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Characterization of energy metabolism phenotypes in GALT-null rats

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Abstract

Characterization of energy metabolism phenotypes in GALT-null rats

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Classic galactosemia (GC) is an autosomal recessive disorder that results from deficiency of Galactose-1-phosphate uridylyltransferease (GALT), the middle enzyme in the Leloir pathway of galactose metabolism. CG has been associated with a range of acute and long-term complications, including delays in both cognitive and physical development. The goal of the following studies was to test whether GALT-null rats exhibit phenotypes relating to energy metabolism. To answer this question, we tested GALT-null Sprague Dawley rats in an attempt to characterize the model's growth rate, food consumption habits, and liver glycogen content. Our results suggest that GALT-null rats experience delayed growth, that smaller body weight is not accounted for by decreased food intake, and that livers from male, but not female, GALT-null rats contain less glycogen. Combined, these data suggest that GALT deficiency has a subtle, but potentially significant, impact on energy metabolism in rats. Additionally, the results described here suggest certain parallels between the model and patients and suggest that the rat may be a suitable model to study the underlying mechanisms of GALT deficiency and test possible interventions for CG.

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Chapter 1: Overview of classic galactosemia and galactose metabolism

Classic Galactosemia

Classic Galactosemia (CG) is a rare metabolic disorder which affects 1:48,000 individuals in the United States (National Newborn Screening Status Report 2014). It results from a profound deficiency of the enzyme Galactose-1-phosphate uridylyltransferase (GALT) which is responsible for converting galactose to glucose via the Leloir pathway. Since individuals with CG cannot fully metabolize galactose, and galactose is produced endogenously and consumed exogenously, GALT deficiency leads to an abnormal accumulation of both galactose and its derivatives throughout the blood and tissues. CG is associated with a wide range of both acute and long-term complications, beginning in infancy and extending into adulthood. Since its discovery in the early 1900's, much research has been performed regarding the various complications that arise throughout the lifetime of affected individuals. Despite this research, the underlying mechanisms surrounding most CG complications remain largely unknown and unfortunately, current treatment is ineffective at preventing or reversing them.

The only current known intervention for CG is to restrict galactose from the diet as soon as possible. This is because one exceptionally problematic time period for individuals with CG is during infancy, when exposure to lactose first occurs through consumption of either breastmilk or dairy formula. Considering the abundance of milk, a source of nutrition which is rich in galactose, in the diet of infants, there are many problems that can occur if the infant is left untreated; these include feeding problems and failure to thrive. However, it has been found that, for a majority of patients, if within the first ten days of life the diet is galactose-restricted, neonatal death can be prevented (Berry, 1993). For this reason, CG is routinely screened for at birth in the United States and many other developed nations as part of the newborn screening test (Levy and Hammersen, 1978). Unfortunately; this is not the case worldwide and in some populations individuals are only diagnosed following the onset of clinical symptoms (Jumbo-

Lucioni et al., 2012). When CG is identified, the infant is usually put on a soy-formula or another low-galactose diet.

However, despite a galactose-restricted diet, for most patients complications begin in childhood and extend through adulthood. These complications most commonly include delays in both cognitive and physical development. There have been reports of tremors, dysmetria, ataxia, ovarian failure, growth delays, and cataracts in the patient population (Bosch, 2006),(Fridovich-Keil et al., 2011).

In order to dissect these underlying mechanisms and test possible interventions, an experimental model is needed that accurately recapitulates at least some phenotypes of CG. In the 1960's a unicellular yeast model was created that lacked the orthologous enzyme which breaks down galactose in yeast. In this model, the yeast were found to not grow on medium supplied with galactose confirming that GALT is responsible for the conversion (Douglas and Hawthorne, 1964),(Douglas and Hawthorne, 1966). A fruit fly model was then produced and able to recapitulate aspects of the disorder (Kushner et al., 2010). Primarily, the fly model showed long term complications independent of exogenous galactose. However, a mammalian model that more closely matches the physiological pathways in humans was still needed. Nancy Leslie and colleagues produced a knockout mouse mode (Leslie et al., 1996)l. While this model exhibited similar biochemical markers to those seen in patients, it failed to recapitulate the acute toxicity or other complications seen in patients. More recently, Lai and colleagues have also created a GALT-null mouse model and have begun to characterize it (Tang et al., 2014).

In an attempt to create a larger mammalian model of GALT deficiency that might better mimic patient outcomes the Fridovich-Keil lab partnered with Dr. Aron Geurts and colleagues at the Medical College of Wisconsin to create a CRISPR-Cas9 gene edited GALT-null Sprague-

Dawley rat. We now have a model with a frame shift mutation (*rGALT*^{m3}), targeting the active site of the GALT enzyme (See Figure 1.1). Analysis of liver lysate from these mutant rats shows no enzymatic activity present in comparison to the wild type (see Table 1.1).

The investigations described in this thesis were designed to help characterize this rat model. This research will hopefully provide further insight into the timing and mechanisms of outcomes of GALT deficiency. In the future we hope this model will be suitable to test possible interventions that can be later used to improve patient outcomes. The following chapters discuss the characterization of a possible growth phenotype, the effect of galactose being present in the post-wean diet, and the liver's glycogen content in GALT-null rats.

Galactose Metabolism

As stated earlier, galactose in the diet comes primarily from the lactose found in milk and many dairy products. Once ingested, the body works to hydrolyze lactose into glucose and galactose. These monosaccharides are then absorbed into the blood stream and transported to the liver and other organs where they are metabolized (Coelho et al., 2017).

Galactose enters the Leloir pathway in the alpha configuration (α-D-galactose) and is then converted into Galactose-1-phosphate (Gal-1-P) by galactokinase (GALK). GALT is the enzyme that uses Gal-1-P and uridine diphosphate-glucose and converts them into Glucose-1-phosphate (Glc-1-P) and uridine diphosphate-galactose. The Glc-1-P is then converted to glucose-6-phosphate, which can be used in glycolysis. UDP-glucose and UDP-galactose can be used as substrates for glycosylation reactions and UDP-glucose can be used to make glycogen. See Figure 1.2.

Figure 1.1. Mutations present in Sprague-Dawley GALT-null (M3) rats that were created by CRISPR-Cas9 gene editing of the *GALT* locus. The mutation is a two base pair insertion resulting in a frame shift and a nonfunctional protein product or nonsense-mediated decay.

