, transcription starts from a hair cycle-specific promoter before exon 2 (short arrowhead labeled A, a). Small arrows show the positions of PCR primers used to selectively amplify the exon 1A and PS1A regions. (From Waterland et al., 2003)

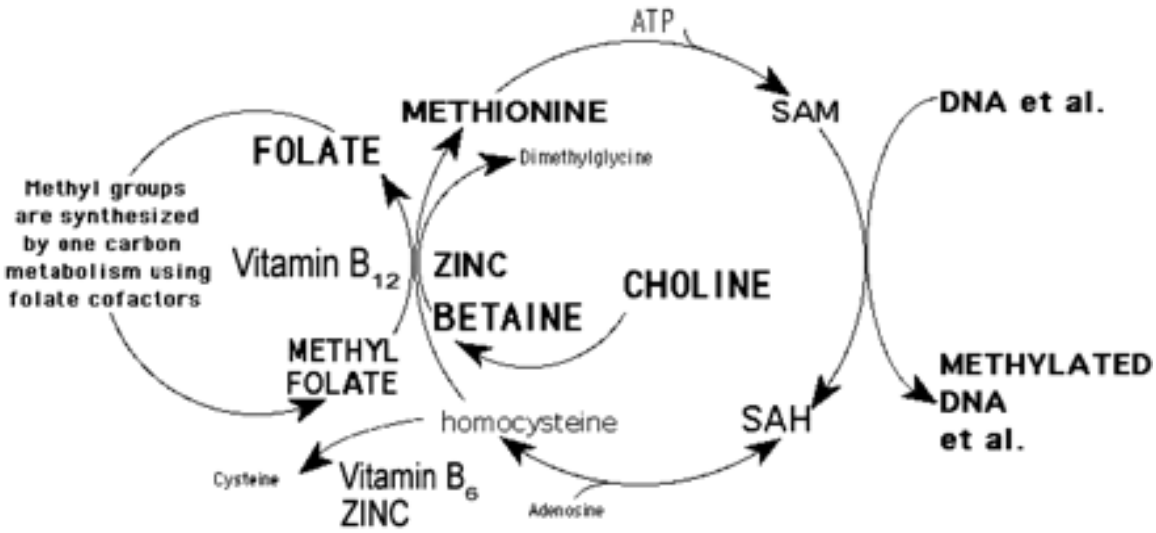


Figure 5: Methyl metabolism showing some major metabolic intermediates, cofactors and dietary sources of methyl groups. ATP, adenosine triphosphate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine. (Taken from Cooney et al, 2002).

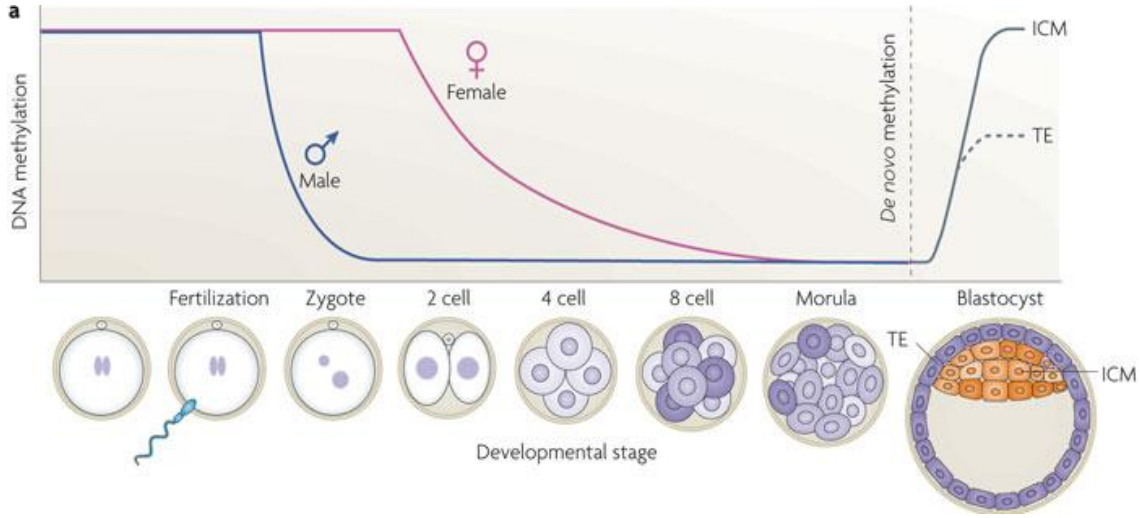


Figure 6: Active demethylation in the zygotic paternal genome. Shortly after a sperm fertilizes an egg, the paternal genome rapidly undergoes genome-wide active DNA demethylation and remains demethylated following multiple rounds of cell division. During this time, the maternal genome experiences gradual, passive demethylation. *De novo* methylation patterns are established by the DNA methyltransferases DNMT3A and DNMT3B during the development of the blastocyst. (From Wu et al., 2010)

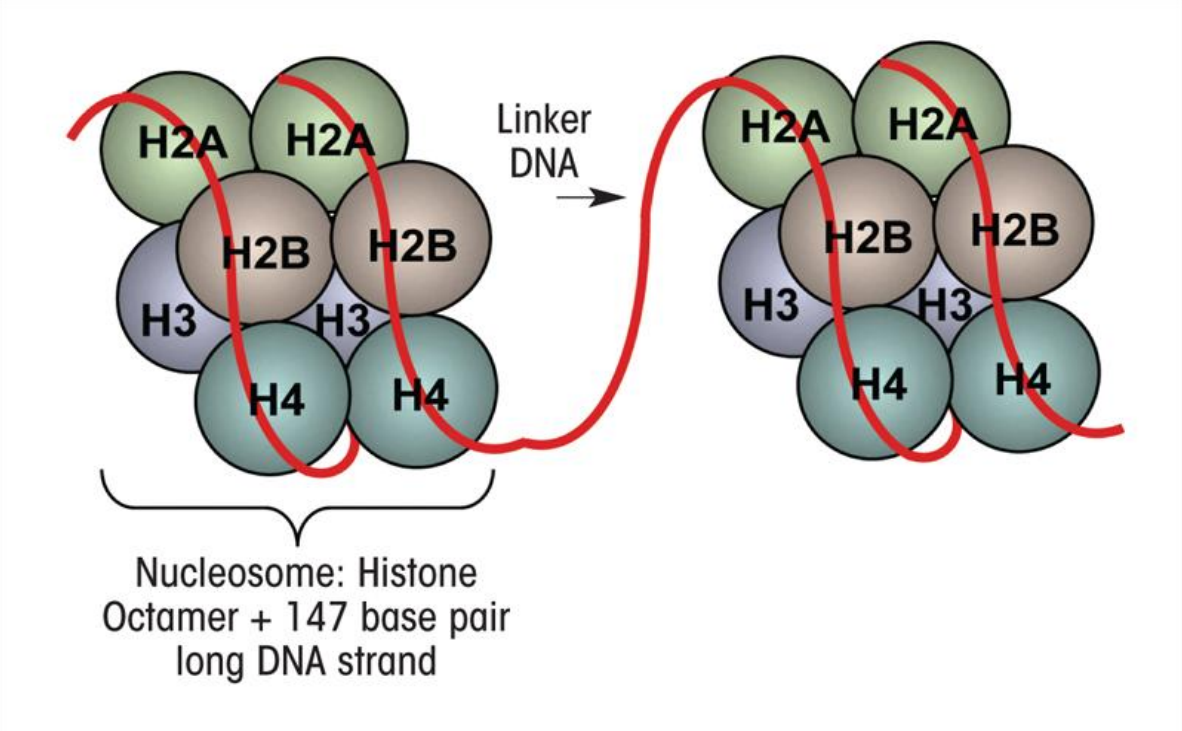


Figure7: The four types of histones (H2A, H2B, H3, H4) with DNA wrapped around them makes up one nucleosome. Various types of molecular "marks" such as methyl groups can be added or removed from these in order to activate or silence a gene. (From Starkman et al., 2012)

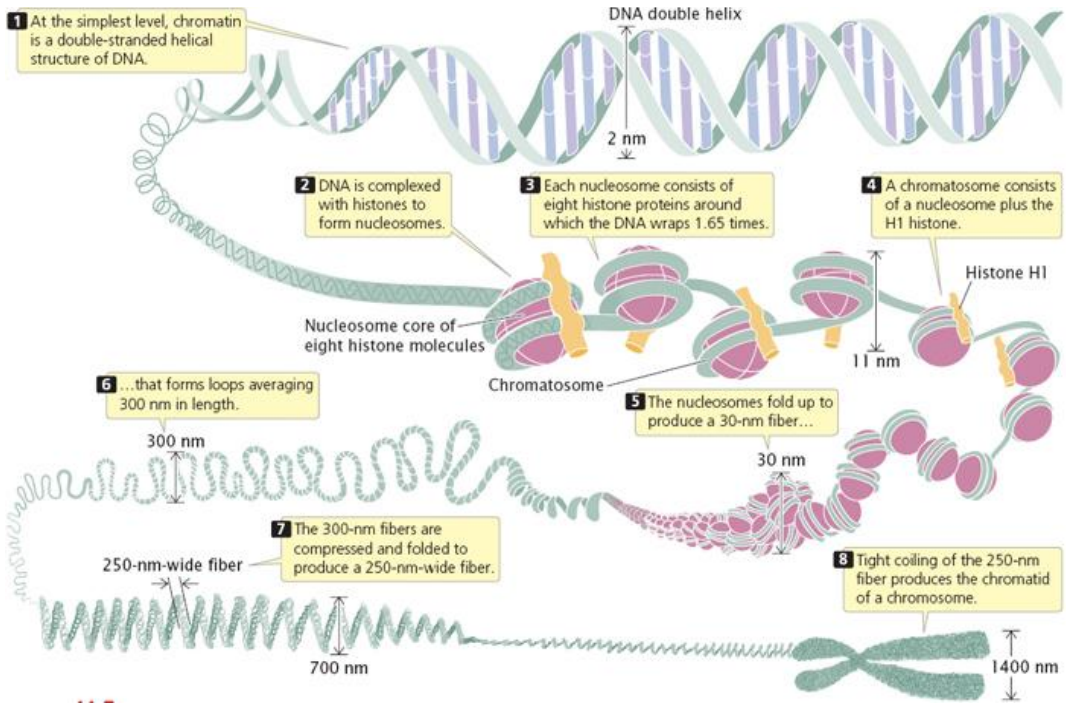


Figure 8: Chromosomal arrangement (from Pray, 2008)

CHAPTER 2

BEHAVIORAL PHENOTYPE OF THE VIABLE YELLOW MOUSE

Abstract

Epigenetics may help clarify some behavioral disorders that are not readily explained by classical genetics. The best characterized animal model in which an epigenetic change in a gene is inherited and leads to well defined pathological outcomes is the viable yellow (A^{vy}) mouse, which possesses coat colors ranging from fully yellow to mottled to completely brown (pseudoagouti) depending on degree of methylation of an intracisternal A particle (IAP) inserted in the promoter region of the *agouti* gene. Yellow A^{vy} mice with a demethylated IAP develop "yellow obese syndrome", typified by obesity and diabetes; whereas, brown coat color A^{vy}, siblings, with a more extensively methylated IAP, and black (*a/a*) pelage siblings, lacking the IAP insertion, remain healthy. Here, we examined whether behavioral differences exist between such mice as they age. We compared the body weight and various behavioral patterns in brown and yellow morph (A^{vy}/a) and black coat color (*a/a*) mice at 6, 10, 15, 20, and 24 weeks of age. By 24 weeks of age, yellow mice weighed more than their brown and black coat color siblings and demonstrated marked behavioral alterations, including decreased ambulatory time, decreased distance traveled but more time at rest and engaged in stereotypic behaviors, such as grooming. However, brown coat color mice exhibited delayed ability to locate concealed food and impaired reference memory ratio compared to yellow and black coat color siblings. These findings suggest that an epigenetic change within a select gene can result in striking weight differences and behavioral alterations.

Introduction

Studies on epigenetics are beginning to influence our interpretation of how animals behave and develop (Bredy et al., 2010; Crews, 2010; Curley and Mashoodh, 2010; Meaney, 2010; Miller, 2010; Svrakic and Cloninger, 2010; Weaver et al., 2004; Zhang and Meaney, 2010). In the past, most differences in behavior were ascribed to polymorphism in either select genes or to physiochemical differences of unknown etiology (Bray, 2008; Hamer, 2002; Inoue and Lupski, 2003; Jabbi et al., 2007; Kreek and LaForge, 2007; Lee, 2007; Mancama et al., 2003; Mantione et al., 2010; Proudnikov et al., 2008; Zhou et al., 2008; Zill et al., 2002). Behavioral epigenetics provides a means of understanding how environmental factors, including maternal care and diet, alter heritable changes in gene expression without changing DNA sequence and hence the origin of behavioral alterations and increased susceptibility to diseases that are not readily explained by conventional genetic mechanisms (Meaney, 2010; Weaver et al., 2004; Zhang and Meaney, 2010). In humans, genetically identical (monozygotic) twins provide a means to study epigenetic effects on behavioral patterns, as marked differences can frequently be observed to distinguish such individuals (Alter et al., 2008; Kaminsky et al., 2008; Zwijnenburg et al., 2010). However, it is not feasible in humans to control for the myriad of environmental factors that might still contribute to behavioral differences in genetically identical twins. Thus, the impact of epigenetics on behavior is best studied by using an inbred animal model, where the subjects can be maintained under uniform environmental conditions.

The best characterized of all animal models in which an epigenetic change in a gene provides a well-defined pathological outcomes is the viable yellow (A^{vy}) mouse.

Crucially, the gene modification underpinning the diseases can also be inherited and modulated by environment. The coat color of these mice is governed by the expression of the *agouti (A)* gene (Duhl et al., 1994). Mice with the A^{vy} allele have a fragment of retrovirus, termed an intracisternal A particle (IAP) inserted in the pseudoexon 1A (PS1A) region of the *agouti* locus (Duhl et al., 1994). As a result, *agouti* expression comes under control of a promoter in the long terminal repeat (LTR) of the IAP proximal to the agouti gene, whose ability to drive expression is correlated with the degree of IAP methylation. Hence, A^{vy} mice have coat colors ranging from yellow (hypomethylation of the A^{vy} IAP) to mottled (yellow with varying degrees of agouti patches) to completely pseudoagouti (full hypermethylation of the A^{vy} IAP). Mice expressing the hypomethylated A^{vy} IAP exhibit "yellow obese syndrome", typified by obesity, insulin resistance, hyperglycemia, and in select strains of mice, increased predilection for cancer (Wolff et al., 1986).

Most intriguingly, dietary addition of nutrients likely to enrich the pool of methyl donors and vitamin co-factors required for methylation or genistein to *a/a* mothers increases methylation of the IAP promoter site, such that more offspring are pseudoagouti and less prone to the "yellow-obese syndrome" (Dolinoy et al., 2007; Waterland and Jirtle, 2003). In contrast, dietary exposure of pregnant black females to bisphenol A (BPA) carrying viable yellow fetuses results in more yellow coat color offspring (Dolinoy et al., 2007). These studies illustrate that the environment clearly influences the expression of the A^{vy} locus.

The *agouti* gene product, agouti related protein (AGRP) (also known as agouti signaling protein, ASIP), is primarily an antagonist of the melanocortin receptors (MCR)

(Michaud et al., 1994; Wolff et al., 1999). In the wild type mouse, only MC1R expressed by hair follicles is antagonized by agouti, while a second receptor, MC4R, which occurs predominantly in the hypothalamus, is unaffected unless the agouti gene product is expressed ectopically (Cone et al., 1996). Mice with the "yellow obese syndrome" exhibit ubiquitous expression of the agouti gene in all organs (Michaud et al., 1994). Additionally, these mice display yellow coat colors because the agouti gene product inhibits MC1R in the hair follicle throughout the hair cycle and obese because AGRP antagonizes MC4R in the hypothalamus (Huszar et al., 1997). Under normal physiological conditions, activation of MC4R in the hypothalamus leads to satiation and increases energy expenditure in rodents and humans (Graham et al., 1997; Ollmann et al., 1997).

