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The Effect of Gluten on Puberty Onset and Glycemic Response in Female Rats

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THE EFFECT OF GLUTEN ON PUBERTY ONSET AND GLYCEMIC RESPONSE IN FEMALE RATS

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ABSTRACT

The Effect of Gluten on Puberty Onset and

Glycemic Response in Female Rats

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Introduction: While individuals with gluten sensitivities are instructed to follow a strict gluten-free diet, misinformed concerns over the consumption of gluten products has led to a rise in gluten avoidance diets, without a diagnosis of celiac disease. Gluten-free products, and therefore the diet as a whole, have been found to have twice as much fat, reduced carbohydrates and fiber, and approximately one-third less protein (Miranda 2014). Research on the effects of gluten-free diets on adolescent development is lacking. With recorded nutrient deficiencies in those that abstain from gluten, detrimental effects could be seen in adolescents who are put on non-prescribed gluten-free diets. Body mass is one factor in puberty onset, and may occur earlier in individuals consuming a gluten-free diet, from lower quality ingredients used in gluten-free products.

Purpose: To evaluate the effects of gluten on weight gain, body composition, glycemic response, and puberty onset in newly weaned female rats, hypothesizing that gluten restriction causes a faster increase in body fat, triggering an earlier onset of puberty.

Also, that gluten-restriction causes a higher increase in blood glucose than gluten-containing food.

Methods: Sprague-Dawley female weaned rats (n=20), age 23 days, were randomly separated into two groups, and fed either a gluten-free chow or a normal chow diet. Animals were housed separately at the UNLV LACF in a 12-hour light/dark cycle room; water was provided *ad libitum*. Food intake, body weight and blood glucose were recorded daily for two weeks. Blood was collected from a tail clip, and glucose was measured using a Bayer Contour glucose meter and test strips. Rats were checked daily for vaginal opening starting on the 25th day of life. After vaginal opening was confirmed, rats were euthanized and body composition was measured by a DXA scan.

Results: Vaginal opening in the gluten-free group occurred earlier than in the control group (30 ± 2 vs 31 ± 2 days for gluten-free vs control respectively, p=0.01). Percent body fat was increased in the gluten-free group compared with controls (13.8 ± 0.39 vs 10.4 ± 0.72, p=0.015), however, weight gain (86 ± 2 vs 84 ± 3 g), food intake (236 ± 30 vs 215 ± 24 g), and daily blood glucose (83 - 86 mg/dL vs 80 mg/dL - 85 mg/dL for gluten-free and control respectively) were not significantly different between the two groups.

Conclusion: Vaginal opening, and hence onset of puberty, occurred sooner in rats fed the gluten-free diet. Percent body fat was higher in the gluten-free group, consistent with the suggestion that increased body fat plays a role in the decreasing age of menarche in adolescent females. These results reinforce the importance of diet and how it can affect growth and maturation.

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CHAPTER 1

INTRODUCTION

Adolescent years are the most important for consuming appropriate nutrient quantity. While breast milk and colostrum help transition the infant into a state of enteral nutrition, by promoting a functioning gastrointestinal (GI) tract and development of enzymes, post-weaning, the intestinal lumen builds new structures and increases functionality. Restrictive diets during pregnancy and the first years of life can retard growth, including of the stomach and intestines. The majority of skeletal development also occurs before puberty, therefore making childhood nutrition vital. Diets limiting consumption of certain food items, such as the gluten-free diet (GFD), may have detrimental effects on growth and maturation.

Celiac disease is a genetically linked autoimmune disease characterized by the inability of an individual to digest gluten, a protein found in wheat, rye, and barley, possibly causing damage to the small intestine. Misinformed concerns over the consumption of gluten products causing such issues as obesity and Autism in children, has lead to a rise in gluten avoidance diets, not contributed to a celiac disease diagnosis. A survey found that the top three reasons parents implemented a gluten-free diet in their child without confirmed celiac disease was due to irritability or poor temper, diarrhea, and weight issues (Tanpowpong 2012).

Individuals with gluten sensitivities are instructed to follow a strict gluten-free diet. Gluten is the normal property that allows wheat flour to leaven, when it combines with yeast, allowing bread to rise. Removing gluten to make gluten-free (GF) food items causes a change in product volume, cell structure, and texture (Capriles 2014). Poor

mouth feel and flavor can lead to lower intake of quality foods in children who are picky eaters. Also, gluten-free products tend to vary in micronutrient composition due to less than optimal wheat replacements.

Gluten-free products have lower levels of fiber and micronutrients compared to items with gluten, since wheat replacements tend to be refined flours, and are generally not enriched or fortified (Capriles 2014). A study that looked at a three-day food recall and evaluated gluten-free nutritional differences between their gluten counterparts found that due to differences in processing, gluten-free products, and therefore the diet as a whole, have twice as much fat, reduced carbohydrates and fiber, and approximately one-third less protein (Miranda 2014).

Lower micronutrient intake, higher calories, and intake of less than optimal food items can lead to issues in growing children, especially those on non-prescribed gluten free diets. Research on the effects of gluten-free diets on adolescent development is lacking. With recorded nutrient deficiencies in those that abstain from gluten, detrimental effects could be seen in adolescents that are put on non-prescribed gluten-free diets. The findings that body mass is connected with puberty onset may show earlier onset in individuals consuming a gluten-free diet, from the possible consumption of hypercaloric foods.

Research Hypothesis

The research focused on answering the question: Does the restriction of gluten from the diet effect adolescent development and initiate puberty sooner than a diet containing gluten?

Hypothesis 1: Gluten restriction causes a faster gain of body fat, signaling an earlier onset of puberty than a non-restricted diet.

Null hypothesis 1: Gluten restriction does not cause a faster increase in body fat than a non-restrictive diet, and has no effect on puberty onset.

Hypothesis 2: Gluten restriction causes a higher increase in blood glucose than gluten containing food.

Null hypothesis 2: Gluten restriction and gluten containing food will show no differences on blood glucose.

CHAPTER 2

THEORETICAL AND EMPIRICAL REVIEWS

Based on early research in the 1960s by Marshall and Tanner who observed variations in the age and time between the stages of development in female girls, norms (known as the Tanner Stages 1-5) were created for the timing of puberty events that are still utilized in Pediatrics today. The first signs of puberty generally appear between 8 ½ and 13 years of age in most (95%) girls, with full maturation reached between ages 11.8 and 18.9 years, and menarche around 13 years old (Marshall & Tanner 1969). Since their research was published, the timing of pubertal development has steadily decreased, showing girls maturing at a much earlier age. This change is due to the many factors that control the onset of puberty. Genetics, hormones, environmental influence, and even the prenatal environment of the womb and maternal nutrition during lactation, can all affect the age at which an adolescent initiates maturation.

A neuropeptide, Kisspeptin, has been identified as a necessity for successful puberty. Kisspeptin has been found to stimulate the release of Gonadotropin Releasing Hormone (GnRH), a major hormone released from the hypothalamus that influences the start of menses. Research has manipulated the genome to knockout the *Kiss1* gene in mice, which is how it was identified as a marker for puberty onset control; without the gene, mice had impairment or complete loss of puberty and eventually developed hypogonadotropic hypogonadism (Takumi 2015). Using this information, Takumi et. al looked further at control of the gene and possible influences on it by maternal diet during lactation. Two groups of dams were used: one fed a high-fat chow (HFD), the

other a normal chow, following birth of pups to the day of weaning. Looking at the differences in *Kiss1* expression in the arcuate nucleus, the pups in the HFD group showed a significantly higher expression (305.80 ± 31.90) than the pups in the normal diet group (198.00 ± 18.15) (Takumi 2015). The higher expression of the *Kiss1* gene lead to significantly earlier vaginal opening of the HFD group (30.86 ± 0.27) than the normal diet group (31.19 ± 0.37) (Takumi 2015).

Frisch et. al. studied the effects of high-fat diet on estrus in 42 weaned baby rats. In rats fed a high-fat diet (24.6% of total calories), estrus was found to occur at day 33.3 of age compared to 37.4 in rats on a low-fat diet (5.0%) (Frisch 1975). This was due to a fast increase in body weight and overall body fat. It's hypothesized that the typical high-energy, high-fat American diet causes metabolic effects, which can alter the timing of puberty in adolescents.

Leptin, the hormone secreted by adipose tissue that controls satiety, has also been studied as a possible influence on puberty onset because of its receptor located on kisspeptin, and the relation between body fat and puberty onset. Ahima et. al. studied the effects of daily injections of recombinant leptin on the onset of puberty in post-weaned baby mice, and found that those with injected leptin had an earlier vaginal opening, estrus, and cycling, compared to controls (1997). The leptin-injected mice however, did not differ in body weight gain compared to the mice that were injected with a saline solution (Ahima 1997). Takumi et. al. also injected baby rat pups with either leptin or a vehicle, from day 15 to day 21 of life (weaning age), and then explored differences in body weight and expression of *Kiss1* neurons. Takumi found no significant differences in each test (2015). Takumi's research though only examined the

Kiss1 expression on day of weaning, which has been shown to be the lowest time of expression in general. Expression of the gene tends to occur post-weaning, therefore, a study with leptin injections until puberty onset, like Ahima et. al, may have shown different results.

A formula created by D. Frances Edwards and R. Kay, after studying differences in time elapsed between weaning and vaginal opening in rats of varying litter sizes, stated that the weight at vaginal opening was influenced by litter effect of male and females reared together, time of vaginal opening, and weight at weaning:

$$W_{vo\_} = a_j + bW_w + ct_{vo} + \text{residual (1985).}$$

Previous research manipulating litter size determined that rats in small litters, approximately 4, tend to have overweight phenotypes, while rats in large litters, approximately 20, tend to have lean phenotypes (Smith & Spencer 2012). Edwards and Kay recorded weight at weaning and vaginal opening, as well as time in days elapsed between the events in 81 female baby rats, and found that the mean weight at vaginal opening was higher for rats from small litters (106.5 ± 2.2 g) and smaller for rats in larger litter (98.8 ± 2.1 g) (1985). Also, time elapsed between the two events was significant; the smaller litter with higher body weight had vaginal opening approximately 1.4 ± 0.3 days sooner than the larger litter (Edwards & Kay 1985). Smith & Spencer also evaluated litter size effect, and looked at its effect on puberty onset.

Smith & Spencer randomly redistributed baby rats between dams to create varying litters of small, control, and large size, with 4, 12, and 20 pups respectively (2012). Male pups in the larger litter, considered underfed because of competition during nursing, showed slower puberty onset than control and the small litter (Smith & Spencer 2012).

Female pups in the small litter, considered over-fed because of more availability to nurse, reached puberty faster than control (Smith & Spencer 2012). Though food intake was not recorded, after pups were weaned and given equal access to chow, differences in body weights were still observed. Thus, babies that were fed by guardians rather than allowed to feed themselves, may have higher than needed intakes and continue on this trend in later stages of life, possibly setting up the individual for unhealthy weight gain.