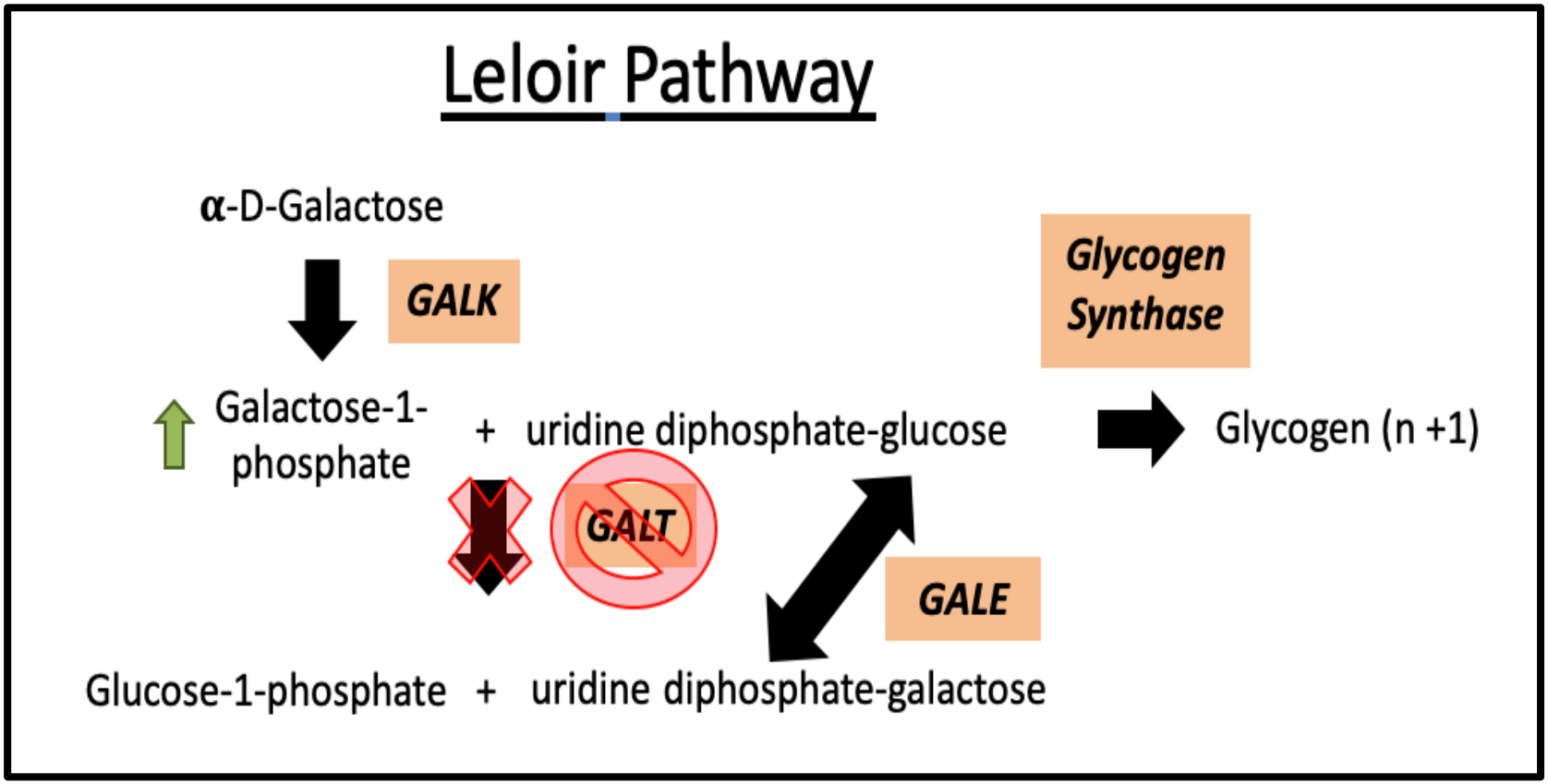

Figure 1.2. The Leloir Pathway. This is the pathway which is primarily perturbed in patients with classic galactosemia.

Chapter 2: Investigation of growth phenotype exhibited in GALT-null rats

2.1 Introduction

The aim of the investigation described here was to characterize any apparent growth delays in GALT-null rats, from birth through adulthood. There have been reports of both decreased height and weight in CG patients. In a study of 40 classical galactosemia children, it was found that prenatal growth was normal, but postnatal growth was affected (Panis et al). Additionally, in another study of 30 CG patients, it was found that height was decreased and body composition was abnormal (Panis et al). Considering these phenotypes relating to growth that we see in the patient population, we wanted to test whether our rat model would recapitulate the phenotype. The results of this research will be used to further narrow down investigations regarding the consequences of GALT deficiency in rats. Specifically, we want to better understand timing and mechanisms of the negative outcomes associated with GALT deficiency so that we can pursue rational approaches to improved intervention for patients.

2.2 Materials and Methods

All animal procedures including breeding and phenotypic characterization were performed at Emory University using protocols approved by the Institutional Animal Care and Use Committee (this includes both the protocol used in this chapter and in subsequent chapters).

Creation of rat model

GALT-null Sprague Dawley rats were generated by gene editing at the *GALT* locus using Clustered Regularly Interspaced Short Palindromic Repeats Cas9 nucleases (CRISPR/Cas9) as described previously (Gaj et al., 2013). Specifically, guide RNAs targeting the rat *GALT* gene were designed, synthesized, and validated by transfection into rat C6 astroglioma cells as

described previously (Geurts et al., 2010). The rat *GALT* target sequence was GCCTGGCAGTGGGGATGGGGG within exon 6. These constructs were injected into the one-cell stage Sprague Dawley (SD/Crl; Charles River Laboratories) embryos and subsequently transferred to pseudopregnant SD females. Pups were screened for CRISPR/Cas9-induced mutations using the Cell-I Surveyor Nuclease assay as described previously using PCR primers flanking the target site (Geurts et al., 2010). Primer sequences were as followed: GALT_F: 5'-CAGTACCCTTGGGTGCAGGTC -3'; GALT_R: 5'- ATCGCTCTTCACGCTGGGCA -3'; A single founder with deletions in *GALT* was identified, confirmed by Sanger sequencing and bred to establish mutant rat lines of which one gave rise to the SD-*GALT-M3* strain. Analysis of liver lysate from these mutant rats shows no GALT enzymatic activity present in comparison to the wild type (see Table 2.1).

Table 2.1 Enzymatic Expression in GALT-null (M3) Sprague-Dawley rats

GENOTYPE	**GALK activity** (pmol/ μg/ min)	**GALT activity** (pmol/ μg/ min)	**GALE activity** (pmol/ μg/ min)
WT/WT	138.53 ± 55.56	44.12 ± 13.47	**59.85 ± 19.66**
M3/M3	**43.54 ± 14.65**	**-0.13 ± 0.84**	**70.29 ± 11.11**

Source: Dr. Jenna Daenzer

Sample size

The total sample size of all rats (including both male and female wild type, *rGALT^{m3}* homozygotes, *rGALT^{m3}* heterozygotes) was 727 unique rats. All rats were genotyped as young pups. The specific mutation named *rGALT^{m3}* is illustrated in Figure. 1.1. All 727 rats were

weighed and treated in the same manner until they were euthanized or transferred to another project.

Aside from the number of rats per cage, which was always within IACUC-approved limits, all rats were housed and handled the same way. All rats pre-wean had unlimited access to mother's milk, solid food (once they could reach it) and water. After weaning, the rats had unlimited access to solid food and water.

Weighing the Rats

The day the rats were born was counted as Day zero and then each day afterwards was counted in order. Each rat's age was represented by the number of days after birth. The first day, Day zero, the rats were not weighed. Instead, the cage was examined from the outside to look for and count the number of pups in the litter. The weighing process was started on Day one, and the rats were then weighed daily at least until weaning.

Between Days 19 and 24, the pups were weaned from their mother. The pups were separated by gender and generally two, or three pups were placed in a new cage, depending on the size and gender distribution of the available pups. The rats were then weighed for ten consecutive days post wean. A large metal bowl with a paper towel was placed on the same electronic scale and zeroed. Each rat was then picked up, the ear button was read, and then the rat was transferred to the bowl. The weight was recorded to the nearest gram, and the rat returned back to its cage.

After ten days post-wean, rats were weighed weekly. The weighing procedure was as described above for the ten days post-wean period and all weights were recorded to the nearest gram.

Analysis of Growth Rates

The primary objective was to evaluate the effect of genotype (GALT+ vs. GALT-null) in shaping a rat's growth trajectory from birth to 375 days of age. In other words, the aim was to address how and if the slope of a rat's growth curve over time is affected by GALT activity. To do so, two subject-level random intercept models of weight by age, genotype, and litter size, one using male data only and one using female data only were fitted. These models included piecewise linear splines so that the slope of the rat-specific curves is allowed to change at specified time points. Terms representing the interaction of these linear splines with genotype were also included in the models. Three knots (time points at which slope can change) were selected for each model, and the locations of these knots were determined by fitting a linear piecewise spline model of weight against age, controlling for genotype and litter size. Potential knot locations from birth to 375 days by increments of one week were tested. Optimal knots for both the male model and female model were selected on the basis of AIC. To evaluate the overall effect of genotype on weight, we fit additional subject-level random intercept models of weight on age (including linear spline terms), genotype, and litter size for each sex, removed the genotype term, and refit these models. A likelihood ratio test was then performed to assess the significance of the genotype effect in both the male and female models. These analyses were performed by Taylor Fischer, MS.

2.3 Results

Figure 2.1 depicts growth curves for all genotype and gender combinations. However, we also see that the largest determinant of mass is gender, with post-wean males being larger than females. We also see that genotype is a significant modifier of growth for both females and males. It also appears that the difference is more significant in males than females. Figure 2.2 depicts the growth curves of GALT+ (both wild type and $GALT^{m3}$ heterozygous rats) and GALT-null ($GALT^{m3}$ homozygous rats). Again, we see that GALT-null rats appear to be significantly smaller when compared to GALT+ rats, and that this effect continues to be exaggerated in males. Tables 2.2 and 2.3 are results from the linear regression model. Table 2.2 shows that growth rate for GALT+ male rats appears to be greater than that of GALT-null male rats during each measured time interval, but that the difference decreases with time. Table 2.3 shows that the growth rate for GALT+ female rats appears to be greater than that of GALT-null female rats only during the period two to seven weeks.