To our knowledge, no previous studies have assessed whether behavioral disparities exist among genetically identical but epigenetically diverging A^{vy}/a mice that possess contrasting coat colors and whether such mice differ from their black (*a/a*) siblings that lack the *agouti* locus. A number of relatively simple test systems are now available that provide a comprehensive and automated analysis of behavioral patterns, including simultaneous assessment of spatial working and reference-memory performance and anxiety-like /exploratory behavior testing (Kuc et al., 2006). Here, we have applied such tests on these mice as they aged and gained weight.

Materials and Methods

Animals

All animal experiments were approved by the University of Missouri ACUC committee and performed in accordance with NIH Animal Care and Use Guidelines. To produce brown and yellow coat color viable yellow (A^{vy}/a) and black (a/a) coat color offspring for these studies, eight to ten week old C57Bl/6J (a/a) females were bred to ten to twelve week old (A^{vy}/a) males, with the original founder mice provided by Dr. George L. Wolff (Arkansas Children's Nutrition Center, Little Rock, AR). Black coat color (a/a) females were employed for this breeding scheme, as confounding transgenerational effects occur through the maternal germline in A^{vy} mice, with yellow-coat color females more likely to birth yellow-coat color offspring than A^{vy} mice with agouti markings (Blewitt et al., 2006; Morgan et al., 1999). The offspring that resulted from this cross include black (a/a), pseudoagouti (brown) (A^{vy}/a,Y_0), brown with yellow stripes (A^{vy}/a, Y_1-Y_2), yellow with brown stripes (A^{vy}/a , Y_3-Y_4), and all yellow (Y_5) coat color (Dolinoy et al., 2007). Those with browner coat colors (Y_0-Y_2) demonstrate increased methylation in the agouti locus; whereas, those with increasing degrees of yellow coat color (Y_3-Y_5) exhibit a lowered degree of methylation in the *agouti* locus. Thus, epigenetic changes in DNA methylation (Cooney et al., 2002; Waterland and Jirtle, 2003; Wolff et al., 1998) and accompanying, histone modifications (Dolinoy et al., 2010) of the *agouti* locus dictate both coat color outcome and predilection for adult onset disease. These mice are otherwise genetically identical and presumably epigenetically similar. For phenotypic analyses purposes, the mice were classified into three groups based on their coat color: black (a/a), brown to brown with slight yellow pattern (Y_0-Y_2), yellow with

slight brown pattern to all yellow (Y_3-Y_5). The body weights of the offspring were measured at the time of the behavioral trials at 6, 10, 15, 20, and 24 weeks of age, which spans the onset of puberty through sexual maturity. For these experiments, at 6 weeks of age both female (n=4 black, 9 brown, and 4 yellow) and male (n=8 black, 10 brown, and 10 yellow) mice were analyzed for behavioral characteristics. Similar analyses were made on the mice at age 10 weeks (n = 5 black, 7 brown, and 3 yellow females; 7 black, 10 brown, and 10 yellow males), 15 weeks (n = 8 black, 8 brown, and 8 yellow females; 6 black, 8 brown, and 6 yellow males), 20 weeks (n = 7 black, 7 brown, and 4 yellow females; 9 black, 8 brown, and 7 yellow males), and 24 weeks (n = 5 black, 10 yellow, and 10 brown females; 4 black, 6 brown, and 5 yellow males) were employed. As some animals died of unknown causes during course of the study, they were replaced by age and coat-color matched counterparts. All of the mice used for these studies were maintained on AIN 93 control diet with 7% corn oil (Harlan-Teklad, Madison, WI). The mice were provided *ad libitum* access to food and water and were maintained at 71 ± 1°C and 45 ± 2% humidity.

Non-food restriction behavioral analyses:

After prior habituation and acclimation for three min, the behaviors of the mice were assessed with the Activity Pro and hole floor board adaptor (Med Associates, St Albans, VT). Specifically, this behavioral apparatus tracks the animals by using 16 evenly spaced infrared (I/R) transmitters and receivers positioned at the periphery of the four sides of the chamber. The X and Y coordinates are mapped based on the I/R beam array, as the receivers detect the location and general activity of the animals based on the

patterns that the mice create when they break the overhead I/R beams and those within the hole floor board. This apparatus includes four chambers (measuring 43.2 x 43.2 x 30.5 cm), which permits four mice to be tested simultaneously. Mice of varying coat colors were randomly tested in one of the four chambers to minimize any confounding results due to the testing chamber. At each behavioral trial, mice were tested for 16 min durations in accordance with the recommendations provided by the company. Their behavioral responses, including time ambulatory, stationary, distance traveled, and amount of time engaged in stereotypic behaviors, were quantified by using the Activity Monitor Software (SOF-811) version 6.03 (Med Associates) and video recorded for additional confirmation and documentation purposes. To prevent contamination and confounding odors, the mazes were treated with 70% ethanol in-between behavioral trials. For the hole floor board, the control diet (Harlan-Teklad) was concealed in the same four holes to test the spatial working and reference-memory performances, as indicated by the amount of time it took the mice to located food from a single hole and all four baited holes (Kuc et al., 2006).

Food Restriction Behavioral Testing:

In order to test whether the learning and memory performance in the ActivityPro Maze was due to actual learning and memory deficiencies or food-motivated, we additionally tested the mice in this maze after short food depravation, as described previously (Kuc et al., 2006). To prevent any prior experience influencing the observed results, additional adult (20 weeks of age) mice were housed singly at least one week prior to trials. A total of 11 female and 14 male brown (Y_1-Y_2) and 15 female and 11

male yellow (Y_4-Y_5) coat color mice were used in these trials. The objective of this test was to measure the mouse's ability to remember which four out of 16 equidistant holes were baited with accessible food (i.e. Bacon Yummies; BioServ, Frenchtown, NJ). We used the same testing apparatus as in the non-food restriction trials (i.e. ActivityPro Open Field Maze with hole floor board inserts, 44.5 x 44.5 x 30.5 cm from MedAssociates, St Albans, VT). On the night prior to testing, as with the olfaction testing, food was removed at 19:00 hrs from each cage and a single Bacon Yummy was placed in the cage to allow the mice to acclimate to this novel food. Testing was conducted from 7:00 to 9:00AM the following morning. On the morning of the initial day of testing, the mice were moved from the animal to the testing room and allowed to acclimate to this new environment for 30 min. After this acclimation period, mice were placed in one of the four sound attenuated chambers. Each animal was randomized as to which chamber it was placed in, but the chamber for a given animal was constant across the four trial days and for the probe testing that was performed four weeks later. Extra maze cues were provided via a triangle, circle, rectangle, and star placed every 90 degrees. Four equidistant holes of 16 were baited with the Bacon Yummy, and these were accessible to the animal. To prevent olfaction alone being a cue in detecting the food, the remaining 14 holes with food had a screen placed above to prevent the animals from actually accessing the food (Kuc et al., 2006). Of the 16 holes, numbers 5, 10, 11, and 16 were the ones baited with a single 190 mg bacon-flavored treat for all of the trials (Bacon Yummy). The mouse was transferred to the chamber in an opaque container and released in the center of the maze for the 300 sec trial. Following the 300 sec trial or until the mouse had located all four baited holes, it was placed back in the home cage and permitted to rest for

10 min in between trials. The mouse was tested three times a day for four consecutive days. All animals were provided access to food one hour after all behavioral trails were completed and given food *ad libitum* until the beginning of the dark phase of the light cycle. Between each trial, the testing apparatus was wiped down with 70% ethyl alcohol. Latency, reference memory ratio, working memory ratio, and time until retrieval of all four holes or Task were recorded for each trial.

Olfactory Testing:

To determine whether any learning and memory behaviors involving recovery of food from the hole floor board were due to true learning and memory deficiencies or variation in ability to smell the food, olfactory testing was performed on the yellow and brown coat color A^{vy}/a mice. This test measured the amount of time required for mice to locate food by olfaction alone, as the food was concealed under approximately 1cm of aspen bedding, as described previously (Yang and Crawley, 2009). Eight yellow coat colored and eight brown coat colored mice of each sex from 16 to 18 weeks of age were used for testing. Mice were housed single caged at least two days prior to the trial and provisioned with a Bacon Yummy a day to acquaint the animals with the novel food and thereby prevent a neophobic response. The evening prior to testing (approximately 12 h prior), food was removed from each cage to encourage foraging during the trial. On the next morning, mice were brought from the animal room to the testing room and allowed to acclimate for a half-hour prior to the trial. Two opaque cages were filled approximately 1 cm high with aspen bedding with one cage being maintained as detailed for the acclimation period but without any food, and the other cage for testing included

one Bacon Yummy hidden under the bedding. A mouse was then transferred from its home cage into the acclimation cage with a plastic isolation lid on top to discourage escape and minimize other sounds and smells, and the animal was allowed to explore the cage for 10 min. After the acclimation period, the mouse was then transferred to the testing cage and a stopwatch was started as soon as the mouse had all four paws touching the bedding. The isolation lid was at this point placed on the cage. The mouse was provided 10 min to locate the Bacon Yummy, and the trial ended when the mouse recovered the food piece with both front paws.

Elevated Plus Maze:

Exploratory and anxiety-related behaviors were assessed by using elevated-plus maze (EPM) (Fountain et al., 2008; Jasarevic et al., 2011). The elevated plus maze was constructed of black polypropylene in a plus configuration with two opposite open arms (30 cm), a middle platform (5 x 5 cm), and two opposing closed arms (30 cm). The maze was supported 100 cm above the floor by a stand constructed of polypropylene. Each mouse was placed on the center of the platform and allowed to explore the maze for 5 min. Nine female and 14 male brown mice, and 15 female and 11 male yellow mice were used in this experiment. After each test, the apparatus was cleaned with 70% EtOH. Each trial was recorded with EthoVision XT, which automatically scores total time spent in open, center, closed arms, number of closed and open arm entries and center entries, as well as proportion of total time spend in open arms [Open / (Open + Closed)], total distance traveled, and amount of time spent freezing. Arm entry was defined as both front

paws and shoulders placed into the area. On the occasion a mouse jumped off the maze, it was gently placed back on the center and the trial was continued.

Barnes Maze:

The Barnes maze was used to test spatial learning and memory (Barnes, 1979), but modified for smaller rodents, including mice, as described previously (Jasarevic et al., 2011). This dry-land, circular maze measures a rodent's ability to learn intra-maze spatial cues to escape the platform into a home cage (Barnes, 1979). A mildly aversive stimuli, bright lights, was employed to encourage the animal to navigate the maze to the escape cage.

The maze is comprised of a circular, polypropylene platform (95 cm diameter), with 12 escape-holes placed every 30˚ and surrounded by a 50 cm high black barrier to prevent escape and visualization of extra-maze cues, such as objects on the walls of the testing room that the animals might use to locate the target exit hole (Harrison et al., 2006). Consequently, this maze design contrasts with that used for rats where the maze is a flat disk and the spatial cues are placed on the walls of the testing room. The maze was placed on a polypropylene stand to elevate it 100 cm above the floor. A small polypropylene ramp led the animal to the correct exit hole with attached escape cage. This ramp was of the same color and texture as the blind exit holes. Four intramaze spatial geometric cues (triangle, square, circle, and star) were placed at the same height (~10 cm) every 90°. Two 100 watt lights were suspended ~150 cm above the platform to motivate the mice, to escape from the brightly lit open surface of the platform to the safety of the escape cage.

Each mouse was assigned an escape-hole number, with numbers for consecutively tested mice alternated by 90°, i.e. 3, 6, 9, and 12, to eliminate odor cues. Across the seven day trial period, the escape hole and intramaze visual cues remained constant for a given animal. At the beginning of each training day, the maze was disinfected with 70% ethanol to eliminate olfactory cues for consecutively tested mice. The assigned exit hole number and the positions of the spatial cues relative to the escape hole remained fixed for the seven day trial period for any individual animal across all acquisition trials and the reversal trial, although as detailed further below in the reversal trial, the exit hole was shifted 180° relative to the initial escape hole. On each trial day, animals were transferred from the vivarium to the testing room 30 min prior to behavioral testing for habituation and to reduce any anxiety associated with the new surroundings. All testing occurred in the light phase (between ~1200 and 1500 h CST), and animals were returned to the animal room immediately after testing. Animals were tested for seven consecutive days with two-trial evaluations per day for 5 min (300 sec) each, with a 30-min inter-trial interval. At the beginning of the trial, the mouse was placed in the center of the maze, but randomly relative to the location of the spatial cues, in an opaque starting box to allow the tracking system (described below) to detect the center body-point of the animal. The box was lifted, and a trial initiated once the mouse had begun to move in the maze. If the animal failed to enter the escape box within 5 min, the experimenter gently guided the animal to the escape-hole.

Twenty-four hours after the last acquisition training day, reversal trials were also run to examine retention of spatial memory and the ability to learn the position of a new escape route. This would mean that the mouse would have to remember its new escape

route while learning that its old escape route was no longer valid. This trial consisted of dividing the Barnes maze into four quadrants and the target exit was shifted to be 180° from the previous exit hole assigned to each animal. The percentage of time spent in each quadrant was recorded, with the time spent in the quadrant containing the original escape-hole indicative of the learning and memory ability of the animal. The reversal trial was also performed for seven consecutive days.

Each trial was recorded with an EthoVision XT video camera (Noldus Technologies, Leesburg, VA), and latency or time to enter escape-hole and path length from the center to the escape hole was tracked by using accompanying automated tracking EthoVision XT software. Since each animal was tested twice per day, latency performance was averaged across the two trials within the same day for each individual. The random search strategy (coded 3) was defined as localized searches of holes separated by maze center crosses, i.e. the animal demonstrated no specific pattern when moving from one hole to the next. Serial search strategy (coded 1) was defined as a systematic search of consecutive holes in a clockwise or counterclockwise direction as the animal consistently traveled along the periphery of the maze. Finally, direct search strategy (coded 2) was defined as navigating directly from the center of the maze to the target escape hole without crossing the center of the maze more than once and with three or fewer errors to blind ending holes.

Statistical Analysis:

Mouse body weight, olfactory, and ActivityPro Maze and EPM behaviors, including total distance traveled, time ambulatory, time resting, and time engaged in

stereotypical behaviors (such as grooming), and the hole-floor board learning and memory behavioral assessments, including latency (amount of time required to find food in a single hole), inter-response time (time elapsed between entries into holes baited with food), and reference memory ratio (total correct entries into holes "baited" with food/over total entries) were studied at 6, 10, 15, 20, and 24 weeks of age. All dependent variables were analyzed with the repeated measures GLM procedures of SAS Software Analysis version 9.2 (SAS Institute, Cary, NC, USA). For all dependent variables, sources of variation that were considered included, coat color, sex and color × sex interaction, and the repeated measures of age. At each age point, dependent variables were further analyzed by using GLM procedure of SAS, fitting color, sex and color by sex interaction as main effects with and without body weight as a covariate. When main effects were detected, the difference between treatment groups was determined by using the Fisher Least Significant Difference analysis. The association between body weight and all behavior perimeters was also analyzed by using correlation procedure of SAS after removing coat color, sex, and their interaction effects. The data were analyzed as a split plot in space and time (Steel, 1996). The linear statistical model contained the fixed effects of diet, sex, day, coat color and all possible interactions with diet, sex, day, and coat color. In the analysis, the individual pup within diet and sex was used as the denominator of F for the main plot effects. Day and interactions of day with main plot effects were tested by using the residual mean square as the denominator of F. Mean differences were determined by using Fisher's protected Least Significant Difference (LSD).

