The impact of maternal protein intake and litter size on organ and stress axis development in the house mouse (*Mus musculus*)

by

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Abstract

The phenotype of an individual is determined by interactions between its genotype and its surrounding environment. Through these interactions, an individual can express a greater range of characteristics than dictated by its genotype alone. There is strong evidence that the early-life environment can have a lasting impact on an individual's organs and physiological characteristics. The quality and quantity of food that an individual is exposed to via its mother can both impact the development of form and function of organs and be used to 'predict' its future environment and thus, determine the optimal endpoint for development in what is known as the a predictive adaptive response (PAR). This response is predicted to play a vital role in shaping an individual's life history strategy. These changes in phenotype may even be transferred across generations. However, few studies have evaluated the relative importance of the quality of an individual's mothers diet verses the quantity of food that an individual had available during development (determined by the size of the litter it was born into) on its subsequent phenotype. With my thesis I attempted to close this gap by evaluating the relative impacts of early-life food quality and quantity broadly, on the size of organs (chapter 1) and specifically, on HPA axis development (chapter 2) throughout the life on an animal.

I investigated the effects of maternal diet quantity and litter size on an individual's organ and stress axis development in semi-natural populations of house mice (*Mus musculus*). Parents were assigned a high (20%) or low (10%) protein diet. After birth, the size of the litter than each pup (F1 generation) was born into was monitored and after weaning, each pup was assigned to a

protein diet that matched (HH, LL) or did not match (HL, LH) that of it parents to evaluate possible predictive adaptive response.

For chapter 1, I measured absolute and relative organ mass of (heart, liver, spleen, kidney, abdominal fat, and testis) of the mice at three stages of life for the F1 generation (4 weeks, 8 weeks and 1 year). I also collected organ mass data for the F2 generation at 4 weeks to evaluated transgenerational effects. I found results following a possible PAR in F1 males at 8 weeks. The matched diet groups had significantly higher abdominal fat compared to the mismatched groups, potentially indicating an advantage for early breeding. Countering a PAR, the liver and kidney of both sexes and the spleen of males as well as the abdominal fat for females were mainly influenced by an individual's diet after weaning. Individuals consuming a high protein diet in adulthood displayed higher kidney and liver mass but lower spleen and abdominal fat mass. Diet quantity had a positive effect on development, with mice born into larger litters displaying higher body masses both at weaning and in females at 1 year of age. Being born into a small litter may have stimulated the development of a "grow now pay later phenotype" that stimulated rapid mass deposition when food was abundant.

For chapter 2, I measured liver and hippocampal glucocorticoid receptor (GR) levels as well as hair glucocorticoids (GC) levels. I collected data on GR and GC level for F1 mice at 4 weeks and 1 year. Though maternal diet quantity and quality show significant effects on offspring development in laboratory studies, stress axis GR development wasn't affected in wild derived mice maintain under conditions that mimic the wild. However, hair GC levels were higher in the HH group than other treatment groups. This may reflect the costs of reproduction in these females that appeared to have high reproductive performance.

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List of Abbreviations

PAR	Predictive Adaptive Response
GC	Glucocorticoid
GR	Glucocorticoid receptor
HPA	Hypothalamus-Pituitary-Adrenal

CHAPTER ONE

The impact of maternal protein intake and litter size on organ development in the house mouse (*Mus musculus*)

Introduction

Environment can play an influential role in determining an individual's phenotype (Dewitt et al. 1998; Mousseau and Fox 1998). It can determine an individual's risk of predation, its probability of finding a mate and the quantity and quality of food that it consumes. Stress, temperature, and diet, among other variables, can alter gene expression in a manner that produces a greater range of phenotypes than dictated by genotype alone (e.g., Young 2003; Uriu-Adams and Keen 2010; Park-York et al. 2012; Riek and Geiser 2012; Rueda-Clausen et al. 2012). Although these flexible responses to environment can occur throughout the life of an individual, many of the most formative responses are thought to occur during development, as organs and physiological systems develop and gain functionality in utero and through the post-natal suckling period (Fowden et al. 2006). There is strong evidence that early life environment can have a lasting impact on the physiology of an organism (e.g., Liu 1997; Gluckman and Hanson 2004; Gluckman et al. 2005) But, most studies have been conducted under tightly controlled conditions. Beyond measures of body mass (Teplitsky et al. 2008; Ozgul et al. 2010), there are few data available describing the long-term importance of the developmental environment on the physiological phenotype of animals in free-living populations.

Among the early life variables that have been evaluated, both the quantity and quality of nutrients that an individual's mother consumes during gestation and lactation have received the most attention (Hendry et al. 2001; Lycett et al. 1998). Early developmental effects can impact the developmental trajectory of the body's organs and have lasting effects on physiology (Hales and Barker 1992; Lummaa and Clutton-Brock 2002). Several studies have shown that dietary cues from the mother effect organ size and function. For example, caloric and selenium restriction by ewes negatively influence the development of liver, pancreas, small intestine,

spleen, lung and heart and reduce adipose deposition in offspring (Reed et al. 2008). Protein-restricted laboratory rats produce offspring with selective growth retardation. Specifically, the relative masses (organ mass/ body mass) of the heart and kidney were constant, and the relative masses of the liver and spleen were lower in the young of protein-restricted mothers than in the young of control mothers (Desai et al. 1996). Associated with reduction in the relative size of the liver, liver enzymatic activity changed in a manner that facilitated greater adipose deposition associated with reduced glycolysis, insulin sensitivity and increased gluconeogenesis (Desai et al. 1995). Early-life dietary effects can also be trans-generational (Bertram et al. 2008). For instance, both maternal and grand-maternal protein intake can program glucose and insulin sensitivity in lab rats (Zambrano et al. 2005).

In mammals, development can also be influenced by the amount of nutrients that mothers partition to their young. Nutrient allocation is based on an interaction between maternal capacity and offspring demand. For example, Hayden et al. (1980) showed that mammary development, and later milk yield, was positively correlated with placental lactogens in goats. Placental lactogens are produced in the placenta and thus, circulating concentrations of placental lactogens are directly correlated with the number of placentas and number of offspring in utero (Duah et al. 2013). Yet, studies in both dairy cattle and goats have shown that increased milking frequency, akin to increased suckling, increases the volume of milk produced by stimulating a proliferation of secretory tissue (e.g., Knight et al. 1990; Wilde et al. 1987; Capuco et al. 2003; Duah et al. 2013). But despite mechanisms that match litter size to nutrient allocation, the size of an individual at birth and at independence is often negatively correlated with its number of littermates (Smith et al. 1994). These effects also impact organ development and function. For example, López-Soldado et al. (2006) found that the offspring of rats rearing 16 young had substantially smaller livers and marginally smaller brains at weaning than the offspring of rats rearing 8 young. These differences were associated with reduced non-esterified fatty acid, triacylglycerol, and very lower density lipoprotein production and reduced adiposity in males reared in a litter of 16 versus 8 offspring. In contrast, litter size has no effect on lipid metabolism in females (López-Soldado et al. 2006). Following a glucose challenge, both males and females raised in a litter of 16 displayed reduced plasma insulin up to at least 16 months old (López-Soldado et al. 2006). The effects of being born into a larger litter on phenotype are thought to be consistent with maternal food deviation (López-Soldado et al. 2006), but the interactions

between food quantity and quality on phenotype have rarely been explored. In species with large and variable litter size, as in many rodents, the impact of litter size on phenotype may be particularly important.

Evaluating differences in organ mass can be a valuable method of exploring variation in phenotype among individuals, as differences in absolute and relative mass often correlate with differences in physiology (e.g., Desai et al. 1995; Desai et al. 1996; López-Soldado et al. 2006; Chappell et al. 1999; Russell and Chappell 2007) and fitness (Rezende et al. 2009; Baker et al. 2004). With this investigation, I compare the relative effects of maternal and grand-maternal protein intake and maternal litter size on an individual's body mass and organ mass in house mice (*Mus musculcus*) maintained in enclosures designed to mimic the natural social and environmental conditions experienced by wild mice.

The laboratory mouse has been an important model for studying maternal effects (e.g., Krackow and Hoeck 1989; Ozanne and Hales 2004; Johnson and Speakman 2001; Speakman and Król 2005). Yet, the laboratory mouse has been subject to approximately 100 years of selection for docility. Lab mice experience few of the natural stressors that typify the life of any free-living animal. The stress response is an important mediator of the changes in phenotype that occur in response to maternal diet and other environmental variables experienced during development (Harper 2008). A heightened responsiveness to stress in wild verses laboratory mice suggests that the stress response has been dampened by artificial selection (Drickamer 1996). Thus, it's probable that the impact of nutritional environment on early development in laboratory mice may not be a good mimic of processes in their wild counter parts or other mammals (Smith 1972; Smith et al. 1994). Indeed, in the wild, it is likely advantageous for an individual to display phenotypic plasticity throughout its life to respond to varied thermal, dietary, and social conditions (Naya et al. 2007; Kristan and Hammond 2003; Swallow et al. 2005).

For this study, I ask if the quality and quantity of food that offspring consume during development has persistent effects on offspring phenotype by comparing dietary treatment and litter size to body mass and relative organ mass just after weaning, just before the onset of reproduction, and as an animal nears 1 year of age. Mice were maintained in enclosures designed to mimic natural home range sizes and group sizes of free ranging house mice. Parents

were offered a low (10%, L) or high (20%, H) protein mouse chow. These levels have been studied extensively in laboratory mice (Fox et al. 2006) and 10% has been shown to be inadequate for optimal growth and reproduction (Nutrient Requirements of Laboratory Animals, Fourth Revised Edition, 1995). At weaning, mice were kept on the same diet as their parents (LL, HH) or given the alternate diet (LH, HL) mimicking differences in forage selection or environment that an individual may experience after weaning in the wild. Maternal food quality, or relative protein intake, is predicted to effect body mass and relative organ mass at weaning. If these effects persist after weaning, mice born to high protein mothers (HH, HL) will have persistent differences in body mass and relative organ mass compared to mice born to low protein mothers (LH, LL). If litter size has persistent effects on body mass and relative organ mass, correlations between body mass and relative organ mass described at weaning will persist through the life of the animal.

Material and methods

Study organism

The house mouse was the selected for this investigation because the effects of maternal diet on offspring physiology have been well documented in the laboratory counterpart of this species (e.g., Derrickson and Lowas 2007, Drickamer and Meikle 1987; Gheorghe et al. 2009). In addition, several investigators have shown that this species can readily be maintained under conditions that mimic the wild (e.g., Manning and Wakeland 1992; Perony et al. 2012; Meagher et al. 2000). Under semi-natural conditions, it is possible to follow animals throughout their life and determine if a mother's diet continues to play a formative and persistent role in determining offspring phenotype when individuals experience the complexity of a natural social setting.

The house mice used to found the populations described herein were obtained from out-bred colonies of mice maintained by Dr. Wayne Potts at the University of Utah. The ancestors of these mice were originally collected in Gainesville, FL and outbred under laboratory condition for 13 generations before being shipped to Auburn University for this study.

Housing and identification

Mice were maintained in 10 semi-natural enclosures on the Auburn University campus. These enclosures were designed to expose the mice to the ambient conditions that wild mice would experience if they were residing in a barn. The ten enclosures are divided between 2 side-by-side wooden buildings. Each building has a solid roof and windows covered with hardware cloth to expose the animals to ambient temperature and humidity but also, provide cover and prevent predation. Each enclosure is 5 meters squared and lined with a one-meter high aluminum flashing to prevent escapes. When the mice first arrived, I transitioned the mice onto their experimental diets within one week of arrival and kept them segregated by sex for 8 weeks before breeding groups were established. Ten breeding groups were initiated with 3 male and 7 female adult mice each. This group size mimics natural group sizes, or demes, common to wild house mice (Crowcroft and Rowe 1963; Krackow 2003). The skewed sex ratio reduced male aggression and mimicked a common social structure in natural demes, with few dominant males per group (e.g., Berry 1981; DeFries and McClearn 1970; Reimer and Petras 1967; Bronson 1979). Adult mice were pit tagged (Biomark, Inc, Boise, ID. Model: HPT12) and ear punched for individual identification.

Diet

Mice were fed a 20% (high, H) or a 10% (low, L) protein diet custom made by TestDiet (Purina Animal Nutrition, LLC., St. Louis, MO). These diets were isocaloric. Cornstarch was used to make up the difference in the caloric content of the L diet relative to the H diet (Table 1). The diets were otherwise similar and designed to meet the complete nutritional requirements of laboratory rodents (based on a modification of TestDiet AIN-93G). Before breeding, the F0 generation mice were randomly assigned to the H or L diet, exposing young to these diets via their parents and as they began to eat solid food after weaning. After weaning at 4 weeks, when all young should be fully weaned (König and Markl 1987), F1 generation mice were either maintained on the same diet as their parents (matched diet, HH or LL) or switched to the alternative diet (mismatched diet LH or HL) (fig. 1).

Breeding setup and collection dates

I placed the founding populations of mice in mixed sex groups, as described above. I waited 2 months after the first births before collecting data. I collected F1 mice to evaluate the effects of

treatment at weaning, just prior to the onset of reproduction, and at adulthood (10 months to 1 year of age). Cannibalism, abandonment, and mortality were common in first litters born in each enclosure. This may be attributed in part to primiparous mothers displaying inefficient nutrient transfer to their young (Weber et al. 2013). Because these observations are common in mice, I do not believe this poor success is attributable to diet. Two months after the first pups were born (September 2012), I retained 1 male and 1 female from each litter after weaning and moved them into a juvenile enclosure where they were maintained on a diet that was either matched or mismatched to their mothers diet. These animals were sacrificed at 8 weeks (in November 2012) to determine the impact of treatment on phenotype before the onset or reproduction. Finally, four months after the first litters were born (November 2012), I collected up to 1 male and 1 female from each litter at 4 weeks to evaluate organ characteristics (December 2012 to March 2013) and retained up to 4 young per litter (2 male, 2 female) from each litter to found the F1 breeding populations. To ensure that all females were in similar non-reproductive states, males were removed from each enclosure when females were approximately 8-10 months old (1-2 months before collection) to end all breeding activity. Females were collected at 10 months to 1 year of age, between September 2013 and December 2013.