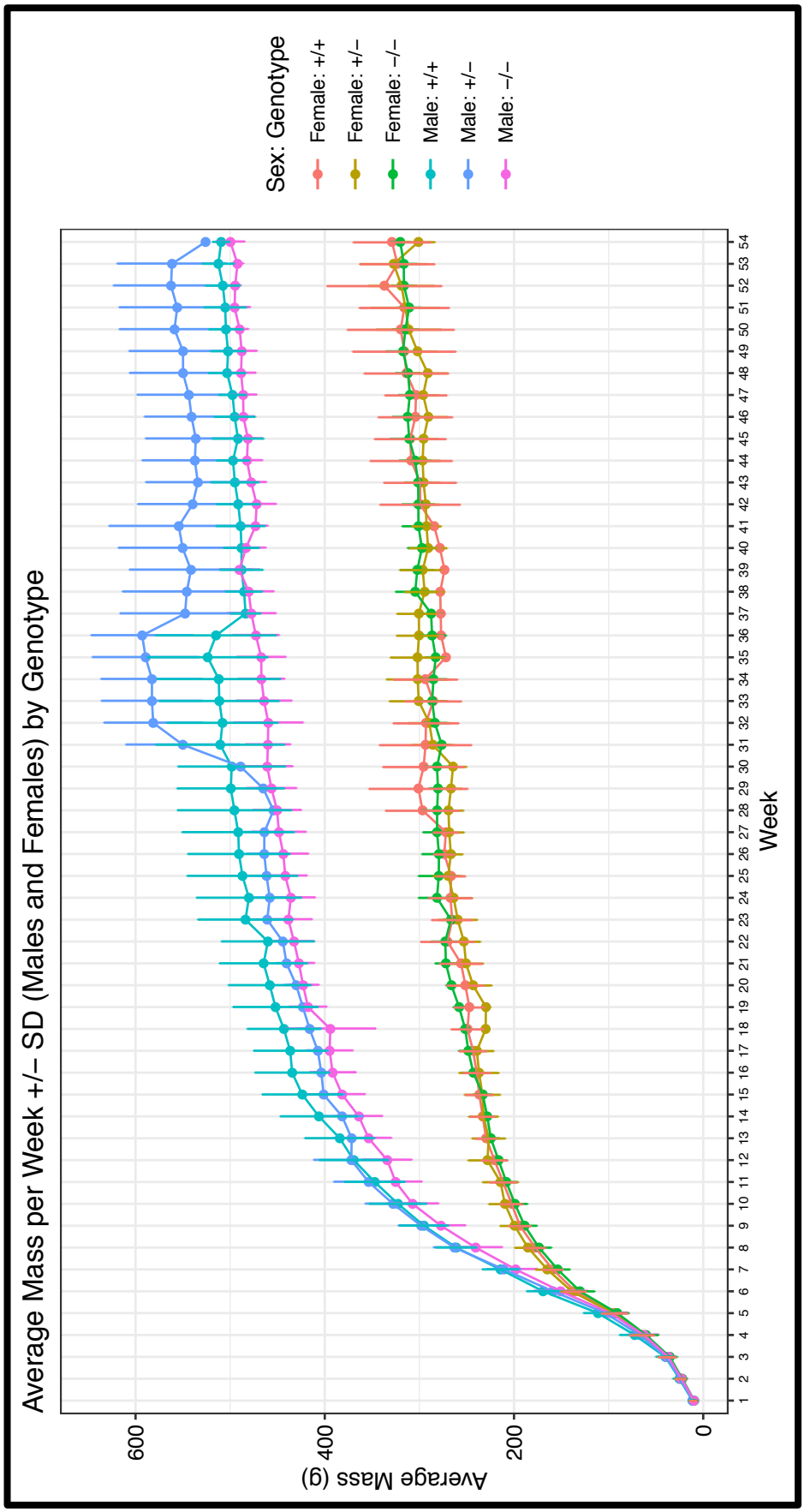

Figure 2.1. Average mass per week for each genotype and gender combination. (+/+) indicates wild type rats, (+/-) indicates *GALT*^{m3} heterozygous rats, and (-/-) indicates *GALT*^{m3} homozygous rats.

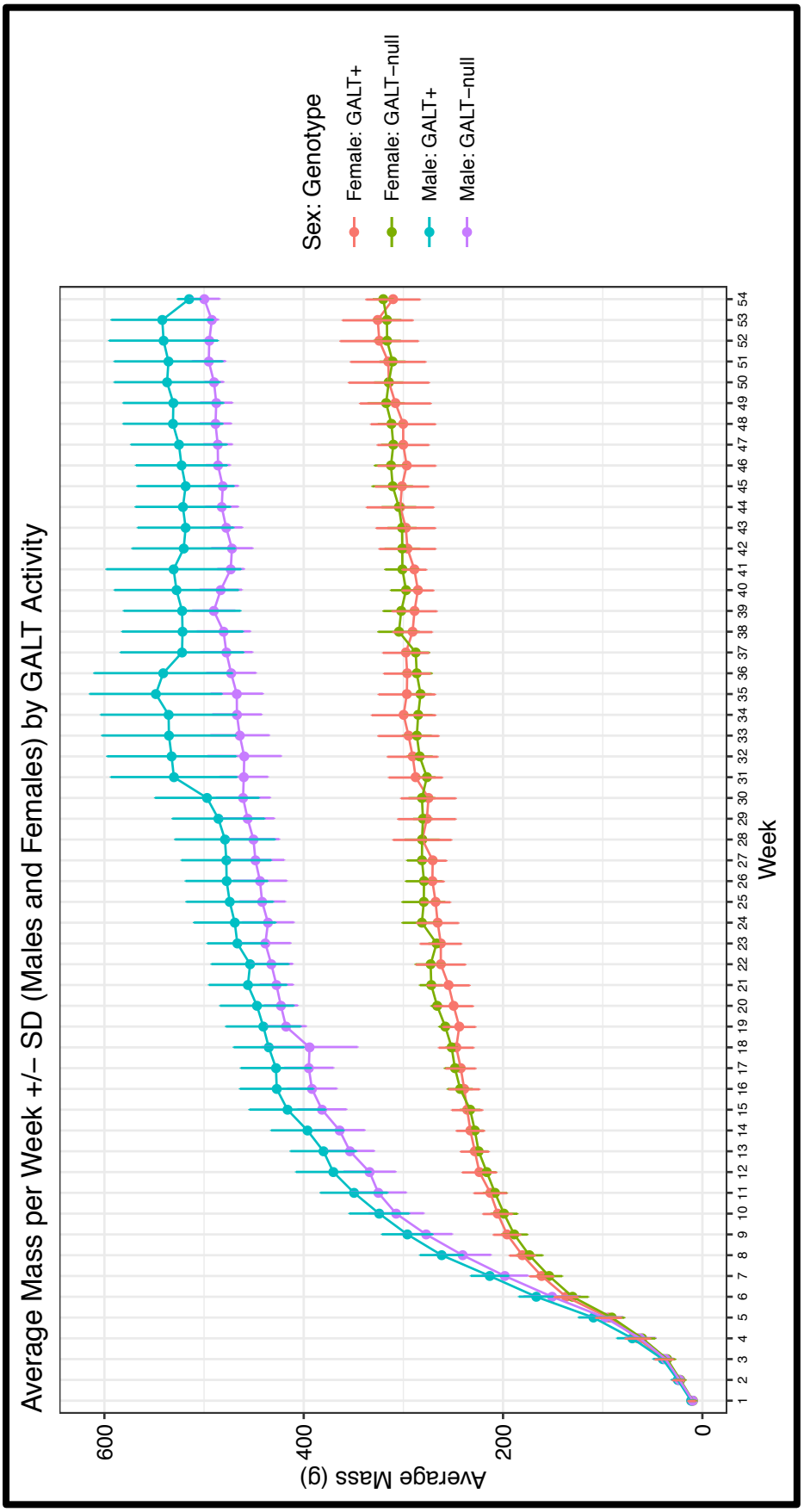

Figure 2.2. Average mass per week for each gender by GALT activity. GALT+ indicates grouping of both wild type and *GALT*^{m3} heterozygous rats. GALT-null indicates *GALT*^{m3} homozygous rats.

Table 2.2 Estimated slope of trajectory curves by genotype and age in male rats.								
	Day [0, 14)		Day [14, 56)		Day [56, 98)		Day [98,375]	
	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
Genotype								
GALT+	0.5161	(0.4205, 0.6117)	6.1713	(6.1155, 6.2271)	3.6924	(3.6212, 3.7636)	0.4744	(0.4594, 0.4894)
GALT-null	0.5161	(0.4205, 0.6117)	5.6405	(5.5746, 5.7063)	3.4287	(3.3426, 3.5148)	0.4283	(0.4077, 0.4488)

Table 2.3 Estimated slope of trajectory curves by genotype and age in female rats.								
	Day [0, 14)		Day [14, 49)		Day [49, 77)		Day [77,375]	
	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI	Estimate	95% CI
Genotype								
GALT+	1.2360	1.1773 1.2946	4.1813	4.1387 4.2239	2.1552	2.0945 2.2158	0.3299	0.3223 0.3375
GALT-null	1.2360	1.1773 1.2946	4.0067	3.9560 4.0574	2.3842	2.3100 2.4585	0.3568	0.3480 0.3656

Table 2.2 and 2.3. Slope trajectory model data for male and female rats. GALT+ indicates grouping of both wild type and *GALT*^{m3} heterozygous rats. GALT-null indicates *GALT*^{m3} homozygous rats. The model was fitted with random intercept to control for correlation among observations from the same rat. It was assumed that the effect of age on weight varied across time and genotype. These estimates are adjusted for the effects of litter size.