Three discrete search strategies for the escape hole were defined as described previously (Jašarević et al., 2011). The first, coded 1, was serial, the second (2) was direct, the third (3) (random). The data were analyzed by using a repeated measurement design with PROC GLIMMIX and SAS version 9.2 software analyses (SAS Institute, Cary, NC). As above, the analyses were done with testing individual unit as the denominator of F. Both methods used a cumulative logit link and a multinomial distribution, i.e. all three search strategies were included in this analysis. The hole-floor board adaptor also measures task behavior, which is the amount of time required for the mice to retrieve food from all four holes. However, in the non-food restriction and food restriction trials, not all mice completed this objective within the 960, 300, or 600 sec trial period, depending on the experimental protocol. As such, this data was additionally analyzed by using a Lifetest procedure (SAS software analysis). Mean differences were determined by using the Fisher Least Significant Difference. All data are expressed as least squares means ± SEM and $P<0.05$ was considered significant.

Results:

Body Weight

Body weight of each mouse was measured prior to testing in the maze at the five different time-points. When mice of the three different coat-color groups were compared in terms of weight gain over time, no sex differences were observed. That is, females gained weight at the same rate as males in all three coat color groups (Fig. 9). Thus, for the body weight comparisons, data for the two sexes within the same coat color group were combined. From ten weeks onwards, the yellow mice were heavier than their black

siblings, and at 20 and 24 weeks, they were also significantly heavier than their brown littermates ($P<0.001$) (Fig. 10).

Ambulatory and Stereotypical Behaviors:

There were no differences between males and females in any of the coat color groups in amount of time the mice were ambulatory or moving around the maze, stationary, distance traveled, or engaged in stereotypic behaviors, in particular grooming. Therefore, data for the two sexes with the same classification of coat color were again analyzed together. Over time, it became evident that the yellow mice became less active, moving around less than their brown and black siblings (Fig. 11A). They also travelled lesser distances, although all groups of mice covered less ground when they were 24 weeks of age than they had done earlier (Fig. 11B, Table 1). Yellow mice spent slightly more time at rest or immobile compared to other groups ($P<0.0471$; Fig. 11C, Table 1), but the most striking differences between the three color coat groups was in stereotypical behavior, mainly grooming, (Fig. 11D, Table 1). Specifically, the yellow mice engaged in such behaviors for more time than the other two groups, especially at 24 weeks ($P<0.0001$). At this age, the yellow A^{vy}/a mice spent about as much time in stereotypical behavior as they did moving around the maze.

Learning and Memory Behaviors in Non-Food Restriction Behavioral Trials:

For the learning and memory behaviors, there were no overall differences between males and females across the coat color groups. Thus, for each of the behaviors, the two sexes within coat color were analyzed together.

Latency:

Latency represents the amount of time required to complete a task, in this case for a mouse to retrieve food from one of the four "baited" holes. These values were highly variable at each time- point. Times were shortest when the mice were youngest, i.e. at 6 weeks. There were no differences in latency times between coat color groups at 6, 10, 15, and 20 weeks of age, although at 24 weeks, the black (*a/a*) mice displayed increased latency compared to the A^{vy}/a brown coat color mice (267.7 ± 52.2 s vs. 85.1 ± 42.6 s, respectively; $P<0.007$) (Fig. 12A). No differences were evident between any of the other groups at this age. Moreover, when the overall averages across ages were compared, there were no differences in latency amongst any of the coat color groups (Table 2), suggesting that the values at 24 weeks for a/a mice might represent a sudden, age-onset change in latency behavior.

Task Behavior:

Task behavior is defined here as the time required for each mouse to locate food concealed in all of the four holes in the hole-floor board maze. No differences in task behavior were evident between groups at 6, 10, and 24 weeks of age (Fig. 12B). However, the A^{vy}/a brown mice took longer to find the hidden food at 15 and 20 weeks, especially compared to yellow coat color mice (Fig. 12B). When mean values across mouse age groups were compared, the brown mice took longer to locate the food in all four holes compared to yellow and black coat color mice (Table 2). However, this test is complicated by the fact that some mice in each of the groups, but particularly the brown

morphs, failed to locate the food in all four holes in the pre-set time of 960 sec (16 min). To determine whether these failures affected the comparisons, we performed a Lifetest analysis. This statistical method considers those animals in each group and time point that passed the test, i.e. located all four holes, versus those that failed to complete the task during the allotted time. Based on this assessment, the brown coat color mice again performed worse than the black and yellow mice at 15 and 20 weeks, but no differences were observed at the other time points, including the later one at 25 weeks (Table 3).

Reference Memory Ratio (RMR):

In the context of this chapter, RMR is the number of times a mouse entered a baited hole (correct entries) relative to the total number of entries into all holes. Values here were relatively constant over time and varied little between coat color groups except at 15 weeks, where the brown A^{vy}/a mice appeared to perform somewhat worse than the yellow mice (0.33 ± 0.03 vs. 0.46 ± 0.04, respectively, $P<0.05$) (Fig. 12C). When the average values across ages were compared, brown mice continued to display decreased RMR compared to yellow coat color mice (0.36 ± 0.02 vs. 0.41 ± 0.02, respectively, $P<0.05$) (Table 2). Again, RMR values are presumably complicated by the fact that some of the brown morphs were unable to locate all the pellets in their allotted time.

Inter-response time (IRT):

IRT is the average time that elapsed between entries into holes baited with food. No differences in IRT were observed at 6, 10, or 15 weeks of age for any of the groups, but at 20 and 24 weeks of age, the brown mice performed worse than either their yellow

A^{vy}/a or black *a/a* siblings (Fig. 12D). When data for all ages were compared the brown mice still demonstrated increased IRT compared to yellow and black coat color mice (Brown: 183.1 ± 12.6 s, Yellow: 135.5 ± 15.4 s, and Black: 116.4 ± 14.7 s, $P<0.01$) (Table 2).

Correlation of Weight with Ambulatory and Stereotypic Behaviors:

At all time -points, body weight across all three groups demonstrated a negative correlation with amount of time the mice moved around the maze (Table 4). R^2 values ranged from 0.13 at 6 weeks to 0.32 at 24 weeks of age (P values 0.01 to 0.0001, respectively) (Table 4). However, in the heavier yellow coat color (A^{vy}/a) mice (Fig. 2), it was only at 24 weeks of age, that weight significantly correlated with time ambulatory (R^2 and P values, 0.52 and 0.002, respectively) (Table 5). In other words, although the yellow mice became heavier and tended to move less than their siblings, weight/ambulatory activity values were not necessarily correlated for individual yellow mice until they were older than 20 weeks of age.

There was a poor correlation between the amount of time spent inert and body weight of 6 and 10 week old mice (Table 4), but the link to body weight became much stronger at 15 weeks and beyond (Table 4). Again, the yellow mice, when considered separately, were anomalous. Body weight, when all three groups were considered, negatively correlated with distance travelled at 6, 15, 20, and 24 weeks of age (Table 4), but did not correlate at any age in the yellow mice alone (Table 5). On the other hand, stereotypic behavior of all mice (Table 4), including the yellow mice alone (Table 5), strongly correlated with body weight.

Clearly, body weight influenced some behavioral parameters. However, when these analyses were adjusted by body weight, coat color continued to influence stereotypic behavioral differences in yellow coat color mice compared to black and brown mice at 15, 20, and 24 weeks of age ($P<0.05$) (Fig. 10). Additionally, differences in mobility and time stationary based on coat color persisted persist when body weight was run as a covariate ($P<0.05$) (Fig. 10).

Correlation of Weight with Learning and Memory Behaviors:

When all three groups were considered, the only learning and memory response that negatively correlated with body weight was the amount of time required for the mice to retrieve food from all four holes in the hole floor board maze. This correlation was only evident at 24 weeks of age with a R^2 of 0.12 and a P value of 0.03 (Table 6).

On the other hand, when only the yellow coat color mice were considered in the analyses, there was significant negative correlation between body mass and learning and memory assessments (latency, task, RMR, and IRT) for at least one time- point (Table 7). For example, latency negatively correlated with body weight at 15 and 24 weeks (R^2 values 0.61 and 0.37; P values 0.004 and 0.01, respectively). Similarly, body weight also appeared to have a negative impact on the time required for older yellow mice to locate all the food concealed in the maze (R^2 values, 0.31 at 24 weeks of age and 0.55 at 20 weeks of age; P values 0.03 to 0.009, respectively) (Table 7).

The data were also analyzed with adjustment by body weight to determine how much influence coat color alone exerted on these behavioral parameters. When the data

were analyzed in this manner, coat color continued to influence task behavior at 15 and 20 weeks, IRT at 10, 20, and 24 weeks, and RMR at 15 weeks of age (Fig. 12).

Learning and Memory Traits in Food-Restricted Mice:

To determine if the initial results obtained in the ActivityPro Maze with mice that had had full access to food prior to the behavioral trials were affected by a lack of motivation to seek out food, the test was repeated with a separate group of brown and yellow coat color male and female mice that had been food-deprived 12 h prior to the behavioral testing (Kuc et al., 2006). In contrast to the previous experimental design, sex differences were observed among food restricted mice, and so data across sexes could not be pooled as it had previously. Nevertheless, the only parameters that differed with these behavioral trials were time to locate all four holes (Task; $P<0.0455$), between coat color in males, (Fig. 13D) and the average time spent between correct holes (inter-response time), within sex and between coat color (Fig. 13A,B). Female brown mice took less time to find each baited hole (IRT) than yellow coat color females on Day 1 of testing (25.9 ± 15.9s; 68.3 ± 11.3s; $P<0.0334$). Male brown mice also took less time to find each baited hole on Day 1 (39.8 ± 12.1s) and Day 3 (40.0 ± 12.1s) of testing compared to yellow coat color males (62.9 ± 12.1s; 81.8 ± 12.1s; $P<0.0446$ and $P<0.0168$). Though not easily seen in Figure 13, there was a statistical difference between time to complete the Task on Day 3 between brown and yellow males with brown males taking less time to complete the task ($P<0.0455$). Overall, brown coat color mice performed better when it came to moving from one baited hole to the next, suggesting they were at least more motivated to continue searching for more treats.

Olfaction Testing:

To determine if the above differences in food retrieval in the ActivityPro Maze were due to differences in ability to detect the odor of the food concealed in the hole floor board, we measured the olfactory responses of male brown and yellow coat color mice of both sexes (Yang and Crawley, 2009). As illustrated in Figure 14, brown and yellow coat color females did not differ in their ability to locate the food. However, brown coat color males required more time to retrieve the concealed treat than yellow coat color males (356.32 ± 76.34 vs. 121.23 ± 76.34 sec, respectively, $P<0.04$). Additionally, yellow coat color males performed better than yellow coat color females (121.23 ± 76.34 vs. 309.43 ± 81.61 sec., respectively, $P<0.037$). Thus, the differences observed between brown and yellow coat color mice in the non-food restricted behavioral trials could be due to olfactory deficiencies in the brown coat color mice, which gave yellow coat color mice an advantage.

Exploratory and Anxiety-like Behaviors:

To determine whether any of the groups of mice differed in anxiety-like and exploratory behaviors, they were tested in the elevated plus (EPM) maze (Fountain et al., 2008; Jasarevic et al., 2011). During these tests, brown coat color females traveled a greater distance than yellow coat color females (20.8 ± 3.66 vs. 39.0 ± 4.88 cm; $P<0.004$) (Fig. 15) but no differences were observed between brown and yellow coat color males. Within the yellow coat color group, females covered less distance than the males (20.8 ± 3.66 vs. 26.8 ± 3.66 cm; $P<0.05$). Minor differences were noted between females but not between males in time spent immobile. Yellow coat color females remained immobile

slightly longer than brown coat color females (35.3 ± 3.65 vs. 18.3 ± 4.9 sec; P<0.008) (Fig. 15). No differences were detected between coat color groups in the overall time spent in the open versus closed arms, but yellow coat color males spent more time in the open arms (29.3 ± 3.64 vs. 19.3 ± 3.64 sec; P<0.05), a behavior presumed to be indicative of greater exploratory activity, and less time in the closed arms (indicative of decreased anxiety-like behaviors) than yellow coat color females (23.4 ± 15.17 vs. 35.0 ± 15.16 sec.; P<0.031) (Fig. 15). There were no other differences observed between sexes or coat color pairings.

Memory and Navigational Behavior Assessments in the Barnes Maze:

Since there was no significant difference in latency, i.e. time to find the correct escape hole, between males and females for any of the three coat colors, the data were collapsed to include both sexes within a coat color group. Yellow coat color mice exhibited greater latency or time to solve the maze (111.1 ± 10.9 s) than either brown (87.5 ± 12.3 s) or black coat color mice (104.5 ± 10.2 s; P<0.05) for both) (Fig. 16). The numbers of errors made increased across all groups and sexes until Day 6 when values decreased (Days 1-7, respectively: 5.58 ± 0.585, 7.0 ± 0.587, 7.55 ± 0.587, 8.04 ± 0.587, 6.94 ± 0.587, 5.99 ± 0.587, 5.33 ± 0.587; P<0.0004) (Fig. 17). To our surprise, regardless of coat color and sex, almost all of the mice predominantly employed the serial search strategy (Fig. 19), and therefore, it was not possible to compare relative use of the three different search strategies across trial days or between the initial and reversal trials.

Black mice traveled further (921.33 ± 81.9 cm), and spent more time resting (44.56 ± 6.25 s) and moving (92.05 ± 9.7 s) compared to brown (605.49 ± 99.0 cm; 22.9

± 7.51 s; 47.84 ± 11.8 s) and yellow (669.48 ± 86.4 cm; 29.23 ± 6.64 s; 62.0 ± 10.24 s) counterparts on Day 1 (P<0.0352). Black (102.33 ± 14.4 cm) and yellow (124.8 ± 15.1 cm) mice traveled further on Day 5 than brown mice (59.01 ± 17.58 cm; P<0.0097; P<0.042). Yellow mice spent more time moving (72.09 ± 10.24 s) than their brown counterparts on Day 5 (40.27 ± 11.91 s; P<0.0437). Black mice traveled faster (velocity) (17.31 ± 2.03 cm/s) than their yellow counterparts on Day 7 (10.74 ± 2.12 cm/s; P<0.026). The only significant difference for the entire 7-day training period overall was that black (*a/a;* 62.5 ± 6.9 s) and brown (57 ± 8.3 s) coat color mice spent more time moving around the maze than yellow (60.9 ± 7.4 s) coat color mice (black vs. yellow: P<0.01;brown vs. yellow: P<0.035; Fig. 18). No differences based on coat color and sex were evident in the reversal protocol. This is illustrated when comparing how much time mice spent near their old or new escape hole in relation to total time spent in the maze (Fig 20). In other words, although mice spent more time near their new escape hole compared to the old one, this time difference did not change significantly over the course of seven days (P<0.245).

Discussion:

Herein, we have compared the body weight and behavioral responses in yellow and brown morphs that expressed the *agouti* (A^{vy}/a) gene to non-agouti or black (*a/a*) mice. Somewhat similar types of behavioral assessment have previously been performed in deer mice and rats, which include the use of the EPM and Barnes Maze (Cottle and Price, 1987; Hayssen, 1997; Keeler, 1942). However, this study is the first that has compared behavioral responses of inbred, sibling mice with differing epigenetic changes.