Finally, I collected F2 pups to determine if the impacts of maternal nutrition on body mass and organ phenotype are transgenerational. The F1 weanlings retained to found the F1 breeding populations were divided such that same sex siblings were assigned to alternate diets and mixed sex siblings assigned to the same diet were not kept in the same enclosure. One male and one female pup were collected from each F2 litter that survived to weaning between June 2013 and September 2013.

The putative mother of each litter and litter size were identified based on the recorded locations of the animals and newborn pups each day during husbandry. The female found on the nest the greatest number of days was designated as the mother of the litter. When the likelihood of being a litter's mother was similar between two females, I considered both the number of days the female was on the nest during the first few days post-partum and the time between reproductive bouts, and then chose the most likely candidate at the mother of the litter.

Body mass and organ mass data

The body and organ mass of F1 and F2 mice was determined at 4 weeks of age (F1 and F2 mice), 8 weeks of age (F1 mice), and at approximately 1 year (F1 mice). Data collected at 4 weeks reflects the impact of maternal diet (H or L, for F1 and F2 mice) and maternal/grand-maternal treatment group (HH, HL, LH, or LL, for F2 only) on phenotype. Data collected at 8 weeks reflects the short-term impacts of dietary treatment, in addition to maternal diet (HH, HL, LH, or LL), on phenotype. As these animals were collected just prior to the onset of reproduction (Miller et al. 2002), the phenotype of these animals is expected to impact the timing of first reproduction. Finally, data collected at approximately 1 year, reflects the cumulative effect of maternal and adult diet as well as life's stressors, including breeding, social interactions, and aging on phenotype. Males were removed from the females, while the females remained in the enclosures 1-2 months before collection so that all returned to a non-reproductive state.

All animals included in this study were euthanized with isoflurane followed by decapitation. The sex and body mass of each individual was recorded and then the heart (not collected for 8 week samples), liver, kidney, spleen, abdominal fat (including mesenteric, epididymal, and perirenal depots), and the testes of males were carefully collected, extraneous material was removed, and then each tissue was quickly weighed to avoid desiccation (Mettler Toledo, NewClassic MF, Model: MS104S/03). The female reproductive tract was not weighed because it is difficult to collect and clean consistently.

Statistical analysis

All analyses were completed using R i386 v.3.1.1 (http://www.r-project.org/). To determine the impact of dietary treatment on organ development, I ran a linear model to test for the effects treatment and litter size have on body mass and organ mass. Significance was set at α of $P < 0.05$. Because prior work has shown that males and females often display different developmental trajectories (Swallow et al. 2005), these effects were evaluated independently for male and female mice. Independent models were also run for absolute and relative organ mass (organ mass/ body mass). By evaluating both, it is possible to determine if organ mass increased at the same rate as body mass. Absolute organ mass is reported in the tables. Relative organ mass is emphasized throughout the results and discussion sections.

Results

Treatment effects

At four weeks of age, shortly after weaning, there was no significant effect of protein treatment on body mass (Table 2). Male mice born to low protein mothers displayed higher relative abdominal fat than mice born to high protein mothers (Table 2, fig. 2a). Given that body mass did not differ between groups, this result suggests that lean mass of males born to low protein males was likely lower than that of high protein males. The relative masses of the heart, kidney and liver of both males and females and the relative testis mass of male mice were similar between treatment groups (Table 2). Litter size had a significant effect on body mass and relative abdominal fat mass in both males and females, with body mass increasing with number of litter mates in males and females (fig. 2b and 3a) and relative fat mass decreasing with number of littermates in males (fig. 2c) and increasing with number of littermates in females (fig. 3b). Testis mass also increased with litter size in males (fig. 2d).

At 8-weeks, just prior to the onset of reproductive age and one month after mice were weaned and moved onto their adult diet (match (LL, HH) or mismatch (HL, LH) to maternal diet) the body mass of male and female mice did not differ between dietary groups. However, the relative masses of the kidneys differed between groups in both males and females and relative mass of the liver, abdominal fat and spleen differed between dietary treatment for males but not females. The relative kidney mass in males was greater for those individuals consuming a high protein diet after weaning (HH, LH), relative to males consuming a low protein diet (HL, LL) (Tables 3 & 4; fig. 4a). The relative kidney mass in females was greater for those individuals consuming a high protein diet after weaning (HH, LH), relative to females under the influence of low maternal protein diet and consuming the same diet in adulthood (LL) while the HH group was also higher than females that switched into a low protein diet in adulthood (HL) (Tables 3 & 4; fig. 5). The relative mass of the spleen in males was lower in those consuming a high protein in adulthood (HH, LH) than those switching into a low protein diet after being under the influence of high maternal protein diets (HL) (Tables 3 & 4; fig. 4b). These results suggest that adult diet is most influential on male and female kidney as well as male spleen phenotype at 8-weeks. The relative mass of the male liver was highest for HH males, intermediate for the mismatched groups (HL, LH) and lowest for LL males (Tables 3 & 4; fig. 4c), suggesting diet

during development and adulthood has cumulative effects on liver phenotype. Finally, the relative mass of abdominal fat was greatest in both the HH and LL groups suggesting that those males exposed to consistent food quality through their life synthesized more body fat relative to their size than males exposed to a mismatched diet (Tables 3 & 4; fig. 4d). Litter size had no impact on body mass or organ mass at 8-weeks.

At approximately 1 year of age, I found no significant effect of dietary treatment or litter size on body mass or organ mass of males (Tables 5 & 6). In females, body mass and relative heart and spleen mass did not differ with dietary treatment, but relative liver and abdominal fat mass did. The liver was relatively higher in mass for females consuming a high protein diet (HH, LH) in adulthood relative to females switching to consuming a low protein diet after experiencing maternal high protein diet (HL) (Tables 5 & 6, fig. 6a). The relative mass of abdominal fat of females was lower for females consuming a high protein diet (HH, LH) in adulthood relative to females under constant effect of low protein maternal and adult diets (LL) (Tables 5 & 6, fig. 6b). Similar to 4-week old females, female body mass continued to display a positive correlation with litter size as females neared 1 year of age (Tables 5 & 6, fig. 6c).

For the F2 generation at 4-weeks of age, the body masses of males and females were not impacted by either maternal or grandmaternal diet (Tables 7 & 8), but both male and female body mass increased with litter size, as it did for F1 mice (Tables 7 & 8, fig. 7a, fig. 8a). Males that switched to high adult protein diets after experiencing low maternal protein diets showed higher relative liver mass than those that maintained a low protein diet as adults (LH > LL) (Tables 7 & 8; fig. 7b). Females born to mothers consuming high protein diet (HH, LH) also had relatively heavier livers and kidneys than female born to mothers consuming a low protein diet (HL, LL), suggesting the grandmaternal diet had little, if any, impact on liver or kidney mass (Tables 7 & 8; fig. 8b,c). No other effect of maternal or grandmaternal diet were observed (Tables 7 & 8).

Discussion

With this investigation I assessed the relative persistence of early-life dietary environment effects on an individual's body mass and organ mass at 3 points during the first year of its life.

Specifically, I evaluated maternal protein intake, which influences the quality of the nutrients transferred (Derrickson and Lowas 2007), and litter size, which influences the quantity of nutrients transferred (López-Soldado et al. 2006) to an individual up to weaning. The results indicate that maternal protein intake influences fat deposition in males from weaning to the onset of reproduction. Otherwise, maternal protein intake had little to no impact on body mass or organ phenotype. In contrast, the protein content of a mouse's diet after it was weaned had several effects on organ phenotype both at 8 weeks and 1 year. Assuming that relative mass of organs affects reproductive performance (Speakman and McQueenie 1996), the quality of food an individual consumes at the time of reproduction is likely have a greater impact on fitness than the quality of the diet that the individual was exposed to early in life. Litter size was correlated with the mass of select organs at weaning. Interestingly, in addition for litter size to be positively correlated with the body mass of both sexes at 4-weeks (F1 and F2 generation), F1 females also showed the same correlation as they neared 1 year of age. This result suggests that litter size may have more persistent effects on phenotype than maternal diet for female mice.

Dietary quality – maternal protein intake

Body mass and relative organ mass data were first collected at 4 weeks, after I assumed all mice had been weaned. During this time, pups were exposed to the diet of their mothers in utero and via mother's milk during suckling. In the enclosures, pups were first seen venturing from their nest, and potentially consuming some food, at 12 days of age (B. Moorer, pers. obs.). Thus, phenotype at 4-weeks could be influenced by both maternal diet and the same food that pups are starting to consume independently. Early-protein availability affected relative abdominal fat in males, but not females. After weaning, the only residual effects that maternal diet had on offspring were that males that consumed an adult diet that did not match that of their mothers carried less body fat and males that consumed mismatched diets had liver masses that were intermediate to the high and low protein matched mice. Both of these effects were found at 8-weeks but not 1 year. Animals that have greater body mass and greater adiposity often begin breeding at a younger age than those with lower fat deposition (e.g., Crocker et al. 2001; Dobson 1992; Dobson and Michener 1995). This finding is consistent with research showing body fat being linked to reproductive maturity and fertility (e.g., Bronson and Manning 1991; Kaplowitz 2008; Frisch 1984). It has been proposed that early life environment is used to predict future

conditions and that the phenotype of some organs are programmed to maximize fitness under similar conditions as predicted (Gluckman and Hanson 2004) In this case, animals that successfully predict their future environment likely have the opportunity to begin breeding earlier than mice animals that experience a dietary mismatch. Early breeding individuals have a fitness advantage over late breeding animals, as their phenotypes become infused into the population before late breeding phenotypes (Oli 2003).

Protein intake after weaning influenced the phenotype of several organs. Male and females consuming high protein diet had heavier kidneys and females consuming high protein diet had heavier livers than mice on the low protein diet. The same diet was also associated with a lower relative spleen mass in males and a lower relative abdominal fat mass in females. Dietary protein levels are positively correlated with increased nitrogenous waste. Furthermore, blood urea nitrogen levels, glomerular filtration and nitrogen filtration rate in mice are also positively correlated with protein intake (Hammond and Janes 1998). Since production and excretion of urea are linked, the masses of kidney and liver showing similar trends due to changes in dietary protein is expected (Hammond and Janes 1998). Spleen mass has been shown to increases in response to both pathogen exposure (Hadidi et al. 2008) and stress (Blanchard et al. 1995). Because difference in pathogen exposure is unlikely between groups, lower spleen mass in high protein males could reflect lower stress than in low protein males. Lower body fat in females consuming higher protein is corroborated by prior findings (Meckling and Sherfey 2007).

Several studies have shown that, at least in laboratory rodents kept under strictly controlled conditions, the impact of a mother's diet on offspring metabolic phenotype can be trans-generational (Bertram et al. 2008; Zambrano et al. 2005). In the F2 generation, grandmaternal-maternal diet matching had a role in male liver mass while maternal diet influenced female liver and kidney mass at weaning. What the mother of these young had consumed during development had a limited effect on offspring organ phenotype. Although organ mass is a less sensitive measure of physiological difference between groups when compared to variables such as hormone levels or gene expression, these findings suggest that the diet of an individual's grandmother may have only have a slight impact on performance in animals living in complex, free ranging environments.

Dietary quantity – litter size

Litter size impacted body mass (F1, F2; female F1's only a trend) and relative abdominal fat deposition (F1) of male and females mice and testis mass in male mice (F1) at weaning. Surprisingly, the effect on females persisted until females were 1 year of age. In all of these cases, the relationship between body mass and litter size was positive. In many animals, including rodents (Laurien-Kehnen and Trillmich 2003; Drummond et al. 2000; Koskela 1998), larger litter or clutch size is almost invariably associated with reduced body mass. The consistency of this effect across sexes and generations lends support to this finding under the conditions of this experiment. Thus, I believe the relationship is not an artifact of small sample size or type I statistical error. Early-life calorie restriction has been show to program phenotypes that have efficient growth and rapid mass deposition when increased food is available (e. g., Metcalfe and Monaghan 2001; Metcalfe and Monaghan 2003; Ozanne and Hales 2004). In this study, communal suckling and solid food consumption before 4-weeks of age were common. Unfortunately, I was unable to weigh the mice at birth, or any other point before weaning. This, I must assume body mass was negatively correlated with litter size at birth (Drummond et al. 2000; Andersen et al. 2011). I predict that mice born into a large litter were able to over compensate for a poor early start due to the abundance of nutritional resources available to them in this study, including other lactating females and ad lib food. It would be fascinating to determine if this effect is also true in populations of house mice that are truly wild. I predict that litter size would also be positively correlated with body mass in mice living in demes near an abundant food resource and/or having multiple females lactating at once.

The positive correlation between body mass and litter size at weaning was associated with greater fat deposition by females born into large litters while causing less fat deposition by males born into larger litters. These findings suggest that females compensated in part by depositing fat, while males compensated in part by depositing lean mass (i.e. muscle). This result was similar to the responses in lab rats, where compensatory growth caused higher body weight rats than the control group (Bieswal et al. 2006). Interesting though, male rats had higher fat mass than controls (Bieswal et al. 2006). Female fecundity often increases with body size (Roff 1992) and fat reserves (Thomas 1982) and thus, it is possible that rapid compensatory growth shifted mice toward a phenotype that would improve short-term survival chances (Metcalfe and Monaghan 2003) and reproductive success (Kirkwood and Austad 2000), even if it results in a

reduced lifespan (e.g., Metcalfe and Monaghan 2001; Metcalfe and Monaghan 2003; Rollo 2002). Future work will evaluate this relationship.