2.4 Discussion

Based on the results that growth patterns of both male and female *rGALT*^{m3} heterozygotes did not seem to differ from their wild type counterparts we combined heterozygotes with wild type rat growth data for the remainder of the analyses. Homozygous rats carrying the *rGALT*^{m3} mutation were then compared to non-homozygous rats. The two groups differed significantly from each other with regard to growth. Consistent with these findings, CG is an autosomal recessive condition so that individuals carrying only one mutant allele of GALT do not exhibit phenotypes characteristic of the disorder. Additionally, the growth phenotype seen here is relatively subtle and the growth differences seem to decrease over time. This too is consistent with the growth delay we see in patients. While the patients experience growth delays around puberty, adults fall within a normal range (Berry, 1993).

Classic galactosemia is a very complex metabolic disorder, affecting multiple aspects of development, and there are limitations in using solely the growth phenotype to determine the suitability of the GALT-null Sprague-Dawley rats as a model organism. More investigation is underway to test the accuracy of this model for other phenotypes. The results presented here provide encouragement that these strains of rat may provide an effective model organism to study at least some features of classic galactosemia.

Chapter 3: Investigation of the effect of diet and cryptic galactose on post-wean growth in GALT-null rats

3.1 Introduction

The aim of the investigation described here is two-fold. First, we wanted to ask if GALT-null rats grow slower because they consume less food. Second, we wanted to characterize the effect of cryptic dietary galactose exposure, post-wean, on the growth phenotype of GALT-null rats. As stated previously, the only existing intervention for patients is to move them to a galactose-restricted diet. Knowing this, we wanted to investigate the similar interventions in rats. The results of this research will be used to further narrow down investigations regarding the effects of diet and possibly varying food consumption habits of the GALT-null rat model. Specifically, we want to better understand the timing and mechanisms of the negative outcomes associated with GALT deficiency so that we can pursue rational approaches to improved intervention for patients.

3.2 Materials and Methods

Sample Size

The total sample size of all rats in this component of the study (including both male and female wild type, *rGALT*^{m3} homozygotes, and *rGALT*^{m3} heterozygotes) was 76 rats. Around Day 10, the rats were genotyped. The specific mutation for each genotype is described in Fig. 1.1. All 76 rats were weighed and treated in the same manner until they were euthanized or transferred to another project in which case, the weighing was stopped.

Aside from the number of rats per cage, all rats were housed and handled the same way. All rats pre-wean had unlimited access to mother's milk, and, when they could reach it, also solid food and water. After weaning, the rats had unlimited access to solid food and water.

Diet

The two diets that were used in this study were 5001 and 5012 from LabDiet®. The nutritional facts from each are outlined in Table 3.1. 5001 is the standard diet provided by the Emory Division of Animal Resources (DAR) that we used for all rats unless otherwise noted; 5012 was used as a low-galactose alternative diet. In HPLC analysis of 5012, we found that the diet contained 10-fold less galactose than 5001 (See Table 3.2). All post-wean rats had ad libitum access to chow and drinking water.

Table 3.1. Contents of LabDiet® 5001 and 5012.

Contents	**5001**	**5012**
Starch, % of Composition	31.9	39.5
Glucose, % of Composition	0.22	0.29
Fructose, % of Composition	0.3	0.34
Sucrose, % of Composition	3.7	3.38
Lactose, % of Composition	2.01	0
Calories Provided by Protein, %	28.51	27.07
Calories Provided by Fat, %	13.5	13.24
Calories Provided by Carbohydrates, %	58	59.69

Table 3.2. Contents of LabDiet® 5001 and 5012 measured with and without lactase.

Sample	μmol galactose/ gram food	% galactose
5001 + Lactase	51.43	0.93
5001 – Lactase	2.72	0.05
5012 + Lactase	4.62	0.08
5012 – Lactase	0.26	0.00

Weighing the Food and Rats

The total amount of food per cage was weighed three times per week. Animals were weighed as described in section 2.2 (pg. 15).

Analysis

All recorded weights of food were per cage. Due to limited resources being available to house the rats, there was often more than one rat in a cage (however co-housed rats were always the same genotype and gender). Therefore, the amount of food consumed in the cage was divided by the total change in weight of all rats in the cage. An Anova test was then run to determine any significant difference between GALT+ and GALT-null rats in the total amount of food eaten per day and the proportion of food consumed per total change in weight.

3.3 Results

We first asked whether we see a change in the subtle growth phenotype (described in Chapter 2) when the rat is placed on a galactose-restricted diet, LabDiet® 5012, at weaning.

After plotting growth curves, we observed similar growth between those rats on 5001 and those on 5012 (See Figure 3.1). We then wanted to ask if GALT-null rats grow slower because they consume less food**.** We saw no meaningful difference in food consumption between homozygotes, heterozygotes, or wild type rats of a given gender (See Figure 3.2). Finally, we asked whether there was a difference in the amount of food consumed by those rats eating 5001 and those eating 5012 diets. Here a statistically significant difference was found. Specifically, we found that for days 21-70, the average amount of food eaten was 43.5 ± 6.99 and 102.5 ± 10.75 grams of food per rat per two days for 5001 and 5012 respectively (this included both genders and all genotypes). Given these data, we wondered whether the observed difference may result from differences in the body weight, or that larger individuals may just eat more. To control for this, we divided the amount food consumed by the weight of the rat. Comparing these values, we still saw a significant difference between the amount of food consumed by those rats on 5001 and those on 5012. Specifically, we saw that GALT+ rats on 5001 and 5012 appeared to eat similar amounts, while GALT-null rats on 5012 ate significantly more food than did their counterparts on the 5001 diet (See Figure 3.2). Despite this, there was no statistically significant difference between the growth curves of rats on 5001 and rats on 5012 for a given age, genotype, and gender.

Figure 3.1. Growth curves comparing rats consuming LabDiet 5001 and 5012 between 21-70 days of age. This shows the relationship between GALT-null and wild type rats' growth trajectories for the seven weeks after rats stop nursing and begin to eat solid food ad libitum. A mixed effects model was made and an Anova test was used to determine no significant difference was detected between the growth rates of rats of a given genotype on each diet. There was no need to adjust for litter size since all data was recorded post-wean.

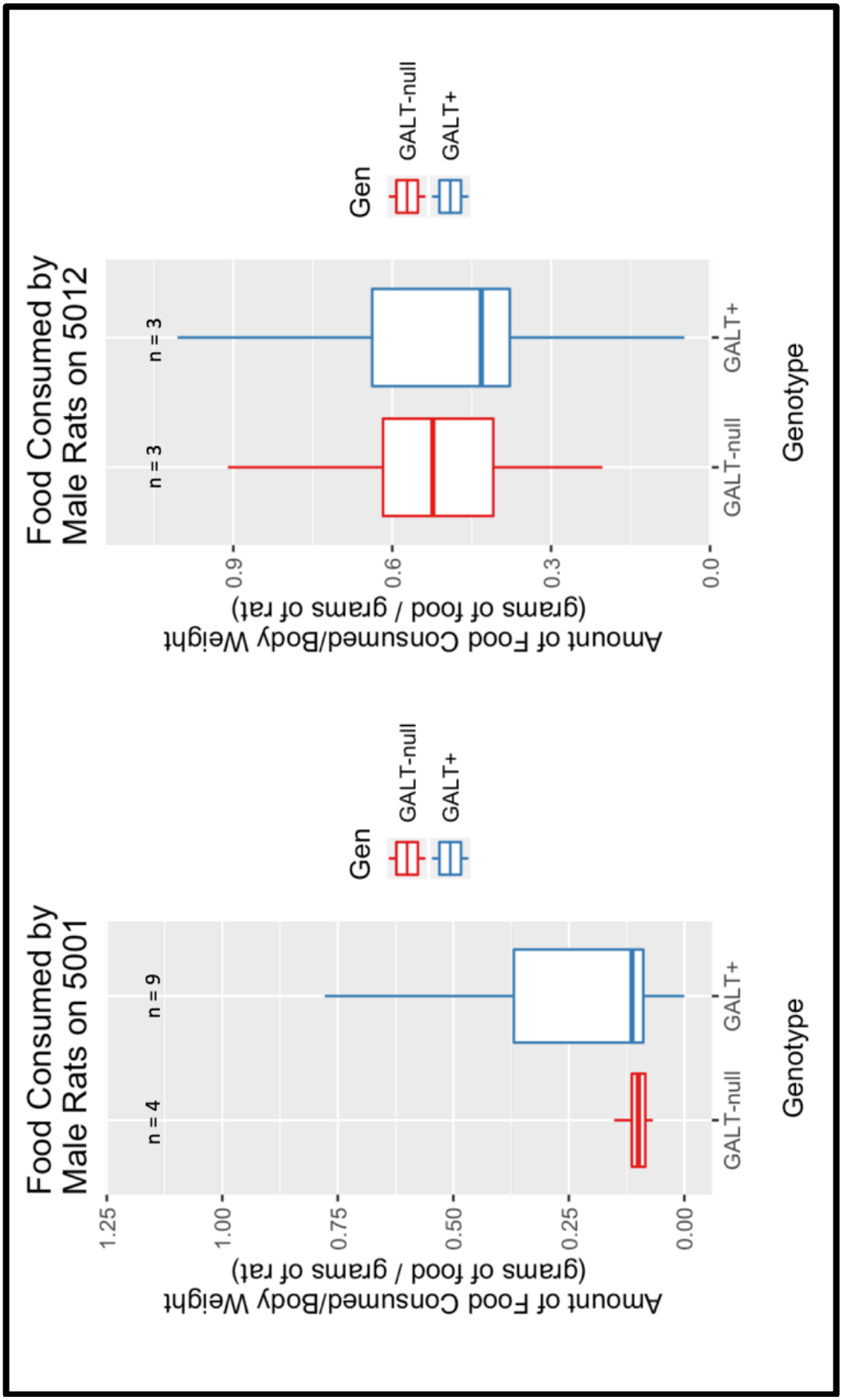

Figure 3.2. Ratio of food consumed per body weight for GALT-null and wild type male rats on 5001 and 5012 diets from day 21-70. An Anova test was run on a mixed effects model to determine that there was no significant difference in the amount of food consumed per body weight between GALT+ and GALT-null male rats for a given diet. Data for females was insufficient. Cohort included four GALT-null rats on 5001 diet, nine GALT+ rats on 5001 diet, three GALT-null rats on 5012 diet, and three GALT+ rats on 5001 diet in the given age range.