While high-energy, high-fat diets can lead to a faster increase in weight change and change timing of puberty, under-feeding can also have effects on development and maturation. In one study, three groups of female pregnant Wistar rats were used: one control, one induced early by intra-uterine growth retardation, and one that was manipulated as a food restricted litter, which was of larger size, n=18 (Engelbregt 2000). Intra-uterine growth retardation (IUGR) caused the pups to be born smaller than normal (minus two standard deviations of the mean of the weight of control pups), simulating birth size of larger litters (Engelbregt 2000). After being offered a normal diet post-weaning, rats were checked for vaginal opening and body weight gain, and showed that rats in the food-restricted larger litter had the slowest rate of weight gain (Engelbregt 2000). Pre-maturely born rats were significantly delayed in puberty onset compared to control (37.4 ± 2.7 compared to 36.1 ± 1.5), though the food restricted rats showed no difference in day of vaginal opening (Engelbregt 2000). This leads to the hypothesis that weight isn't the necessary factor for puberty onset, but body composition in relation to fat percentage, could have more control. Since rats in the food restricted group were the smallest, but did not show delay of vaginal opening, they could have caught-up in body fat gain post-weaning when they were given the same access to food as the other test

groups. The IUGR rats were stopped in womb from developing completing like the other rats, which could have changed the number of adipose tissue cells that each was born with, thus not allowing for higher amounts of fat to be retained and causing a lower weight gain as well as a delay in puberty.

Similar to under-feeding, exclusion diets, like the gluten-free diet, need to be researched for possible implications on development. Research is available for macro- and micro-nutrient differences between gluten-free products and their standard counterparts. Polito et. al. studied the growth, both height and weight, in 17 children with celiac disease, who had a history of being on a gluten-free diet. From a three-day dietary recalls, researchers found an excess in calories, animal protein, and lipid intake, as well as a deficit in complex carbohydrates and vegetable proteins (Polito 1992). The researchers associated these dietary issues with the less than optimal food choices the children were making: mostly meats, cheeses, chocolate, fried potatoes, etc., since the modified wheat products most likely didn't appeal to the children's taste (Polito 1992).

In addition to the micronutrient differences and macronutrient proportions in a product, the protein digestibility and amino acid content can affect nutritional value. Burns et. al examined the differences in weight change in post-weaned beagles and rats given diets varying in protein levels, from 0% to 20%. The researchers also looked at protein utilization, by measuring nitrogen balance, of three different protein sources: soy, casein, and wheat gluten (Burns 1982). Five total experiments were performed. In the first experiment, research showed that the dogs on higher levels of protein actually consumed more food than those on lower to no protein (Burns 1982). This could be because of higher amounts of fat and carbohydrates, satisfying the dogs sooner than

those on the protein rich diet. Wheat gluten promoted changes in growth the least out of the three sources, though overall food intake was less in the wheat gluten, thus research cannot be used to signify wheat gluten as a unnecessary protein for growth.

Blazina et. al examined the differences in bone mineral density and Body Mass Index (BMI) for age of 55 children and adolescents with a celiac diagnosis, who had been on a strict gluten-free diet for two years (2010). Adherence to the GFD was determined by absence of endomysium antibodies for the past two years, though it was not stated where this information was obtained. The researchers also concluded that most of their subjects who followed the GFD for more than two years "had normal pubertal development," and that delayed pubertal development occurred in those with different heights, which influenced bone mineral density and puberty onset (Blazina 2010). Researchers did not identify what markers pubertal development represented, though only height and weight were measured, and no questions of menstruation or bodily development were discussed.

Low intake of calcium and vitamin D can affect a growing child's skeletal growth. Blazina et. al. examined the differences in bone mineral density of children on a strict gluten-free diet compared to children on a not strict diet (2010). The researchers found that though individuals on a not strict diet can have lower bone mineral density because of lack of absorption, due to inflamed mucosa of the small intestine, individuals on a strict gluten-free diet also have lower bone mineral density due to lower intake of calcium and vitamin D. Calcium intake was only 85% of the recommended intake in the strict GFD group (Blazina 2010). Serum vitamin D levels were recorded and found to be significantly deficient, below 50 nmol/L, for all subjects from December to mid April,

when sun exposure is minimal (Blazina 2010). Low calcium and vitamin D not only put younger individuals at increased risk of fractures and bone plate developmental issues, but also increase their risk of developing osteoporosis.

Research has assessed the differences in glycemic response between standard foods and gluten-free versions, and also between different types of wheat alternative foods. One study tested postprandial glucose response of three different gluten-free pastas in non-celiac and celiac subjects. All individuals were fed a meal that included 50 grams of carbohydrates, from rice flour, corn flour, or a mix of both. The pasta made with rice flour showed the greatest increase in blood glucose after consumption, compared with the corn flour pasta and the mix of rice and corn flour pasta (Bacchetti 2014). Rice is digested more quickly, which is why it showed a higher effect on blood glucose. Celiac participants had a higher glycemic effect compared to the control group, which could be due to differences in A1C (blood test showing average blood glucose over past two to three months; percentage of hemoglobin coated with sugar), intestinal lumen, or even pancreatic function, since there is a strong association between celiac and Type 1 Diabetes.

Gluten-free products are also digested differently and have been found to have negative effects on metabolic responses. Various pastas and breads, regular and gluten-free, as well as quinoa, were submerged in pepsin and pancreatic α-amylase in a test tube to simulate digestion, and then evaluated by how much sugar was diffused (Berti 2004). *In vitro* results showed significant differences in the gluten-free bread versus the standard bread product, with higher observed effects on Area Under the Curves (AUC) of digested starch over 5 hours from the gluten-free product (4426 ± 656

mg min dl^{-1}) than the standard bread (3914 ± 191 mg min dl^{-1}) (Berti 2004). No differences were seen between the two types of pasta and the quinoa (Berti 2004). This research shows that there can be great differences even between gluten-free products themselves.

Berti et. al. also looked at the various food products *in vivo* and evaluated glucose, insulin, free fatty acids, and triglyceride blood level effects from 50 grams of carbohydrate of each item (2004). Sample size was small: seven in the "healthy" subject group, and six celiac participants. The AUC of glucose response was much higher in the celiac subjects (2261 ± 2473 mg min dl^{-1} for GF pasta) compared to the "healthy" subject (1127 ± 758 mg min dl^{-1} for GF pasta) (Berti 2004). During processing, removing gluten from a wheat based product causes a structural change so that the starch is more accessible, and thus decreases the rate of starch availability, causing a larger increase in glycemic response (Berti 2004). It's possible that the differences in GI integrity could contribute to the differences in glycemic response. Celiac disease is also associated with a higher incidence of type 1 diabetes, therefore making the glycemic response of gluten an important aspect for future research's focus.

In relation to the increased trend of individual's choosing a gluten-free diet due to lack of information, a recent study showed that individual's claiming to be "gluten sensitive" could not correctly identify gluten-free food items from standard items with gluten. Researchers studied 35 individuals with self-diagnosed gluten sensitivities (non-celiac gluten sensitivity) who were maintaining a gluten-free diet. During the experiment, subjects were given 10 bags of 10 grams of either gluten-containing flour or gluten-free flour inside, and were instructed to sprinkle the flour on top of meals like pasta and

soup, to use one bag each day for a 10-day challenge (Zanini 2015). The experiment was repeated so that each individual experienced each type of flour. Two weeks were given in between the experiments as a "washout period" (Zanini 2015). Participants were asked to record a food log, and answer the question at the end of the experiment, "Do you think that gluten was in the sachets labeled A, or in those labeled B?" (Zanini 2015). Out of the 35 subjects tested, six stated they experienced no symptoms during the entire experiment; 17 experienced symptoms with gluten-free flour and 12 with gluten flour (Zanini 2015). Zanini et. al. hypothesized that the individuals who experienced gastrointestinal issues with the gluten-free flour may have intolerances to other ingredients in these and gluten products, or may be sensitive to FODMAPs (Fermented Oligo- Di- Mono- saccharides And Polyols) which have been shown to trigger similar symptoms as celiac disease in persons' with Irritable Bowel Syndrome (IBS) (2015). This study provides further evidence that individuals should not self-prescribe gluten-restricted diets; a celiac diagnosis or instruction from a Physician or other qualified Registered Dietitian Nutritionist should be obtained before major changes be made, that could possibly lead to nutrient deficiencies.

"Female Puberty: A Comprehensive Guide for Clinicians" looks at the trending decrease of puberty onset in children from a positive evolutional standpoint stating that it's related to the increased availability of proper nutrition, which has lead to improved status and earlier body weight gain (2014). Early onset of puberty however, has been linked to "obesity, adult-onset diabetes, breast cancer, disordered eating, and anxiety," as well as increased adolescent risk-taking behavior, teenage sexual intercourse, pregnancy, and STD exposure (Smith & Spencer 2012).

Using previous data collected on 60 Italian gluten-free products, Pellegrini et. al. expanded her research by comparing nutrition information to gluten-containing counterparts. Pellegrini et. al. found that “potassium, phosphorus, calcium, iron, zinc, and B vitamins of almost all types of GF bread and pasta” tested, showed lower levels than standard products (2015). Also, 46 out of the 60 products tested were considered high sodium containing items, having greater than 400-500 mg sodium per 100 g of food (Pellegrini 2015). The American Heart Association recommends limiting sodium to less than 1,500 mg per day; most Americans consume more than twice this amount. This shows that salt is used to make gluten-free foods more appealing to a society that is accustomed to salty tasting food items. Consuming higher sodium gluten-free products increases an individual’s blood pressure, thus putting strain on the heart. These factors not only have negative implications for an individual in a state of maturation, but also put an individual at a higher risk of cardiovascular disease, the number one killer in the United States.

Data on the effects of gluten restriction on development and puberty onset is lacking. Though research has not yet focused on the effect of gluten restriction on maturation, nutrient deficiencies created from these products and differences in macronutrient content can shed light on possible effects during childhood. Nutrient deficiencies caused by lower quality macronutrients and elimination of needed fortified vitamins and minerals from gluten-free products can lead to stunted bone growth, excessive weight gain, and decrease age of puberty onset, potentially leading to disease in an adolescent’s future. Focusing research on the effect of gluten restriction

on body weight change, vaginal opening, and blood glucose can provide parents with additional evidence-based material when deciding how to structure their child's diet.

CHAPTER 3

METHODOLOGY

Materials

Twenty Sprague-Dawley female newly weaned rats, age 23 days, were obtained from Taconic Biosciences, Inc (Taconic Biosciences, Rensselaer, NY). Pups had similar starting weight. Original weight was recorded with an average of 59 ± 5 grams, upon receiving.