First, we considered weight correlations with coat color after dividing the A^{vy}/a mice into predominantly yellow and brown groupings without attempting to include data for the intermediate, mottled, Y_3 mice. Differences in body weight had emerged between black and yellow- coated mice at 10 weeks (after puberty but before adulthood) and became more evident as the mice aged. In particular, the black (non-agouti) mice weighed the least and yellow (A^{vy}/a) the most, while the brown exhibited intermediate values. Many previous studies dating back to the 1960s have indicated that yellow coat color mice become obese compared to their brown and black siblings (Dickie, 1969; Wolff, 1978; Wolff et al., 1986) and that the differences become exacerbated with increasing age (Wolff, 1978) (Fig. 2). It has been proposed that the increase in body weight observed in yellow coat color mice is due to a combination of increased food intake, increased ability to utilize and store nutritional components in the form of fat, and broad-scoped metabolic changes resulting from direct endocrine actions of the *agouti* gene product (Wolff et al., 1986; Yen et al., 1994). One likely underlying cause of the associated obesity in yellow coat color A^{vy}/a mice may be sustained antagonism of hypothalamic-expressed melanocortin receptor 4 (MC4-R) by agouti signaling protein (Fan et al., 1997; Huszar et al., 1997; Kiefer et al., 1997). An additional possibility that has not been considered previously is that some of the behaviors noted here, notably lowered locomotor activity and increased lethargy, also worsen the observed obesity phenotype.

As our studies revealed, yellow coat color mice were generally more lethargic than brown (A^{vy}/a) and black (a/a) mice (Table 1), with the differences beginning to emerge by about 15 weeks of age and becoming significant by 20 weeks, which coincides with the emergence of body weight differences between brown *versus* yellow coat color

A^{vy}/a mice. The yellow mice spent less time ambulatory, rested more, travelled less, and engaged in more stereotypic behaviors than their black and brown siblings. Thus, the general languor of the yellow coat color mice might contribute to their obesity rather than the reverse.

In addition to differences in overall locomotor activity, there was a strong correlation between body weight and behavioral performance in the ActivtyPro maze when all mice were considered (Table 4). These differences became more pronounced as the mice aged and were most obvious at 24 weeks, even though the mice had not been food-restricted in this instance (Fig. 11). However, the correlations between weight and ambulatory behavior were weakest for the yellow mice, suggesting that the ectopically expressed agouti signaling protein and not simply mouse weight was a driving cause of the behavioral abnormalities in this group of animals. If differences in behavior had only been observed between yellow and black morphs, the relatively higher expression of the *agouti* gene in the yellow coat color mice, might have accounted for the changes. However, both yellow and brown color mice carry the A^{vy}/a gene, but in the former case it is unmethylated leading to its promiscuous, ectopic expression; whereas, in the latter, this allele is predominantly expressed by the hair follicles and then only selectively. Thus, early epigenetic changes in the *agouti* locus might govern the phenotypic differences in behavior patterns that emerge with age.

The most striking feature in the behavior of the yellow coat A^{vy}/a mice was the increase in time that they engaged in stereotypic behaviors, especially grooming, relative to their brown or black counterparts. As stereotypic behaviors presumably require more energy than when the animal is stationary, this activity seems unlikely to contribute to

weight gain. Again, these differences manifested at 15 weeks of age. Curiously, in deer mice (*Peromyscus maniculatus gracilis*), the opposite is observed. In this outbred species, non-agouti/black coat colored mice displayed more stereotypic behaviors and increased body weight compared to their *agouti* counterparts (Hayssen, 2001, 1997). In my work, when data from all mice, including those with yellow coat color, were considered, there was a strong positive correlation between body weight and time engaged in stereotypic behaviors (Tables 4 and 5). In other words as the heavier the mouse the more likely it was to behave in this manner. In farm mink (*Neovison vison)*, there is a strong genetic component governing the extent to which animals display stereotypic behaviors (Hansen et al., 2010), but in this instance it was the leaner minks that behaved most often in this manner. Indeed, the increased energy demands of this activity is believed to contribute to the leaner phenotype (Hansen et al., 2010).

The brown coat color (A^{vy}/a) mice unexpectedly performed the worst on the learning and memory assessments employed, as exemplified by prolonged time for task behavior and IRT compared to the yellow and black coat color mice (Fig. 12, Table 2). Additionally, the brown mice had the lowest RMR, suggesting that they were aimlessly wandering from one hole to the next, compared to the yellow and black (*a/a*) mice, which appeared more purposeful in their ability to locate the "baited" holes. These results might be due to the food-based method employed to assess learning and memory. Perhaps, the yellow mice outperformed their brown counterparts because of increased food motivation rather than because they were smarter. However, if such is the case, it is not clear why the leaner black mice had approximately the same response times as the obese yellow mice. While minimal correlation of body weight and these learning and memory parameters

existed when all three groups were combined, yellow coat color mice displayed a strong negative correlation between body weight and task behavior that began at about 15 weeks of age. Most likely, the metabolic state of the animal and other internal factors underpin motivation and performance of the mice in these learning and memory response tests. Presumably, the methylation status of the *agouti* gene and its ectopic expression is a major contributing factor.

In a second set of experiments in which the mice were food-restricted, brown coat color mice did perform better than their yellow counterparts in IRT (inter-response time; average time spent between each baited hole) and, in males, Task (time spent finding baited holes) (Fig 13). This better performance in terms of the shorter time to retrieve food from baited holes after food had been restricted for 12 h might be expected, as the leaner, brown mice have fewer fat reserves on which to draw energy than the heavier, yellow mice. This improvement held for both sexes, but what makes the data puzzling and contributes to the aforementioned idea that the mouse's learning and memory is controlled to an extent by their methylation status, is that only the brown coat color males performed better in Task compared to their yellow counterparts (Fig 13D). This was only statistically significant on Day 3 of the trial. It could be concluded that the male mice, with their more exploratory-like behavior (Fig 15D) in open areas were more at ease searching for the treats than the females. Both coat color males performed similarly when it came to Task, signifying that the yellow coat color males usually found all four baited holes at the same rate as their brown counterparts.

In order to ensure that the differences that were seen when employing the hole floor board insert was indeed due to learning and memory and not olfactory difference,

the viable yellow's sense of smell was tested. Yellow coat colored males were the only mice to perform significantly better than either the females or brown coat color males (Fig 14). This is most likely due to an increased desire to find the hidden treat and not to any increase in olfaction. As yellow males tend to be the heaviest when comparing them against either coat color female or brown males, it would follow that part of this could be due to an increase desire to consume food. This would be another byproduct of a possible change in methylation status of *agouti,* affecting the yellow coat color mouse's brain.

The Barnes maze, a widely used maze to assess spatial learning and behavior assessment (Barnes, 1979 and Jasarevic et al., 2011 and 2012), was employed to compare the outcomes that had been anticipated from the ActivityPro test, which measures food-motivated learning memory and behaviors (Kuc et al., 2006). The ActivityPro maze data illustrate that yellow coat color mice were overall more lethargic , as evidenced by decreased time spent moving over the seven day trial period (Fig 18C), than their black and brown counterparts. In the case of the Barnes Maze, differences in latency may be attributed to use of contrasting search strategies. However, all coat color groups predominantly employed the serial search strategy throughout and there was no apparent change in strategy over the 7-day test period (Fig. 11). A similar search strategy was observed with C57BlJ6 mice in previous studies (O'Leary et al, 2011; O'Leary and Brown, 2012). Other variables that may impact latency include time spent resting, error rate and velocity, although all three of these measures were similar across all groups. In short, it is unclear why the yellow coat color mice exhibited greater latency compared to brown and black coat color groups. The failure of the mice to learn the location of the escape hole could be attributed to their getting used to the maze and becoming more

interested in searching for other escape routes or hiding spots, instead of going directly back to the home cage. This is based on anecdotal evidence and would have to be verified in a later experiment.

In summary, we have demonstrated herein that there are striking body weight and behavioral differences between non-agouti and *agouti* mice. Moreover, the data also demonstrate that there are marked differences in body weight and behavioral responses between yellow and brown coat color A^{vy}/a mice, both of which express the *agouti* gene. In the former it is unmethylated and ubiquitously expressed while in the latter it is maintained in a methylated state and its expression pattern predominantly confined to the hair follicles. These findings suggest not only does genetics but also epigenetics, including DNA methylation, governs body weight and behavior in mice. Body weight differences might account for some but not all of the behavioral alterations. Future studies in comparative and human psychology are needed to understand this emerging field of behavioral epigenetics and its interplay with classic genetics.

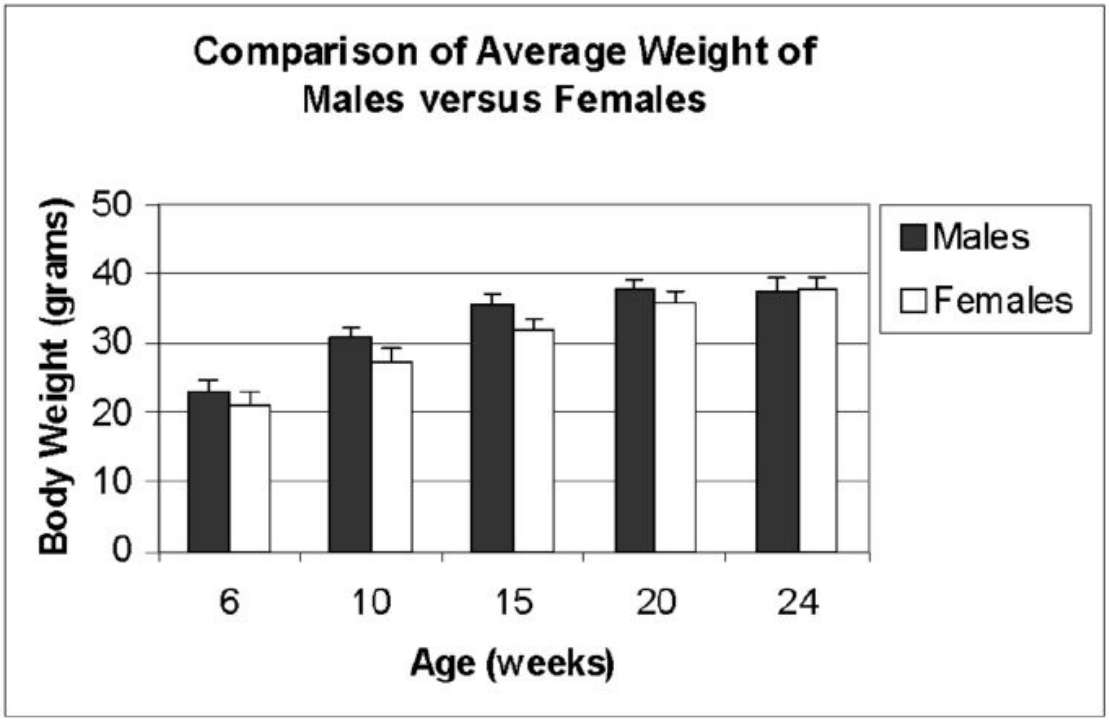


Figure 9: Body weight between males and females with all three coat color groups combined. When each of the individual ages is compared, there were no significant differences between males versus females.

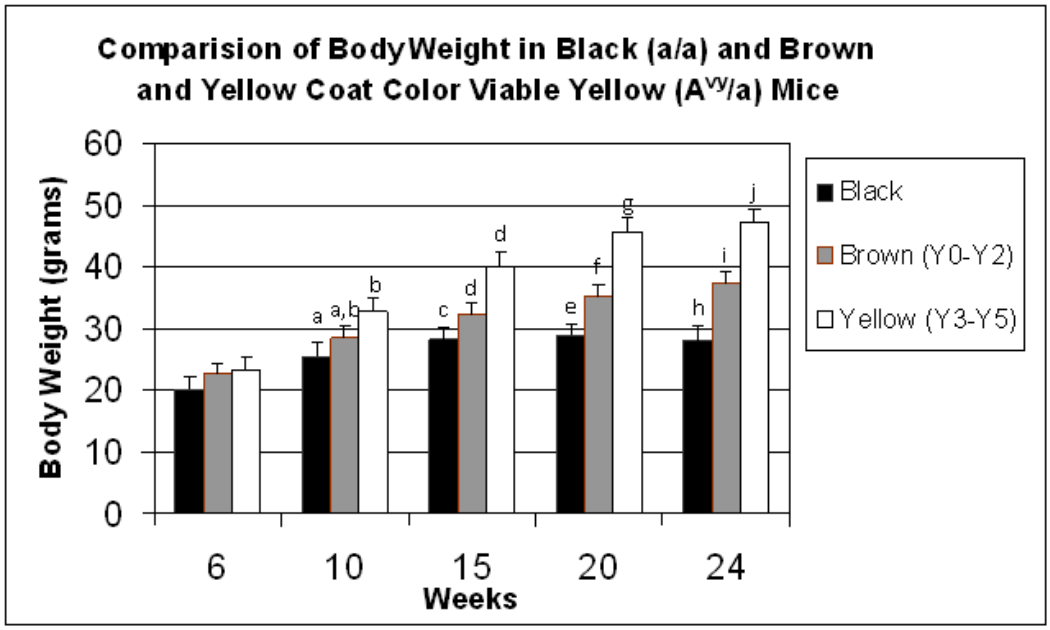


Figure 10: Body weight in black (*a/a*), brown (A^{vy}/a), and yellow (A^{vy}/a) coat color mice. Error bars= SEM. Within the same age, values with different superscripts are significantly different at P<0.05 for 10 weeks of age, and P<0.01 for 15, 20, and 24 weeks of age.

Table 1: Average ambulatory and stereotypic behavior responses in black (*a/a*), brown and yellow coat color viable yellow (A^{vy}/a) mice. Values with different superscripts are different at P<0.05. In general, the yellow coat color A^{vy}/a mice spent less time ambulatory, more time at rest or in engaged in stereotypic behaviors, such as grooming, and overall travelled less distance than brown (A^{vy}/a) and black (*a/a*) coat color mice.

Coat Color	Time Ambulatory (s)	Distance Traveled (cm)	Time at Rest (s)	Time Engaged in Stereotypic Behaviors (s)
Yellow (A^{vy}/a, Y4-5)	94.9 ± 10.3[a]	2668.6 ± 188.5[a]	547.3 ± 13.6[a]	100.0 ± 6.6[a]
Brown (A^{vy}/a, Y0-2)	147.3 ± 8.4[b]	3306.2 ± 153.5[b]	510.4 ± 11.1[b]	72.3 ± 5.4[b]
Black (*a/a*)	132.3 ± 9.8[b]	3238.7 ± 179.2[b]	473.6 ± 13.0[c]	63.2 ± 6.3[b]

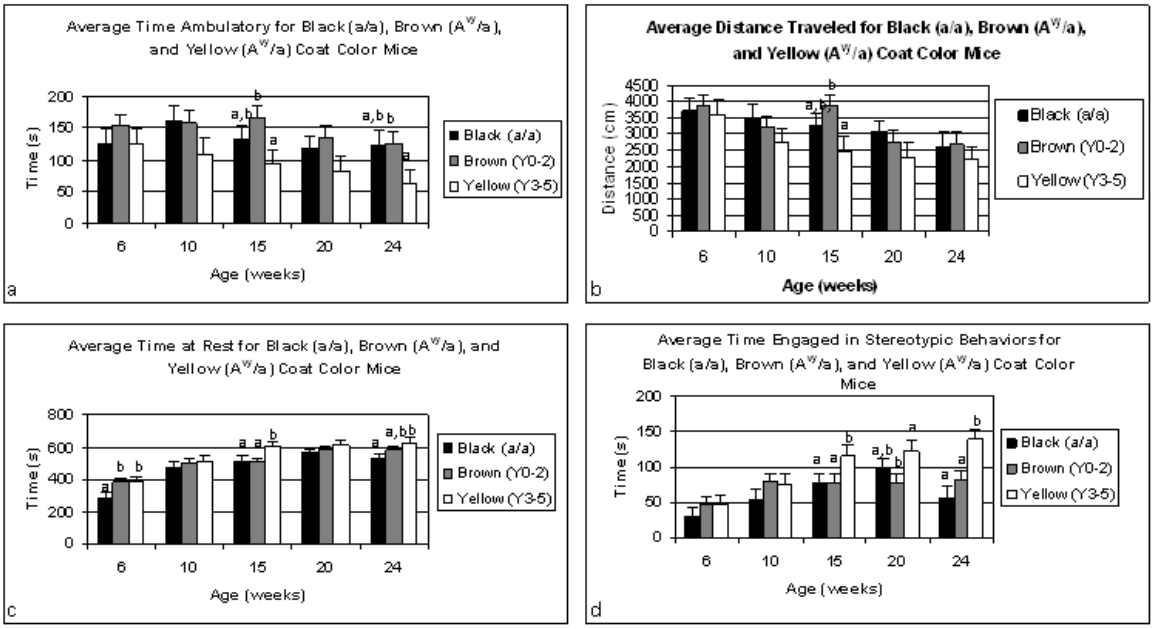


Figure 11: Ambulatory and Stereotypic behavior responses in black (*a/a*), brown (A^{vy}/a), and yellow (A^{vy}/a) coat color mice. a) Average time ambulatory, b) Average distance traveled, c) Average time at rest, and d) Average time engaged in stereotypic behaviors. Error bars= SEM. For each behavior assessed, values with different superscripts are different at $P<0.05$.