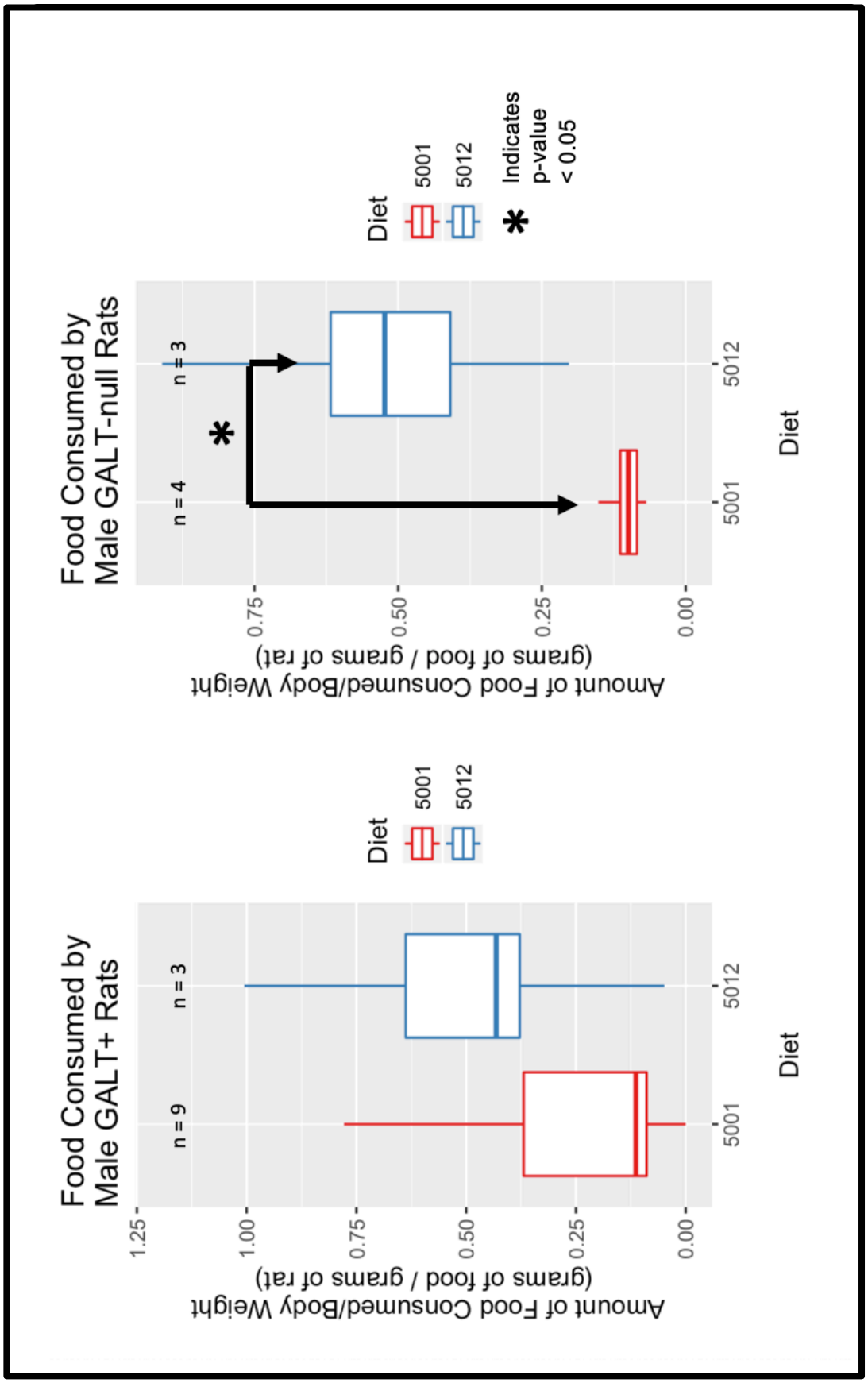

Figure 3.3. Ratio of food consumed per body weight for GALT-null and wild type male rats on 5001 and 5012 diets from day 21-70. An Anova test was run on a mixed effects model to determine that there was a significant difference in the amount of food consumed per body weight between diets for a given GALT activity. Data for females was insufficient. Cohort included four GALT-null rats on 5001 diet, nine GALT+ rats on 5001 diet, three GALT-null rats on 5012 diet, and three GALT+ rats on 5001 diet in the given age range.

3.4 Discussion

We observed no difference in amount of food consumed by male GALT-null and GALT+ rats (See Figure 3.2). This is interesting since this could suggest some underlying difference in energy metabolism between GALT-null and GALT+ rats. Specifically, this suggests that the difference in growth curves described in Chapter 2 are not due to a decrease in food consumption. It also appears that both GALT-null and GALT+ rats preferred LabDiet 5012 (See Figure 3.3).

One possible explanation for the observed results may be that in order to prevent a difference in growth rate, galactose-restriction is needed at the time of birth, rather than after weaning. Considering the pups in this study nursed from their mothers for the first three weeks of life (and therefore were not galactose-restricted), it is possible that early exposure alone may be sufficient to cause the observed growth phenotype. Yet another possible explanation is that the mechanisms through which symptoms arise still occur, regardless of dietary galactose restriction. This is what is seen in patients, where long-term symptoms, including growth delay, persist despite early dietary intervention (Bosch, 2006)(Fridovich-Keil et al., 2011). It may be necessary in the future to begin feeding animals a low-galactose diet immediately after birth. Restricting galactose consumption from birth may provide valuable insight into whether or not outcomes arise due to endogenously produced versus exogenous galactose exposure.

Additionally, some data regarding those rats on LabDiet 5012 had to be discarded. This food disturbance happened mostly with females and was the reason that the data for LabDiet 5012 in Figures 3.2 and 3.3 was based on what was observed in male rats. A limitation to this study was the limited sample size and further research may be necessary to increase sample size and determine whether this phenomenon is real and potentially seen in females. Regardless, the other results from this study suggest that restricting galactose from the post-wean diet of GALT-

null rats has no real effect on the growth outcome previously observed when trace galactose is present in the diet.

Chapter 4: Investigation into the liver's glycogen content in GALT-null rats

4.1 Introduction

The aim of this investigation was to test whether glycogen storage differed between our GALT-null and control rats. One of the byproducts of GALT is glucose-1-phosphate, a possible starting point for glycogen synthesis. In the glycogen synthesis pathway, glucose-1-phosphate is first converted to uridine-diphosphate-glucose, which is later converted to glycogen via glycogen synthase (See Figure 1.2). Additionally, anecdotal evidence from patients suggests that individuals with CG have difficulties gaining weight, despite normal caloric intake (Edmonds et al., 1952). One possible explanation for this outcome may be glycogen deficiencies in the liver because it has been found that glycogen storage diseases result in growth issues (Rake et al., 2006). Considering these data, we wanted to ask whether there were differences between mutant and wild type weanlings' (defined as animals who were just weaned from their mother) storage of glycogen. To investigate this, we measured glycogen content of the liver of these animals. The liver was the focus of this investigation given that it is one of the principal places in the body where glycogen synthesis and storage occurs.

The results of this research will be used to focus future studies of energy metabolism in GALT deficient rats. Specifically, we want to better understand how GALT deficiency impacts energy metabolism in the form of glycogen storage. This knowledge will hopefully give us the ability to pursue rational approaches to improved understanding of the disorder and intervention for patients.

4.2 Materials and Methods

Tissue Collection

Animals were euthanized via CO2 inhalation and opened with a U-shaped incision. Livers were excised, weighed, and then a portion from the left lateral lobe was flash frozen on dry ice. See Figure 4.1.

