Postweaning consumption of a high-carbohydrate diet mutes the anti-diabetic potential of dietary polyphenols in the Nile Rat

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Abstract

Type II Diabetes Mellitus (T2DM), obesity, and heart disease have been linked to diet and lifestyle. Because of the increased prevalence of such chronic diseases, it is critical to explore their pathogenesis and consequences as a way to deter prevention and enhance treatment. Dietary polyphenols have shown promise in improving health and preventing chronic diseases such as T2DM. This study sought to investigate two interrelated questions: 1. How do dietary polyphenols and diet compositions affect T2DM development in the NR? and 2. What is the underlying pathophysiology of T2DM in the NR (independent of diet)? In the current study, fifty male Nile Rats (NR) were primed for 2wks at 3wks of age on standard diabetogenic hiCHO diet. After priming, the NR were divided into 5 groups (n=10 per group): 1. control with no phenolics (60:20:20, CHO:fat:protein %energy), 2. curcumin (150ug GAE/kcal, 70:10:20), 3. bergamot (40ug GAE/kcal), 4. palm fruit juice (1187ug GAE/kcal), and 5. green coffee bean (210 ug GAE/kcal). Throughout the study, random blood glucose (RBG), fasting blood glucose (FBG) and oral glucose tolerance (OGTT) were monitored at 4wks and 8wks; all NR were necropsied at 8wks and their organs were harvested and weighed. Results indicated that hiCHO priming may have damaged the NR metabolism to the point where none of the dietary interventions were able to reverse T2DM, suggesting that early priming with hiCHO diet may have muted the potential effects of the polyphenols. Results also suggested that the 30min OGTT was the most sensitive screen for pre-diabetes; RBG was the most indicative of terminal pathophysiology related to liver damage; FBG was the least sensitive screen, but the most pronounced for indicating late-stage T2DM in the NR. These findings may be clinically relevant for enhancing procedures in the Hayes Lab and for screening patients.

Abbreviations:
Type II Diabetes Mellitus (T2DM), Metabolic Syndrome (MetS), Nile Rat (NR), high carbohydrate (hiCHO), Oral Glucose Tolerance Test (OGTT), Random blood glucose (RBG), Fasting blood glucose (FBG), Fasting body weight (FBW), Gallic acid equivalence (GAE), Glycemic Load (GL), Glycemic Index (GI), Palm fruit juice (PFJ), Green coffee bean (GCB), Bergamot (BERG), Nonalcoholic fatty liver disease (NAFLD), Total cholesterol (TC), Total triglycerides (TG), Liver (Liv), reactive oxygen species (ROS)
Introduction

The popular saying advocates: “You are what you eat”. Thus, researchers have begun to dig into the complex relationships between nutrition, disease, genetics, and overall health (Burdge & Lillycrop, 2010). Poor nutrition can lead to well-known undesirable consequences like tooth decay, early-onset of puberty, and food allergies (Weichselbaum & Buttriss 2011). More recently, global issues like obesity, diabetes mellitus, and heart disease have become attributed to diet and lifestyle as well. The emerging idea that nutrition could determine the onset of chronic diseases is gaining attention because nutrition is a modifiable factor ("Diet, nutrition and the prevention of chronic diseases," 2002). Studying the elements of nutrition that act to potentially prevent chronic disease, reverse illness, and increase lifespan, is important for improving the overall wellness of human populations.

Phytonutrients, phenolics, and health. In recent decades, much attention has been given to polyphenols, a subgroup of phytochemicals found in fruits, vegetables, legumes, grains, and plant beverages. A burst of interest in the potential beneficial effects of dietary polyphenols on health and disease prevention has led to a variety of studies exploring the subject (Pandey & Rizvi 2009). Many of the positive effects of fruits and vegetables are have been attributed largely to the antioxidant polyphenols they contain (Rahman, Biswas, & Kirkham 2006). So far, 8,000 different plant-derived polyphenols have been identified, and many are under the scrutiny of international research. Polyphenols have been implicated in cardio-vascular protection, anti-cancer therapy, anti-diabetic activity, anti-aging activity, and neurological protection (Pandey & Rizvi 2009). Some studies have suggested that some dietary polyphenols alter the pattern of glucose uptake in the small intestine, which may lead to improved glucose tolerance (Johnston et al. 2003).