Table 2: Average learning and memory responses in black (*a/a*), brown and yellow coat color viable yellow (A^{vy}/a) mice. Values with different superscripts are different at P<0.05. In general, the yellow coat color A^{vy}/a mice spent less time ambulatory, more time at rest or in engaged in stereotypic behaviors, such as grooming, and overall travelled less distance than brown (A^{vy}/a) and black (*a/a*) coat color mice.

Coat Color	Latency (s)	Task Behavior (s)	Reference Memory Ratio (RMR) (s)	Inter-response time (IRT) (s)
Yellow (A^{vy}/a, Y3-5)	125.8 ± 22.5	526.6 ± 40.3[a]	0.41 ± 0.02[a]	135.5 ± 15.4[a]
Brown (A^{vy}/a, Y0-2)	85.2 ± 18.4	685.4 ± 32.8[b]	0.36 ± 0.02[b]	183.1 ± 12.6[b]
Black (*a/a*)	118.7 ± 21.4	580.9 ± 38.3[a]	0.37 ± 0.02[a,b]	116.4 ± 14.7[a]

Table 3: Confidence interval analysis for task behavior responses (seconds) in black (*a/a*), brown and yellow coat color viable yellow (A^{vy}/a) mice. The values represent how long it took 25%, 50%, and 75% of the mice to complete the task. Since some of the animals failed to complete the behavior in the requisite time, for some ages and groups, there were not sufficient number of mice to determine the 50 and 75 percentile values. In these cases, it is designated as n/a= not applicable. Based on the Lifetest procedure, which accounts for these failures, values in the same time-period with different superscripts in the 25% column are significantly different at $P<0.05$.

	25%			50%			75%		
Age	Black	Brown	Yellow	Black	Brown	Yellow	Black	Brown	Yellow
6	280	154	178.5	383	449	318.5	577	793	457
10	6	38	137	148	559	261	403	798	596
15	370^{a}	903.5^{b}	330^{a}	n/a	n/a	700	n/a	n/a	n/a
20	444^{a}	847^{b}	375^{a}	652	n/a	404	n/a	n/a	n/a
24	577	729.5	393	n/a	n/a	624	n/a	n/a	n/a

Table 4: Correlation of weight with ambulatory and stereotypic behaviors for all three coat colors combined for Non-Food Restricted Maze.

Behavior	Age	Intercept	Slope	R^2	P-value
Time Ambulatory	6	293.0	-7.03	0.13	0.01
	10	285.6	-4.98	0.16	0.01
	15	266.5	-4.01	0.25	0.0009
	20	223.4	-3.03	0.22	0.001
	24	218.0	-3.07	0.32	0.0001
Time at Rest	6	197.3	7.19	0.06	0.11
	10	383.4	4.37	0.08	0.07
	15	350.1	5.64	0.33	<0.0001
	20	462.1	3.3	0.28	0.0003
	24	396.0	4.82	0.23	0.001
Time Engaged in Stereotypic Behaviors	6	-46.9	4.00	0.32	<0.0001
	10	-38.1	4.02	0.26	0.0007
	15	-45.7	4.03	0.62	<0.0001
	20	-15.4	3.2	0.3	0.0002
	24	-44.0	3.69	0.45	<0.0001
Distance Traveled	6	6643.6	-131.44	0.21	0.002
	10	4464.6	-43.36	0.07	0.1
	15	5389.7	-63.48	0.10	0.05
	20	4011.3	-34.6	0.12	0.03
	24	3618.9	-29.3	0.11	0.03

Table 5: Correlation of weight with ambulatory and stereotypic behaviors in yellow coat color (A^{vy}/a) mice for Non-Food Restricted Maze.

Behavior	Age	Intercept	Slope	R^2	P-value
Time Ambulatory	6	361.2	-10.58	0.27	0.08
	10	251.7	-4.48	0.13	0.21
	15	200.1	-2.66	0.21	0.16
	20	237.7	-3.35	0.26	0.11
	24	240.6	-3.77	0.52	0.002
Time at Rest	6	88.1	12.54	0.23	0.11
	10	682.4	-4.4	0.07	0.37
	15	533.6	1.79	0.14	0.25
	20	455.6	3.26	0.28	0.09
	24	632.9	-0.09	0.0003	0.95
Time Engaged in Stereotypic Behaviors	6	-76.8	5.41	0.49	0.01
	10	-59.3	4.53	0.48	0.009
	15	-46.3	4.01	0.73	0.0009
	20	-134.4	5.65	0.76	0.0005
	24	-118.0	5.47	0.49	0.003
Distance Traveled	6	7472.2	-174.18	0.28	0.07
	10	2707.0	1.73	0.0001	0.97
	15	2447.6	1.13	0.0002	0.96
	20	2158.9	3.85	0.002	0.88
	24	1592.4	12.85	0.02	0.60

Table 6: Correlation with weight and learning and memory behaviors for all three coat color groups combined for Non-Food Restricted Maze.

Behavior	Age	Intercept	Slope	R^2	P-value
Latency	6	24.1	0.13	0.0003	0.90
	10	193.7	-2.03	0.004	0.70
	15	195.9	-3.49	0.06	0.12
	20	217.8	-2.86	0.03	0.30
	24	300.3	-4.09	0.05	0.18
Task	6	513.7	-2.98	0.002	0.77
	10	465.5	-2.47	0.0002	0.77
	15	195.9	-3.49	0.06	0.12
	20	928.1	-5.67	0.04	0.19
	24	1042.9	-8.01	0.12	0.03
Reference Memory Ratio (RMR)	6	0.4	-.0003	0.002	0.93
	10	0.4	0.001	0.007	0.61
	15	0.5	-0.003	0.05	0.16
	20	0.3	0.002	0.03	0.29
	24	0.4	0.0003	0.0002	0.92
Inter-response time (IRT)	6	138.9	-1.28	0.005	0.64
	10	262.3	-3.4	0.04	0.24
	15	269.8	-3.19	0.06	0.14
	20	140.9	-0.13	0.0001	0.94
	24	98.9	1.63	0.02	0.32

Table 7: Correlation with weight and learning and memory behaviors in yellow coat color (A^{vy}/a) mice in Non-Food Restricted Maze.

Behavior	Age	Intercept	Slope	R^2	P-value
Latency	6	-0.5	0.57	0.07	0.40
	10	424.8	-6.8	0.02	0.63
	15	449.2	-8.98	0.61	0.004
	20	847.9	-15.67	0.27	0.10
	24	860.0	-15.37	0.37	0.01
Task	6	-212.7	26.2	0.13	0.25
	10	1023.6	-18.9	0.15	0.19
	15	1315.2	-17.9	0.52	0.01
	20	1791.7	-25.53	0.55	0.009
	24	1608.0	-20.65	0.31	0.03
Reference Memory Ratio (RMR)	6	0.5	-0.006	0.1	0.31
	10	0.7	-0.009	0.22	0.1
	15	0.9	-0.10	0.38	0.04
	20	0.2	0.004	0.06	0.46
	24	0.9	-0.009	0.18	0.12
Inter-response time (IRT)	6	34.4	2.25	0.02	0.69
	10	532.7	-10.8	0.52	0.005
	15	360.3	-4.42	0.11	0.30
	20	89.8	-0.04	0.00004	0.98
	24	-189.3	7.00	0.19	0.10

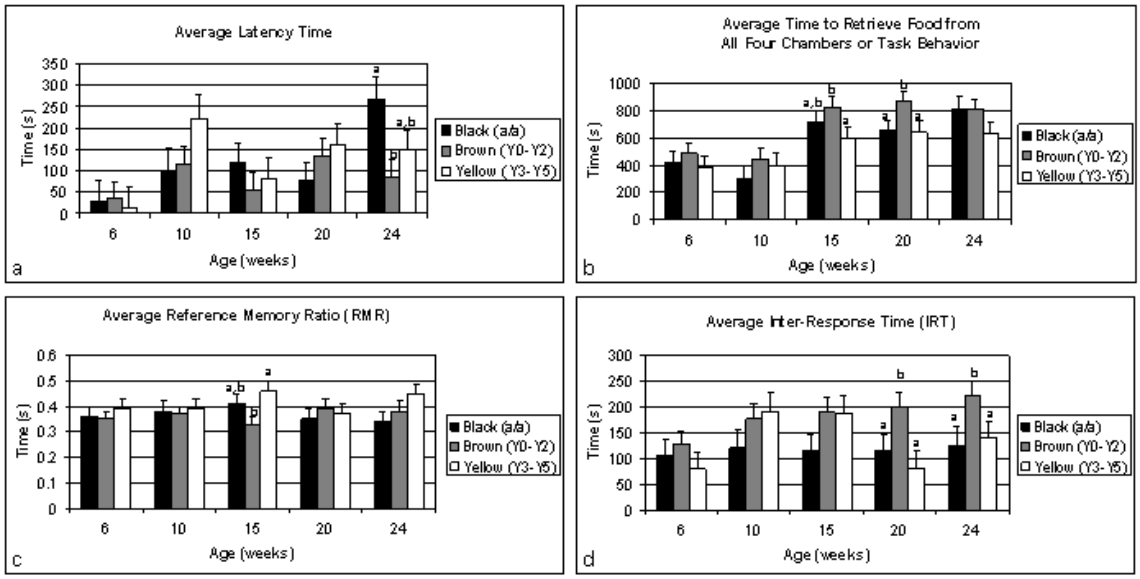


Figure 12: Learning and memory response behaviors in non-food restriction trials for black (*a/a*), brown (A^{vy}/a), and yellow (A^{vy}/a) coat color mice. a) Average latency time, b) Average Task time, c) Average RMR, and d) Average IRT. Error bars= SEM. For each behavioral parameter, values with different superscripts are different at $P<0.05$.

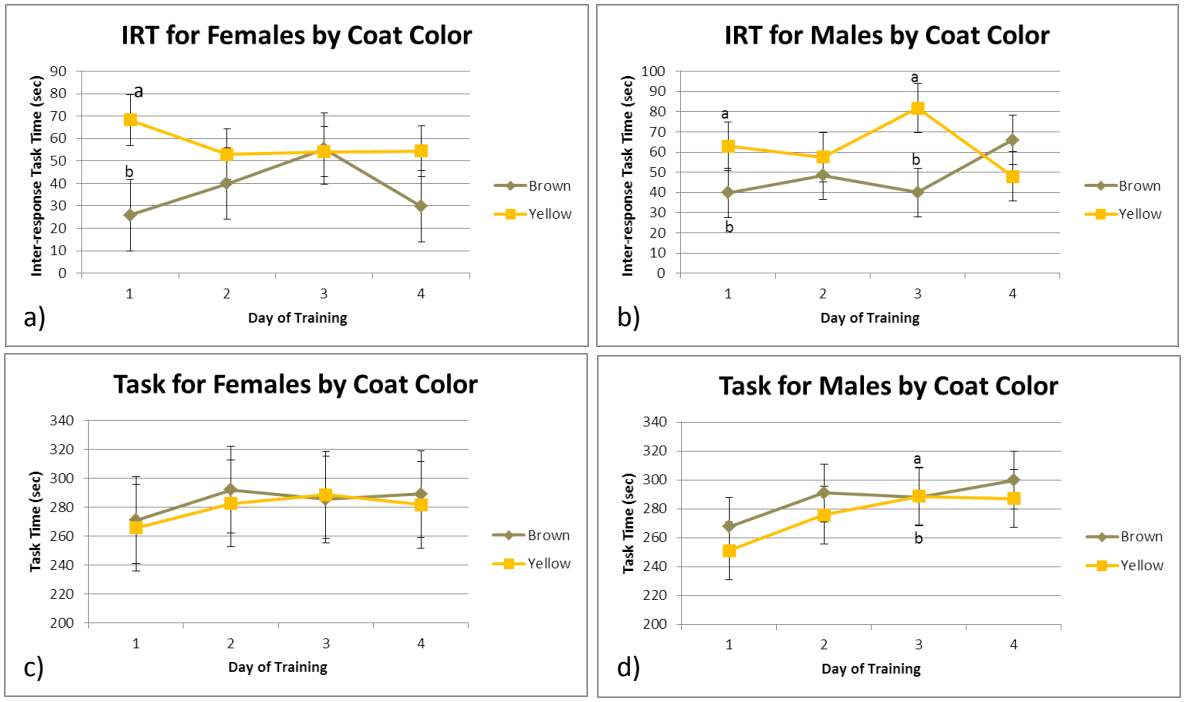


Figure 13: Learning and memory response behaviors in food restriction trials for brown (A^{vy}/a) and yellow (A^{vy}/a) coat color mice. The only two variables that differed over time with this testing method were a,b) task and c,d) inter-response time (IRT). Within sex, those with different letter superscripts are significantly different ($P<0.05$).

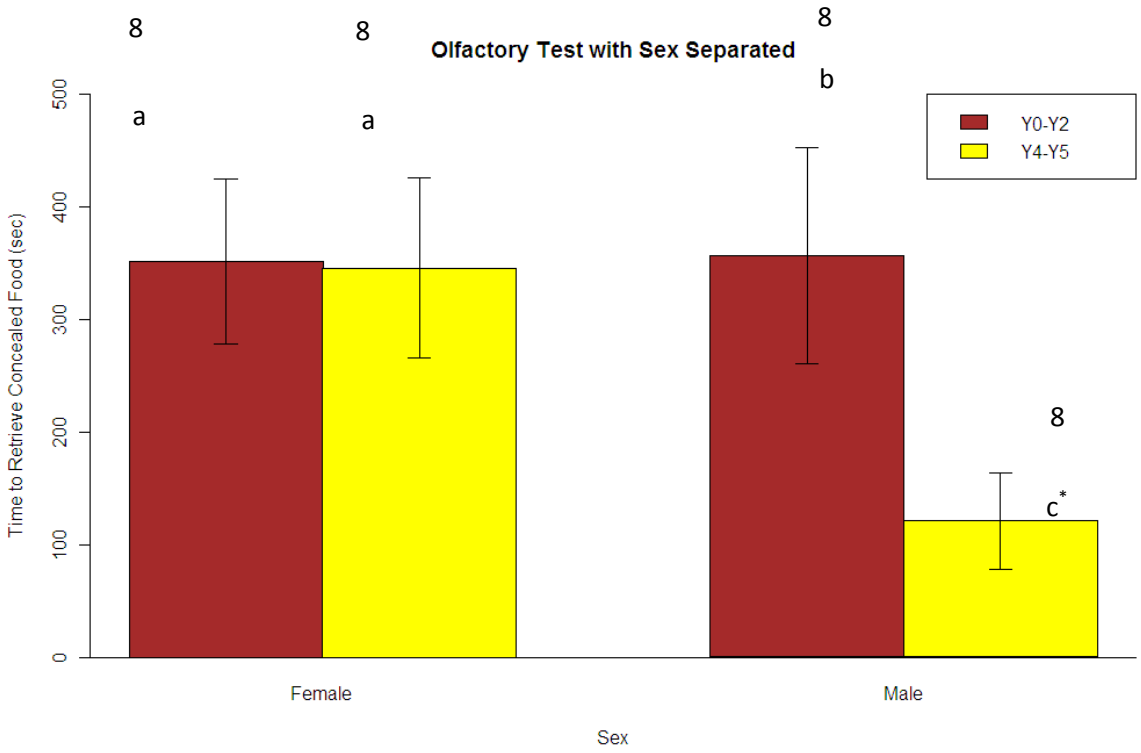


Figure 14: Olfaction testing results for brown and yellow (A^{vy}/a) coat color mice. This test measures the amount of time required for each animal to retrieve a food treat (Bacon Yummy) concealed under the bedding. Number of above graphs equals replicates for each group. Within sex, those with different letter superscripts are significantly different (P<0.04). Within coat color, those with different character superscripts are significantly different (P<0.04).