Assay Protocol

In order to measure the glycogen content in the liver of the rats, the Glycogen Assay Kit II from Abcam® was used. All included reagents, Glycogen Hydrolysis Buffer (GHB), Developmental Enzyme Mix (DEM), and the probe, were prepared according to the protocol provided by Abcam®.

Standard Preparation

The glycogen standard provided in the kit was diluted and plated to form a 0, 0.4, 0.8, 1.2, 1.6, and 2 ug/well Glycogen Standards with background controls. Each well was then adjusted to 50 µl with GHB. 2 µl of the kit's Hydrolysis Enzyme Mix (HEM) was then added to half of all standard wells (the half lacking the HEM were used as a background control for the standard) and the plate was allowed to incubate at room temperature for 30 minutes. A Reaction Mix containing the kit's Glycogen Development Buffer (GDB), Development Enzyme Mix (DEM), and a probe was then prepared and added to all wells containing the standard. Once again, the plate was left to incubate at room temperature for 30 minutes before being run on a microplate reader measuring OD450 nm.

Sample Preparation

The liver tissues used in this study came from animals recently euthanized. The livers were extracted and the left lateral lobe was isolated. A sagittal slice about 2 mm in width was then collected from this lobe and frozen for later use. For this assay, these tissues were removed from -80 freezer and allowed to thaw on ice. Considering that glycogen is not always evenly distributed throughout the liver (Ekman), the tissue was then cut to isolate an approximately 10 mg liver sample from the middle of the sagittal slice. Deionized water was then added to adjust the solution to 1mg tissue/20 µl deionized water. Samples were then homogenized on ice with an electric hand grinder for 2 minutes, and then moved to a refrigerated room and mixed vigorously with glass beads for an additional 10 minutes. The homogenates were then boiled for 10 minutes to inactivate any enzymes in the homogenate. These homogenates were then centrifuged at 14000 rpm for 15 minutes and the supernatant containing the desired soluble material was isolated. A 1:50 and 1:100 dilution of the supernatant was then made and plated to form sample background controls (those lacking HEM) and samples to measure glycogen (to which 2 µl of HEM was added). The plate was then allowed to incubate at room temperature for 30 minutes. A Reaction Mix containing the kit's Glycogen Development Buffer (GDB), Development Enzyme Mix (DEM), and a probe was then prepared and added to both sample background controls and samples to measure glycogen. Once again, the plate was left to incubate at room temperature for 30 minutes before being run on a microplate reader measuring OD450 nm.

4.3 Results

The samples were read at OD450 nm and the corresponding glycogen content was recorded. After blank and standard values were subtracted from the sample values, the technical replicates were averaged. These averages for a given individual were then compared using a Tukey HSD test across genotypes and genders. The average glycogen content for female mutants, female wild types, male mutants, and male wild types are included in Figure 4.2.

Table 4.2 contains adjusted p-values comparing given genotype and genders. As Table 4.2 shows, it appears that wild type males and females do differ significantly, while there appeared to be no meaningful difference between mutant males and females. It also appears that wild type males seem to differ significantly from GALT-null males, but no meaningful difference was observed between wild type females and GALT-null females. The most seemingly similar genotype/gender groups were GALT-null males and wild type females.

Table 4.2: Adjusted p-values for comparing glycogen content between male and female GALT-null and wild type rats.

Comparison	**Adjusted p-value**
Female WT – Female M3	.4213108
Male M3 – Female M3	.6651906
Male WT – Female M3	.2755087
Male M3 – Female WT	.9866887
Male WT – Female WT	.0164883
Male WT – Male M3	.0459145

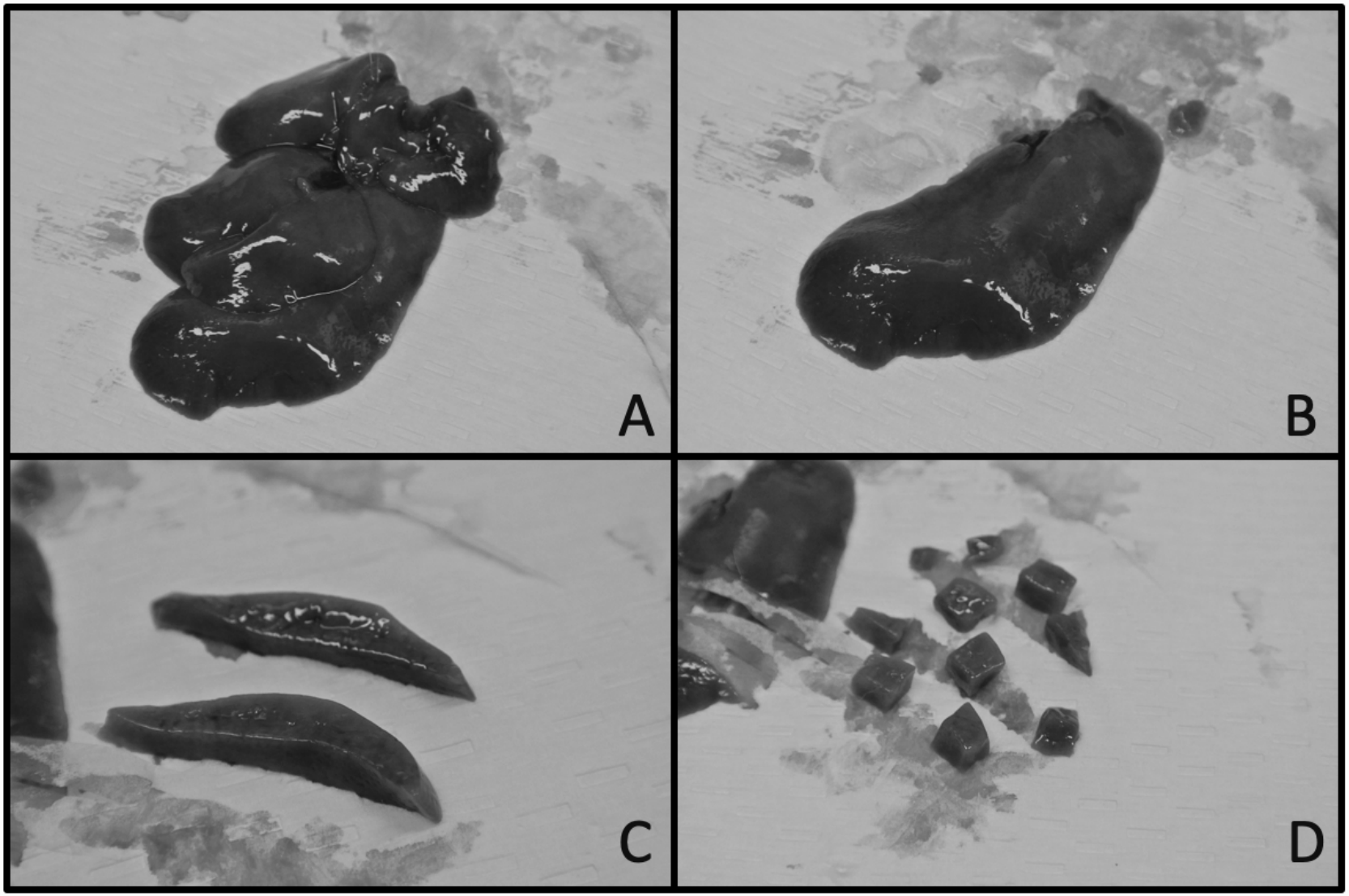

Figure 4.1. Liver tissue collection. Panel A depicts the liver whole directly after removal. Panel B depicts the isolated left lateral lobe of the liver. Panel C depicts the sagital slices taken from the left lateral lobe. Panel D depicts the approximately 2 mm thick pieces from the middle of the sagital slice that were used in the assay.