Metabolism and other biological processes are thought to release reactive oxygen species (ROS) that can damage the body and alter the development of an organism (Rahman, Biswas, & Kirkham 2006). Polyphenols contain a phenolic ring with hydroxyl constituents, which give them unique antioxidant properties. They are able to seize destructive free radicals by donating hydrogens as electron donors. In this way, they have
the ability to prevent ROS from damaging cells (Sambanthamurthi et al. 2011). As more polyphenols are being discovered, their unique molecular effects are beginning to stir the interest of nutritionists. Curcumin, a yellow polyphenol compound found in the root of Curcumma longa (turmeric), has been used widely as a condiment in Asian dishes for centuries. It has been studied in various in vivo and in vitro environments to measure its potential antioxidant effect (Rahman, Biswas, & Kirkham 2006). In vitro, curcumin has been shown to protect human keratinocytes, fibroblasts, and NG 108-15 cells (a mouse neuroblastoma-rat glioma hybrid cell line) from reactive hydrogen peroxide damage. In a mouse model, studies have suggested that curcumin prevents the development of Alzheimer’s disease by reducing the number of oxidized proteins and proinflammatory cytokines. The radical scavenger property of curcumin has been proposed as a prevention method for Alzheimer’s (Lim et al. 2001).

There has also been a budding interest in palm fruit water-soluble extract (palm fruit juice, PFJ), derived from the oil palm Elaeis guineensis, which has shown promise in polyphenolic benefits (Balasundram et al. 2005). Mirroring the favorable effects of other polyphenols in vivo, palm fruit extract also seems to be a free radical scavenger with antioxidant properties. To date, only a few studies have been launched to explore the potential disease-preventative and health-enhancing properties of palm fruit extract. The African Nile Rat (Arvicanthis niloticus) spontaneously develops diabetes when fed standard rat chow in captivity. One study from Brandeis University in the Nile Rat showed that palm fruit juice can protect against, and even reverse the onset of type 2 diabetes (Sambanthamurthi et al. 2011) (Bolsinger et al. 2014). Providing PFJ (1800 ppm GAE) as the liquid (in place of drinking water) to 12-week-old pre-diabetic male Nile Rats for 17wks, fasting blood glucose levels fell from 70 mg/dl to 49 mg/dl after treatment. The control study, which was only given water, had an increase of fasting blood glucose levels from 49 mg/dl to 139 mg/dl, a diabetic level, after 17wks.

Another study performed on mice suggested that the consumption of the oil palm phenolics (OPP) in PFJ reduced the effects of an atherogenic diet. The extract was shown to up-regulate antioxidant genes in the heart, decreased turnover of metabolites in the liver, and decreased inflammation. These findings suggested that OPP can help attenuate atherosclerosis (Leow et al. 2013). Other provocative studies also suggest that palm fruit
extract protects against tumors, cardiovascular diseases, diabetic retinopathy, and high blood pressure (Noda et al. 2010). Much more awaits to be discovered about the effects and mechanisms of this polyphenolic cocktail in vivo (Sambanthamurthi et al. 2011).

Another recently studied source of polyphenols is bergamot (*Citrus bergamia*), which comes from a citrus-fruit peel found exclusively in the Calabria region in Italy. Studies in rats and humans have indicated that bergamot significantly reduced the risk of coronary artery disease and other cardiovascular diseases in hyperlipemic (elevated lipid levels in blood) individuals by lowering LDL cholesterol (Mollace et al. 2011). A class of polyphenols present in bergamot called flavonoids has also been documented to enhance the activity of superoxide dismutase, an enzyme network involved in eliminating superoxide radicals (Mollace et al. 2011).