General Conditions

Rats were randomly assigned into one of two groups (n= 10/group) and fed either a normal chow diet (control) or gluten-free chow (treatment) (initial weight = 58.9 ± 3.9 vs. 58.4 ±5.9 for control and treatment respectively, p=0.39), both formulated by LabDiet (LabDiet, St. Louis, MO). LabDiet's formula 5053 was used as the standard chow. Wheat products and wheat-byproducts are removed to create a formula free of gluten ingredients.

Animals were housed in the UNLV LACF in separate cages so that daily food intake could be recorded. Housing is set in a 12-hour light/dark cycle room; water was provided *ad libitum*.

Study Design

The body weight of each rat was recorded between 3 pm and 5 pm each afternoon. Food that was not consumed from the previous days feeding was collected and weighed. Separate containers were used to weigh food to reduce possible cross-contamination of gluten containing food into non-wheat food.

Blood glucose was recorded daily using a Bayer Contour glucose meter

(Ascensia Diabetes Care, Parsippany, NJ) and test strips. Blood was collected from a tail clip (0.5 mL from the tip of tail). The first drop of blood was discarded; the second drop collected on a test strip and analyzed.

Rats were checked daily for vaginal opening starting on day 25 of life, by restraining the rat in one hand, on its back, and observing the vaginal cavity. The opening could be visualized by sight; it is sealed closed until hormonal stimulation. After vaginal opening was confirmed, rats were euthanized and body composition was measured by a dual energy x-ray absorptiometry (DXA) scan with small animal software (Lunar Prodigy, General Electric).

Statistical Analysis

Age at vaginal opening, glucose, weight gain, body fat, and food intake were analyzed. Values that were skewed due to technical or experimenter errors were excluded from data analysis. A one-tailed, independent t-test was used to analyze differences between the two groups for weight gain (grams), age at vaginal opening, and feed efficiency. Statistically significant effects were set at $p<0.05$.

CHAPTER 4

RESULTS

Sample Characteristics

Descriptive data for the animals is summarized in table 1. The total sample size was 20 rats (n=10). The age was controlled: 23 days old upon receiving. The mean starting weight for the control group was 58.9 ± 4 g; treatment group was 58.4 ± 6 g (p=0.39).

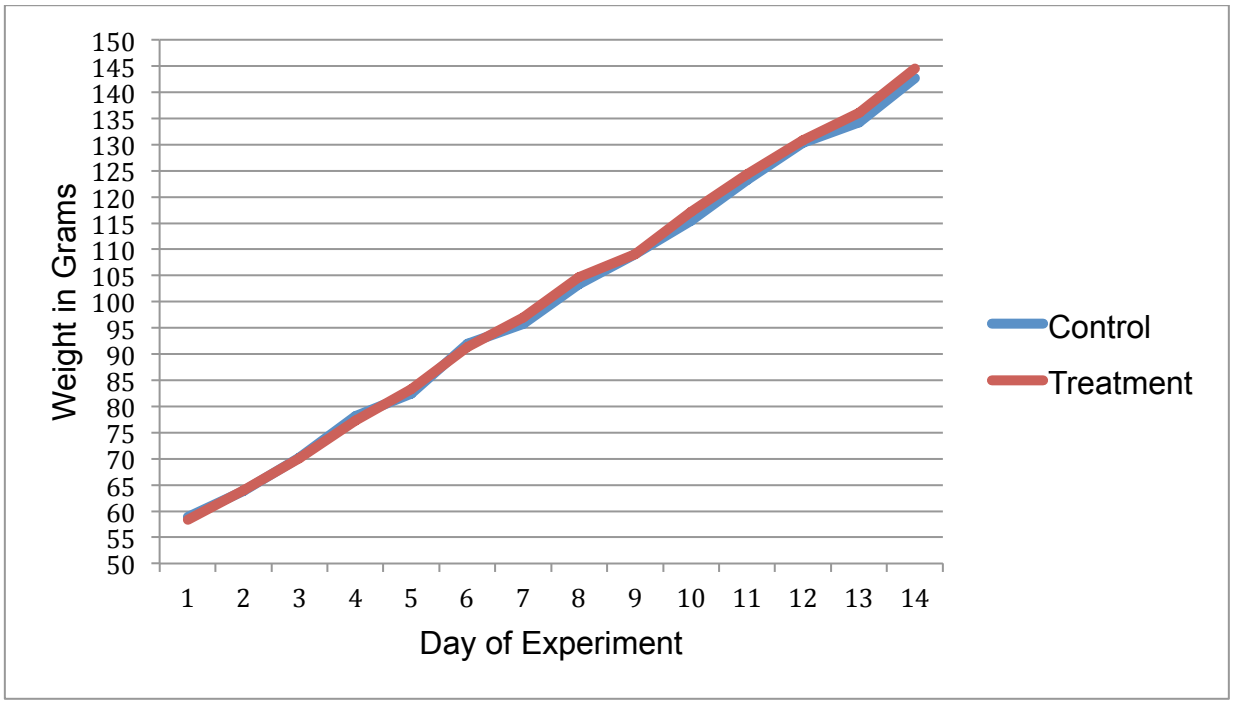
Table 1. Descriptive statistics for animals randomly assigned to control/treatment group.

Category	Sample (n)	Pre-Treatment Weight Mean (grams)	Post-Treatment Weight Mean (grams)	Percent Weight Change	Total Weight Gain Across Time (grams)	Age at Vaginal Opening (days)
Control	10	58.9 (S.D. 3.9)	142.7 (S.D. 11.1)	59%	83.9 (S.D. 9.7)	31 (S.D. 2)
Treatment	10	58.4 (S.D. 5.9)	144.5 (S.D. 8.5)	60%	86.2 (S.D. 6.3)	30 (S.D. 2)

Post-treatment weight was 142.7 ± 11 g for the control, compared to 144.5 ± 8.5 g for the treatment group. The treatment group gained more weight across time (86.1 ± 6 g or 60% of original weight) than the control group (83.8 ± 10 g or 59% of original weight), though the weight change was not significant (p=0.29). Figure 1 expresses the similarity in growth of each test group.

Age of vaginal opening differed significantly between the two groups. The control group (M= 31 ± 2 days) was significantly delayed (p=0.01) to reach this measured marker for puberty onset than the treatment group (M=30 ± 2).

Figure 1. Weight Gained Across Time

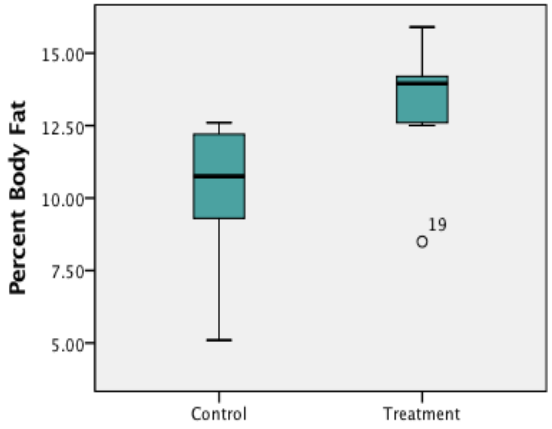


Percent body fat was determined by a DXA scan (Table 2). Mean differences in percent body fat between the two groups are shown in Figure 2. Although Rat 19 was found to be a significant outlier (Q_1 – (1.5xIQR)= 12.65-(1.5x1.975) = 10.35; rat 19= 8.5% body fat), rats in the gluten-restricted group had a significantly higher percentage of body fat (M=13.4, SE= 0.39), than rats fed a standard diet (M= 10.4, SE = 0.72), ($p = 0.015$).

Table 2. Percent Body Fat Results

	PERCENT BODY FAT (CONTROL 1-10)	PERCENT BODY FAT (TREATMENT 11-20)
	12.2	14
	10.8	14.1
	12.6	12.5
	10.1	15.9
	10.7	12.6
	8.8	13.9
	5.1	14.2
	11.5	12.8
	12.6	8.5
	9.3	15.6
MEAN	**10.4**	**13.4**
S.D.	**2.3**	**2.1**

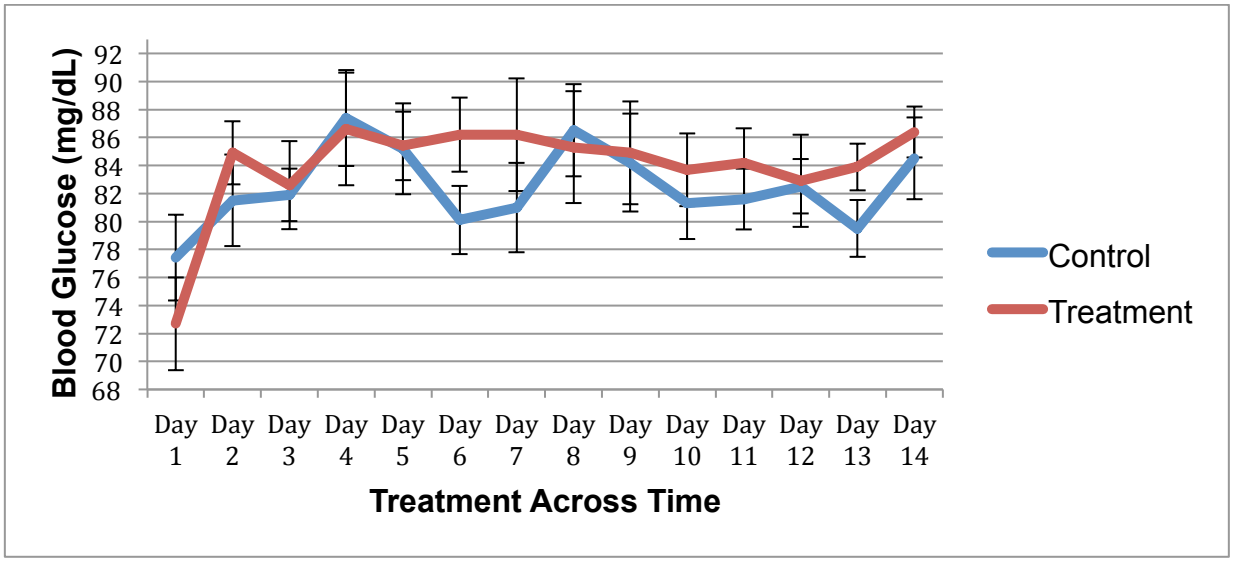
Figure 2. Differences in Percent Body Fat



Blood Glucose and Food Intake

The mean of the initial blood glucose was 77 mg/dL for the control group, while the treatment group had a mean glucose of 73 mg/dL. Figure 3 displays the average blood glucose values, with standard error bars, between the two groups across the time of the experiment. The treatment group showed a quartile range between 83 mg/dL and 86 mg/dL, while the control group had more fluctuation, ranging between 80 mg/dL and 85 mg/dL. Although there were day-to day fluctuations in glucose, the daily differences in glucose between groups were not significant (Appendix I).