Conclusions

With this investigation, I showed that both the quality (protein manipulation) and quantity (litter size) of nutritional resources available to mice in early-life can impact body mass and organ size, with most effects only apparent until weaning. Many of these effects were sex-specific. The predictability of food resources appears to play an important role in determining male body fat, and possibly age at first reproduction. Litter size has an impact on body mass that appears to persist well into adulthood in females and thus, litter size may have formative effects on female performance throughout her life. Among the most interesting results of this study was that body mass was positively correlated with litter size at 4 weeks. Given the relatively unique practice of communal suckling in mice and ability to thrive when near an abundant food source in this study and in the wild, these observations reflect a common life history modification towards a rapid reproduction phenotype. Further work is needed to explore this relationship.

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Table 1: Macronutrient composition of high and low protein diets.

	High protein diet	Low protein diet
Protein	20.6%	10.1%
Fats	16.4%	16.2%
Carbohydrates	63.0%	73.7%
Energy	3.88 kcal/g	3.95 kcal/g

Table 2: Body mass, absolute and relative masses of organs for 4-week old F1 mice reared by mothers consuming a high (H) and low (L) protein diet. Means, standard errors, and the effects of maternal protein intake and litter size on each variable are given. All comparisons were completed using a linear models procedure. Significant results are bold.

Variable	n	*Mean + Standard Error*		Comparison (P-value)	
		H group	L group	Diet	Litter size
MALES					
Body mass (g)	18	10.6 ± 0.4	11.0 ± 0.6	0.152	**0.011**
Absolute mass (g):					
heart	16	0.086 ± 0.004	0.092 ± 0.003	0.203	0.345
kidney	18	0.169 ± 0.004	0.162 ± 0.008	0.053	0.044
liver	16	0.419 ± 0.028	0.496 ± 0.015	0.821	0.414
abdominal fat	18	0.102 ± 0.027	0.112 ± 0.021	0.091	**0.035**
testis	13	0.058 ± 0.004	0.058 ± 0.007	0.069	**0.004**
Relative mass (%):					
heart	16	0.820 ± 0.040	0.797 ± 0.042	0.297	0.179
kidney	18	1.61 ± 0.05	1.487 ± 0.041	0.713	0.129
liver	16	3.95 ± 0.237	4.29 ± 0.10	0.972	0.589
abdominal fat	18	0.917 ± 0.200	1.12 ± 0.32	**0.006**	**0.001**
testis	13	0.550 ± 0.020	0.512 ± 0.045	0.064	**0.018**
FEMALES					
Body mass (g)	23	10.0 ± 0.5	10.6 ± 0.3	0.433	**0.048**
Absolute mass (g):					
heart	18	0.078 ± 0.003	0.093 ± 0.003	**0.009**	0.259
kidney	22	0.165 ± 0.008	0.170 ± 0.005	0.635	0.204
liver	18	0.395 ± 0.041	0.441 ± 0.024	0.483	0.318
abdominal fat	23	0.057 ± 0.011	0.095 ± 0.024	0.421	**0.027**
Relative mass (%):					
heart	18	0.781 ± 0.043	0.870 ± 0.029	0.128	0.837
kidney	22	1.651 ± 0.061	1.619 ± 0.036	0.682	0.660
liver	18	3.906 ± 0.248	4.096 ± 0.142	0.589	0.560
abdominal fat	23	0.557 ± 0.082	0.826 ± 0.173	0.431	**0.038**

Table 3: Body mass and the absolute and relative mass of organs for 8-week old F1 mice relative to dietary treatment group (HH, HL, LH, LL). Means and standard errors are given.

Variable	n	HH	HL	LH	LL
MALES					
Body mass (g)	31	15.8 ± 0.7	15.6 ± 0.6	15.2 ± 0.8	16.2 ± 0.5
Absolute mass (g):					
kidney	31	0.303 ± 0.013	0.241 ± 0.015	0.292 ± 0.019	0.258 ± 0.017
liver	31	0.785 ± 0.032	0.744 ± 0.053	0.695 ± 0.064	0.688 ± 0.042
abdominal fat	31	0.106 ± 0.021	0.050 ± 0.009	0.061 ± 0.006	0.139 ± 0.022
spleen	31	0.032 ± 0.002	0.054 ± 0.009	0.039 ± 0.004	0.049 ± 0.008
Relative mass (%):					
kidney	31	1.92 ± 0.05	1.54 ± 0.06	1.92 ± 0.05	1.59 ± 0.07
liver	31	4.97 ± 0.13	4.73 ± 0.21	4.55 ± 0.29	4.24 ± 0.16
abdominal fat	31	0.668 ± 0.137	0.319 ± 0.054	0.399 ± 0.021	0.864 ± 0.141
spleen	31	0.203 ± 0.011	0.336 ± 0.050	0.256 ± 0.024	0.300 ± 0.042
FEMALES					
Body mass (g)	39	12.2 ± 0.2	12.6 ± 0.6	12.7 ± 0.2	12.8 ± 0.5
Absolute mass (g):					
kidney	39	0.198 ± 0.004	0.183 ± 0.006	0.199 ± 0.007	0.184 ± 0.007
liver	39	0.475 ± 0.014	0.488 ± 0.038	0.465 ± 0.021	0.464 ± 0.027
abdominal fat	39	0.087 ± 0.014	0.057 ± 0.012	0.035 ± 0.008	0.062 ± 0.013
spleen	39	0.019 ± 0.001	0.019 ± 0.004	0.021 ± 0.003	0.024 ± 0.005
Relative mass (%):					
kidney	42	1.62 ± 0.03	1.47 ± 0.03	1.58 ± 0.19	1.45 ± 0.04
liver	42	3.89 ± 0.10	3.86 ± 0.13	3.68 ± 0.20	3.63 ± 0.15
abdominal fat	42	0.708 ± 0.114	0.462 ± 0.098	0.286 ± 0.073	0.486 ± 0.100
spleen	42	0.157 ± 0.006	0.147 ± 0.026	0.169 ± 0.026	0.184 ± 0.036

Table 4: Result (P-valves) of statistical comparisons of body mass and the absolute and relative masses of organs for 8-week old F1 mice by dietary treatment (HH, LL, HL, LH) and litter size. All comparisons were completed using a linear models procedure. Significant results are bold.

Variable	*Dietary treatment*						Litter
	HH-HL	HH-LH	HH-LL	HL-LH	HL-LL	LH-LL	size
MALES							
Body mass	0.825	0.547	0.873	0.662	0.759	0.352	0.852
Absolute mass:							
kidney	**0.010**	0.638	0.124	0.173	0.719	0.197	0.822
liver	0.632	0.112	0.089	0.215	0.18	0.921	0.271
abdominal fat	**0.010**	0.544	0.062	0.216	**0.001**	**0.004**	0.183
spleen	**0.024**	0.719	0.727	0.059	0.202	0.39	0.183
Relative mass:							
kidney	**<0.001**	0.872	**0.004**	**0.005**	0.777	**0.001**	0.806
liver	0.459	**0.043**	**0.005**	0.131	**0.022**	0.310	0.118
abdominal fat	**0.009**	0.608	0.059	0.176	**0.001**	**0.005**	0.159
spleen	**0.010**	0.817	0.714	**0.044**	0.130	0.469	0.137
FEMALES							
Body mass	0.512	0.604	0.462	0.96	0.817	0.847	0.874
Absolute mass:							
kidney	0.084	0.706	0.075	0.408	0.641	0.157	0.351
liver	0.707	0.971	0.962	0.826	0.825	0.992	0.642
abdominal fat	0.117	0.107	0.509	0.633	0.616	0.287	0.571
spleen	0.951	0.456	0.216	0.507	0.261	0.640	0.462
Relative mass:							
kidney	**0.003**	0.209	**0.001**	0.364	0.323	**0.042**	0.245
liver	0.815	0.687	0.527	0.823	0.666	0.831	0.371
abdominal fat	0.097	0.086	0.421	0.603	0.666	0.299	0.583
spleen	0.717	0.396	0.193	0.296	0.141	0.673	0.316

Table 5: Body mass and the absolute and relative mass of organs for 1-year old F1 mice relative to dietary treatment group (HH, HL, LH, LL). Means and standard errors are given.

Variable	n	HH	HL	LH	LL
MALES					
Body mass (g)	13	26.1 ± 1.1	25.5 ± 4.0	24.6 ± 1.9	27.2 ± 2.5
Absolute mass (g):					
heart	13	0.156 ± 0.011	0.145 ± 0.003	0.159 ± 0.032	0.161 ± 0.009
kidney	11	0.420 ± 0.045	0.345 ± 0.015	0.394 ± 0.008	0.397 ± 0.050
liver	11	1.25 ± 0.16	0.909 ± 0.074	0.981 ± 0.111	1.25 ± 0.13
abdominal fat	13	0.390 ± 0.094	0.821 ± 0.747	0.245 ± 0.052	0.666 ± 0.364
spleen	13	0.040 ± 0.002	0.046 ± 0.0167	0.037 ± 0.001	0.055 ± 0.008
Relative mass (%):					
heart	13	0.600 ± 0.035	0.584 ± 0.106	0.637 ± 0.092	0.601 ± 0.041
kidney	11	1.57 ± 0.13	1.38 ± 0.16	1.73 ± 0.04	1.47 ± 0.17
liver	11	4.66 ± 0.44	3.61 ± 0.27	4.29 ± 0.3	4.61 ± 0.37
abdominal fat	13	1.47 ± 0.32	2.83 ± 2.49	0.976 ± 0.135	2.19 ± 1.00
spleen	13	0.152 ± 0.008	0.174 ± 0.038	0.152 ± 0.016	0.209 ± 0.037
FEMALES					
Body mass (g)	36	26.6 ± 1.3	25.8 ± 0.3	26.8 ± 1.3	26.0 ± 0.7
Absolute mass (g):					
heart	36	0.193 ± 0.01	0.166 ± 0.009	0.184 ± 0.009	0.175 ± 0.006
kidney	36	0.432 ± 0.02	0.350 ± 0.025	0.397 ± 0.026	0.367 ± 0.015
liver	36	1.42 ± 0.13	1.10 ± 0.04	1.41 ± 0.08	1.27 ± 0.08
abdominal fat	36	0.380 ± 0.052	0.712 ± 0.115	0.415 ± 0.075	0.979 ± 0.197
spleen	36	0.093 ± 0.031	0.073 ± 0.015	0.065 ± 0.012	0.058 ± 0.004
Relative mass (%):					
heart	36	0.732 ± 0.067	0.644 ± 0.040	0.700 ± 0.039	0.676 ± 0.021
kidney	36	1.63 ± 0.11	1.36 ± 0.10	1.50 ± 0.10	1.41 ± 0.04
liver	36	5.32 ± 0.25	4.27 ± 0.17	5.30 ± 0.19	4.88 ± 0.29
abdominal fat	36	1.45 ± 0.27	2.75 ± 0.45	2.16 ± 0.64	3.68 ± 0.71
spleen	36	0.356 ± 0.126	0.283 ± 0.061	0.238 ± 0.038	0.226 ± 0.016

Table 6: Result (P-valves) of statistical comparisons of body mass and the absolute and relative masses of organs for 1-year old F1 mice by dietary treatment (HH, LL, HL, LH) and litter size. All comparisons were completed using a linear models procedure. Significant results are bold.

Variable	*Dietary treatment*						Litter size
	HH-HL	HH-LH	HH-LL	HL-LH	HL-LL	LH-LL	
MALES							
Body mass	0.928	0.998	0.767	0.937	0.843	0.788	0.765
Absolute mass:							
heart	0.922	0.709	0.595	0.684	0.559	0.860	0.424
kidney	0.541	0.962	0.839	0.668	0.459	0.837	0.679
liver	0.270	0.715	0.759	0.567	0.214	0.561	0.552
abdominal fat	0.425	0.796	0.788	0.357	0.341	0.977	0.786
spleen	0.574	0.725	0.539	0.430	0.941	0.403	0.947
Relative mass:							
heart	0.948	0.769	0.430	0.845	0.490	0.623	0.520
kidney	0.585	0.701	0.574	0.449	0.329	0.935	0.705
liver	0.196	0.986	0.377	0.292	0.086	0.484	0.643
abdominal fat	0.451	0.778	0.789	0.368	0.362	0.995	0.765
spleen	0.554	0.648	0.294	0.370	0.619	0.200	0.659
FEMALES							
Body mass	0.950	0.738	0.800	0.605	0.814	0.379	**0.018**
Absolute mass:							
heart	0.129	0.740	0.275	0.091	0.445	0.259	0.361
kidney	0.114	0.465	0.189	0.199	0.574	0.385	0.976
liver	0.126	0.961	0.423	**0.031**	0.251	0.208	0.267
abdominal fat	0.306	0.968	**0.043**	0.171	0.228	**0.004**	0.502
spleen	0.751	0.409	0.111	0.529	0.110	0.247	0.110
Relative mass:							
heart	0.157	0.628	0.393	0.174	0.359	0.587	0.258
kidney	0.064	0.310	0.161	0.187	0.395	0.569	0.069
liver	0.083	0.904	0.486	**0.025**	0.116	0.392	0.554
abdominal fat	0.274	0.953	**0.037**	0.114	0.228	**0.002**	0.324
spleen	0.747	0.303	0.124	0.373	0.129	0.450	0.318

Table 7: Body mass and the absolute and relative mass of organs for 4-week old F2 mice relative to dietary treatment group (HH, HL, LH, LL). Means and standard errors are given.