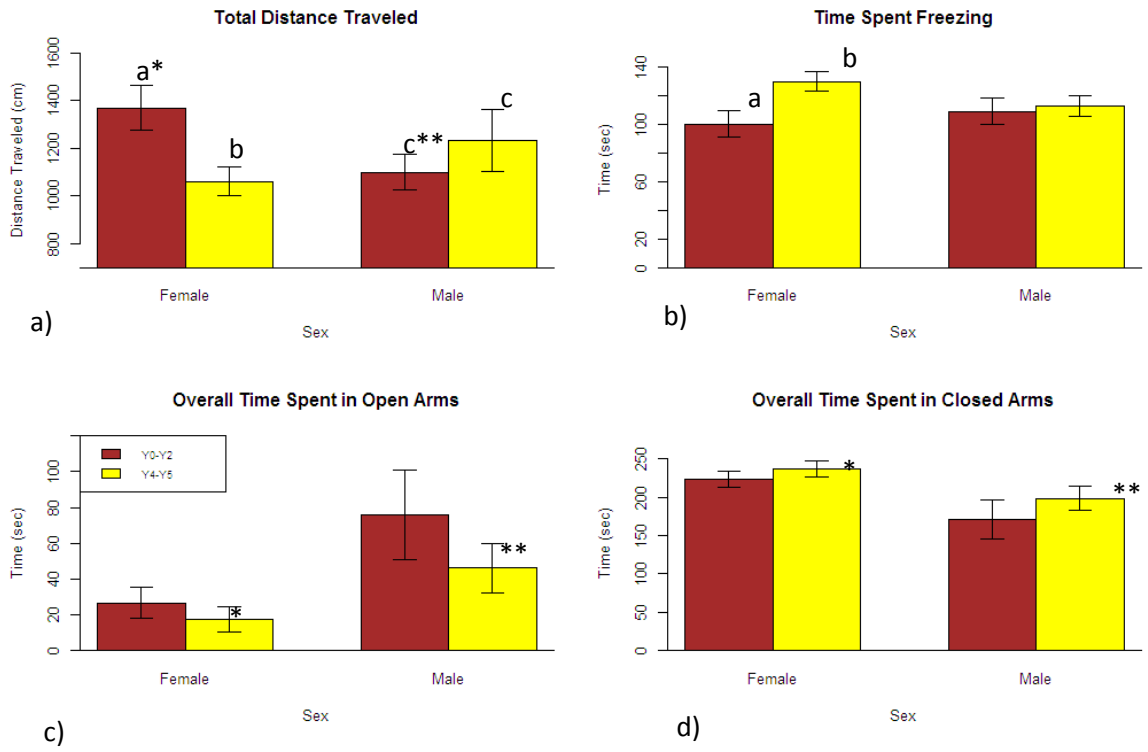


Figure 15: Exploratory and anxiety-like behavior results in the EPM. a) Total distance each coat color and sex traveled in the 5 minute trials in the EPM; b) total amount of time each coat color and sex spent freezing; c) overall time each coat color and sex spent in the open arms of the maze; and d) Overall time each coat color and sex spent in the closed arms of the maze. Within sex, those with different letter superscripts are significantly different ($P<0.05$). Within coat color, those with different character superscripts are significantly different ($P<0.05$).

Figure 16: Latency by coat color. Yellow coat color mice exhibited greater latency or time to solve the Barnes maze than brown and black coat color mice ($P<0.05$).

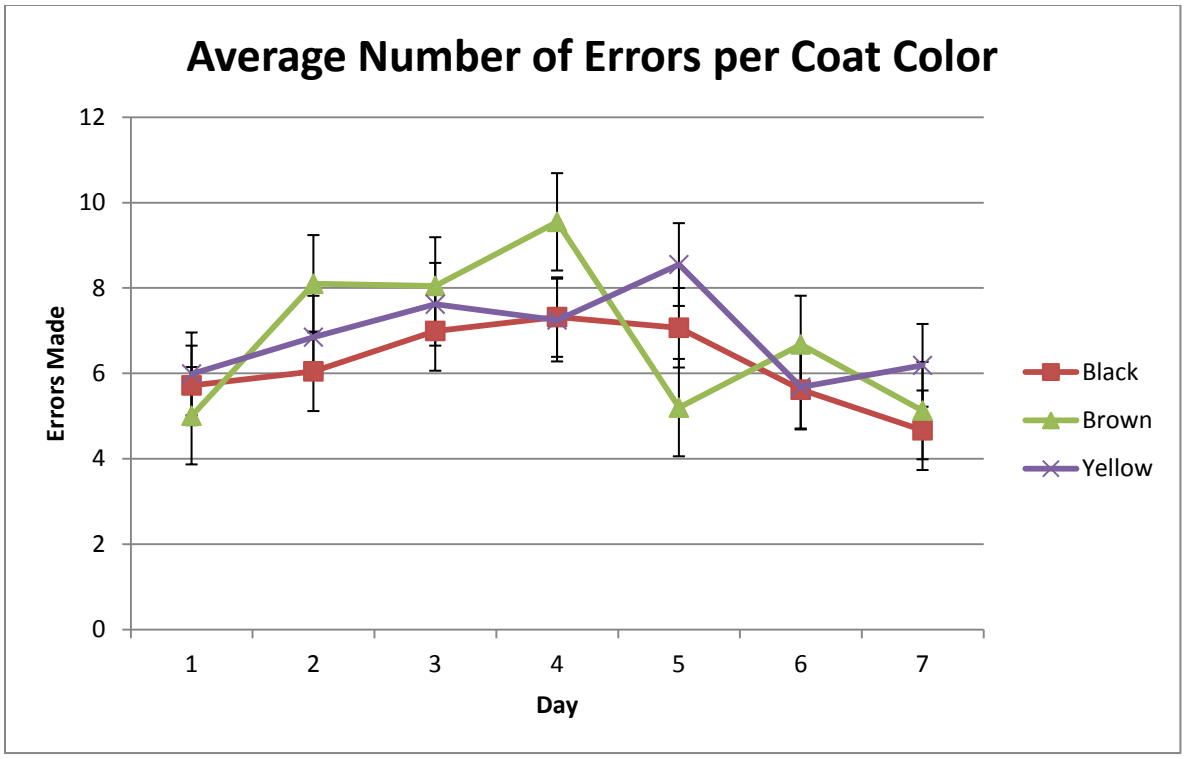


Figure 17: Average number of errors per coat color. Number of errors mice made increased across all groups and sexes in the Barnes maze until Day 64 when it decreased ($P<0.0004$).

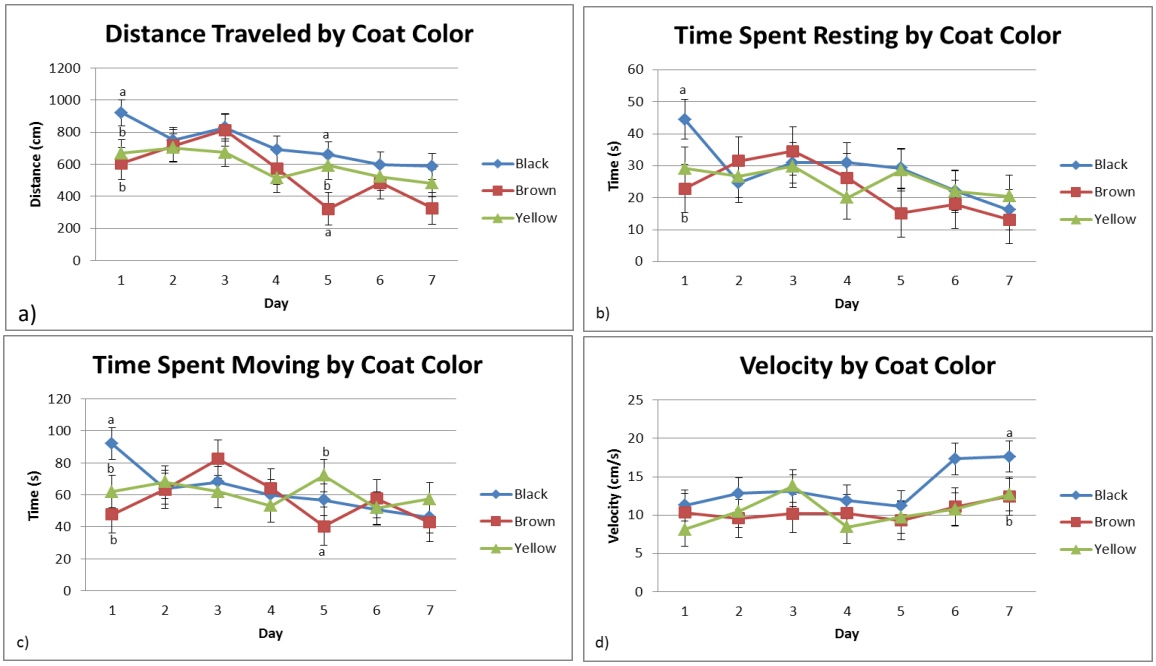


Figure 18: Barnes Maze movement. Different superscripts within same day are significantly different. Black mice traveled further, and spent more time resting and moving compared to brown and yellow counterparts on Day 1 ($P<0.0352$). a.) Black and yellow mice traveled further on Day 5 than brown mice ($P<0.0097$; $P<0.042$) c) Yellow mice spent more time moving than their brown counterparts on Day 5 ($P<0.0437$) d) Black mice traveled faster than their yellow counterparts on Day 7 ($P<0.026$).

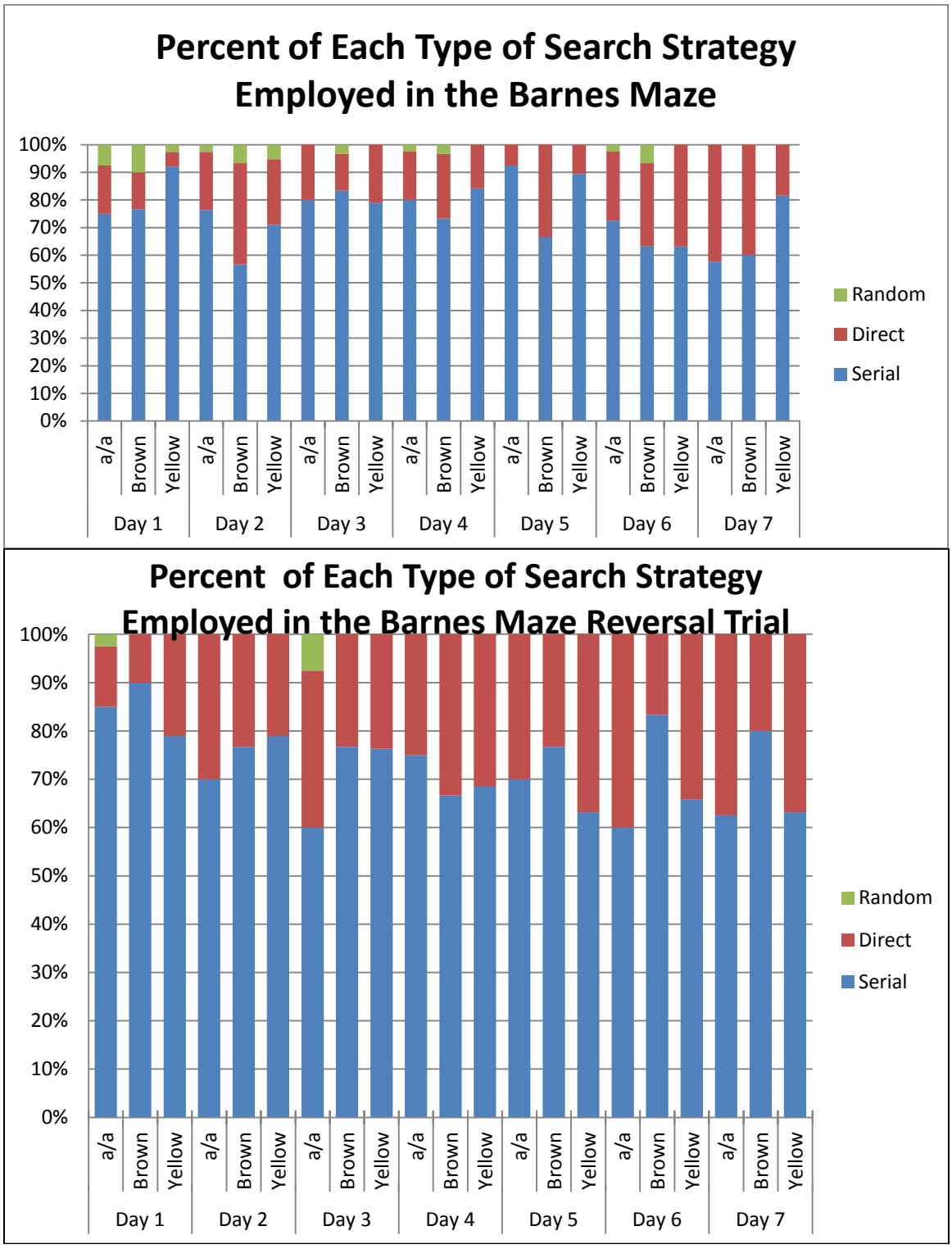


Figure 19: Barnes Maze search strategy. No differences between coat colors, across days, or between trials for search strategies. Graph depicts each search strategy type employed as a percentage of the whole column.

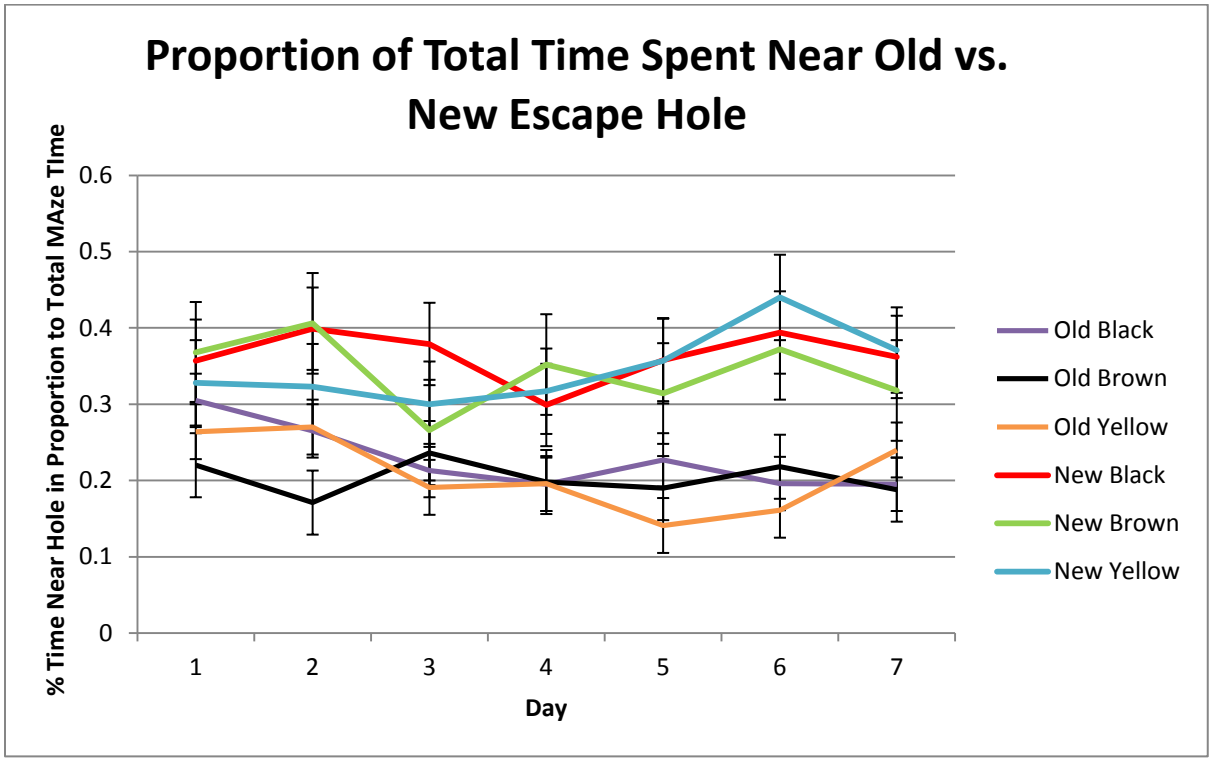


Figure 20: Proportion of total time spent near escape holes. Time spent near old and new escape hole as a proportion of time spent in Barnes maze per day did not change significantly over time.