Coffee beans have also shown promising affects. Coffee is one of the most commonly consumed beverages in the world (Hu 2005). It is very important to analyze the positive and negative potential effects of coffee because it influences the lives of millions of people. Some of the key components of coffee beans are chlorogenic acids (CGA), natural esters composed of caffeic acid and quinic acid. CGA are antioxidants, and have been thought to slow the release of glucose into the bloodstream after a meal (Johnston et al. 2003). One epidemiological study examined the association between routine coffee consumption and the risk of T2DM (Hu 2005). It brought together evidence that consumption of coffee generally has an inverse relationship with the risk of T2DM. It also suggested that the components of coffee other than caffeine, like magnesium, may increase insulin sensitivity and secretion. A study on mice also found a positive correlation between green coffee bean extract and weight loss and fat loss (Shimoda et al. 2006). The triglyceride (TG) levels in the liver were decreased and weight loss was noted, but only when all the components of the coffee bean extract were together and not isolated. It indicated that the coffee extract might inhibit fat absorption and activation of hepatic fat metabolism. One study was conducted to explore whether CGA in coffee modulate glucose uptake, gastrointestinal hormone release, and insulin secretion in humans (Johnston et al. 2003). The results confirmed previously suggested effects of CGA on mice, attenuating glucose uptake by the small intestine. The authors of
this study offered a novel potential function of CGA: to delay glucose absorption rates in the upper intestine and shift the site of absorption further down the intestines.

**Nile rat diabetes model.** The present study used the African Nile Rat (Arvicanthis niloticus) as the model for diet-induced diabetes type II (T2DM) and metabolic syndrome (MetS). This animal is native to the Nile River delta and in sub-Saharan Africa where it feeds on vegetative plants and grass seeds. In the 1990s, a team in the United States began to study this species for sleep and circadian rhythm studies, due to the diurnal nature of the rats (Chaabo et al., 2010). In captivity, the African Nile rats were fed regular chow and were spontaneously developing diabetes. Because of the fairly consistent onset, the rat was determined to be an optimal model for diet-induced T2DM (Noda et al. 2010). In fact, almost all male Nile rats fed standard chow develop diabetes in their first year, and sometimes even as early as 6-10 weeks. To date, the rodents seem to express all aspects of MetS, including abdominal fat accumulation, hyperinsulinemia, hyperglycemia, hypertriglyceridemia, and hypertension (Chaabo et al. 2010). Studies on necropsied Nile rats supported these findings as well by measuring accumulation of fats in organs and relative functioning of kidneys, pancreas, and heart (Noda et al. 2010, Bolsinger 2013).

**Diet compositions.** To study the development of diabetes in the Nile rat and try to reverse it, one must be able to induce MetS and T2DM consistently in the model. A previous study from Brandeis University investigated the impact of glycemic load, a number that estimates how much of a specific food will raise the postprandial blood glucose level (Bolsinger et al. 2013). This study found that the Nile rat developed hyperglycemia most aggressively and quickly when it was fed high-carbohydrate diet (hiCHO 70:10:20). Contrary to the thought that fat-intake may induce obesity, other observations point to hiCHO as the inducer of obesity and diabetes more so than fat (Bolsinger, 2013). Bolsinger’s results suggest that the modCHO diet induced the most symptoms that reflect the onset of metabolic syndrome like steatosis and dyslipidemia. The hiFat (10:70:20) diet did not induce these symptoms and seemed to prevent the onset of diabetes, reducing the incidence considerably. The hiCHO diet very clearly induced diabetes, but 70%energy exceeds a realistic ratio of carbohydrates that would be present
in a human diet. But the modCHO (40:43:17), which most resembles the American diet (50:35:15) induced MetS and T2DM as well.

Glycemic load (GL) is an estimate of how much a certain food will raise the blood glucose after ingestion of various sources of glucose. GL is based on the glycemic index (GI), the number assigned to a certain food based on its ability to raise blood glucose when consumed, multiplied by the grams of carbohydrate in the food that the subject has ingested. Adding to the glycemic load story, adding fiber to a diet is a way of reducing the glycemic load, and therefore could be a mechanism for preventing these chronic syndromes. The current study used these past observations as the backbone for the diets that were fed to the NR in the present study. The priming diet (70:10:20) contains 70% energy as CHO and served to induce diabetes in 2wks after the separation of the NR from their siblings. The base diet, which was paired with various polyphenols, was hiCHO-modFat (60:20:20) that utilized the damaging combination of carbohydrates and fat. Though for some time polyphenols have not been considered as part of dietary fibers (Bravo et al. 1994), recent studies have shown that phenols affect blood lipids in vivo in a similar manner as soluble dietary fiber (Ruiz-Roso 2010).