Variable	n	HH	HL	LH	LL
MALES					
Body mass (g)	89	11.7 ± 0.4	11.9 ± 0.5	11.8 ± 0.3	11.7 ± 0.4
Absolute mass in g:					
heart	42	0.077 ± 0.004	0.078 ± 0.004	0.072 ± 0.002	0.072 ± 0.003
kidney	42	0.170 ± 0.003	0.152 ± 0.007	0.180 ± 0.008	0.169 ± 0.012
liver	89	0.427 ± 0.033	0.461 ± 0.022	0.466 ± 0.013	0.420 ± 0.015
abdominal fat	37	0.130 ± NA	0.177 ± 0.035	0.141 ± 0.023	0.171 ± 0.034
spleen	41	0.026 ± 0.005	0.029 ± 0.003	0.023 ± 0.001	0.027 ± 0.002
testis	33	0.060 ± NA	0.070 ± 0.009	0.068 ± 0.006	0.067 ± 0.006
Relative mass (%):					
heart	42	0.645 ± 0.031	0.667 ± 0.044	0.619 ± 0.011	0.623 ± 0.010
kidney	42	1.41 ± 0.12	1.35 ± 0.05	1.54 ± 0.08	1.41 ± 0.09
liver	89	3.64 ± 0.25	3.92 ± 0.18	3.94 ± 0.06	3.64 ± 0.12
abdominal fat	37	1.20 ± NA	1.35 ± 0.22	1.13 ± 0.14	1.40 ± 0.22
spleen	41	0.221 ± 0.068	0.251 ± 0.032	0.204 ± 0.011	0.236 ± 0.017
testis	33	0.557 ± NA	0.567 ± 0.086	0.561 ± 0.038	0.557 ± 0.052
FEMALES					
Body mass (g)	82	11.3 ± 0.5	11.0 ± 0.4	11.8 ± 0.2	11.4 ± 0.4
Absolute mass in g:					
heart	39	0.066 ± 0.003	0.071 ± 0.002	0.071 ± 0.001	0.071 ± 0.002
kidney	37	0.157 ± 0.005	0.139 ± 0.006	0.169 ± 0.004	0.154 ± 0.006
liver	82	0.461 ± 0.021	0.411 ± 0.016	0.455 ± 0.012	0.402 ± 0.017
abdominal fat	32	0.103 ± 0.033	0.255 ± 0.108	0.135 ± 0.022	0.246 ± 0.068
spleen	39	0.024 ± 0.001	0.030 ± 0.006	0.024 ± 0.001	0.027 ± 0.001
Relative mass (%):					
heart	39	0.634 ± 0.038	0.640 ± 0.043	0.635 ± 0.019	0.611 ± 0.026
kidney	37	1.48 ± 0.08	1.25 ± 0.05	1.47 ± 0.03	1.29 ± 0.06
liver	82	4.14 ± 0.25	3.75 ± 0.12	3.88 ± 0.07	3.56 ± 0.10
abdominal fat	32	0.930 ± 0.278	2.17 ± 0.10	1.13 ± 0.17	2.19 ± 0.77
spleen	39	0.225 ± 0.018	0.269 ± 0.044	0.212 ± 0.014	0.232 ± 0.019

Table 8: Result (P-valves) of statistical comparisons of body mass and the absolute and relative masses of organs for 4-week old F2 mice by maternal dietary treatment (HH, LL, HL, LH) and litter size. All comparisons were completed using a linear models procedure. Significant results are bold.

Variable	*Diet Comparisons*						
	HH-HL	HH-LH	HH-LL	HL-LH	HL-LL	LH-LL	Litter size
MALES							
Body mass	0.964	0.750	0.714	0.530	0.515	0.908	**0.036**
Absolute mass:							
heart	0.930	0.389	0.418	0.137	0.195	0.946	0.360
kidney	0.411	0.921	0.751	0.063	0.391	0.403	0.330
liver	0.487	0.502	0.727	0.925	0.101	0.063	0.201
abdominal fat	0.888	0.817	0.970	0.313	0.800	0.445	0.472
spleen	0.499	0.857	0.700	0.108	0.6110	0.2430	0.628
testis	0.850	0.645	0.634	0.525	.537	.939	0.116
Relative mass:							
heart	0.651	0.816	0.853	0.185	0.258	0.930	0.557
kidney	0.816	0.567	0.983	0.110	0.663	0.291	0.874
liver	0.281	0.215	0.946	0.843	0.111	**0.035**	0.629
abdominal fat	0.964	0.763	0.938	0.344	0.932	0.285	0.644
spleen	0.438	0.990	0.5540	0.148	0.750	0.2260	0.259
testis	0.698	0.560	.554	0.644	0.646	.945	0.184
FEMALES							
Body mass	0.445	0.822	0.624	0.186	0.684	0.274	**<0.001**
Absolute mass:							
heart	0.332	0.283	0.282	0.961	0.983	0.931	0.471
kidney	0.083	0.277	0.662	**0.001**	0.117	0.057	0.284
liver	0.080	0.571	**0.021**	0.077	0.645	**0.005**	**0.017**
abdominal fat	0.118	0.550	0.104	0.168	0.933	0.130	0.392
spleen	0.212	0.964	0.540	0.101	0.406	0.368	0.742
Relative mass:							
heart	0.745	0.678	0.822	0.959	0.504	0.352	0.093
kidney	**0.017**	0.868	**0.043**	**0.001**	0.554	**0.004**	0.258
liver	0.089	0.201	**0.006**	0.394	0.289	**0.014**	0.531
spleen	0.246	0.904	0.771	0.108	0.287	0.576	0.349
abdominal fat	0.153	0.517	0.109	0.254	0.953	0.158	0.126

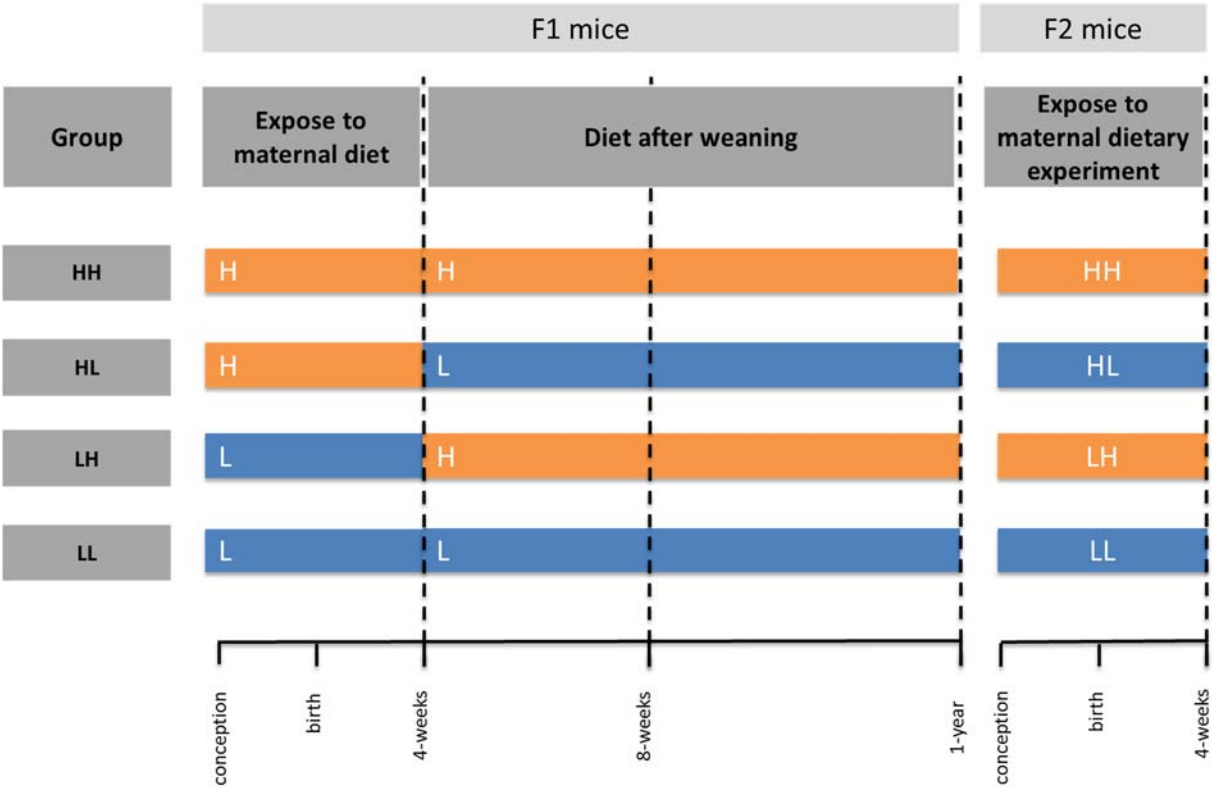

Figure 1. Timeline, dietary treatment groups, and collection events (dash lines) for this experiment (H=High protein diet: 20%; L=Low protein diet 10%)

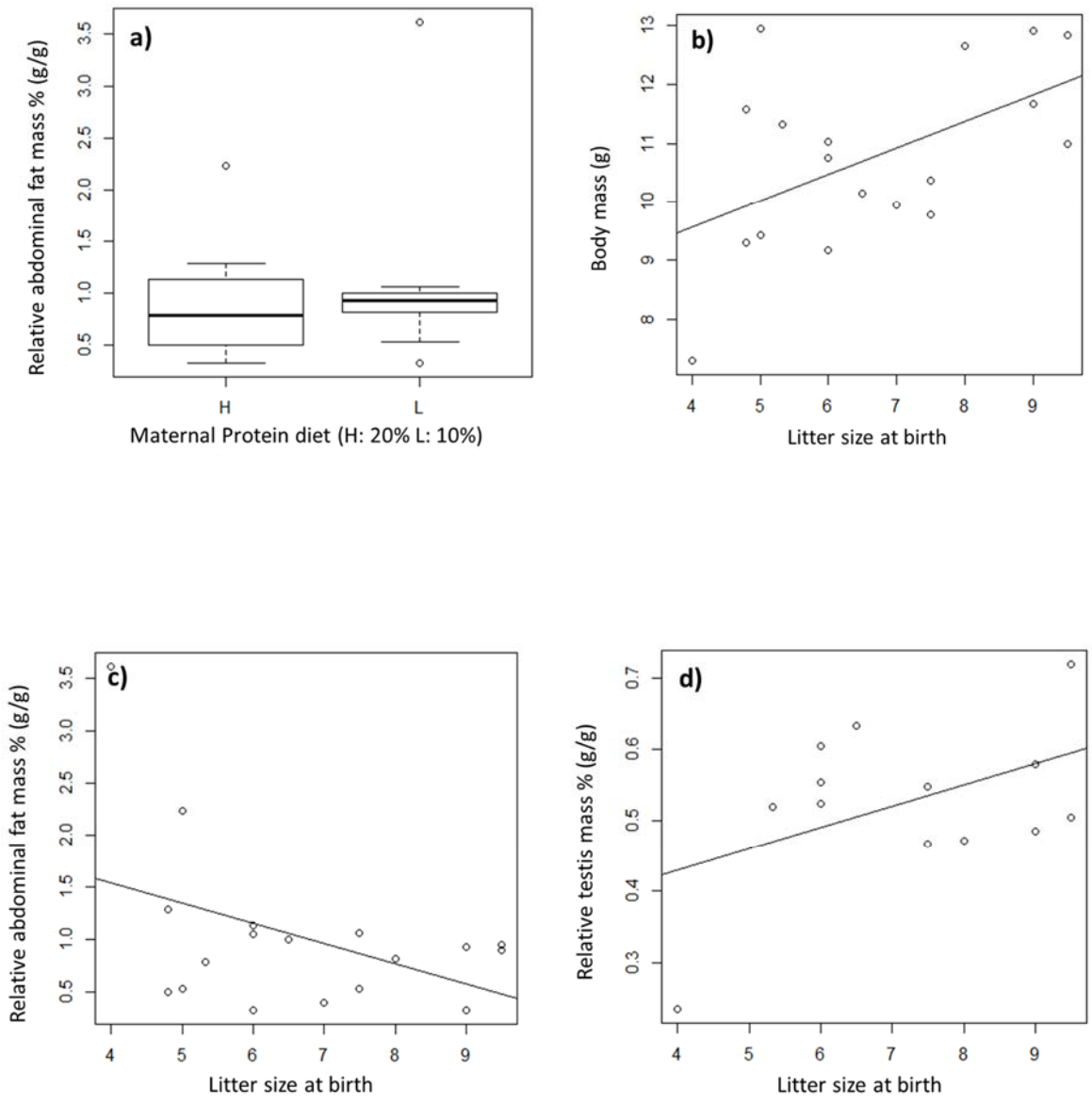

Figure 2. Comparison of maternal protein intake (a) and litter size (b-d) on the body mass and relative organ mass in F1 males at 4-weeks. Data for body mass (b), relative abdominal fat mass (a, c), and testis mass (d) are given. Only significant differences are shown.

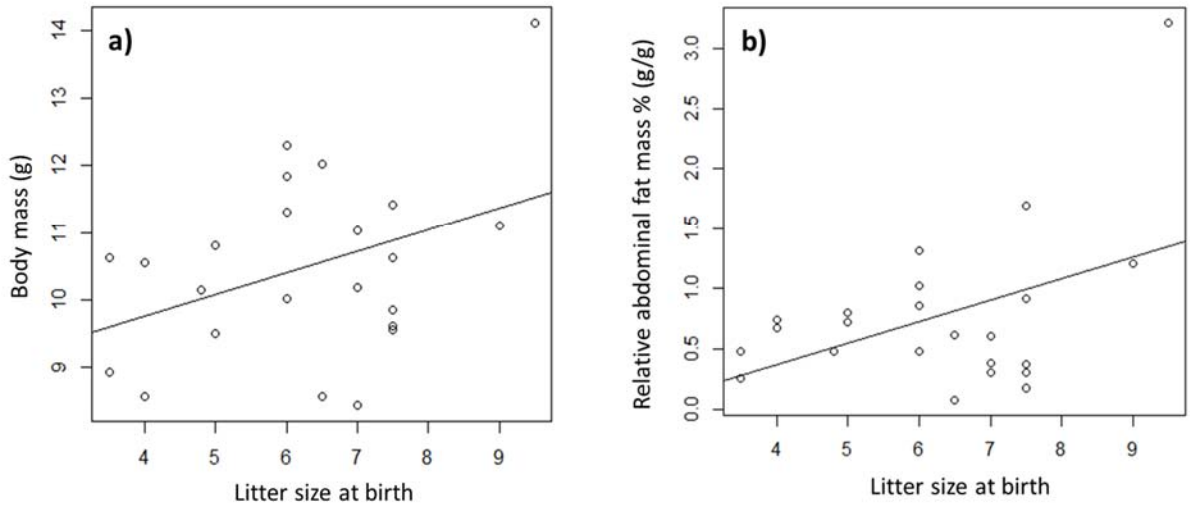

Figure 3. Comparison of litter size (a-b) on the body mass and relative organ mass in F1 females at 4-weeks. Data for body mass (a) and relative abdominal fat mass (b) are given. Only significant differences are shown.