CHAPTER 3

EFFECTS OF POST WEANING DIET ON PHENOTYPIC ASPECTS IN VIABLE YELLOW MICE

Abstract

The viable yellow (A^{vy}/a) mouse strain (Wolff et al., 1978;Wolff et al., 1986) has provided one of the best models for studying epigenetic inheritance in mammals (Cooney et al., 2002;Wolff et al., 1998). A^{vy} mice have also been useful in understanding "metabolic syndrome", as animals with the more extreme yellow coat color (see Fig. 1) develop "yellow obese syndrome" characterized by adult-onset obesity, accompanied by diabetes (Wolff et al., 1986; Wolff et al., 1999; Klebig et al., 1995). In contrast, their A^{vy}/a brown-coated (pseudoagouti) siblings tend to remain essentially healthy, even though they are genetically identical to their yellow coat color siblings on the background of an inbred strain, C57Bl6J (Wolff et al., 1986;Wolff et al., 1999). Intriguingly, the coat color of these mice and their associated risk for disease can be controlled by the diet provided to the dam during the peri-conception to parturition period (Cooney et al., 2002; Wolff et al., 1998; Waterland and Jirtle, 2003). These findings indicate that even subtle changes in nutrient composition of standard mouse diets can enhance or reduce the risks of her offspring developing adult-onset diseases (developmental origin of health and disease, DOHaD), presumably by modulating epigenetic modifications on genes encoding key metabolic enzymes and hormones (Godfrey et al., 2011;Lillycrop et al., 2011;Weaver, 2009).

Introduction:

The various coat color patterns of A^{vy}/a mice originate from differences in the control of expression of the *agouti* (*A*) gene, and specifically the A^{vy} allele (Cooney et al., 2002; Wolff et al., 1998; Waterland and Jirtle, 2003; Morgan et al., 1999). Mice (Bultman et al., 1992; Miller et al., 1993) and various animals, including humans (Kwon et al., 1994; Wilson et al., 1995), possess at least one *agouti* gene locus, which encodes for the agouti signaling protein (ASIP). Mouse ASIP is a 131 amino acid that acts in a paracrine manner to bind to the melanocortin-1 receptor (MC1R), which prevents alpha-MSH signaling. The suppression of this pathway down-regulates synthesis of brown/black pigments and increases synthesis of yellow/red (pheomelanin) pigments during the mid-stage of the hair follicle cycle (Cone et al., 1996). The coat color patterns in the siblings range from full yellow to pseudoagouti (brown), with mottled patterning observed in the mice intermediate between these extremes, and these differences arise due to the labile state of the A^{vy} allele and its variable expression. The increased appetite and obesity of the yellower mice arises from ectopic expression of ASIP outside the hair follicle, especially in the hypothalamus (Bultman et al., 1992), where it antagonizes the melanocortin 4 receptor (MC4R) (Cone et al., 1996), and the pancreas (Mansour et al., 2010).

Analysis of the A^{vy} and the related A^{iapy} allele has demonstrated an inserted retrotransposon (an intracisternal A particle or IAP) not present in the wild type allele that is located upstream (in pseudoexon 1a) of the customary (exon 2) transcriptional start site of the wild type *A* gene (Morgan et al., 1999; Michaud et al., 1994). This viral insertion contains a cryptic promoter in its 5′ long-term repeat (LTR) region capable of

usurping control of the agouti gene expression and thereby preventing transcriptional factor control that typically confine the production of ASIP to the hair follicles with systemic expression instead resulting (Cooney et al., 2002; Wolff et al., 1998;Duhl et al., 1994;Chen et al., 1996; Michaud et al., 1994). The fur of the mice is completely brown (pseudoagouti) when the cryptic promoter within the IAP is silent, but yellow when the promoter is fully unmethylated and active. Thus, the diverse phenotypes observed in these genetically identical sibling is directly correlated with the degree of cytosine methylation of the 5′ LTR of the IAP, with CpG island hypermethylation leading to the pseudoagouti phenotype and hypomethylation associated with yellow fur and broad ectopic expression of ASIP. When the A^{vy} allele and methylation status is passed through the male germ line, there is presumed to be complete erasure of the methylation marks, and then variable methylation patterns are re-established during development of the F1 embryos, resulting in litters where the pups can range from completely yellow to fully brown (Morgan et al., 1999). Subsequent studies from several groups of investigators showed that a diet enriched in various methyl donors and cofactors, such as Mg^{+2}, which are essential for the methyl cycle to operate, when provided to *a/a* pregnant dams carrying A^{vy}/a conceptuses influences coat color. Specifically, a greater proportion of pseudoagouti offspring are born and these have an accompanying decreased risk for adult-onset diseases, presumably because of increased CpG methylation within the IAP sequence within the A^{vy} allele occurring during conceptus development when the methylation marks are established (Cooney et al., 2002; Wolff et al., 1998;Cropley et al., 2010;Waterland and Jirtle, 2003). These findings indicate that the maternal uterine

environment can modulate epigenetic marks introduced during the very early stages of embryonic development.

Other maternal supplemented diets have also been reported to be capable of shifting the coat colors within litters born to *a/a* dams after they had been bred to A^{vy}/a males (Anderson et al., 2012; Dolinoy et al., 2007; Dolinoy et al., 2006). The phytoestrogen genistein (G), fed at a concentration of 250 mg/kg feed weight (fw), has been reported to shift the coat color balance towards greater percentage of brown coat color and more healthy offspring (Dolinoy et al., 2006), whereas relatively high (50 mg /kg fw) feed concentration of the endocrine disrupting compound (EDC) bisphenol A (BPA), favored the birth of more yellow, presumably more unhealthy mice with a hypomethylated IAP (Dolinoy et al., 2007). In a more recent paper, extremely low doses of BPA (50 µg and 50 ng/kg fw) were reported to influence coat color redistribution compared to controls, but in an inconsistent manner (Anderson et al., 2012). However, our laboratory has recently been unable to confirm these results (Rosenfeld et al., 2013). Despite the contradictions observed with endocrine disrupting compounds, such as genistein and BPA, on coat color, there have been no doubts expressed regarding gestational effects of a maternally administered, methyl-rich diet on coat color of viable yellow mice. However, as far as we are aware there are no studies exploring whether or not, a post-weaning methyl -rich diet fed to the offspring has any effect on coat color and animal health. Possibly there is sufficient plasticity in the methylation pattern of the IAP site that it would be susceptible to post-weaning epigenetic modification. This question has considerable relevance, as it would suggest that changing the quality and quantity of nutrients in children and adult's diet might also be used to prevent adult diseases,

particularly for those infants at risk because of an inadequate maternal diet during pregnancy. There have been a handful studies that have examined the effects of post-weaning diet, on various diseases, including liver cancer in F344 male rats brought on by a methyl deficient diet (Davis et al., 2000); Mikol et al. (1983); Pogribny, 2006; Wilson et al., 1984). This idea of diet preventing or causing certain diseases to occur later in life will be further discussed below.

One of the earliest studies demonstrating the protective effects of a methyl diet component, choline, was done in pancreatectomized dogs (Newberne, 1986). When these dogs were concurrently treated with insulin and lecithin (a choline derivative), they did not develop hepatic lipidosis (fatty liver); whereas, the pancreatectomized dogs not provided this methyl supplement developed this disease. In mice, post-weaning diet may change the renal expression of imprinted genes, in particular insulin-like growth factor 2 (*Igf2*) in mice (Waterland et al., 2006). When hybrid C57 mice were placed on a methyl donor-deficient post-weaning diet that lacked folic acid, vitamin B12, methionine and choline, they exhibit inappropriate expression of maternal *Igf2* and a corresponding decrease in the normally expressed paternal *Igf2* allele. The overall effect was the mice consuming this diet had decreased expression of *Igf2* compared to control counterparts fed a well-balanced diet.

A diet deficient in key methyl donors, including choline and methionine, leads to increase incidence of hepatic carcinogenesis in F344 male rats (Mikol et al., 1983; Pogribny et al., 2006; Wilson et al., 1984). In contrast, female rats for uncertain reasons appear are more resistant to these diet-induced hepatic cancer (Saito et al., 1991). Male rats on this hypomethylated diet exhibit increased hepatic expression of proto-oncogenes,

such as *Myc*, *Fos*, *Hras1*, within several days, and this pattern positively correlates with the duration of the diet (Christman et al., 1993; Wainfan et al., 1989; Wainfan and Poirier, 1992). Replenishment of methyl donors in the diet results in re-methylation of several cytosines in CpG islands of hepatic DNA (Pogribny et al., 2007) and may reverse the early stages of liver carcinogenesis in these rats (Verma et al., 2003). However, some of the methylation changes induced by the methyl deficient diets fed to weaned offspring can persist for up to 14 months in rats (Locker et al., 1986). and may reverse the early stages of liver carcinogenesis in these rats (Verma et al., 2003). However, some of the methylation changes induced by the methyl deficient diets fed to weaned offspring can persist for up to 14 months in rats (Locker et al., 1986).

These studies demonstrate that there might be malleability in shifting the methylation status of select genes, such as those involved in cancer, by tinkering with nutritional factors (Cooney et al., 2002; Pogribny et al., 2007; Pogribny et al., 2008; Pogribny et al., 2009; Wolff et al., 1998). As such, we sought to determine whether placement of yellow coat color A^{vy} mice on a methyl supplemented diet would reduce their risk for obesity and hyperglycemia. Additionally, we sought to determine how the various control diets, NIH-31 and AIN 93G with 7% corn oil, that have been used in the former A^{vy} studies, affects post-weaning outcomes.

Materials and Methods:

Animals:

The NIH guidelines for the care and use of laboratory animals were followed and all experiments were approved by the Animal Care and Use Committee (ACUC). Female

C57Bl6J *a/a* females on a phytoestrogen-free AIN 93G control diet were bred to A^{vy}/a males. This breeding scheme leads to A^{vy}/a mice with coat colors ranging from all brown or pseudoagouti, Y_0, to all yellow, Y_5 with intermediary patterns. From the offspring born, ten weanling, 3 week old mice from varying litters for each sex and coat color (brown, Y_0-Y_2 or yellow, Y_4-Y_5) A^{vy}/a C57Bl6J mice were used for these studies (Ounpraseuth et al., 2009). Mice were housed with a same sex but opposite colored mouse throughout the duration of the experiment with both placed at 4 weeks of age on one of the three diets: AIN93G (AIN), NIH-31 (NIH), or methyl-supplemented (Methyl) diet. The two former diets have been used in past experiments (Cooney et al., 2002; Dolinoy et al., 2007; Wolff et al., 1998). Food and water was provided on *ad libitum* basis, and the animals were housed in polypropylene cages and provided filtered water in glass water bottles to reduce exposure to the estrogenic compound, bisphenol A. The room temperature was 70-75°C and humidity range was 40-45%.The AIN diet, as can be seen in Table 2, is a refined, soy-free gestational diet that has more calories than either the NIH or Methyl diet. Mice were maintained on their assigned diets for the duration of the experiments.

Measurement of body weight and blood glucose concentrations in A^{vy}/a mice:

In order to determine if the diets altered body weight or blood glucose concentrations, both measurements were taken once a week at 1300 hrs from 4 to 12 weeks of age. This time- point was chosen as it would presumably mimic fasting blood glucose concentrations as other studies and our work indicate laboratory mice eat primarily during the dark cycle when the lights go off at 1900 hrs (Fuse et al, 2012).

Samples were then drawn on an every other week basis until 32 weeks of age at which point mice were culled and liver samples collected. Blood was sampled from the saphenous vein, and blood glucose concentration was determined by using the Bayer Contour Glucometer (Bayer, Tarrytown, NY).

Statistics:

The body weight and serum glucose concentration data were analyzed by using a repeated measurement design with PROC GLIMMIX and SAS version 9.2 software analyses (SAS Institute, Cary, NC). In order to determine the potential effects of the variables detailed below on body weight and serum glucose concentrations, the data were grouped into two age categories 4-6 weeks of age (weaning to puberty) and 7 to 32 weeks of age (puberty to adulthood). For all dependent variables, sources of variation that were considered included, coat color, sex and the interaction of coat color x sex, and the repeated measures of time for both variables. At each age, dependent variables were further analyzed by using GLM procedure of SAS, fitting coat color, sex, and interaction of coat color x sex interaction as main effects. When main effects were detected, the difference between treatment groups was determined by using the Fisher Least Significant Difference analysis. Mean differences were determined by using the Fisher Least Significant Difference. All data are expressed as least squares means ± SEM and $P<0.05$ was considered significant.

Results:

Body Weight

The weekly data were grouped into three time points: weaning (4 to 9 weeks of age), puberty (10 to14 weeks of age), and adulthood (15 to 32 weeks of age). No differences were observed at weaning. By puberty, NIH and Methyl brown coat color males (31.46 ± 1.8 g, and 34.97 ± 1.8 g, respectively) and females from these same diet groups (21.22 ± 1.8 g and 26.1 3± 1.8 g, respectively) weighed significantly less than AIN brown males (41.3 ± 1.8 g; $P<0.007$ and $P<0.028$) and females (34.28 ± 1. 8g; $P<0.0001$ and $P<0.0098$). These same differences between NIH and AIN brown coat color males (41.8± 2.0 g and 49.34± 2.0 g; $P<0.05$) and females (32.08 ± 2.03 g and 48.05 ± 2.14 g; $P<0.009$), and Methyl and AIN brown coat color males (46.71 ± 2.03 g) and females (40.13 ± 2.27 g), persisted through adulthood; $P<0.0001$ and $P<0.012$). Comparison of coat color groups within diet revealed that females also showed weight differences within AIN diet between brown versus yellow coat colors at puberty (34.28 ± 1.8 g and 40.5 ± 1.8 g; $P<0.037$) and adulthood (48.05 ± 1.8g and 54.53 ± 1.98 g; $P<0.03$; Fig. 13). No other differences were noted between males of contrasting diet groups.