**T2DM pathogenesis and NAFLD.** The findings that demonstrate the potentially destructive effects of hiCHO are reflected in the pathology of organ systems. A fascinating aspect of the T2DM/MetS onset is the association with severe liver damage and inflammation (Loria 2013). In 1906, the term “Hepatogenous diabetes” was coined to discuss the high incidence of diabetes in individuals with cirrhosis. Recent clinical observations show that individuals with T2DM have a lower life expectancy associated with vascular complications and renal disease, but also linked to cirrhosis and hepatocellular carcinomas, the most common type of liver cancer. Evidence is accumulating to suggest that non-alcoholic fatty liver disease (NAFLD), the most common liver disease in the Western world, may be closely linked to the onset of T2DM and insulin resistance (IR). These findings have been observed in the Nile Rat as well (unpublished data). NAFLD combines two clinical and histological entities: non-alcoholic fatty liver (NAFL, steatosis hepatis) and steatohepatitis (NASH) (Firneisz 2014). Hepatic steatosis is defined by the accumulation of triglycerides and other lipids in hepatocytes. More specifically, it is defined as a “intrahepatic fat content above 5.5%” or
when over 5% of the hepatocytes show macrovesicular fat (fat vesicles that are large enough to distort nuclei) on histological analysis. Traditionally, there is a way to grade the severity of hepatic steatosis from mild (10%) to severe (over 30%), though experts are challenging histological analysis because other factors may influence the severity as well.

What may be the reason for the correlation between hyperglycemia, insulin resistance, and NAFLD? The pathogenesis of NAFLD is largely unknown (Bozkurt 2012). However, it is thought to be linked to the biochemical event that accumulates the triglycerols (TAG) in the hepatocytes (Firneisz 2014). The liver is known to regulate glucose and lipid metabolism (Loria 2013). Donnelly et al. aimed to directly find the biological sources of hepatic and plasma lipoprotein triglycerols in NAFLD (Donnelly et al. 2005). Using gas chromatography and mass spectrometry, they showed that triglycerols from the intestine are delivered to the liver, where they may be secreted as lipoproteins (Firneisz, 2014). The liver is also able to synthesize triglycerols using fatty acids and glycerol. Evidence indicates that 60% of the TAG comes from the non-esterified fatty acid pool (free fatty acids released from adipose or synthesized in liver from excess glucose). This energy pool is generally used as fuel for tissues during fasting conditions (Ferrannini 1997). It is also considered that adipose tissue releases 80% of the fatty acids found in the non-esterified fatty acid pool. This report also indicated that 25% of the TG comes from hepatic de novo lipogenesis, a process that converts dietary carbohydrate to fat (Firneisz, 2014). Data suggest that hyperglycemia and hyperinsulinemia induce the activation of certain transcription factors in the liver which active genes involved with de novo lipogenesis, resulting in increased lipogenesis in the liver. In other words, hepatic de novo lipogenesis increases when insulin-resistance and NAFLD emerge.

An interesting example of the relationship between NAFLD and T2DM can be observed with respect to gestational diabetes (Firneisz 2014). It has been shown that women who develop gestational diabetes are at increased risk of developing diabetes years later (Bozkurt et al. 2012). Bozkurt et al concluded that increased hepatic lipid storage is an early and frequent indicator in insulin-resistant women who have a history of gestational diabetes. The indication that insulin-resistant women with recent
gestational diabetes have increased risk for fatty liver could be not only an important
association of general MetS and NAFL, but also a potential indicator for predicting the
onset of T2DM or the severity of liver damage. Though this particular study focused on
gestational diabetes, which may have a different mechanism of onset than traditional
T2DM due to the energy demands of pregnancy, the relationship between the risk of
developing T2DM after pregnancy and increased hepatic fat content shows relevance to
the ongoing mystery of NAFLD and MetS and IR.