Figure 3. Average Blood Glucose Across Time



Nutrition facts and ingredients for each food type are shown in appendix II. The gluten containing ingredients were eliminated from the standard chow, the feed used for the control group, to make the feed for the treatment group. Protein, carbohydrates, and fat were maintained at equal percentages and contributed to the same caloric breakdown (control: 3.43 kcal/g; treatment: 3.44 kcal/g). The total food consumed for the control group was M=215.4 ± 23.9 g, while the treatment group consumed M=235.7± 29.7 g. Total food consumption between the two groups across the time of the experiment was not significant ($p=0.053$). Food efficiency, weight gained per calorie consumed, was also not significant ($p=0.137$).

CHAPTER 5

DISCUSSION

Gluten-free products were designed for individuals with celiac disease who could not physically digest the gluten protein. Individuals without a celiac diagnosis started eliminating gluten from their diets based on the mistaken assumption that gluten was an unhealthy protein, which caused stomach upset or weight gain. Since research on the effects of gluten restriction is minimal, following an unnecessary gluten-free diet may lead to physical problems, especially in developing adolescents.

Previous research studying the potential factors that influence puberty onset have looked at genetic, physiological, and environmental factors with limited data focusing on special diets, in particular the gluten-free diet (Takumi 2015; Engelbregt 2000; Ahima 1997). When gluten was restricted from the diet of post-weaned female rats, total food consumption and overall weight gain was similar to rats fed a gluten product, but body fat was significantly higher: 13.8% in treatment compared to 10.4% in control ($p < 0.05$). Body fat, rather than weight, has been previously examined as an indicator for early onset of puberty (Engelbregt 2000), and the significantly higher body fat did lead to pups maturing faster.

In newly weaned rats fed a high-fat diet (24.6% of total calories), estrus was found to occur at day 33.3 of age compared to 37.4 in rats on a low-fat diet (5.0%) (Frisch 1975), ostensibly due to a rapid increase in body weight and overall body fat. Similarly, mean weight at vaginal opening was higher for rats from small litters (106.5 ± 2.2 g) than rats from larger litter (98.8 ± 2.1 g), and rats from the smaller litter with higher body weight had vaginal opening approximately 1.4 ± 0.3 days sooner than rats from the

larger litter (Edwards & Kay 1985). Conversely, pre-maturely born rats were significantly smaller than normal and puberty onset was delayed compared to control animals (Engelbregt 2000). The rats fed the gluten-restricted diet had significantly earlier vaginal opening, used as a marker for puberty onset, compared to rats fed a standard chow. Vaginal opening in the treatment group occurred in 30 ± 2 days compared with 31 ± 2 days in the control group.

While differences in micronutrient content has been shown from replacing wheat with less quality ingredients, focuses on the elimination of the gluten-containing ingredients, maintaining nutrient quality, was unknown. When rat feed was produced to examine this difference, overall food intake, feed efficiency, and blood glucose were not significantly affected. The total food consumed for the control group was M=215.4 ± 23.9 g, while the treatment group consumed M=235.7± 29.7 g, and was not significant ($p=0.053$) across the time of the experiment. Since calories were kept consistent per gram of food as well, feed efficiency could be calculated and properly compared. Even with the almost significant differences in food consumption, feed efficiency was not significant ($p=0.137$). This can suggest that the slight difference in ingredients in the feed, the elimination of wheat products, could be a contributing factor to the differences in overall body fat.

Micronutrient content is controlled in animal feed to meet daily recommendations, though food made for human consumption is not always fortified or enriched. Gluten-free products have lower levels of fiber and micronutrients compared to items with gluten, since wheat replacements tend to be refined flours, and are generally not enriched or fortified (Capriles 2014). Due to differences in processing, gluten-free

products, and therefore the diet as a whole, have twice as much fat, reduced carbohydrates and fiber, and approximately one-third less protein (Miranda 2014). In the current study, body fat were significantly higher in rats fed food that had wheat ingredients eliminated, but maintained the same ratio of macronutrients and micronutrients. Differences in bone mineral density, nutrition related blood levels, growth rate, and overall weight could therefore be affected in individuals that consume gluten-free products that are not properly held to high standards to replace missing vitamins and minerals.

Obesity and overweight, particularly higher amounts of adipose tissue and abdominal obesity, are linked to higher precedence of developing diabetes and metabolic syndrome. Differences in the effect on blood glucose and area under the glucose tolerance test curve have been seen between gluten-free products and their counterparts, though research has mainly looked at gluten-free products in individuals with celiac disease (Berti 2004; Bacchetti 2014). When rats without a celiac diagnosis were fed gluten-free chow, their blood glucose levels were not significantly different than the control. The average blood glucose levels across time are displayed in Figure 3. The treatment group tended to average between 83 mg/dL and 86 mg/dL, while the control group had more fluctuation, averaging between 80 mg/dL and 85 mg/dL, however the daily differences were not significant. The blood glucose was recorded daily during the nocturnal state of the rats, which was meant to simulate a fasting state. Blood glucose tended to be in the normal range, 70-100 mg/dL, though some rats did have glucose levels out of range, which could have skewed averages. From an

endocrinology view, the treatment group had more favorable levels, since the spiking levels as seen in the control group might create a higher risk for developing diabetes.

Limitations of the Study

Since the research was performed on animals, certain aspects of data collection could not be performed. The study looked at only a short time period of the life of the rat, though it has been correlated that 13.2 rat days is equivalent to 1 human year (Sengupta 2013). Further growth was still expected in the rats, since the average full-grown female Sprague-Dawley rat weights between 250 to 300 grams (Sengupta 2013).

Initial body fat could not be obtained, since animals had to lay still in a particular way for proper measurement. Having both pre- and post-body fat measurements would have provided further details on growth in the animals.

Food was available at all times for the rats, which could have caused error in blood glucose collection. Rats tended to awaken when data collection was conducted and may have consumed food before blood sampling was performed. Though it typically takes 15 minutes for the digestive system to start to breakdown carbohydrates and glucose to enter the blood stream, data collection of all twenty rats sometimes took up to one hour. Controlling food intake to control for actual fasted states might show different results on blood glucose.

Conclusion and Recommendations for Future Research

Though this research focused primarily on the exclusion of gluten, human food equivalents, since they are not enriched and fortified like the rat chow studied, have been found to vary in micronutrients as well as macronutrients, suggesting possible further issues. Future research might look into the specific mineral and vitamin

differences when eliminating gluten-containing products to see if other influences on puberty may exist. Also, research controlling feed during the entire development stage is also important to study, to see if differences in body fat corrected, or if the continuation of gluten restriction led to even higher gains of body fat.

The results of this study indicated a significant effect of gluten restriction on total body fat and puberty onset in weanling rats. Vaginal opening, and hence onset of puberty, occurred sooner in rats fed the gluten-free diet. Percent body fat was higher in the gluten-free group, consistent with the suggestion that increased body fat plays a role in the decreasing age of menarche in adolescent females. Further studies, along with the results of this research, can help reinforce the importance of diet and its effects on growth and maturation.

APPENDIX I: RAW DATA

GROUP	TOTAL WEIGHT GAIN	PERCENT WEIGHT CHANGE	TOTAL FOOD CONSUMED (GRAMS)	FOOD EFFICIENCY	BODY FAT (%)	DAY OF LIFE OF VAGINAL OPENING
Control						
1	85.6	62%	203	1.45	12.2	31
2	87.1	57%	220.8	1.35	10.8	31
3	78.8	55%	224.8	1.20	12.6	30
4	83.4	58%	250.5	1.14	10.1	32
5	96.5	63%	222.3	1.49	10.7	34
6	81.8	59%	213.9	1.31	8.8	31
7	61	53%	156.5	1.34	5.1	27
8	85.8	59%	221.6	1.33	11.5	31
9	94.5	61%	224.3	1.45	12.6	31
10	83.7	59%	215.9	1.33	9.3	31
MEAN	83.8	59%	215.36	1.34	10.3	31
S.D.	9.68	0.03	23.90	0.11	2.27	1.73
Treatment						
11	87.4	62%	197.4	1.52	14	31
12	90.1	58%	264.7	1.17	14.1	31
13	72.6	52%	283.8	0.88	12.5	30
14	88.2	59%	227	1.34	15.9	30
15	79.3	59%	197.7	1.38	12.6	31
16	90.7	62%	219.5	1.42	13.9	31
17	94.1	58%	230.9	1.40	14.2	27
18	87.7	62%	236.6	1.28	12.8	29
19	83.3	62%	226.1	1.27	8.5	29
20	88.3	62%	273.7	1.11	15.6	27
MEAN	86.2	60%	235.7	1.28	13.4	30
S.D.	6.24	0.03	29.75	0.19	2.07	1.58
p-value	0.2976	0.1580	0.0530	0.1371	0.0151	0.0112

WEIGHT (GRAMS)

SUBJECT	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7
1	53	57	64.5	73	74.5	85	87.9
2	65.5	68.5	75	82.5	88	98	99.9
3	63.5	69	75	83.5	88.5	98.5	102.3
4	60.5	66	72	80	86	95.5	100.7
5	55.5	63	67	75	82.5	90.7	97
6	57.5	62	68	75	78.7	88.5	91.5
7	55	60.5	66	74	73.5	82	84
8	59.5	64.5	70	79	83	94.5	97.5
9	61	66	73.5	81	86.7	97	101.5
10	58	62.5	71	78.5	82.5	89.7	95.2
11	54.5	60	66.5	72.5	79	87	93
12	65	71	79	88	93	102	105.2
13	66	71	76.5	82.5	88.5	95.5	99.5
14	61	67	73	81	87	96.2	99.5
15	56	60	64	71.5	78	84	89.2
16	55	60.5	66	74	81	89	104.7
17	67	72.5	79	85.5	92.5	102.1	105.5
18	53	59.5	66	73.5	78	85.7	89.5
19	51	57	63	69.5	75	83.7	88.5
20	55	61.5	67.5	75.5	81	88	95.5

WEIGHT CONTINUED

SUBJECT	DAY 8	DAY 9	DAY 10	DAY 11	DAY 12	DAY 13	DAY 14
1	96.7	100.7	106.7	115.5	121	126.7	138.6
2	109	114.7	122.4	127.3	132.5	139.7	152.6
3	109.7	116.2	123	131	139	140.8	142.3
4	108.5	115	120.5	123.6	133.5	135.6	143.9
5	106.3	110.2	116.7	124.4	132.6	140.9	152
6	96.5	102.2	108.5	119	124.4	131.3	139.3
7	90	94.5	98.2	110	114.5	113.8	116
8	107.7	113.2	118.3	128.3	133.2	137.4	145.3
9	109.5	116.5	125.5	131.2	142	144.5	155.5
10	100	107.4	114.5	122.4	130.5	131.8	141.7
11	103	107.7	113.7	122.5	129.6	135.8	141.9
12	114	119.4	127.7	133.3	138.2	145.5	155.1
13	106	111.5	117.7	126.5	134.2	135.3	138.6
14	109.2	116.5	121.2	128.5	135	138.6	149.2
15	96.9	101.5	109.2	114.4	121.1	127.1	135.3
16	101.4	109.5	117.5	125.4	131.8	135.4	145.7
17	114.7	109.2	127.5	134.8	145.2	151.2	161.1
18	98.6	104.5	112	119.7	123.6	130.4	140.7
19	98.2	101	110	115.7	122.7	129.6	134.3
20	104.3	109.7	115	122.7	126.6	131.5	143.3