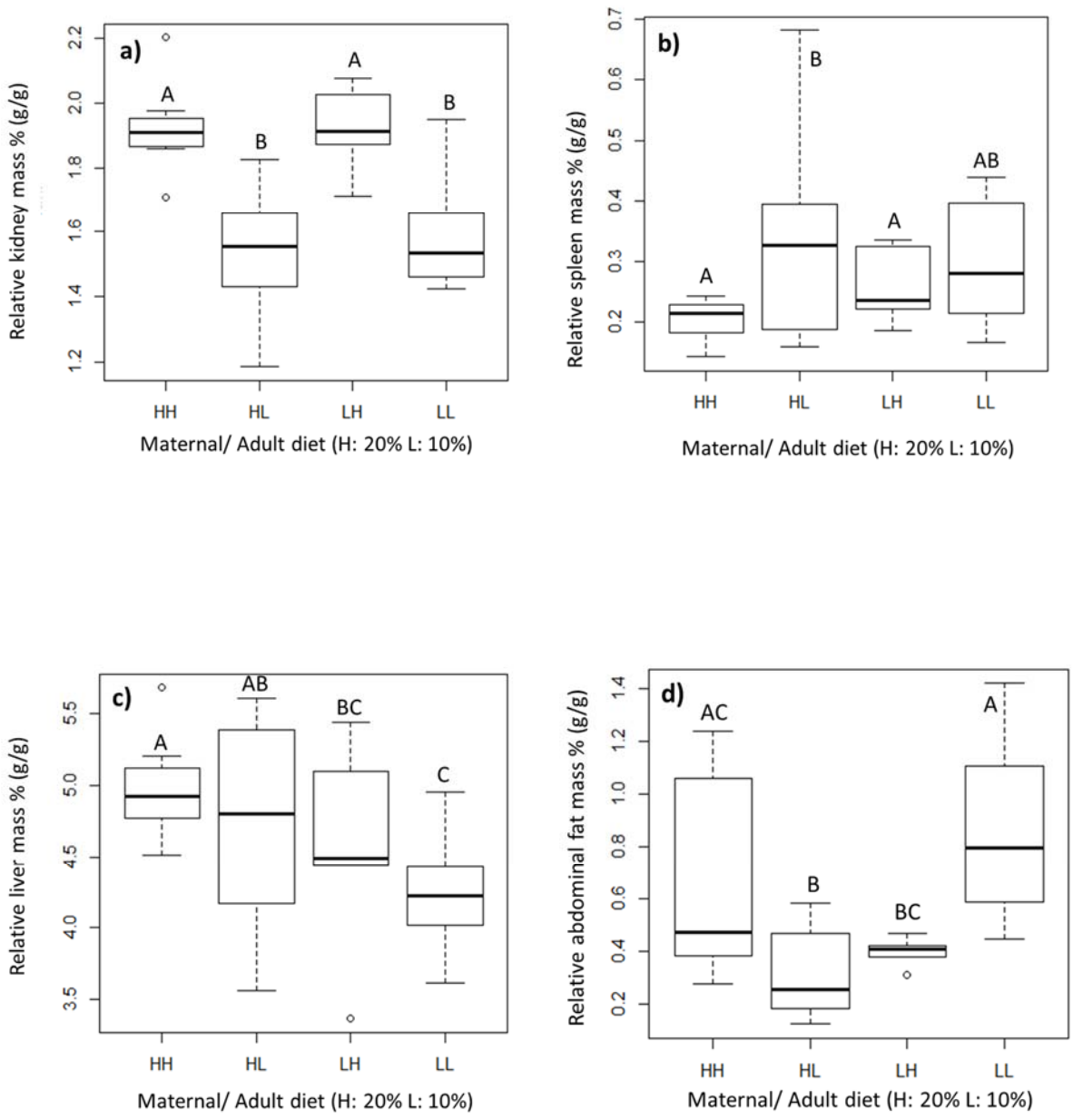

Figure 4**.** Comparison of maternal protein intake (a-d) on the relative organ mass in F1 males at 8-weeks. Data for relative kidney mass (a), relative spleen mass (b), relative liver mass (c), and relative abdominal fat mass (d) are given. Only significant differences are shown.

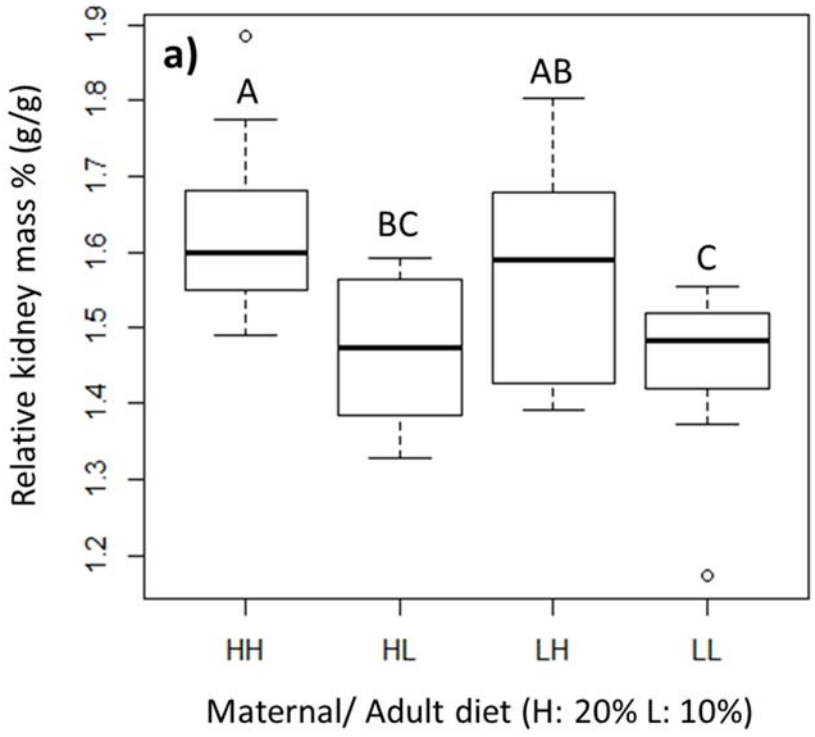

Figure 5. Comparison of maternal protein intake on relative kidney mass in F1 females at 8-weeks. Only significant differences are shown.

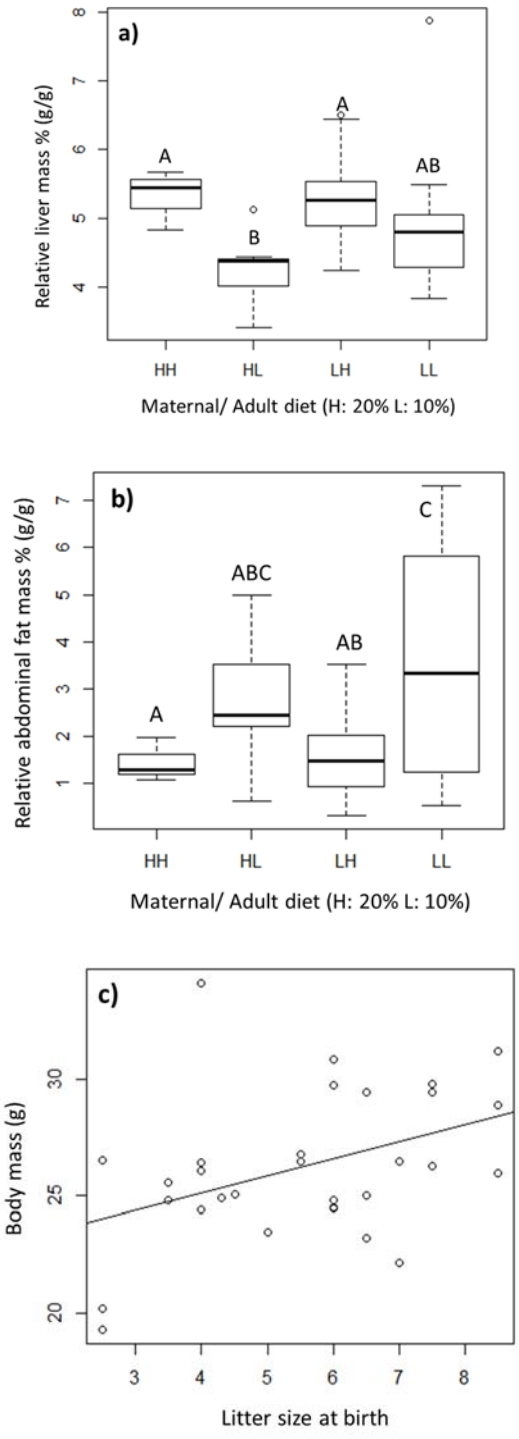

Figure 6. Comparison of maternal protein intake (a, b) and litter size (c) on the body mass and relative organ mass in F1 adult females at approximately 1 year of age. Data for relative liver mass (a), relative abdominal fat mass (b), and body mass (c) are given. Only significant differences are shown.

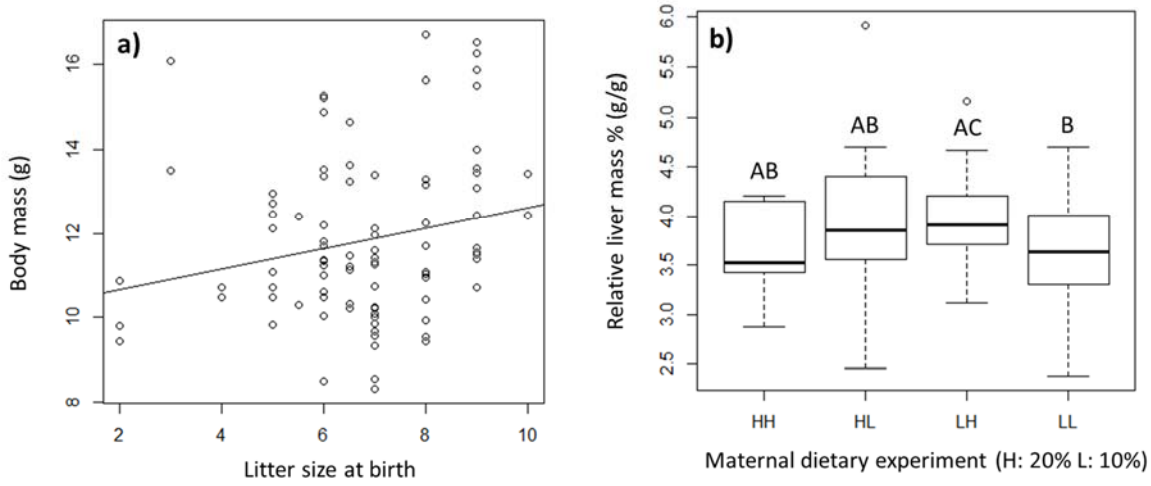

Figure 7. Comparison of litter size (a) and maternal protein intake (b) on the body mass and relative organ mass in F2 males at 4-weeks. Data for body mass (a), and relative liver mass (b) are given. Only significant differences are shown.

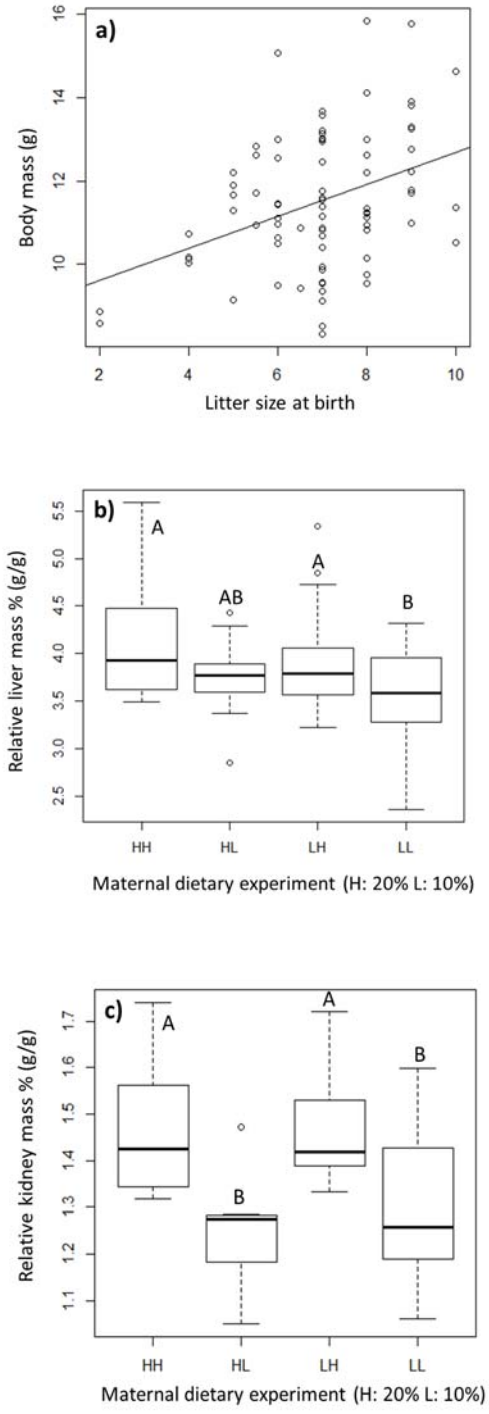

Figure 8. Comparison of litter size (a) and maternal protein intake (b, c) on the body mass and relative organ mass in F2 females at 4-weeks. Data for body mass (a), relative liver mass (b), and relative kidney mass (c) are given. Only significant differences are shown.