**Methods & Materials**

**Study Design.** The current study sought to understand two independent concepts:
the pathophysiology of diabetes in the NR, as well as and the effects of polyphenols and
diets on T2DM in this species. The questions this study sought out to answer were: Do
polyphenols from different sources, often linked to glucose metabolism, equally affect
(prevent, reverse, or delay) the onset of T2DM in the NR? How does diet composition
impact the development of T2DM? What is the relationship between diet and liver
inflammation and NAFLD? What is the best way to determine incidence and severity of
diabetes from blood glucose measurements?

To further investigate the relationship between polyphenols, diet composition, and
pathology on the Nile rat, a study was designed to examine five polyphenols paired with
a previously determined diabetes-inducing diet. Fifty male NR were primed for 2wks on
hiCHO diet (70:10:20, CHO:fat:protein %energy). These were divided into five groups
based on initial 30min OGTT. Four of the groups continued to receive hiCHO diet
(70:10:20) with hypothetically optimal levels of polyphenol supplements, while the
Control was given a second hiCHO-modFat diet (60:20:20). The five groups were:
control (60:20:20), curcumin, bergamot, palm fruit juice, and green coffee bean
(70:10:20). The animals were monitored for 8wks by measuring food intake every other
day, sampling random blood glucose, random body weight, 30 minute OGTT at 4wks,
and behavior patterns. At the eight week, each NR had a 2h OGTT and was sacrificed for
necropsy. Organ weights and observations were monitored at this point to help identify
pathophysiology of their conditions.
The initial hypothesis was that polyphenolic supplements would reduce the incidence of T2DM in the NR after 8wks with comparison to the control. Based on previous studies by Hayes et al, the bergamot and palm fruit juice supplements could be the most promising (unpublished data 2012-2013). However, the secondary hypothesis was that the initial hiCHO and the 8wk hiCHO diet may push the T2DM “past the point of no return”. The results suggested that early priming had a severe impact on the incidence of T2DM in the NR, so severe, in fact, the much of any potential effects of the polyphenols may have been masked by the early exposure to hiCHO. Results also indicated that the most sensitive blood glucose marker was the 30min OGTT, the most representative of pathology was RBG, and the least sensitive was FBG. Diabetic livers were found to be significantly enlarged compared to non-diabetic livers, which points to the development of NAFLD and NASH with hyperglycemia.

**Animals and housing**

Fifty male NR from the Foster Biomedical Research breeding colony were primed for 2wks at weaning (3wks old) on standard diabetogenic hiCHO diet (CHO:Fat:Protein %Energy, 70:10:20). All NR were housed in individual cages in an air-conditioned facility with a 12-hour light cycle (temperature: 68-72F, humidity: 40-60%). These NR were divided into five groups as described above, each of which was given either hiCHO-modFat diet (60:20:20, 5.0 kcal/g) or hiCHO diet (70:10:20, 4.0 kcal/g) with different levels of polyphenol supplements (Table 1). Previous studies suggested that the doubling of fat in the control would not greatly affect the parameters of diabetes but does better approximate human diet composition (unpublished data 2014). The groups were sorted after 2wks of priming so that each one had approximately equal initial 30min OGTT values compared to every other group. There were 2-3 diabetes-prone (DP) NR in each group and 7-8 wild-type (WT) NR (Note: DP are defined as decedents of 5-6 generations of inbreeding high random blood glucose rats. WT NR are randomly bred and not a result of inbreeding). This randomization assigned variability equally within each group, but eliminated accidental bias.

The control group was given semi-hiCHO with diet composition 60:20:20. All other groups were given the diet composition of 70:10:20 with different amounts of
phenolics. The five groups were: 1. control with no phenolics (60:20:20), 2. curcumin (150ug GAE/kcal, 70:10:20), 3. bergamot (40ug GAE/kcal), 4. palm fruit juice (1187ug GAE/kcal), and 5. green coffee bean (210 ug GAE/kcal). All animals were fed for 8wks after 2wks of priming providing food and water ad libitum. Food intake was measured every other day for 8wks and general behavior patterns were observed daily. After 4wks on the experiment, NR were fasted overnight for 16h and were assayed for 30min OGTT and measurement of fasting body weight (FBW). On a non-feeding day at 4wks, random blood glucose (RBG) and random body weight measurements (RBW) were taken. Previous experiments have indicated that RBG is the simplest way to follow the progression of the diabetes. At the eighth week, each NR was again tested for RBG and RBW, then fasted overnight prior to undergoing a 2h OGTT and FBW measurement. At the end of the eighth week, the NR was necropsied under anesthesia after measuring a FBG and FBW. Organ and major fat pad (perirenal-retroperitoneal, epididymal, and interscapular brown fat) weights were recorded. Blood was collected via cardiopuncture for analysis of plasma triglycerides (TG) and plasma total cholesterol (TC). The harvested organs included liver, cecum, and kidney.