BLOOD GLUCOSE VALUES (MG/DL)

SUBJECT	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	DAY 10	DAY 11	DAY 12	DAY 13	DAY 14
1	91	93	82	82	100	82	93	84	78	76	92	88	79	102
2	71	81	82	82	87	81	73	97	85	74	77	84	86	73
3	80	74	84	112	91	88	92	76	98	80	85	87	84	89
4	76	100	76	85	82	87	91	99	100	77	79	75	87	84
5	80	92	85	98	90	89	83	97	91	97	87	75	83	93
6	59	75	85	84	92	65	82	82	77	70	74	92	69	90
7	66	68	76	76	67	73	62	92	68	82	70	75	71	80
8	82	72	84	76	84	83	71	84	94	80	80	86	80	81
9	87	80	93	90	90	80	85	88	73	87	89	79	73	72
10	82	80	72	89	69	73	78	66	78	90	83	84	83	81
11	78	95	81	89	92	95	96	104	106	80	96	100	90	95
12	68	80	82	90	89	85	82	81	92	87	86	85	86	85
13	92	85	76	69	73	69	78	62	94	79	77	86	79	82
14	69	89	80	92	87	93	83	95	73	78	79	86	81	81
15	75	81	67	108	83	88	78	67	69	83	89	75	82	88
16	67	80	98	69	98	84	112	89	90	92	79	79	81	84
17	72	91	80	74	78	75	81	86	76	78	78	80	91	79
18	51	72	82	84	77	89	72	88	74	74	76	73	76	97
19	77	93	101	96	92	92	102	93	88	102	97	98	91	87
20	78	83	79	95	85	92	78	88	87	84	85	67	82	86
p-value	0.13	0.17	0.40	0.44	0.48	0.06	0.13	0.41	0.45	0.26	0.08	0.46	0.10	0.27

PicoLab® Rodent Diet 20 5053*
PicoLab® Rodent Diet 20 EXT IRR 5R53*

DESCRIPTION

PicoLab® Rodent Diet 20 is a 20% protein diet formulated for rat, hamster and mouse breeding colonies. This diet is a complete life cycle diet formulated using managed formulation, delivering Constant Nutrition®. This is paired with the selection of highest quality ingredients to assure minimal inherent biological variation in long-term studies. Irradiation gives reliable microbial control and eliminates the need for autoclaving. Irradiation treatment and special 3-ply packaging provide virtually bacteria-free dietary control.

Features and Benefits

- Managed Formulation delivers Constant Nutrition®
- Precision processing and selection of highest quality ingredients assures Constant Nutrition® quality
- Formulated with 20% protein
- High quality animal protein added to create a superior balance of amino acids for optimum performance
- Recommended for rat breeding colonies and mice not requiring a higher energy diet
- Irradiation gives reliable microbial control and eliminates the need for autoclaving

Product Forms Available	*Catalog #*
• 5053: Oval pellet, (3/8"x5/8"x1")	0007688
• 5R53: Extruded Particle	3002890-742
• Meal (ground pellets), special order	0006939
Other Versions Available	*Catalog #*
• 5061 Pico-Vac® Lab Rodent Diet	0048961

GUARANTEED ANALYSIS

Crude protein not less than	20.0%
Crude fat not less than	4.5%
Crude fiber not more than	6.0%
Ash not more than	7.0%
Moisture not more than	12.0%

INGREDIENTS

Ground corn, dehulled soybean meal, wheat middlings, whole wheat, fish meal, dried beet pulp, wheat germ, cane molasses, brewers dried yeast, ground oats, dehydrated alfalfa meal, soybean oil, whey, calcium carbonate, salt, DL-methionine, menadione dimethylpyrimidinol bisulfite (source of vitamin K), choline chloride, pyridoxine hydrochloride, cholecalciferol, vitamin A acetate, dl-alpha tocopheryl acetate (form of vitamin E), biotin, folic acid, thiamine mononitrate, vitamin B_{12} supplement, nicotinic acid, riboflavin supplement, calcium pantothenate, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, cobalt carbonate, sodium selenite.

FEEDING DIRECTIONS

Feed ad libitum. Provide plenty of fresh clean water at all times.
Rats- All rats will eat varying amounts of feed depending on their genetic origin. Larger strains will eat up to 30 grams per day. Smaller strains will eat up to 15 grams per day. Feeders in rat cages should be designed to hold two to three days supply of feed at one time.
Mice-Adult mice will eat up to 5 grams of pelleted ration daily. Some of the larger strains may eat as much as 8 grams per day per animal. Feed should be available on a free choice basis in wire feeders above the floor of the cage.
Hamsters-Adults will eat up to 14 grams per day.
For information regarding shelf life please visit www.labdiet.com.

03/17/15

CHEMICAL COMPOSITION[1]

Nutrients[2]	
Protein, %	**21.0**
Arginine, %	1.28
Cystine, %	0.36
Glycine, %	0.96
Histidine, %	0.52
Isoleucine, %	0.86
Leucine, %	1.56
Lysine, %	1.17
Methionine, %	0.62
Phenylalanine, %	0.91
Tyrosine, %	0.59
Threonine, %	0.78
Tryptophan, %	0.24
Valine, %	0.96
Serine, %	0.98
Aspartic Acid, %	2.18
Glutamic Acid, %	4.16
Alanine, %	1.19
Proline, %	1.31
Taurine, %	0.03
Fat (ether extract), %	**5.0**
Fat (acid hydrolysis), %	**6.3**
Cholesterol, ppm	142
Linoleic Acid, %	2.14
Linolenic Acid, %	0.27
Arachidonic Acid, %	<0.01
Omega-3 Fatty Acids, %	0.44
Total Saturated Fatty Acids, %	0.78
Total Monounsaturated Fatty Acids, %	0.97
Fiber (Crude), %	**4.5**
Neutral Detergent Fiber[3], %	15.8
Acid Detergent Fiber[4], %	5.8
Nitrogen-Free Extract (by difference), %	**53.5**
Starch, %	28.6
Glucose, %	0.19
Fructose, %	0.24
Sucrose, %	3.24
Lactose, %	1.34
Total Digestible Nutrients,%	**75.1**
Gross Energy, kcal/gm	**4.11**
Physiological Fuel Value[5], kcal/gm	**3.43**
Metabolizable Energy, kcal/gm	**3.04**

Minerals	
Ash, %	**5.9**
Calcium, %	0.81
Phosphorus, %	0.65
Phosphorus (non-phytate), %	0.34
Potassium, %	1.09
Magnesium, %	0.23
Sulfur, %	0.33
Sodium, %	0.30
Chloride, %	0.52
Fluorine, ppm	9.3
Iron, ppm	200
Zinc, ppm	89
Manganese, ppm	84
Copper, ppm	14
Cobalt, ppm	0.71
Iodine, ppm	0.97
Chromium (added), ppm	0.01
Selenium, ppm	0.37

Vitamins	
Carotene, ppm	1.5
Vitamin K,ppm	3.3
Thiamin Hydrochloride, ppm	17
Riboflavin, ppm	8.0
Niacin, ppm	85
Pantothenic Acid, ppm	17
Choline Chloride, ppm	2000
Folic Acid, ppm	3.0
Pyridoxine, ppm	9.6
Biotin, ppm	0.30
B_{12}, mcg/kg	51
Vitamin A, IU/gm	15
Vitamin D_3 (added), IU/gm	2.3
Vitamin E, IU/kg	99
Ascorbic Acid, mg/gm	—

Calories provided by:	
Protein, %	24.496
Fat (ether extract), %	13.123
Carbohydrates, %	62.380

★Product Code

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly.
2. Nutrients expressed as percent of ration except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.
3. NDF = approximately cellulose, hemi-cellulose and lignin.
4. ADF = approximately cellulose and lignin.
5. Physiological Fuel Value (kcal/gm) = Sum of decimal fractions of protein, fat and carbohydrate (use Nitrogen Free Extract) x 4,9,4 kcal/gm respectively.

LabDiet
www.labdiet.com

LabDiet 5053 without Wheat, Low Gluten 57DD

DESCRIPTION

Modified LabDiet® Laboratory 5053 Diet without wheat, low Gluten.

CAUTION: Contains a drug or compound for investigational use only in laboratory research animals or in vitro. Not for human use.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
1/2" Pellet	1817142-209

**Other Forms Available On Request*

INGREDIENTS

Ground Corn, Dehulled Soybean Meal, Ground Oats, Fish Meal, Dried Beet Pulp, Cane Molasses, Brewers Dried Yeast, Dehydrated Alfalfa Meal, Soybean Oil, Dried Whey, Calcium Carbonate, Salt, DL-Methionine,
Mineral and Vitamin Premix (Menadione Dimethylpyrimidinol Bisulfite (Vitamin K), Pyridoxine Hydrochloride, Cholecalciferol (Vitamin D-3), Trace Mineral Px (Manganous Oxide, Zinc Oxide, Ferrous Carbonate, Copper Sulfate, Zinc Sulfate, Calcium Iodate, Calcium Carbonate, Cobalt Carbonate), Vitamin A Acetate, DL-Alpha Tocopheryl Acetate (Form of Vitamin E), Biotin, Thiamin Mononitrate, Folic Acid, Vitamin B-12, Nicotinic Acid, Calcium Pantothenate, Riboflavin), Choline Chloride

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store upon receipt.
For laboratory animal use only; not for human consumption.