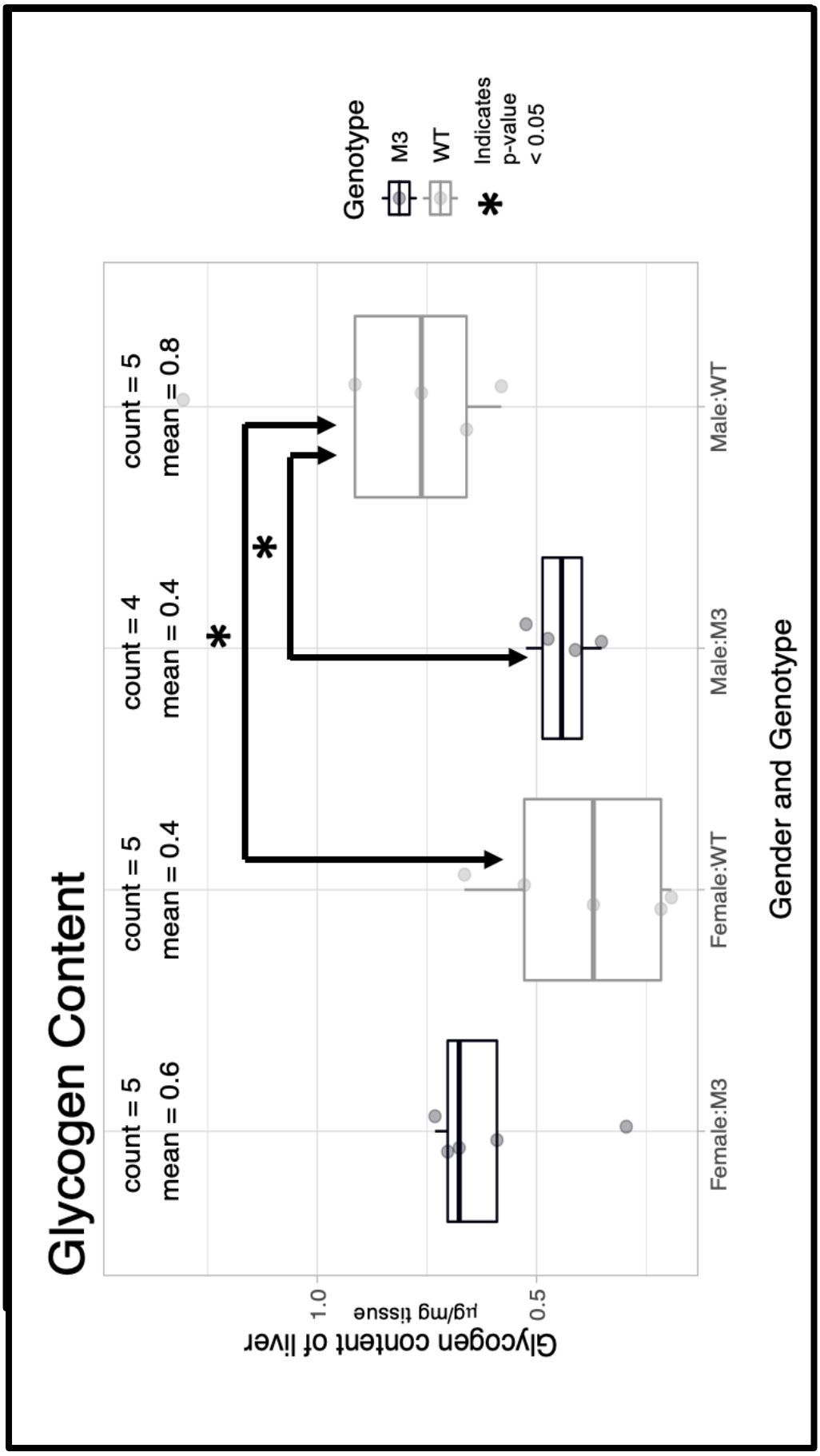

Figure 4.2. Comparison of liver glycogen content between GALT-null and wild type rats. The mean glycogen content of each female mutants, female wild types, male mutants, and male wildtypes were 0.6, 0.4, 0.4, 0.8 µg/mg of tissue respectively.

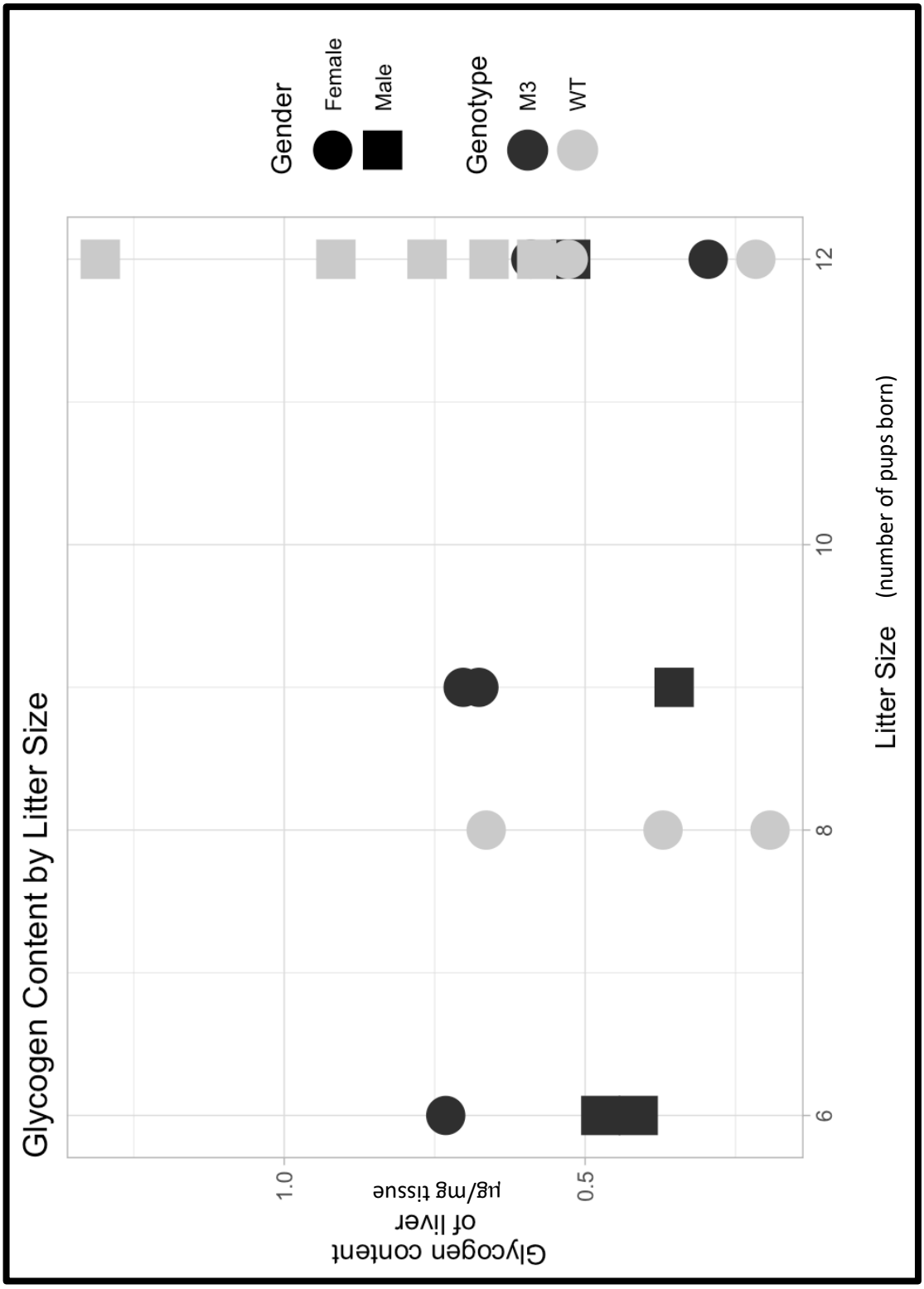

Figure 4.3. Lack of correlation between the liver glycogen content and the litter size of each rat. It appears that there is no meaningful difference between litter size and glycogen content of the liver, regardless of genotype and gender.