**Organ Weight**

Organ weight and fat pads were weighed and expressed as a percentage of total body weight i.e. it was the weight of the organ (in grams) divided by the terminal fasting body weight (in grams). The carcass weight (lean body mass estimate for body muscle and skeletal growth) was determined by weighing the NR after exsanguination and removal of all organs.

**Blood Glucose**

Random blood glucose (RBG) was measured while the NR was under 50% O2/CO2 anesthesia from a drop of tail blood. The blood was obtained by lancet puncture at the lateral vein using the Elite XL glucometer (Bayer Co., Elhart, IN, USA). RBG was assessed on non-feeding days and FBG was assessed after a 16h overnight fast.

**Oral Glucose Tolerance Test (OGTT)**

Rats were fasted for 16h overnight. After assessment of body weight and fasting blood glucose, they were given 1.75g/kg BW of dextrose solution (10.5g in 6mL distilled water) by means of oral gavage, and blood glucose was assessed every 30 minutes for 2h.
**Plasma TG and TC**

Plasma TC and TG were determined spectrophotometrically using infinity kits (Thermo Fisher Scientific Inc., Middletown, VA, USA; TG ref # TR22421, TC ref # TR13421).

**Food Efficiency**

Food efficiency was calculated by dividing body weight gain (g/d) by energy intake (daily food intake in kcal/d) and multiplying the result by 1000. The results were expressed as grams of body weight gained per 1000 kcal consumed. Greater food efficiency reflects greater weight gain per calorie consumed.

**Statistical analysis**

Statistical analysis was performed by the Statistical Package for the Social Sciences (SPSS). Pearson correlations, comparing means, and one-way ANOVA functions were used where appropriate for the study design. The ANOVA was run with a Fischer’s Least Significant Difference test (LSD). A P value of <0.05 was considered statistically significant. A t-test was run using individual measures from diabetic versus non-diabetic NR using SPSS as detailed in results. Graphs and tables were composed in Microsoft Excel for Mac 2011.

**Polyphenol Procurement**

The curcumin used was manufactured by Hindustan Herbals Limited and contained three curcuminoids (95%): curcumin, bisdemethoxy curcumin, and demethoxy curcumin. It revealed a GAE content of 61% of the total product. The bergamot used was obtained from HP Ingredients in Bradenton, FL via Metagenics. It had a GAE content of 40 ug GAE/kcal. The SD-PFJ used was obtained from Malaysian Palm Oil Board and was in spray-dried powder form. It had a GAE content of 49,300 ug GAE/kcal. The Green Coffee Bean was of the Robusta species obtained from New England Tea & Coffee LLC in Malden, MA. It had a GAE content of 210 ug GAE/kcal.
Results

The study was designed to examine four polyphenols paired with a previously determined diabetes-inducing diet (Table 1).

**Body Weights.** Table 2 shows the total body weight gain during the study for each of the 5 diet groups. After randomization based on initial 30min OGTT, the initial body weights were all similar, except for PFJ, which happened to be significantly greater at the start by random chance (p<0.05) as compared to the control diet. After 4wks, the NR maintained nearly equivalent weight gain between groups. After 8wks on the study, NR on curcumin had the highest average body weight, which was significantly greater than NR fed bergamot. Because each diet group in Table 2 included all the NR together, variation was great mainly because non-diabetic NR were lumped with the diabetic NR at that point. Table 3 split out the NR based on degree of diabetes using 8wk 30min OGTT. It was notable that all the “healthy” non-diabetic NR subgroups, except for those fed GCB, had lower body weights than the diabetic NR even at the beginning of the study. Thus, it is evident that the NR that became diabetic by the 8th week of the study already had been affected by the 2wk hiCHO priming with 70:10:20 diet.