10/5/2015

NUTRITIONAL PROFILE [1]

Protein, %	***21.1***
Arginine, %	1.24
Histidine, %	0.51
Isoleucine, %	1.04
Leucine, %	1.70
Lysine, %	1.23
Methionine, %	0.70
Cystine, %	0.30
Phenylalanine, %	0.96
Tyrosine, %	0.64
Threonine, %	0.81
Tryptophan, %	0.26
Valine, %	1.06
Alanine, %	1.36
Aspartic Acid, %	2.63
Glutamic Acid, %	4.23
Glycine, %	1.00
Proline, %	1.42
Serine, %	1.13
Taurine, %	0.03
Fat (ether extract), %	***5.1***
Fat (acid hydrolysis), %	***6.1***
Cholesterol, ppm	152
Linoleic Acid, %	2.28
Linolenic Acid, %	0.27
Arachidonic Acid, %	0.02
Omega-3 Fatty Acids, %	0.44
Total Saturated Fatty Acids, %	0.99
Total Monounsaturated Fatty Acids, %	1.09
Polyunsaturated Fatty Acids, %	2.00
Fiber (max), %	***4.6***
Neutral Detergent Fiber[2], %	13.9
Acid Detergent Fiber[3], %	5.6
Nitrogen-Free Extract (by difference), %	***53.6***
Starch, %	31.04
Glucose, %	0.27
Fructose, %	0.32
Sucrose, %	3.48
Lactose, %	1.34
Total Digestible Nutrients, %	**74.5**

Energy (kcal/g) [4] **3.44**

From:	kcal	%
Protein	0.845	24.5
Fat (ether extract)	0.455	13.2
Carbohydrates	2.144	62.3

Minerals	
Ash, %	**5.6**
Calcium, %	0.80
Phosphorus, %	0.55
Phosphorus (available), %	0.28
Potassium, %	1.04
Magnesium, %	0.20
Sulfur, %	0.35
Sodium, %	0.30
Chloride, %	0.55
Fluorine, ppm	10.8
Iron, ppm	174
Zinc, ppm	78
Manganese, ppm	67
Copper, ppm	12
Cobalt, ppm	0.71
Iodine, ppm	1.02
Chromium (added), ppm	0.74
Selenium, ppm	0.35

Vitamins	
Carotene, ppm	1.6
Vitamin A, IU/g	15
Vitamin D-3 (added), IU/g	2.3
Vitamin E, IU/kg	99
Vitamin K, ppm	3.3
Thiamin Hydrochloride, ppm	16
Riboflavin, ppm	8.1
Niacin, ppm	84
Pantothenic Acid, ppm	17
Folic Acid, ppm	3.0
Pyridoxine, ppm	9.61
Biotin, ppm	0.3
Vitamin B-12, mcg/kg	51
Choline Chloride, ppm	2,000
Ascorbic Acid, ppm	0

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.
2. NDF = approximately cellulose, hemi-cellulose and lignin.
3. ADF = approximately cellulose and lignin.
4. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

ISO 9001:2008 CERTIFIED

TestDiet
www.testdiet.com

APPENDIX III: PROTOCOL FOR ANIMAL CARE AND USE

UNLV IACUC

Approved

DATE:	July 21, 2015
TO:	John Young, Ph.D.
FROM:	UNLV IACUC
PROJECT TITLE:	[758782-3] Effect of Gluten on Puberty Onset and Glycemic Response
REFERENCE #:	
SUBMISSION TYPE:	Revision
ACTION:	APPROVED
DECISION DATE:	July 21, 2015
EXPIRATION DATE:	July 20, 2018

Thank you for your submission of Revision materials for this research project. The UNLV IACUC has APPROVED your submission. All research must be conducted in accordance with this approved submission.

Please note that any revision to previously approved materials must be approved by this committee prior to initiation. Please use the appropriate amendment form for this procedure.

Please report all NON-COMPLIANCE issues regarding this project to this committee.

Annual Review

This project requires annual review by this office. Please use the appropriate annual review form for this procedure.

Renewals

All projects expire three years after the approval date. You must submit and obtain approval for a new project prior to July 20, 2018 to avoid any lapse in approval.

If you have any questions, please contact Kevin Bergeron at (702) 895-5453 or kevin.bergeron@unlv.edu. Please include your project title and reference number in all correspondence with this committee.

Office of Research Integrity - IACUC & IBC
4505 Maryland Parkway . Box 454022 . Las Vegas, Nevada 89154-4022
(702) 895-5453

UNLV **Institutional Animal Care and Use Committee**

PROTOCOL FOR ANIMAL CARE AND USE

1. PROJECT OR EXERCISE TITLE: Effect of Gluten on Puberty Onset and Glycemic Response

2. PRINCIPAL INVESTIGATOR/COURSE DIRECTOR:
NAME and TITLE: Dr. John Young (Professor and Advisory Committee Chair)
DEPARTMENT: Kinesiology and Nutrition Sciences
OFFICE PHONE: 702-895-4626 EMERGENCY PHONE:
LAB CONTACT (TECHNICIAN) and PHONE NUMBER:
LOCATION (BUILDING/LAB#):
E-MAIL:john.young@unlv.edu FAX: 5-1500
Does the PI have direct contact with the animals? ☒ Yes ☐ No

3. LIST RESEARCHERS AND STAFF INVOLVED WITH ANY PROTOCOL PROCEDURES:

Name	Responsibility	Office Phone	Emergency Phone
Bethaney McLaughlin	Student		760-525-8379
Dr. Laura Kruskall	Co-PI	702-895-4985	702-274-0370
Dr. James Navalta	Data analysis	702-895-2344	

Hit tab to add more rows to any of the tables in this form. Use the down arrow to move out of the table to the next field.

4. TRAINING FOR ALL PERSONNEL WORKING WITH ANIMALS ON THIS PROTOCOL:

Name	Role in this protocol (PI, co-PI, student, etc.)	Experience working with species	Training Received
Dr. Jack Young	PI	38 years	1991 UNLV
Dr. Laura Kruskall	Co-PI	13 years	2001 UNLV
Bethaney McLaughlin	Student	1 year	2015 UNLV

5. PROTOCOL STATUS: ☒ New/Renewal ☐ Modification

START DATE: July 2015 ANTICIPATED COMPLETION DATE (3 yr max): August 2015

6. FUNDING SOURCE: ☒ DEPARTMENT ☒ OTHER INTRAMURAL SOURCE
☐ EXTRAMURAL SOURCE SPECIFY SOURCE:
IF FUNDED, GRANT NUMBER:

7. CATEGORY OF ETHICAL CONCERN APPLICABLE TO THIS PROTOCOL (see Appendix A): C

8. JUSTIFICATION: THIS SECTION IS REQUIRED FOR ANY PROJECT IN WHICH THERE IS A POTENTIAL FOR PAIN, DISTRESS, OR DISCOMFORT THAT **CANNOT BE ALLEVIATED**. EXAMPLES OF SUCH PROJECTS INCLUDE INDUCED DISEASE STATES WHICH CAUSE SEVERE SYMPTOMS OR DEATH, SURGICAL STUDIES RESULTING IN SEVERE POST-OPERATIVE DISCOMFORT OR LOSS OF FUNCTION, USE OF SEVERE AND UNAVOIDABLE NOXIOUS STIMULI, AND SO FORTH.

a. Will there be unrelieved pain or stress (defined as lasting for more than a moment, i.e. longer duration or more painful than a needlestick)? ☒ No ☐ Yes

b. If yes, provide justification and an indication of the number of animals, per year, that are going to experience unrelieved pain or stress must be provided.

c. Is death an end-point? ☒ No ☐ Yes

d. If yes, explain why some earlier end-point data cannot be used instead (provide objective supporting data).

9. WILL THE PROJECT OR EXERCISE INVOLVE EXPOSURE OF ANIMALS OR ANIMAL HANDLERS TO:

BIOHAZARDS?	☒ NO	☐ YES	IF YES, SPECIFY:
RADIOISOTOPES?	☒ NO	☐ YES	IF YES, SPECIFY:
CARCINOGENS?	☒ NO	☐ YES	IF YES, SPECIFY:
TOXIC CHEMICALS?	☒ NO	☐ YES	IF YES, SPECIFY:

IF ANY ITEM ANSWERED YES, PLEASE COMPLETE AND **ATTACH SUPPLEMENT FORM 1.** An approved Institutional Biosafety Committee protocol may be required.

10. ITEMS WHICH APPLY TO THIS PROJECT:

☒ Blood and/or tissue collection	☐ Survival surgery
☐ Antibody production and collection	☐ Non-survival surgery
☐ Behavioral studies	☐ Aseptic surgery
☐ Prolonged physical restraint	☐ Multiple surgeries on the same animal
☐ Food or water deprivation	☐ Alleviated pain
☐ Environmental extremes	☐ Unalleviated pain
☐ Electrical stimuli	☐ Anesthetics used
☐ Induction of trauma	☒ Single housing
☐ Work to be done off campus	☐ Immobilizing agents without anesthesia
☐ Field studies	

11. IS SURGERY INVOLVED IN THE PROPOSED PROJECT?
☒ No ☐ Yes IF YES, **ATTACH SUPPLEMENT 2**

12. ARE STRESSFUL OR PAINFUL PROCEDURES, OTHER THAN SURGERY, PART OF THIS PROJECT?
☒ No ☐ Yes IF YES, **ATTACH SUPPLEMENT FORM 3**

13. ARE PROLONGED (MORE THAN A FEW HOURS) PHYSICAL RESTRAINT PROCEDURES PART OF THIS PROJECT?
☒ No ☐ Yes IF YES, **ATTACH SUPPLEMENT FORM 4**

14. WILL GENETICALLY ENGINEERED OR OTHER MUTANT ANIMALS BE PRODUCED OR MAINTAINED THAT MAY BE EXPECTED TO DEVELOP CLINICAL SIGNS AS A RESULT OF THE MUTATION?
☒ No ☐ Yes IF YES, **ATTACH SUPPLEMENT FORM 5. AN APPROVED INSTITUTIONAL BIOSAFETY COMMITTEE PROTOCOL IS ALSO REQUIRED.**

15. WILL TISSUES, CELLS OR OTHER PRODUCTS DERIVED FROM ANIMALS BE SHARED OR A TISSUE BANK ESTABLISHED FOR FUTURE SHARING WITH OTHER INVESTIGATORS DURING OR AFTER THE PROJECT?
☒ No ☐ Yes

16. ARE BEHAVIORAL STUDIES INVOLVED IN THIS PROJECT? ☐ Yes ☒ No
Do any of the following procedures or conditions apply?

Food Reward	☐	
Electrical Shock	☐	Strength and duration:
Food Deprivation	☐	Kcal/day provided:
Water Deprivation	☐	ml/kg provided:

If a conditioning protocol applies, please explain the purpose.

Criteria for monitoring the condition of the animals during food and water deprivation:

17. ARE THERE ANY EXCEPTIONS TO THE GUIDE (*Guide for the Care and Use of Laboratory Animals, National Research Council*)? ☒ Yes ☐ No

If yes, describe the exceptions in detail including the scientific justification for the exception. The most common exceptions include; single housing, lighting, food/water variations, cage changes, temperature, ventilation, or euthanasia method that differs from the guide.

Single housing will be used to accurately measure food intake for each animal used. Research evaluating stress hormone levels and food intake of single-housed versus paired animals found no differences in stress hormone measures, 24-hour food intakes or body weight gain (Boggiano et. al. *Physiology and* Behavior, 95(1-2), 222-228). Food variations, standard gluten chow and gluten-free chow, will be used to see the effects of gluten exclusion on puberty and glycemic response. Both gluten and gluten-free chow will be obtained from Research Diets, who make their standard chow without gluten products, by using corn and casein. Numerous published research projects have used Research Diets chow, including a study looking at the effects of a gluten-free diet on intestinal microbiome (Marietta et.al PLoS ONE 8(11): e78687).