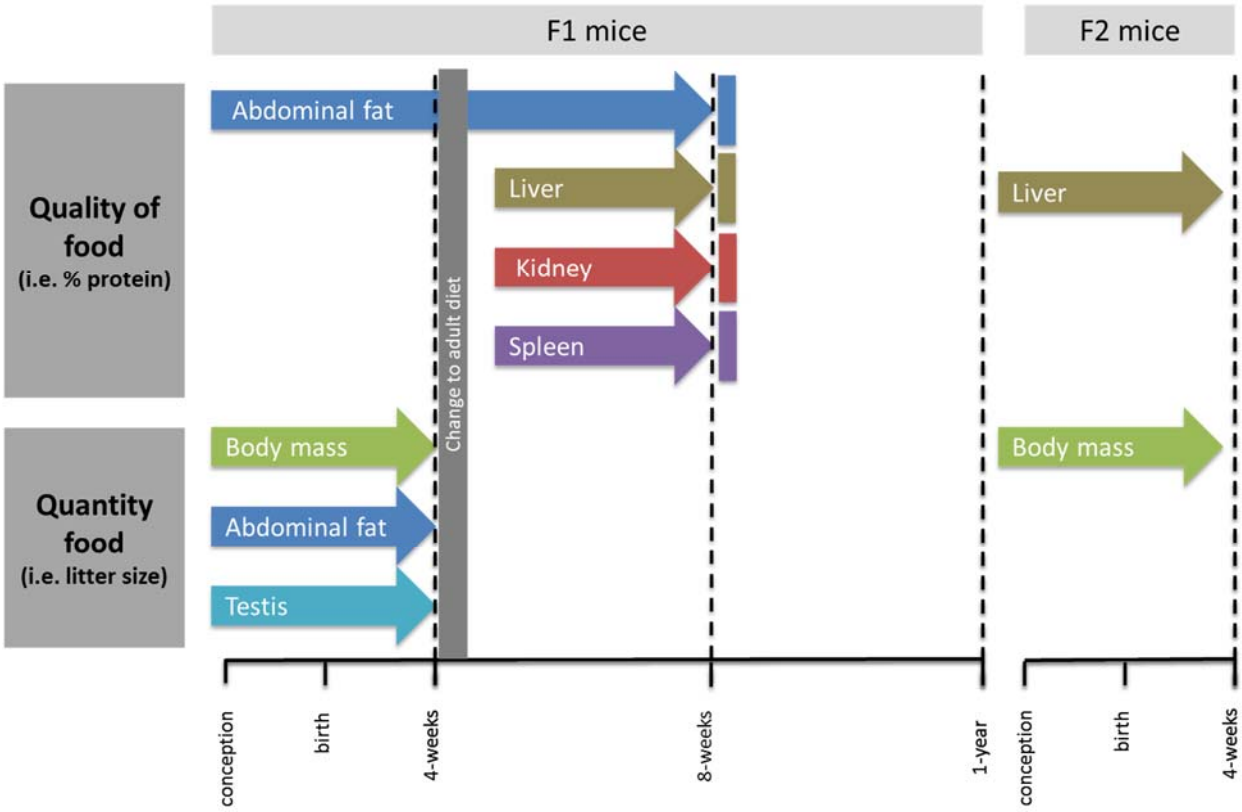

Figure 9. Summary of the significant effects of dietary treatment and litter mass on the body mass and relative organ masses of male mice. Only significant relationships are reported. The dash line notes a sample collection event.

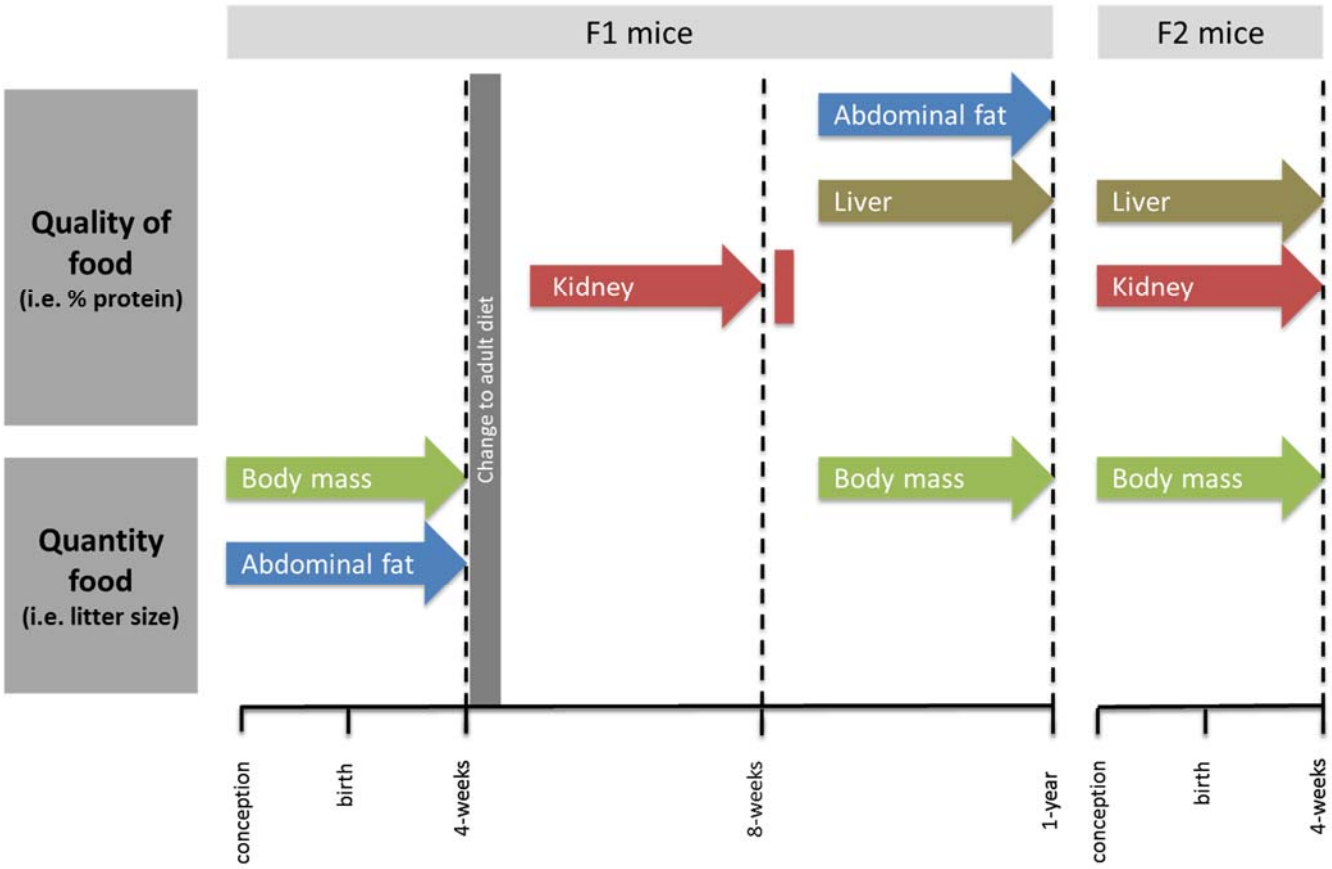

Figure 10. Summary of the significant effects of dietary treatment and litter mass on the body mass and relative organ masses of female mice. Only significant relationships are reported. The dash line notes a sample collection event.

CHAPTER TWO

The impact of maternal protein intake on offspring stress axis development in the house mouse (*Mus musculus*)

Introduction

An individual's phenotype is determined, in part, by its surrounding environment (Via et al. 1995). The environment can play a key role in an individual's survival, fitness and reproductive success. Variables including stress, temperature, diet and others, can change gene expression in a manner that lets an individual express a more diverse range of phenotypes than those purely dictated by their genotype (e. g., Young 2003; Riek and Geiser 2012; Uriu-Adams and Keen 2010; Park-York et al. 2012; Rueda-Clausen et al. 2012). Although flexible responses to environmental conditions can occur throughout the life of an individual, many of the most formative responses occur during development as physiological systems, such as the neuroendocrine axes, gain functionality. Notably, the quality and quantity of food that a mother consumes has been shown to impact the development of offspring metabolic (McMillen and Robinson 2005), cardiovascular (Torrens et al. 2006) and reproductive systems (Zambrano et al. 2005). In chapter one, I found the development of organ masses differed due to maternal and adult diet at different stages in both sexes of house mice (Chen per obs). Many of these organs, either independently or collectively, influence an individual's metabolic output, cardiovascular ability and reproductive success in mammals (e. g., Russell and Chappell 2007; Rezende et al. 2009, Selman et al. 2001; Speakman and McQueenie 1996; Johnston et al. 2007). In addition, both dietary intake restriction (Lesage et al. 2001; Lesage et al. 2006), and protein restriction (Lillycrop et al. 2005; Lillycrop et al. 2007; Langley-Evans et al. 1996) can affect metabolic, cardiovascular and reproductive systems indirectly by altering the stress response via the hypothalamic-pituitary-adrenal (HPA) axis, which has a vital role in many systemic and phenotypic alterations (Tsigos & Chrousos 2002). Though dietary manipulation and HPA axis development have been studied previously, the critical window of effect of dietary factors on the HPA axis in natural environments is still poorly understood.

The HPA axis is the main stress regulatory system in mammals (Veenema et al. 2003). It consists of three main regions, the hypothalamus and pituitary gland, located in the brain, and the adrenal gland, located above the kidneys. Intricate sets of direct effects and feedback interactions occur to regulate the stress response (Smith & Vale 2006). Glucocorticoids (GC), the downstream product of the HPA axis, bind with glucocorticoid receptors (GR) and elicit a specific response in the target tissue. GR in the hippocampus play an important role in negative feedback sensitivity (Liu 1997) and, hippocampus development is shown to be mediated by maternal diet during gestation and suckling, as maternal food restriction decreases the hippocampal GR in rats (Lesage et al. 2001). In addition, hepatic GR influence metabolic output through regulating energy balance (Nieuwenhuizen & Rutters 2008) and gluconeogenesis capacity (Yoon et al. 2001). Significantly higher levels of hepatic GR are observed in rats with mothers under dietary protein restriction (Lillycrop et al. 2005). Higher levels of hepatic GR produce a metabolic phenotype that shows altered stress response in adults with a propensity for cardiovascular and metabolic disease (Seckl 2001). Furthermore, many other studies have also supported the notion that HPA axis development is strongly affected by an individual's early life maternal influence (e. g., Lesage et al. 2006; Levine et al. 1991; Sanchez 2006).

It is posited that maternal effects play a role in adaptive prenatal programming of the HPA axis (e. g., Love and Williams 2008; Weinstock 2005; Weinstock 2008). This pattern is known as a 'Predictive Adaptive Response' (Gluckman and Hanson, 2004; Gluckman et al., 2005). These phenotypic responses are due to an individual's early life environmental cues, which act as a predictor of the future environment, leading to a modified phenotype expression. These adjustments are then favorable for the individual if it is maintained under comparable adult environments (Gluckman et al. 2005). Predictive Adaptive Response has been observed in stress physiology development studies in laboratory mammals (e. g., Seckl 2001; Seckl 2004; Liu 1997; Meaney 2001). On the other hand, metabolic dysfunction has been seen in mismatched developmental and adult environments (Hales & Barker 2001) and its cause may be linked to impaired HPA axis development (Levitt et al. 2000).

A characteristic of individuals with negative maternal effects (ex. less maternal care, maternal diet restriction) is a prolonged stress-induced GC response (Augustyniak et al. 2010; Barbazanges et al. 1996). As decreased hippocampal GR has been linked to these prolonged

stress responses (Maccari et al. 1991), chronic stress in these individuals might be associated with negative feedback insensitivity caused by lowered hippocampal GR (Liu 1997). Because chronic stress levels has also shown to be an indicator of numerous metabolic and reproductive dysfunctions (Tsigos & Chrousos 2002; Chrousos et al. 1998), measuring chronic stress levels in conjunction with GR levels can give us a comprehensive understanding of the development and functionality of an individual's HPA axis. Fairly recently, measurements of GC levels in hair has emerged as a viable option for quantifying the amount of stress an individual has experienced over time (Meyer & Novak 2012; Russell et al. 2012). It has been used to measure chronic stress in a wide variety of mammals, including bears (MacBeth et al. 2010; Bechshoft et al 2011), hyraxes (Koren et al. 2008), dogs (Ouschan et al. 2013) and chipmunks (Martin & Reale 2008).

With this investigation, I evaluated the impact of maternal and adult protein intake on an individual's hepatic and hippocampal GR and hair GC in house mice (*Mus musculcus*) maintained in enclosures designed to mimic the natural social and environmental conditions experienced by wild mice. In addition, I ask if the number of littermates plays a role in stress response development and how it compares to that of maternal diet in determining these stress axis variables.