Serum Glucose Concentrations:

The weekly data were grouped into the same three time-points as detailed above. There were no differences seen in serum glucose concentrations based on diet, coat color, or sex observed at weaning and none whatsoever within. Moreover, for females when looking between diets and coat colors, post-weaning diet and coat color did not impact the serum glucose concentrations ($P<0.6772$ to $P<0.8716$; Fig. 14). On the other hand,

differences emerged between males at puberty within the AIN diet. Specifically, brown coat color males on the AIN and NIH diets exhibited lower serum glucose concentrations than yellow coat color males on these two diets (respective to diets-brown:164.52 ± 13.54 mg/dL and, yellow: 222.43 ± 13.54 mg/dL; $P<0.0028$). Additional differences persisted at maturity with brown coat color males on the AIN diet demonstrating lower serum glucose concentrations than their yellow counterparts on the same diet (150.31±13.54 mg/dL and 199.13 ± 13.54 mg/dL, $P<0.0115$). Comparison across diets revealed that brown coat color males on the AIN and NIH diets had lower serum glucose concentrations than those on the Methyl diet (218.48 ± 13.54 mg/dL; $P<0.0053$ and $P<0.0432$). Additionally, brown males on the AIN diet had lower blood glucose concentrations than their brown counterparts on the NIH (204.8 ± 13.54 mg/dL) and Methyl (205.45 ± 13.54 mg/dL) diets (AIN vs. NIH: $P<0.0049$; AIN vs. Methyl: $P<0.0043$). This was also true for yellow males on the AIN diet versus those on the NIH (204.8 ± 13.54 mg/dL and 240.28 ± 13.54 mg/dL) and Methyl (205.45 ± 13.54 mg/dL) and Methyl (247.15 ± 13.54 mg/dL) diets (AIN vs. NIH: $P<0.0327$; AIN vs. Methyl: $P<0.0129$). Serum glucose concentrations appeared to be lower overall within the males of both coat color groups that had been on the AIN diet compared to those on the other two diets.

Discussion:

In general, mice consuming the AIN- 93G diet gained significantly more weight than those on either the NIH-31 or Methyl diet (Fig. 21). Within diets, yellow coat color females were consistently heavier than brown coat colored counterparts (Fig. 21).

However, female mice showed no differences between diet or coat color when comparing serum glucose concentration results. Male mice on the NIH-31 and Methyl diet, which is based on the former diet, had significantly higher serum glucose concentration levels than those on AIN-93G. Within diets, yellow male mice had significantly higher serum glucose concentrations than brown coat color males when on either of the diets that have been employed as controls in other studies (Cooney et al., 2002; Dolinoy et al., 2007; Wolff et al., 1998), at puberty and adulthood (Fig. 22).

Table 8 provides a possible explanation for these observations. The AIN-93G diet is typically used as a gestational diet and may not be optimal for the lifespan of the animal. As it is designed for pregnant females, this diet is higher in carbohydrates and fat, and hence calories than the NIH-31 diet, which is considered a generic diet for almost all life stages and, hence, lower in calories, fat, and carbohydrates. The methyl-donor diet is based upon the NIH-31 diet which accounts for the similar body weights across time in these two groups. The fact that differences between coat colors emerge around puberty replicates prior studies measuring obesity and serum glucose concentrations in variant A^{vy}/a coat colors (Wolff, 1978; Wolff et al., 1986).

Unexpectedly, after sexual maturity, lower serum glucose concentrations was observed in AIN males compared to males on either NIH or Methyl diets (Fig. 22). It is possible that since serum glucose concentrations were assessed during the mid-morning light cycle, mice on the NIH and Methyl diets consumed more than those on AIN during this point in the light-dark cycle. A previous study within the laboratory had shown that mice ate the same amount of food no matter the diet within a 24h period but this was not parsed out between the light and dark cycle (Sieli et al., 2011). As mice were not fasted

prior to each measurement, the values may vary dependent upon the time of blood sampling relative to food consumption. Female mice, however, showed less variation than males (Fig. 22) which is consistent with previous studies (Wolff et al, 1986;Wolff et al, 1999).

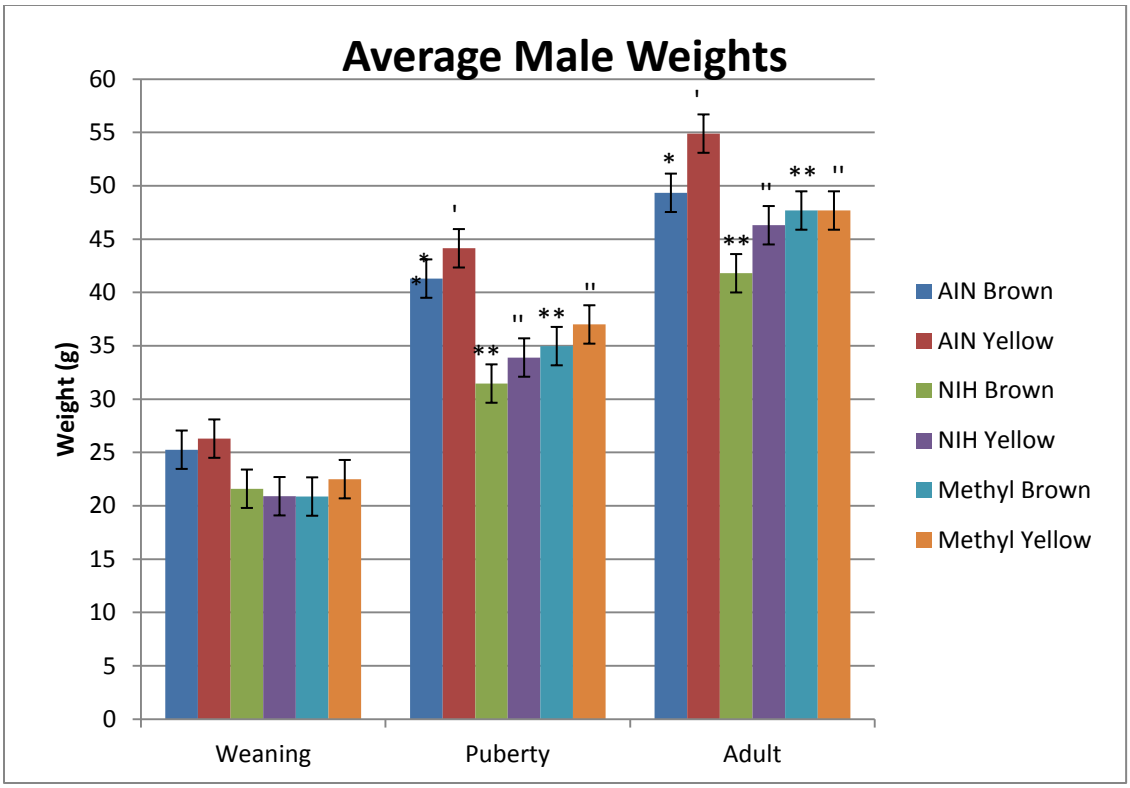
The NIH-31 diet may be a superior control diet compared to AIN93G due to its lower caloric content. Even though the ingredients are not as controlled as in the AIN-93G diet, there were fewer variations within the group when it came to serum glucose concentrations and weight (Figs 21 and 22). The AIN-93G diet with its higher caloric content increased the weight of the mice markedly than those on the NIH-31 diet. This added weight to an already obese mouse likely may impact experimental outcomes by making breeding difficult or resulting in pronounced weight gain primarily due to the particular diet. For endocrine disruptor studies, the NIH-31 diet might be reformulated to ensure phytoestrogens are minimized.

As no major phenotypic differences were observed between the different diets, it can be concluded that feeding the viable yellow mice a diet high in methyl-donors post-weaning has no effect on changing their diabetic status or obesity as they age. The predominant effect the methyl-donor diet has on viable yellow mice appears to be found mainly when they are exposed *in utero* (Cooney et al., 2002; Waterland et al., 2003; Wolff et al., 1998) and are longstanding. These results with A^{vy}/a mice are in contrast to studies with F344 male rats, who develop liver cancer when maintained on a methyl deficient diet, but addition of these key nutrients to adults can reverse their risk (Mikol et al., 1983; Pogribny et al., 2006; Wilson et al., 1984). It is possible though that the post-weaning diets to A^{vy}/a mice leads to epigenetic changes in the absence of overt

phenotypic alterations. A follow-up study examining candidate metabolic genes with either bisulfite sequencing or quantitative real time PCR (qRT-PCR) might explain the difference seen between the male blood serum glucose concentrations. It is possible that the differences seen in serum glucose concentrations could be due to epigenetic changes that are occurring solely in males. Bisulfite sequencing would illustrate the differences between mice that are known to have many methyl marks (brown coat color) and any mice that should have fewer methyl marks but may not (yellow coat color). qRT-PCR would show if there was a decrease in expression of the *agouti* gene based on the possibility of more methyl marks on the yellow mice's DNA. Findings from such an experiment would provide more evidence that what human adults consume could be affecting their epigenome.

Table 8: General Composition of the Three Diets

	AIN-93G	**NIH-31**	**Methyl**
Vit B6	0.7 g/kg	0.009 g/kg	0.009 g/kg
Vit B12	2.5 g/kg	6x10^-5 g/kg	1.5 g/kg
Zinc	1.65 g/kg	0.047 g/kg	0.661 g/kg
Betaine	0	0	15 g/kg
Choline	2.5 g/kg	1.89 g/kg	17.28 g/kg
Fat	7.20%	4.70%	4.50%
CHO	60.10%	46.80%	44.80%
Energy	3.8 Kcal/g	3 Kcal/g	2.9 Kcal/g



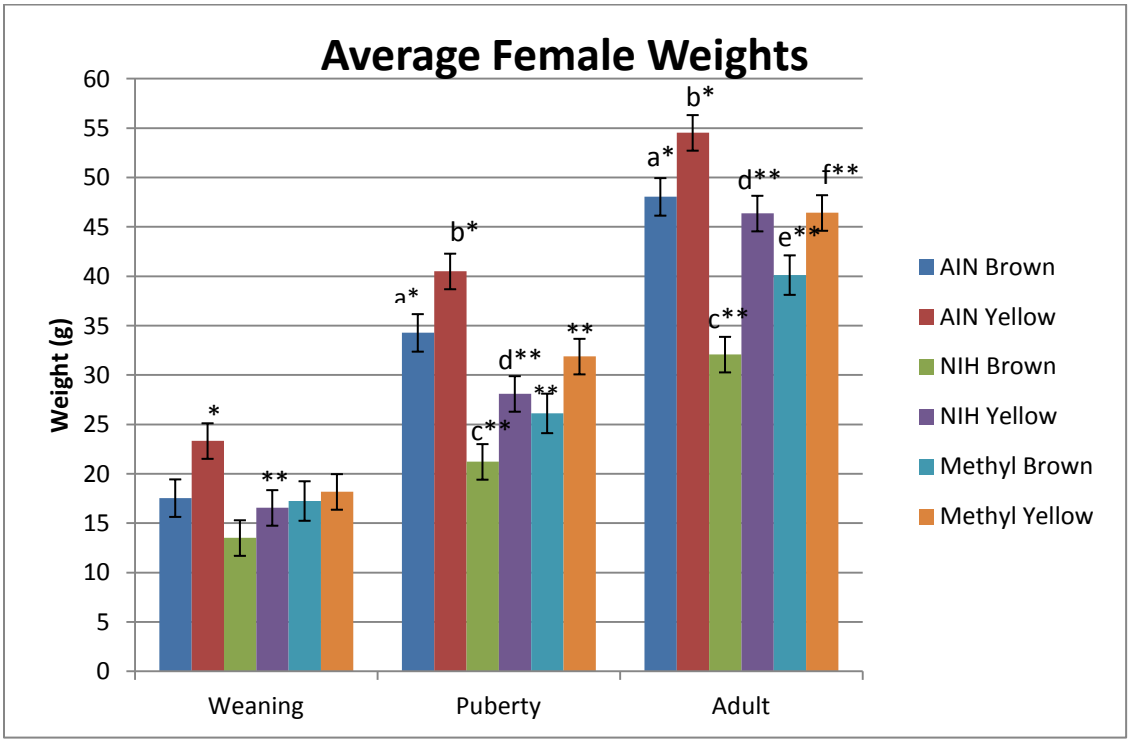


Figure 21: Average weights. [a,b] represent differences within diet. *,**: represents differences between coat color mice.

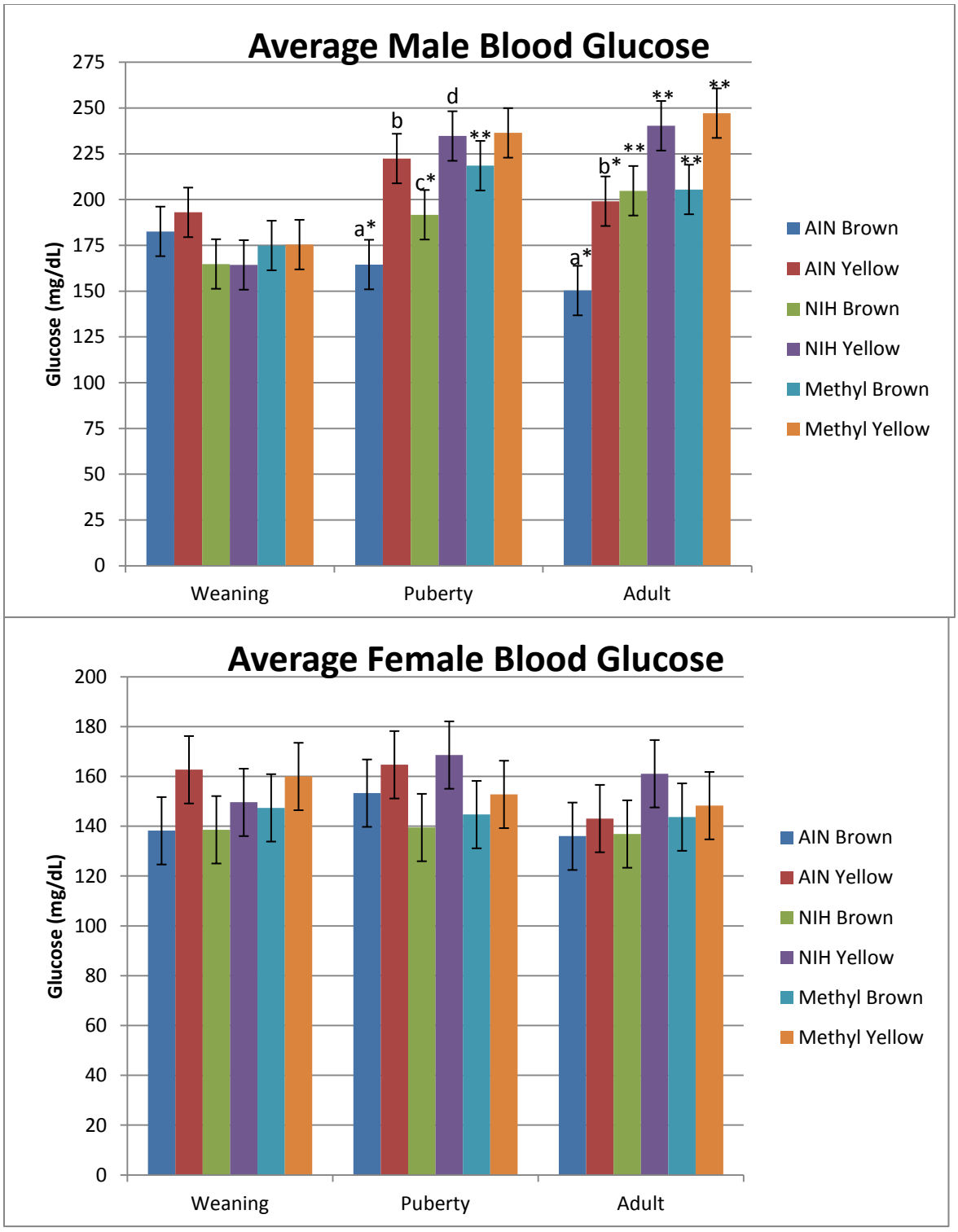


Figure 22: Average serum blood glucose concentration. [a,b] represent differences within diet. *,**: represents differences between coat colors.

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