18. ANIMAL CENSUS AND HOUSING - Use one line/species/pain category. Repeat table rows as needed.

Common Species Name/Scientific Name	Strain/Stock Breed	Sex	Starting Age/Weight	Total # Requested	Pain Category
Rat / Rattus norvegicus	Wistar/Sprague-Dawley	F	21 days; approx. 60 g	20	C

During the course of the study, list the source of the animals and the expected average daily census:

Average Daily Census: 20	Source of the animals: Approved Vendor

Will these animals be bred? If so explain, please include numbers of animals bred and anticipated number of offspring.

NA

19. LOCATION OF ANIMAL HOUSING AND USE AREAS:

ANIMAL HOUSING FACILITY: UNLV LACF
WILL ANIMALS BE REMOVED FROM THE HOUSING FACILITY FOR USE ELSEWHERE?
☐ No ☒ Yes IF YES, SPECIFY LOCATIONS:

Procedures	Building or Site	Room
Non surgical procedures or conditions		
Nonsurvival surgery		
Survival surgery		
Postsurgical/Postanesthesia/Postprocedural Recovery		

Maximum number of hours at one time the animals will be kept in the laboratory/surgery:
☒ 0-12 hrs ☐ 12-24 hrs ☐ over 24 hrs *(Animals kept over 24hours require an establishment of an IACUC approved satellite facility.)*

20. ARE THERE ANY SPECIAL REQUIREMENTS FOR ANIMAL HOUSING, DIETS, RESTRAINT, OR PROCEDURES FOR DISPOSAL? ☒ Yes ☐ No

IF NO, ANIMALS WILL BE MAINTAINED ACCORDING TO THE STANDARD OPERATING PROCEDURES. IF YES, PROVIDE (AN) EXPLANATION(S) FOR EACH PARAMETER INVOLVED (E.G., LIGHT:DARK CYCLES, HUMIDITY, AMBIENT TEMPERATURE, CAGING BEDDING, DIETS, AND SO FORTH):

Rats will be fed either a diet formulated with gluten or a gluten-free diet, both purchased from Research Diets INC. This is needed to determine effects of gluten on weight change, puberty onset, and blood glucose.

21. INSTRUCTIONS FOR DISPOSITION OF SICK OR INJURED ANIMALS

Call Investigator ☐ Veterinarian (or Designee) to Treat ☒ Euthanize ☒

22. INSTRUCTIONS FOR DISPOSITION OF DEAD ANIMALS

Call Investigator ☒ Veterinarian (or Designee) to Necropsy ☐ Bag for Disposal ☒

23. IF WILD OR EXOTIC SPECIES ARE TO BE USED:

a. ARE SPECIAL PERMITS REQUIRED? ☐ Yes ☒ No
IF YES, ATTACH A COPY OF APPLICABLE PERMITS OR PERMIT APPLICATIONS

b. WILL ANIMALS BE OBSERVED, MANIPULATED, OR TRAPPED IN THE WILD? ☐ Yes ☒ No
IF YES, AND THEY HAVE NOT ALREADY BEEN ADDRESSED, DESCRIBE THE FIELD PROCEDURE(S) INCLUDING THE GEOGRAPHIC LOCATION(S) INVOLVED:

24. ADMINISTRATION OF ANESTHETICS AND ANALGESICS.

Species	Drug	Route	Site/location	Volume	Dosage	Frequency
NA						

25. ADMINISTRATION OF EXOGENOUS SUBSTANCES
Complete this section for all drugs, infectious agents, carcinogens, toxins, experimental compounds, etc.

Species	Drug/Substances	Route	Site/Location	Volume	Dosage	Frequency
NA						

All substances must be pharmaceutical grade. If non-pharmaceutical grade substances will be used, please list the substance and explain why a pharmaceutical grade cannot be used:

Other Comments:

26. ANTEMORTUM FLUID/TISSUE COLLECTION

Species	Type of Fluid or Tissue	Volume or Mass	Frequency of Collection	Method or Route of Collection	Anesthesia or Sedation Used?
Rat	Blood	2 drops (2µl)	Daily	Tail Vein	No

27. EUTHANASIA

Species	Method and Secondary Method (if applicable)	Drug (if applicable)	Dose of Drug mg/kg (if applicable)	Route
Rat	CO_2			Inhalation
Rat	Bilateral pneumothorax Note: carcasses must be intact for DEXA scan, so opening chest cavity and severing heart will be done after the DEXA scan is completed			

28. LAY SUMMARY

This summary may be forwarded to the Office of Public Affairs. They may use it to describe your research to the media. The lay summary should be brief and readily understandable to the general public. Provide a summary of the project that explains the need for the research, what the project goals are, and how the use of animals will help reach the stated goals:

Adolescent years are the most important for consuming appropriate nutrient quantity. Diets limiting consumption of certain food items, such as the gluten-free diet, may have detrimental effects on growth and maturation. Research evaluating nutrient differences between gluten-free products and traditional counterparts have found that gluten-free items tend to be higher in fat, have reduced fiber and carbohydrates, and possible differences in protein levels (Miranda

Plant Foods for Human Nutrition, *69*(2), 182-187). Nutrient consumption in individuals on a gluten-free diet has shown below recommended intake of B vitamins, vitamin D, zinc, calcium, fiber, iron, and magnesium, all essential nutrients for a developing child (Lamacchia Nutrients 2014(6), 575-590). The proposed research will evaluate the effects of gluten on weight change, glycemic response, and puberty onset in 21-day-old rats, hypothesizing that gluten restriction causes a faster increase in body weight, signaling the onset of puberty earlier than rats on a non-restricted diet. Glycemic response will also be determined to see if differences arise during a gluten-free diet. Rats tend to reach puberty within 6 weeks of birth, making them ideal subjects for this study. To determine overall changes in body composition and fat stores, a post-mortem procedure will be utilized, therefore making it necessary to use animals in this study.

29. EXPERIMENTAL SUMMARY

ALL 3 QUESTIONS MUST BE ANSWERED.

a. What are the objectives or underlying hypotheses of the experiment?

The proposed research will evaluate the effects of gluten on weight change, body composition, glycemic response, and puberty onset in 21 day old rats, hypothesizing that gluten restriction causes a faster increase in body weight, signaling the onset of puberty earlier than rats on a non-restricted diet.

b. What happens to the animals from start to finish (please give a detailed description of all procedures)?

In this experiment, 20 female weaned rats, age 21 days, with similar starting weight, approximately 60 grams, will be randomly separated into two groups and fed either a gluten-free chow or a normal chow diet. Animals will be housed in the UNLV LACF in separate cages so that daily food intake can be recorded. Rats will be in a controlled temperature setting, with 12 hours of light and 12 hours of dark. At approximately the same time each morning, body weight of each rat, as well as weight of remaining food from the previous nights' feeding, will be recorded.
Blood glucose will be measured daily, at the same time as animals are weighed, to see possible changes in glycemic response between the two groups. Blood will be collected by a tail clip (0.5 mm tip of tail). The first drop of blood will be discarded; the second drop will be collected on a test strip and be analyzed by a standard glucose meter. To sample for blood glucose on successive days, we will attempt to collect blood from the previous tail clip by wetting and removing the scab. A new tail clip will be done if the scab from the previous clip is sufficiently healed to prevent obtaining a drop of blood. Starting on day 10 of the experiment, rats will be checked daily for vaginal opening at the same time as animals are weighed and blood glucose is measured. Upon the day of vaginal opening, rats will be euthanized to determine body composition. After euthanization, via CO_2 inhalation, animals will be transported to BHS 335 where body composition will be determined by dual energy x-ray absorptimerty (DEXA) with small animal software. Animal carcasses will be placed on an absorbent underpad separating the carcass from direct contact with the DEXA surface. Following DEXA scans, carcasses will be returned to LACF for disposal.

c. Why was this species or strain selected?

Rats were selected because the species tend to be a commonly used and convenient model. Since this experiment will look at puberty onset, rats grow and mature very quickly, and therefore can show results in a shorter period of time. Typically a day for a rat is equivalent to approximately 110 human days (Sengupta). Research began using rats to study reproductive physiology and puberty in the 1930s, and thus make a good model to use for this experiment.

d. How many animals will be used and how did you determine that this will be a sufficient number?

i. Number of animals requested:

Twenty; two groups of 10 animals each (10 standard chow and 10 gluten-free chow)

ii. How did you determine that this would be a sufficient number? (Choose at least one)

☐ Statistical power analysis (please describe)

☒ Similar published experiments (cite references)

Group size in research looking at the effects of diet on growth in weanling rats ranges from 4-12 animals per treatment group. Based on this research, we believe 20 rats (n=10 per group) is a sufficient number of animals to demonstrate a treatment effect in this study.

Howe, EE and CL Dooley. Effect of delayed supplementation of wheat gluten with lysine and threonine on its capacity to promote growth in the weanling rat. J. Nutrition 81: 379-382, 1963

Mustin B, D Peace, and CH Anderson. Food intake regulation in the weanling rat: self-selection of protein and energy. J. Nutrition 104: 563-572, 1974

Ashley, DVM and CH Anderson. Food intake regulation in the weanling rat: effects of the most limiting essential amino acids of gluten, casein, and zein on the self-selection of protein and energy. J. Nutrition 105: 1405-1411, 1975
Ashley, DVM and CH Anderson. Correlation between the plasma tryptophan to neutral amino acid ratio and protein intake in the weanling rat. J. Nutrition 105: 1412-1421, 1975

Landmann, WA, CW Dill, and CR Young. Nutritive value of globin-amino acid and complementary globin-cereal mixtures. J Nutrition 110: 2254-226, 1980

Phillips, RD. Estimators of body nitrogen in growing rats fed varying levels and qualities of protein. J. Nutrition 110: 1441-1452, 1980

Phillips, RD. Linear and nonlinear models for measuring protein nutritional quality. J. Nutrition 111: 1058-1066, 1981

Burns RA, MH LeFaivre and JA Milner. Effects of dietary protein quantity and quality on the growth of dogs and rats. J. Nutrition 112: 1843-1853, 1982

Finke, MD, GR DeFoliart, and NJ Benevenga. Use of a four-parameter logistic model to evaluate the protein quality of mixtures of Mormon cricket meal and corn gluten meal in rats. J. Nutrition 117: 1740-1750, 1987

Sampson DA, SC Harrison, SD Clarke, and X Yan. Dietaary protein quality alters ornithine decarboxylase activity but not vitamin B-6 nutritional status in rats. J. Nutrition 125: 2199-2207, 1995

☐ Special consideration for difficult to obtain, rare, or endangered species, explain for clarification

e. For 3-year renewals please describe what has been accomplished in the past 3 years and how this protocol differs from the previously approved protocol (for new protocols indicate N/A):

N/A

30. ASSURANCES (See Appendix B)

I have searched the following sources for alternatives to animal model.