The laboratory mouse has been an important model for looking at maternal effects (e. g., Krackow and Hoeck 1989, Ozanne and Hales 2004, Johnson and Speakman 2001, Speakman and Król 2005). Yet, the laboratory mouse has been subject to approximately 100 years of selection for docility and in laboratory settings, mice encounter few of the natural stressors that any free-living animal experiences daily. The stress response is an important mediator of the changes in phenotype that occur in response to maternal diet and other environmental variables experienced during development (Harper 2008). A heightened sensitivity to intrinsic and extrinsic stress in wild verses laboratory mice implies that the stress response has been dampened by artificial selection (Meagher et al. 2000; Ruff et al. 2013). Therefore, it's probable that the impact of an individual's maternal diet on their phenotype in laboratory mice is possibly not a good predictor of the response of their wild mice or other mammals (Smith 1972; Smith et al. 1994). Indeed, in the wild, it is likely advantageous for an individual to display constant, lifelong phenotypic plasticity to acclimate to fluctuating thermal, dietary, and social conditions (e. g., Naya et al. 2007; Kristan and Hammond 2003; Swallow et al. 2005).

For this study, all mice were maintained in enclosures designed to mimic natural home range sizes and group sizes of free ranging house mice. Parents were offered a low (10%; L) or high (20%, H) protein mouse chow. These levels have been studied extensively in laboratory mice (Fox et al. 2006) and 10% has been shown to be inadequate for optimal growth and reproduction. At weaning, mice were kept on the same diet as their parents (LL, HH) or given the alternate diet (LH, HL). For the mice at weaning, we anticipate similar stress phenotype and response patterns as seen in dietary restriction studies done on rats (e. g., Lesage et al. 2001; Lesage et al. 2006; Lillycrop et al. 2005; Lillycrop et al. 2007; Langley-Evans et al. 1996), with mice born to mothers consuming a lower protein diet having lower Hippocampal GR and higher liver GR as well as Hair GC. For the adults, I base my predictions according to the predictive adaptive response, where early life environment "programs" an individual's phenotype to be more adapted to an adult environment similar to what it experienced during early development and show altered effects if its adult environment differs from its early life environment (Khan et al. 2004, Ozanne and Hales 2004). Therefore, if the HPA axis is controlled by a predictive adaptive response, the stress axis phenotype of the adult mice that stayed on the same diet as their mothers (HH, LL) would differ as they are programmed for different nutritional environments. In addition, it would be expected that adult mice that switched diets (HL, LH) would differ from that of mice that stayed on the same diet (HH, LL) due to a mismatch between the prediction and available resources. In addition, I predict that the size of the litter that an individual was born into will also have significant influence on the stress axis phenotype, particularly at weaning. But this effect is expected to be less prominent as an individual ages. The relative importance of a mother' s and an individual's adult diet as well as litter size to an individual's stress axis phenotype at weaning, just before the onset of reproduction, and at 1 year of age were evaluated.

Materials and methods

Study organism

The house mouse was selected for this investigation because the impacts of maternal diet and physiology have been well studied in the laboratory counterpart of this species (e. g., Derrickson and Lowas 2007, Drickamer and Meikle 1987, Gheorghe et al. 2009). Numerous studies have evaluated the stress response and glucocorticoid receptor development (Michailidou et al. 2008, Hinds et al. 2010). In addition, several investigators have shown that this species can be readily

maintained under conditions that mimic the wild (e. g., Manning 1992, Perony 2012, Meagher et al. 2000). Under these semi-natural conditions, it is possible to determine if a mother's diet plays a formative and persistent role in determining the development and formation of the HPA axis when individuals are experiencing a natural social environment.

The house mice founding the populations described herein were obtained from out bred colonies maintained by Dr. Wayne Potts at the University of Utah. The ancestors of these mice were originally collected in Gainesville, FL and outbred under laboratory condition for 13 generations before being shipped to Auburn University for this study.

Housing and identification

Mice were maintained in 10 semi-natural enclosures on the Auburn University campus. These enclosures were designed to expose the mice to the ambient conditions that wild mice would experience if they were residing in a barn. The ten enclosures are divided between 2 side-by-side wooden buildings. Each building has a solid roof and windows covered with hardware cloth to expose the animals to ambient temperature and humidity while providing cover and preventing predation. Each enclosure is 5 meters squared and lined with a one-meter high aluminum flashing to prevent escapes. When animals first arrived, we transitioned them onto their experimental diets within one week of arrival and kept them segregated by sex for 8 weeks before breeding groups were established. Ten breeding groups were initiated with 3 male and 7 female adult mice each. This group size mimics natural social groups, or demes, common to wild house mice (Crowcroft 1963; Krackow 2003). The skewed sex ratio reduced male aggression and mimicked the social structure of few dominant males found in naturally occurring demes (Berry 1981, DeFries 1970, Reimer 1967, Bronson 1979). Adult mice were pit tagged (Biomark, Inc, Boise, ID. Model: HPT12) and ear punched for individual identification.

Diet

Mice were fed a 20% (high, H) or a 10% (low, L) protein diet custom made by TestDiet (Purina Animal Nutrition, LLC., St. Louis, MO). These diets were isocaloric and cornstarch was used to make up the difference in the caloric content of L diet relative to the H diets (Table 1). The diets were otherwise similar and designed to meet the complete nutritional requirements of laboratory rodents (based on a modification of TestDiet AIN-93G). Before breeding, the F0

generation mice were randomly assigned to the H or L diet, exposing young to these diets via their parents and as they began to eat solid food. After weaning at 4 weeks, when all young should be fully weaned (König and Markl 1987), F1 generation mice either maintained same diet as their parents (matched diet, HH or LL) or switched to the alternative diet (mismatched diet LH or HL) (Fig. 1).

Hair, hippocampal region of brain and liver collection

Brain and liver were collected from the F1 generation at 4 weeks of age to determine the impact of a mother's immediate diet in their offspring and at 1 year of age to observe the effects of an individual's dietary history (HH, HL, LH, LL) on the progression of the HPA axis development. Adult males were removed from each breeding enclosure when females were approximately 8-10 months old (1-2 months before collection) to end all breeding activity and the females were collected at 10 months to 1 year of age, between September 2013 and December 2013. Mice were euthanized with isoflurane followed by decapitation and then the liver was immediately submerged in RNA later solution and stored in -80^{o}C degrees. The brain was flash frozen on dry ice. Hippocampal region of the brains were dissected using a cryostat unit (Thermo Scientific HM 525) in the Foradori lab at the Auburn University Vet School. Hair was collected using scissors and forceps.

Quantitative PCR analysis

Liver and hippocampal RNA was extracted using TRIzol (Invitrogen). I then treated the RNA samples with Turbo DNase (Ambion Inc.) for 30 minutes at 37^{o}C and further purified them using a phenol-chloroform reaction. I converted 2 µg of RNA to cDNA via reverse transcription using qScript XLT cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). We used qPCR to determine levels of Glucocorticoid receptor mRNA expression in the liver and hippocampus of F1 females and used GADPH and B-actin as a house keeping control (Table 1 for primer sequences & references). We used PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD) to complete the qPCR according to the manufacturer's instructions and 0.5 ul of primers at 25 ng/µl concentration per reaction. Fast-3-step cycling was used with conditions of: 30 second initial denaturation at 95^{o}C and 50 cycles of 95^{o}C for 5 seconds followed by 58^{o}C for 15 seconds then 68^{o}C for 20 seconds (Eppendorf Mastercycler eprealplex2).

Hair GC extraction and ELISA analysis

To rid the surface contaminants from the hair, a methanol wash protocol was implemented as in previous studies (Macbeth et al. 2010; Bechshøft et al. 2011). To assure samples were rid of all contaminants, each sample was washed 9 times. Two to three milliliters of methanol was used per wash, sample was then vortexed and centrifuged to remove methanol. Hair was then air dried under a fume hood in a 37ºC water bath (Fisher Scientific, Isotemp water bath 5L-M) and weighted (hair sample with tube – original tube weight). GC extraction was also adjusted based on (Macbeth 2010). 2 to 3 ml methanol was added to each sample and put in a 50 ºC water bath and placed on a rotator (Hoefer, Red rotor PR-70) for 24 hours. Methanol was then filtered (Corning, Spin-X UF 500) and dried. Extracted GC was measured using a corticosterone ELISA kit (Enzo Life Sciences, Inc. Farmingdale, NY) according to the manufacturer's instructions. Plates were read using a Powerwave XS microplate spectrophotometer (Biotek, Inc).

Breeding setup and collection dates

I placed the founding population of mice in mix sex groups, as described above. I waited 2 months after the first births before collecting data. I collected F1 mice to evaluate the effects of treatment at weaning, just prior to the onset of reproduction, and 1 year of age. Cannibalism, abandonment, and mortality were common in first litters born in each enclosure. This may be attributed in part to first time mothers displaying inefficient nutrient transfer to their young (Weber et al. 2013). Because these observations are common in mice, I do not believe this poor success is attributable to diet. Four months after the first litters were born (November 2012), I collected a female from each litter at 4 weeks for sample collection (brain, liver and hair, December 2012 to March 2013) and retained up to 4 young per litter (2 male, 2 female) to raise till adulthood. To ensure that all females were in a similar, non-reproductive state, males were removed from each enclosure when females were approximately 8-10 months old (1-2 months before collection) to end all breeding activity. Females were collected at 10 months to 1 year of age, between September 2013 and December 2013.

The putative mother of each litter was identified based on the recorded locations of the animals each day during husbandry. The female found on the nest greatest number of days was designated as the mother of the litter. When the likelihood of a litter's mother was similar

between two females, I considered both the number of days the female was on the nest during the first few days post-partum and the time between reproductive bouts.

Statistical analysis

Only those females in the F1 generation (4 week old and 10-12 month of age) were included in the analyses. All analyses were completed using R i386 v.3.1.1 (http://www.r-project.org/). To determine the impact of dietary treatment on stress level and HPA axis development, I ran a linear model to test for the effects of the two main variables (dietary treatment & litter size) on Hippocampal and liver GR and hair GC. Bartlett's test was used to determine homoscedasticity of my data. Significance was set at α of $P < 0.05$.

Results

Hippocampal glucocorticoid receptor

At four weeks of age, shortly after weaning, there was no significant difference between the hippocampal glucocorticoid receptor levels of female mice born to high and low protein mothers (Table 3). No significant difference was found in the adult females groups (matched or mismatched to maternal diet; HH, HL, LH, LL) at approximately 10 months to 1 year of age (Table 4, 5). The litter size an individual was born into didn't have any effect at either stage.

Liver glucocorticoid receptor

At four weeks of age, there was no significant difference between the liver glucocorticoid receptor levels of female mice born to high and low protein mothers (Table 3). No significant difference was found in the adult females groups (matched or mismatched to maternal diet; HH, HL, LH, LL) at approximately 10 months to 1 year of age (Table 4, 5). The litter size an individual was born into didn't have any effect at either stage.

Hair GC

At four weeks of age, there was no significant difference between the hair GC levels of female mice born to high and low protein mothers (Table 3). For adult females, we found that the

HH group was significantly higher than the HL group (P = 0.039; Table 4, 5; Fig. 2) and was higher than the LH group, but not significantly (P = 0.058; Table 4, 5). The litter size an individual was born into didn't have any effect at either stage.

Discussion

I evaluated the effects of maternal diet, adult diet and litter size on hepatic and hippocampal GR as well as hair GC at 4 weeks, shortly after weaning and following months of breeding as adults when it neared 1 year of age. I predicted that information gained from a mother's diet would be used to program an individual's phenotype following a predictive adaptive response. The results of this investigation suggest that at least in hepatic and hippocampal GR and hair GC, the diet of an individual's mother and litter size had no impact on its phenotypes at weaning. For adults, only hair GC showed a trend of predictive adaptive response. Across this timeline, only hair GC was influenced by the diet of an individual's mother during development and the cumulative effects of the diet during development and at independence on phenotype.

In my study, the adult female HH group showed the highest level of hair GC. Prior studies show that high GC has negative impacts on body composition (Cabezas et al. 2007). Based on the findings of my study on organ development (Chapter 1) and GR levels, I found no indication that body composition differed between these mice. No difference in GR levels in adults suggests all groups showed normal HPA axis development. Thus, higher hair GC levels in the HH group, which shows that the mice had relatively higher chronic GC levels, could be an indicator of a life history trade off that focuses energy on reproduction (Boonstra et al. 2001). This notion is supported by the higher relative liver mass and lower abdominal fat mass seen in adult females (Chapter 1) as well as higher litters produced, total pups birthed and weaned in F1 adults that were on a high protein adult diet (Hood per obs). As liver is vital to energy metabolism (Brand et al. 1991), higher relative liver size could indicate increased energy expenditure. In addition, lower levels of abdominal fat are often seen in individuals investing highly in reproduction (Naismith et al. 1982; Gerhart et al. 1997). Furthermore, High GC levels could reflect the residual negative effects of reproduction on the body (Speakman 2008). I posit that the high GC levels of the HH group reflect effort to maximizing reproduction.

Phenotype can be impacted by the quantity and quality of solid food that an individual consumes as it transitions from milk to a solid diet. Thus, this transition could allow for difference in the efficiency of growth and tissue accretion, including compensatory growth (Yambayamba et al. 1996; Kamalzadeh et al. 1998). Mice were observed consuming mouse chow as early at 12 days of age in this study (B. Moorer pers obs). Based on this observation, it is possible that differences in stress axis development for mice at 4 weeks of age with mothers from different protein diet levels could be negated or dampened due to possible compensatory growth as many mice started transitioning into solid food prior to 4 weeks of age. Furthermore, although work in laboratory mammals provides evidence that adult stress responses are programmed by their early life environment (e. g. Seckl 2001; Seckl 2004; Liu 1997; Meaney 2001), there have also been studies that show a lack of repeatability of early life stress responses and those during adulthood (Wada et al. 2008), indicating development still shows plasticity after early stages. This type of plasticity is seen in adult mammals in the wild, as they showcase a high level of adaptive response (Scott et al. 2014) and often vary in coping strategies (Cabezas et al. 2007; Blas et al. 2007). Hence, similar GR levels in adult females in my study could be an accumulation of adult adaptive response and alternative coping schemes.