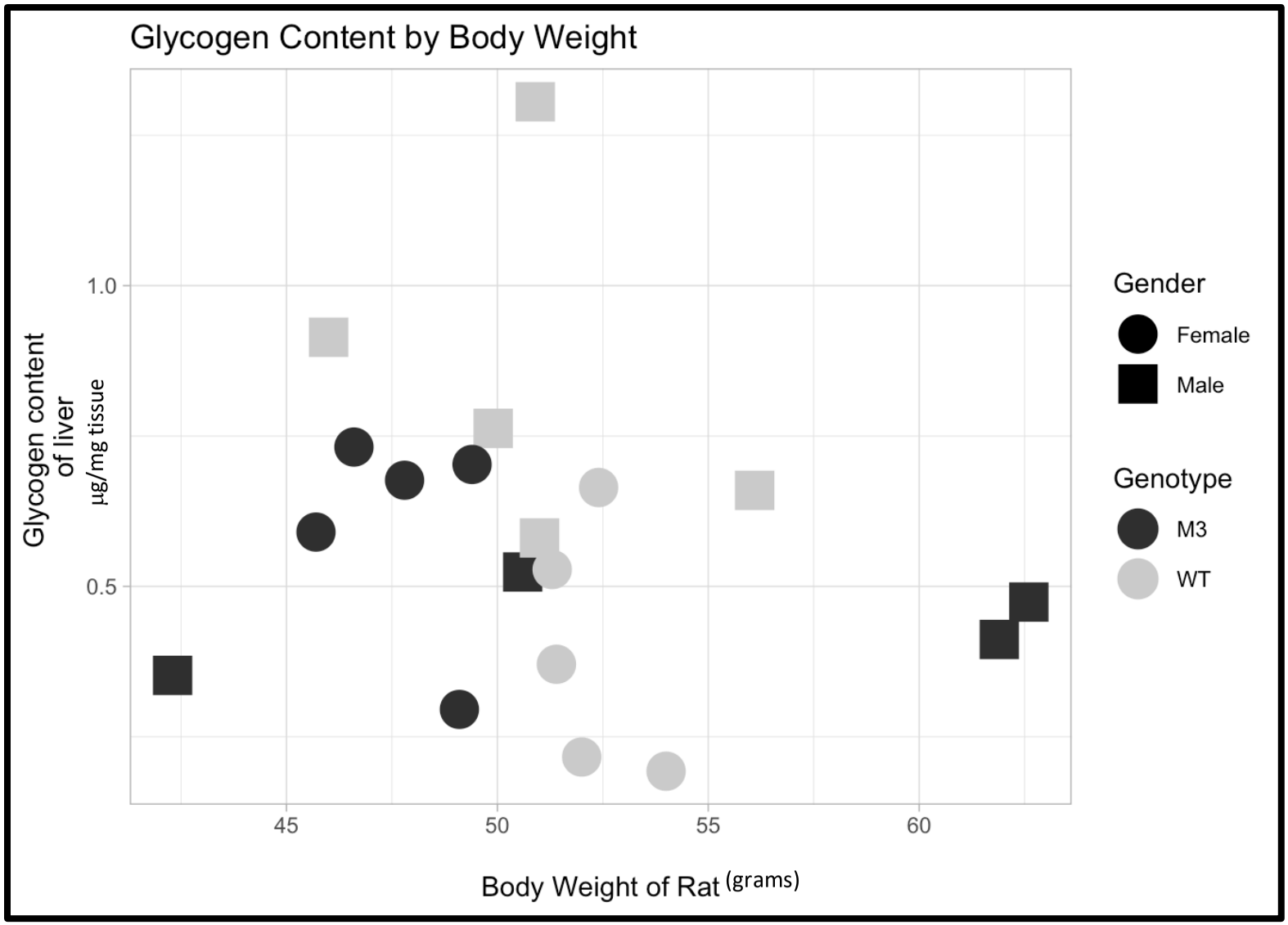

Figure 4.4. Lack of correlation between the liver glycogen content and the body weight of each rat. It appears that there is no meaningful difference between litter size and glycogen content of the liver, regardless of genotype and gender.

4.4 Discussion

While we did find a statistically significant difference when comparing the liver glycogen content between male mutant and wild type weanling rats, we asked whether there possibly existed other confounding variables that could explain the given results. Primarily, we were interested in looking at litter size (the number of pups born in the litter) and the body weight at the time of euthanasia. Figures 5 and 6 show the observed relationship between these possibly confounding variables and the glycogen content of the liver. Both figures seem to show no significant correlation between either variable and glycogen content of the liver. Furthermore, an Anova test was run and no significant difference was detected. This leads us to believe that GALT deficiency and gender are the most likely causes of the detected difference in liver glycogen content.

Limitations of this study are primarily due to small sample size. Additionally, since the rats had unlimited access to food and water, the exact time between the rat's last meal and the time of euthanasia were unknown. Furthermore, it is possible that incomplete extraction of glycogen among samples could have introduced a source of error in the assay. However, if the results found in this preliminary study are real, this model may provide valuable insight into the mechanisms behind the various energy metabolism outcomes in patients. One possible explanation may be that glycogen deficiency plays a role in growth outcomes in patients. Furthermore, this outcome may make the rat model more suitable to test possible interventions in CG.

Chapter 5: General Discussion

Classic Galactosemia (CG) is a metabolic disorder associated with many serious symptoms in patients. Today, the only intervention that exists for those suffering from CG is to place the individual on a galactose restricted diet (Berry, 1993). While this does prevent or resolve the acute and potentially-lethal negative outcomes, there exist more long-term complications which arise regardless of galactose-restriction (Bosch, 2006),(Fridovich-Keil et al., 2011). The underlying mechanisms of CG still remain largely unknown and there is currently no further treatment available for patients. To further knowledge about the disorder, the GALT-null rat model was created. The experiments described here contribute to initial characterization of this GALT-null rat model for classic galactosemia.

The results from these three studies appear to indicate that the rat model may recapitulate at least some of the symptoms seen in patients. However, it should be noted that the phenotypes described here are very subtle and we see no parallel to the acute sensitivity of infant patients with CG exposed to milk. Despite this, data from these studies suggest that GALT plays a role in pathways involved in development perhaps including both glycogen synthesis and storage. Similar to patients, we observe a significant growth delay in the model which appears to decrease with time. We also found interesting data regarding diet habits of GALT-null rats that may signal that they metabolize food differently than GALT+ rats. Finally, we found that male GALT-null rats did have significantly less glycogen than their GALT+ counterparts. However, we cannot explain why the opposite result was observed for female liver samples. While the results from this study were found to be significant, further research should be done to increase the number of animals tested.

In general, the results described here imply certain parallels between the model and patients and suggest that the rat may be a suitable model to study the underlying mechanisms of GALT deficiency and test possible interventions into CG.

References

Berry, G.T. (1993). Classic Galactosemia and Clinical Variant Galactosemia. In GeneReviews®, M.P. Adam, H.H. Ardinger, R.A. Pagon, S.E. Wallace, L.J. Bean, K. Stephens, and A. Amemiya, eds. (Seattle (WA): University of Washington, Seattle), p.

Bosch, A.M. (2006). Classical galactosaemia revisited. J. Inherit. Metab. Dis. *29*, 516–525.

Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somia, N.V., Bogdanove, A.J., and Voytas, D.F. (2011). Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res. *39*, e82–e82.

Coelho, A.I., Rubio-Gozalbo, M.E., Vicente, J.B., and Rivera, I. (2017). Sweet and sour: an update on classic galactosemia. J. Inherit. Metab. Dis. *40*, 325–342.

Douglas, H.C., and Hawthorne, D.C. (1964). Enzymatic Expression and Genetic Linkage of Genes Controlling Galactose Utilization in Saccharomyces. Genetics *49*, 837–844.

Douglas, H.C., and Hawthorne, D.C. (1966). Regulation of Genes Controlling Synthesis of the Galactose Pathway Enzymes in Yeast. Genetics *54*, 911–916.

Edmonds, A.M., Hennigar, G.R., and Crooks, R. (1952). GALACTOSEMIA: Report of Case with Autopsy. Pediatrics *10*, 40–47.

Fridovich-Keil, J.L., Gubbels, C.S., Spencer, J.B., Sanders, R.D., Land, J.A., and Rubio-Gozalbo, E. (2011). Ovarian function in girls and women with GALT-deficiency galactosemia. J. Inherit. Metab. Dis. *34*, 357–366.

Gaj, T., Gersbach, C.A., and Barbas, C.F. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. *31*, 397–405.

Geurts, A.M., Cost, G.J., Rémy, S., Cui, X., Tesson, L., Usal, C., Ménoret, S., Jacob, H.J., Anegon, I., and Buelow, R. (2010). Generation of Gene-Specific Mutated Rats Using Zinc-Finger Nucleases. In Rat Genomics: Methods and Protocols, I. Anegon, ed. (Totowa, NJ: Humana Press), pp. 211–225.

Jumbo-Lucioni, P.P., Garber, K., Kiel, J., Baric, I., Berry, G.T., Bosch, A., Burlina, A., Chiesa, A., Pico, M.L.C., Estrada, S.C., et al. (2012). Diversity of approaches to classic galactosemia around the world: a comparison of diagnosis, intervention, and outcomes. J. Inherit. Metab. Dis. *35*, 1037–1049.

Kushner, R.F., Ryan, E.L., Sefton, J.M.I., Sanders, R.D., Lucioni, P.J., Moberg, K.H., and Fridovich-Keil, J.L. (2010). A Drosophila melanogaster model of classic galactosemia. Dis. Model. Mech. *3*, 618–627.

Leslie, N.D., Yager, K.L., McNamara, P.D., and Segal, S. (1996). A Mouse Model of Galactose-1-Phosphate Uridyl Transferase Deficiency. Biochem. Mol. Med. *59*, 7–12.

Levy, H.L., and Hammersen, G. (1978). Newborn screening for galactosemia and other galactose metabolic defects. J. Pediatr. *92*, 871–877.

Rake, J.P., Visser, G., and Smit, G.P.A. (2006). Disorders of Carbohydrate and Glycogen Metabolism. In Physician's Guide to the Treatment and Follow-Up of Metabolic Diseases, N. Blau, J. Leonard, G.F. Hoffmann, and J.T.R. Clarke, eds. (Berlin, Heidelberg: Springer Berlin Heidelberg), pp. 161–180.

Tang, M., Siddiqi, A., Witt, B., Yuzyuk, T., Johnson, B., Fraser, N., Chen, W., Rascon, R., Yin, X., Goli, H., et al. (2014). Subfertility and growth restriction in a new galactose-1 phosphate uridylyltransferase (GALT) - deficient mouse model. Eur. J. Hum. Genet. *22*, 1172–1179.

National Newborn Screening and Genetics Resource Center. National newborn screening status report. Available online. 2014. Accessed 1-27-19.