Data Source	Searched From (Yr)	Searched To (Yr)	Keywords used in the Search	Date Search Performed
PubMed	1995	2015	Gluten-free, puberty, non-celiac, development; gluten-free and blood glucose	4/18/2015
UNLV Library			Gluten-free, puberty, non-celiac, development; gluten-free and blood glucose	4/18/2015
Google Scholar	1995	2015	Gluten-free, puberty, non-celiac, development; gluten-free and blood glucose	4/18/2015

Alternatives refer to methods or approaches which result in refinement of procedures which lessen pain and/or distress; reduction in numbers of animals required; or replacement of animals with non-whole-animal systems or replacement of one animal species with another, particularly if the substituted species is non-mammalian or invertebrate.
I have determined that the following alternatives are available. Described briefly if applicable:

This study involves diet modification during pre-puberty, to access possible implications on growth and maturation. Other possible models are larger mammalians (rhesus monkey) or humans, which would not be a reasonable alternative model.

[7]

Investigator Certifications

By submitting and digitally signing this package in IRBNet, you agree to the following certifications:

I understand that 'unnecessarily duplicative' research involving laboratory animals is not permitted by the federal animal welfare law and hereby provide assurance that the research proposed herein does not, to the best of my knowledge, unnecessarily duplicate research already reported in the public literature.

I certify that the use of all animals involved in this project will be carried out according to the provisions of the Animal Welfare Act, PHS Policy, the principals of the 'NIH Guide for the Care and Use of Laboratory Animals', and the UNLV Policy Governing The Use of Animals in Research, Teaching and Testing. I agree to notify the UNLV Institutional Animal Care and Use Committee (IACUC) of any substantive changes in the research use of the animals, including the number of animals, species used, or procedures performed.

I understand that the University of Nevada, Las Vegas and its representatives on the IACUC have the authority to suspend any part of my research, should I not be in compliance at any time with USDA, PHS/NIH, or UNLV regulations for animal care and use.

I certify that all personnel having direct animal contact, including the investigator, have or will have, prior to participating in this protocol, been trained in humane and scientifically acceptable procedures in animal handling, administration of anesthetics, analgesics, and euthanasia to be used in this project or are under the direct (in-lab) supervision of a trained individual; and that employees will be allowed adequate time to attend training sessions.

I assure that all named individuals on this project have read and understood the procedures outlined in this protocol as approved by the IACUC.

I certify that I will obtain approval from the IACUC before initiating any significant changes in this study.

I certify that I will notify the IACUC regarding any unexpected study results that impact the animals. Any unanticipated pain or distress, morbidity or mortality will be reported to the attending veterinarian and the IACUC.

I certify that the information provided in this application is accurate to the best of my knowledge. I also understand that should I use the project described in this application as a basis for a proposal for funding (either intramural or extramural), it is my responsibility to ensure that the description of animal use in such funding proposal is identical in principle to that contained in this protocol.

I understand that the Department of Laboratory Animal Care Services is responsible for administering and assigning animal housing space within the central animal facilities. The Manager, LACF will make space assignments for the efficient utilization of space, which may result in investigators sharing animal housing space. I further realize that I must notify the Manager, LACF of animal housing needs in order to insure the availability of suitable space before animals are procured.

Appendix A

CATEGORIES OF ETHICAL CONCERN FOR USE OF VERTEBRATE ANIMALS IN TEACHING AND RESEARCH PROGRAMS

The USDA has established categories of invasiveness for animal research which must be addressed in the protocol. The University reports to the USDA how many USDA animals are used each year in each category.

CATEGORY B: Maintaining animals without experimentally manipulating them in any way. Standard animal care and food, water, and shelter/enclosures are all that the animals experience. Any bleeding, tail clips or other manipulations are not allowed. Some breeding colonies, conditioning periods as well as field studies with only observation and no animal contact would qualify for Category B.

CATEGORY C: Experiments or teaching exercises that involve no or minimal pain or distress to vertebrate animal species. For Category C procedures no pain-relieving drugs or anesthetic drugs are utilized because animals experience, at most, no more than transient slight pain or distress. These manipulations might include minor blood sampling, injections with benign substances (NOT Freund's adjuvant), use of positive rewards for training, tail clips, ear tagging or punching, and euthanasia for tissue collection. If any of these manipulations are done under anesthesia, this would then make them Category D.

((Examples - Holding animals captive for experimental purposes; simple procedures such as observing feed selection preferences; injections of relatively harmless substances such as therapeutic levels of antibiotics, and blood collection from peripheral veins; physical examinations; food and water deprivation for short periods measured in hours; or standard methods of euthanasia which induce rapid unconsciousness, such as anesthetic overdose.)

CATEGORY D: Research studies in which pain or distress is relieved with appropriate anesthetics, analgesics, or tranquilizer drugs (or other means of relieving pain or distress). Surgery must have adequate anesthesia, and if the animal is recovered after surgery and anesthesia, pain control must be instituted. Major surgeries include opening any body cavity, orthopedic procedures, etc.

(Examples: Exposure of blood vessels or implantation of catheters under full anesthesia; behavioral studies on conscious animals which involve short-term stressful restraint; noxious stimuli from which escape is possible; and surgical procedures under deep anesthesia that may result in only some minor post-operative discomfort or pain.)

CATEGORY E: Procedures that involve unrelieved pain or distress. Category E experiments will not normally be approved at the University of Nevada, Las Vegas. The IACUC must have solid scientific justification for withholding agents to alleviate the pain or distress before these studies may be approved. Toxicological or microbial testing or infectious disease research that requires that the study continue until animals show clinical signs or death occurs are an example of Category E. Many such procedures may be expressly prohibited by Federal laws, regulation, or policies. Category E procedures will not be approved for use in teaching exercises.

(Examples - Severe burn or trauma infliction on anaesthetized animals or without the use of analgesics following awakening from anesthesia; application of noxious stimuli (as in shock for behavioral studies), killing by nonapproved methods which may involve severe distress or pain before unconsciousness is obtained.)

Appendix B

Guidelines for Searches to Alternatives to Animal Use

Introduction

The Animal Welfare Act Regulations, Section 2.31 and USDA (Policy 11 and 12) require that a written narrative be provided by the Principle Investigator (PI) to determine whether or not alternatives exist to procedures which may cause pain or distress in animals used for teaching or research.

Definition of Alternatives

Alternatives refer to methods or approaches which result in refinement of procedures which lessen pain and/or distress; reduction in numbers of animals required; or replacement of animals with non-whole-animal systems or replacement of one animal species with another, particularly if the substituted species is non-mammalian or invertebrate.

Animal Welfare Act Regulations

The AWA regulations require the Institutional Animal Care and Use Committee (IACUC) to determine that "the principle investigator has considered alternatives to procedures that may cause more than momentary or slight pain or distress to the animals and has provided a written narrative description of the methods and sources used to determine that alternatives were not available." The PI must provide scientific justification to the IACUC if alternatives are available but not used.

Types of Studies Requiring an Alternatives Search

Not all animal use protocols require an alternatives search. Only studies utilizing procedures which result in more than momentary or slight pain or distress require a search. Examples of studies that require a search include: toxicity and infectious diseases research, tumor induction or transplantation studies, survival and non-survival surgical procedures, pain research, in vivo monoclonal or polygonal antibody production procedures, fluid and/or food restriction, and prolonged restraint. If you are unsure if your study requires an alternative search, either contact one of the institutional veterinarians for advice or just go ahead and do the search. If you fail to do a required search for alternatives, approval of your protocol by the IACUC may be delayed.

Alternatives Narrative

The minimal written narrative should include: the databases searched or other sources consulted, the date of the search and the years covered by the search, and the key words and/or search strategy used by the Principal Investigator when considering alternatives or descriptions of other methods and sources used to determine that no alternatives were available to the painful or distressful procedure. The narrative should be such that the IACUC can readily assess whether the search topics were appropriate and whether the search was sufficiently thorough. Reduction, replacement, and refinement (the three R's) must be addressed, not just animal replacement.

Database and Web Site Searching

In order to perform literature searches that meet regulatory requirements, use key words that would identify alternatives to any and all study procedures that may cause more than momentary or slight pain or distress to the animals. Problems often arise in choosing keywords and search strategies that will yield the most pertinent information. Appropriate search terms or keywords include animal testing alternatives, alternatives, tissue culture, cell culture, simulation, in vitro, and model. Keywords should be selected keeping the 3R principles in mind. The 3 R's represent reduction in the number of animals used, refinement of techniques and procedures that reduce pain and distress, and replacement of animal with non-animal techniques. A list of suggested terminology for alternatives from the UC Center for Animal Alternatives website is listed below (http://www.vetmed.ucdavis.edu/Animal_Alternatives/main.htm). While this list of terms is not exhaustive, you can use it as a model, combining alternatives terms with those specific to your area of research. In your database search, combine appropriate text words from this list with text words that are appropriate to the particular protocol. Although it is important to include relevant keywords, adding many irrelevant keywords that are not likely to identify alternatives with the idea that "more is better" is discouraged and will not improve the likelihood that the IACUC will approve your protocol.

alternative
analges* or sedative*
anesthe* or anaesthe*
animal model
animal testing alternative*
anxiolytic*
artificial*
artificial intelligence systems or AI
assay* or technique* or technic* or method*
bacteria or protozoa
behavioral enrichment
cadaveric model*
computer aided instruction or CAI
computer simulation
computer software or software
culture and (cell or tissue or organ)
digital image*
environmental enrichment
euthanasia
fish or cephalopod*
handling
housing or facility design or caging
interactive
invertebrate*
mannequin or manikin or mannikin
mathematical model*
model*
plastinat*
simulat*
single-cell* organism*
software
train* or educat* or teach*
tranquiliz*
video*
virtual and (surg* or reality)

vitro and (method or model or technique)
welfare or pain or stress or distress

Example of a Keyword Search

This example of a keyword search is for a study involving hibernation in ground squirrels. The keywords to be used in a database search for this project include:

hibernation; ischemia AND alternatives; ground squirrel and other models; less invasive AND catheterization.

REFERENCES

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2. Bacchetti, T., Saturni, L., Turco, I., & Ferretti, G. (2014). The postprandial glucose response to some varieties of commercially available gluten-free pasta: a comparison between healthy and celiac subjects. *Food Funct.*, *5*(11), 3014-3017. Retrieved from http://xlink.rsc.org/?DOI=C4FO00745J

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6. Capriles, V. D., & Arêas, J. A. G. (2014). Novel approaches in gluten-free breadmaking: Interface between food science, nutrition, and health. *Comprehensive Reviews in Food Science and Food Safety*, *13*(5), 871-890.

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