Due to natural and accidental incidents, the sample size for our adults are sub-optimal. This lowered statistical power will decrease the probability detecting a true effect and also reduces the accuracy of whether a significant effect is a true effect (Button et al. 2013). In addition, the feedback mechanism in the HPA axis is an extensive network that collaborates to dampen the stress response and return to the body's basal state. Other than the hippocampus, many other regions such as the hypothalamus and pituitary (Tsigos & Chrousos 2002) as well as enzymes, hormones and protein have been shown to also play a role in negative feedback (Seckl and Walker 2001; Pariante and Lightman 2008). Moreover, as hormone cascades exhibit the multiplier effect ("domino effect" or "snowball effect"), a significantly changed negative effect in the HPA axis may not be detected just by measuring Hippocampal GR alone. If other regions also show similar developmental response as the hippocampus to the diet manipulation I used, the combined effect may cause the HPA axis to lose optimal functions. The dexamethasone suppression test (DST) has been used to determine HPA functionality in mice (Ridder et al. 2005; Snyder et al. 2011). Further evaluation using DST, in higher sample sizes, may show

differentiations in HPA functionality between groups that weren't detected based on hippocampal GR levels.

Similar concepts can be used on the hepatic GR results. As increased hepatic GR can cause a metabolic disorder through altering gluconeogenesis levels, it is important to observe the downstream cascade of the gluconeogenesis pathway elicited by higher levels of hepatic GR. Phosphoenolpyruvate carboxykinase (PEPCK) and Glucose 6-phosphatase (G6P) are both key regulatory enzymes in gluconeogenesis and expression levels of both have been linked to maternal dietary protein (Lillycrop et al. 2007; Vo et al. 2013). As gluconeogenesis is also affected by other factors such as insulin and glucagon expression, the potential downstream effects of altered hepatic GR combined with other mechanisms within an individual must be considered. By looking into the PEPCK and G6P levels in my study animals, possible abnormal gluconeogenesis occurrence may be detected. Follow up studies measuring PEPCK and G6P at a higher sample size could show significant variation that wasn't detected in the hepatic GR levels I observed. Furthermore, diagnosis of metabolic conditions can also be performed as a direct evaluation of body condition of my mice. Fasting glucose tests and radiotelemetry have been used to look at diet induced insulin resistance and hypertension in mice (Samuelsson et al. 2007). In addition to looking at downstream gluconeogenesis enzyme expression, by also doing a cardiovascular function analysis via radiotelemetry as well as an insulin responsiveness analysis through a fasting glucose test at a sample size higher than before, whether dietary treatment in my study caused a significant effect on body condition, which potentially may be influenced by the hepatic GR variation, can be determined.

Conclusions

In my study, I observed significantly higher hair GC levels for the HH group when compared to the mismatched groups for female mice at 1 year of age. Based on results in chapter one and preliminary observations in the lab, this may reflect the costs of reproduction in these females that appeared to have high reproductive performance rather than overall poor body condition. Though maternal diet quantity and quality show significant effects on offspring development in laboratory studies, stress axis GR development wasn't affected in wild derived mice maintain under conditions that mimic the wild. Future studies, with higher sample sizes, directly testing the HPA axis negative feedback sensitivity and downstream gluconeogenesis

enzymes can be done to further the understanding of the effects of maternal diet quantity and quality on the stress axis development.

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Table 1: Macronutrient composition of high and low protein diets.

	High protein diet	Low protein diet
Protein	20.6%	10.1%
Fats	16.4%	16.2%
Carbohydrates	63.0%	73.7%
Energy	3.88 kcal/g	3.95 kcal/g

Table 2: Quantitative polymerase chain reaction (qPCR) primer sequences

Gene	Primer sequence 5' to 3'	TM	Source
Hippocampus GR-F	GCAGTGGAAGGACAGCAC	58	Petropoulos et al. 2014
Hippocampus GR-R	CGAGCTTCCAGGTTCATTC	58	Petropoulos et al. 2014
GAPDH-F	TCGGTGTGAACGGATTTG	59	
GAPDH-F	CCGTGAGTGGAGTCATACTG	57	
β-actin	AGAGGGAAATCGTGCGTGAC	63	Wynne et al. 2012
β-actin	CAATAGTGATGACCTGGCCGT	62	
Liver GR-F	CGGGACCACCTCCCAAACCCCATAATGGCATACCGAA	63	Watts et al. 2005
Liver GR-R		63	

Table 3: Hippocampal GR, hepatic GR and hair GC in F1 4-week old female mice reared by mothers consuming a high (H) and low (L) protein diet. Means, standard errors, and the effects of maternal protein intake and litter size on each variable are given. All comparisons were completed using a linear model procedure. Significant results are bold (Hippocampal and hepatic GR: % of average of all samples; Hair GC: pg/mg).

Variable	n	H group	L group	*Comparisons*	
		mean ± se	mean ± se	diet P	litter size P
Liver GR	36	0.947 ± 0.052	0.963 ± 0.031	0.896	0.193
Hippocampal GR	35	1.01 ± 0.03	0.998 ± 0.039	0.996	0.196
Hair GC	15	185 ± 32	123 ± 8	0.195	0.765

Table 4. Hippocampal GR, hepatic GR and Hair GC for 1 year old adult female mice. Means and standard errors are given (Hippocampal and hepatic GR: % of average of all samples; Hair GC: pg/mg).

Variable	n	HH group mean ± se	HL group mean ± se	LH group mean ± se	LL group mean ± se
Liver GR	31	1.11 ± 0.26	0.914 ± 0.149	1.07 ± 0.12	0.979 ± 0.110
Hippocampal GR	33	1.01 ± 0.09	0.947 ± 0.094	1.03 ± 0.07	0.958 ± 0.065
Hair GC	28	291 ± 25	200 ± 24	197 ± 16	212 ± 26

Table 5: Hippocampal GR, hepatic GR and hair GC in F1 1 year old female mice reared by mothers consuming a high (H) and low (L) protein diet and received a matched (HH, LL) or mismatched (LH, HL) diet compared to their mothers diet after weaning. Means, standard errors, and the effects of maternal protein intake and litter size on each variable are given. All comparisons were completed using a linear model procedure. Significant results are bold (Hippocampal and hepatic GR: % of average of all samples; Hair GC: pg/mg).

Variable	*Comparisons*						
	Diet P values				litter size		
	HH-HL	HH-LH	HH-LL	HL-LH	HL-LL	LH-LL	P
Liver GR	0.333	0.861	0.672	0.329	0.454	0.752	0.267
Hippocampal GR	0.952	0.805	0.305	0.878	0.321	0.103	0.227
Hair GC	**0.039**	0.058*	0.151	0.568	0.304	0.542	0.783

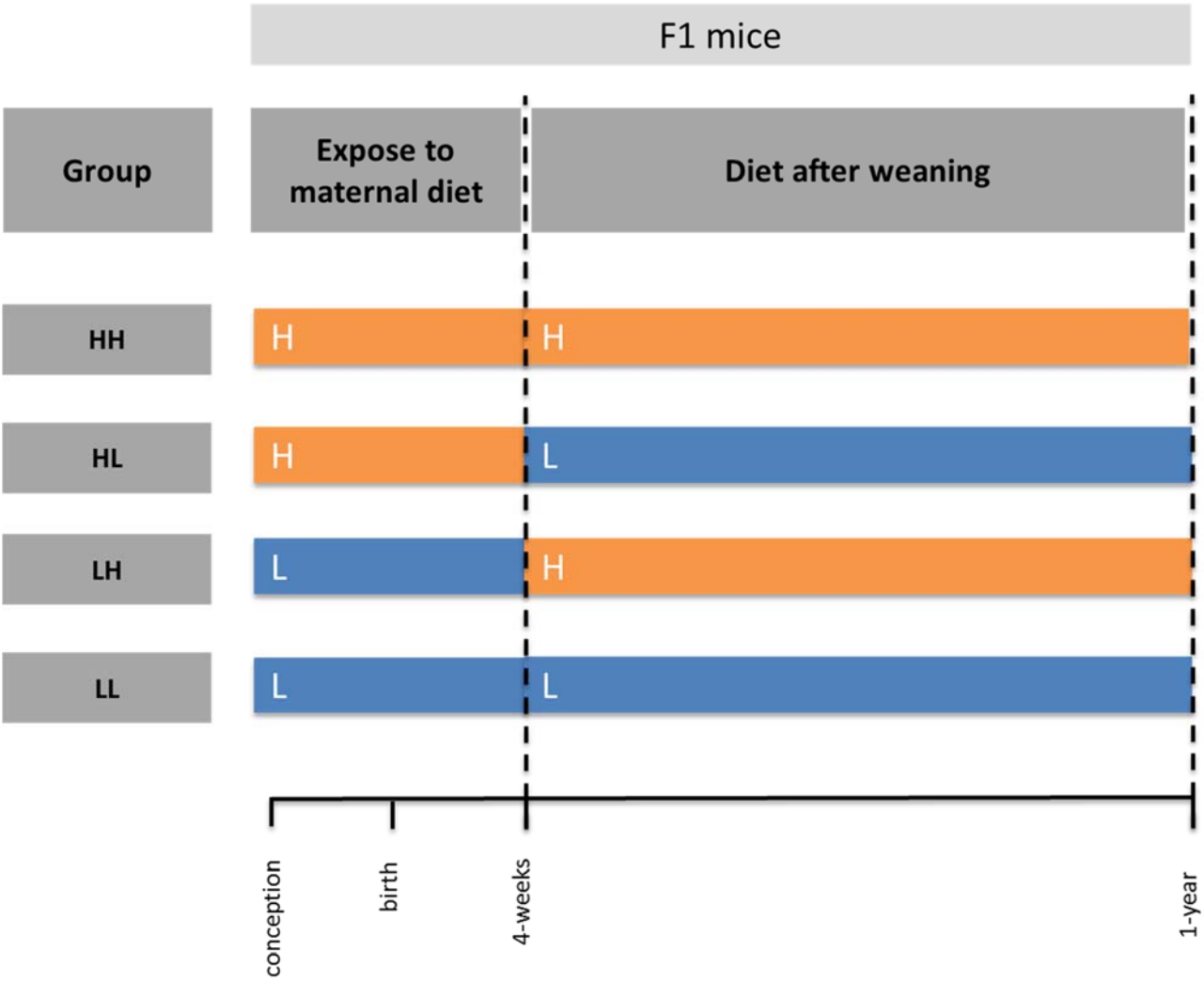

Figure 1. Timeline, dietary treatment groups, and collection events (dash lines) for this experiment (H=High protein diet: 20%; L=Low protein diet 10%)

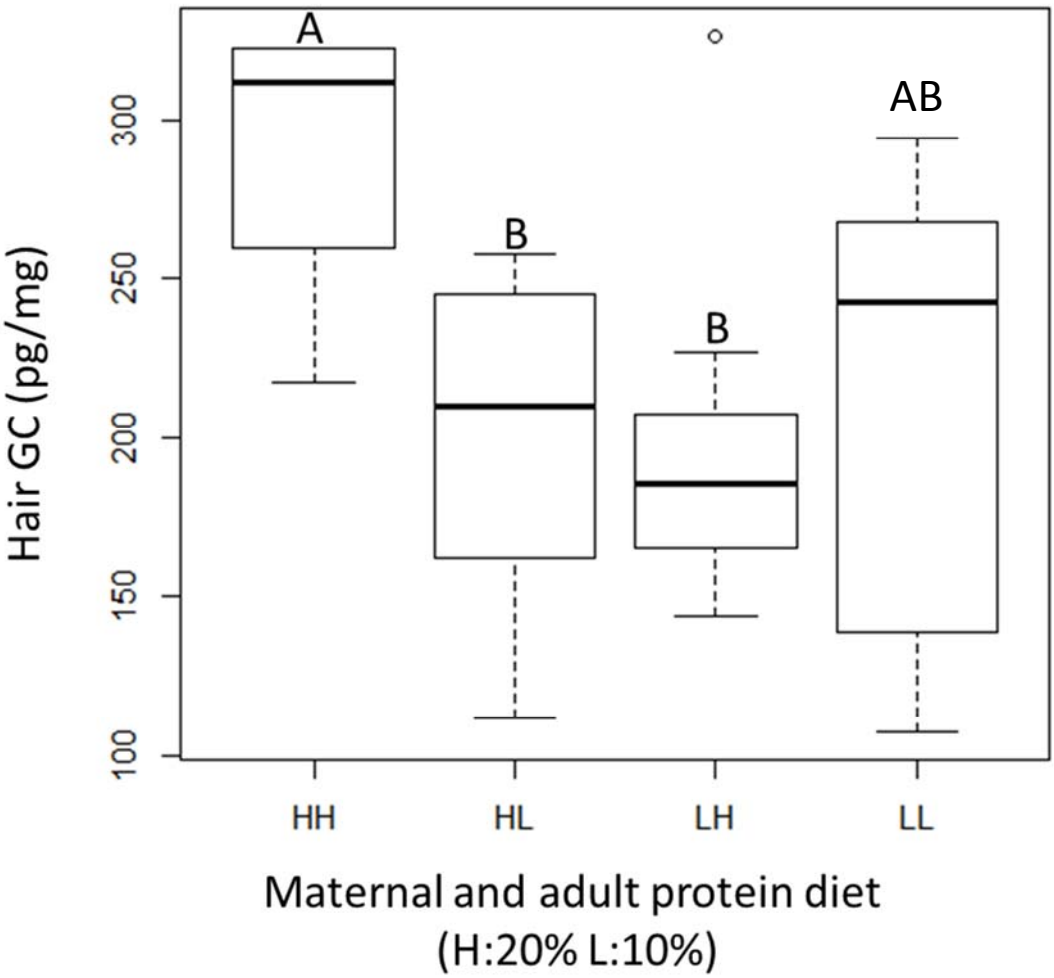

Figure 2. Comparison of maternal/adult protein intake on the Hair GC levels in 1 year old adult female mice. Only significant differences are shown.