In addition, other aspects of body weight were instructive. For instance, the Control NR at 4wks that did not develop diabetes weighed 74 ± 7 g, which was significantly less than the NR that developed diabetes, weighing 91 ± 6 g (p<0.05). In Table 3, it was evident that as time went on, the diabetic NR in general became significantly heavier than non-diabetic NR, except for GCB where all tended to be on the heavy side.

To consider these patterns as a function of diet-influence versus overall diabetic incidence due to genetic variation and susceptibility is an important exercise for understanding diabetes. The main significance of body weights in Table 3 was between diabetic and non-diabetic NR across all diets. However, several patterns were noted: Initially, among non-diabetic NR, those fed PFJ weighed significantly more than the non-diabetic control. The diabetic PFJ-fed NR were also significantly heavier initially than diabetic NR fed GCB and BERG initially. Referring to Table 2, this could be an unexplained error induced during randomization. Therefore, significance of body weights for the PFJ-fed rats should be treated with caution.
Food intake and food efficiency. In Table 2, average food intake was similar between groups (9 ± 1 g/day) for all the polyphenol diets, while the control group was significantly less than all other groups. This indicated that the NR adjusted for the increased calories in the Control diet, since the fat content was higher for that diet. The numbers also demonstrated that on average, the NR that developed diabetes ate more calories per day (Table 3). It was interesting to note that the dietary phenolic supplementation played a role in food intake. Most interestingly, diabetic NR on PFJ ate significantly more than both the diabetic and non-diabetic control NR, possibly because Splenda (a sweetener) was added to that diet to counteract the bitterness of PFJ.

The best measure of food intake is the caloric intake (kcal/d) per NR. This number derives from diet composition (Table 1), where each diet contained 5.0 or 4.0 kcal/g, based on the dry weight of the prepared food. There was no significant difference between the caloric intake of the overall groups (Table 2). This indicated again that the NR fed the Control diet were still consuming a similar amount of calories (compared to the experimental groups) even though they were consuming significantly less food by dry weight but with greater caloric density. Table 3 demonstrates that all the diabetic NR in each group, regardless of diet, ate more kcal/d than the non-diabetic rats on average, but these differences overall were not significant, however this was only significant in the case of curcumin and BERG.

Food efficiency measures how well the food ingested is converted into weight gain measured as the NR body mass. Specifically, it measured how much weight was gained per 1000 kcal consumed. This number was usually greater for the diabetic NR presumably because of adipose expansion, which trended higher, since body length (linear growth) was typically unaffected. PFJ-fed NR had significantly lower food efficiency than either control or curcumin-fed NR (Table 2). However, the lowest food efficiency occurred in the non-diabetic BERG-fed NR, which were significantly less efficient than non-diabetic Controls (Table 3). The general trend, excluding Control, seen in Table 3, was that the diabetic NR had higher food efficiency than non-diabetic NR, when comparing within diet groups given the various polyphenols.
Blood Glucose

**Random Blood Glucose (RBG).** Table 3 reveals that RBG at 4wks did not differ significantly between diets or between diabetic and non-diabetic NR although the RBG was found to be higher in diabetic NR. After 8wks, significance between several non-diabetic RBG and diabetic RBG was observed. Most notably, non-diabetic PFJ and non-diabetic Bergamot had the lowest RBG. Diabetic GCB and PFJ had the highest RBG.

**Oral Glucose Tolerance Test (OGTT).** Table 2 revealed no significant differences between initial, 4wk, or 8wk OGTT, whereas the split out in Table 3 based on 30min OGTT revealed certain differences in diabetic and non-diabetic NR. For example, the 30min OGTT separated the non-diabetic from the diabetic NR in each diet group, with the 6 diabetic PFJ and 6 diabetic Bergamot NR being the most responsive. This pattern continued at 4wk and 8wk OGTT, with the 30min response being the peak activity for all NR, and hence explains the reason for using this value to split the NR into diabetic and non-diabetic subgroups. Diabetic Control, BERG and PFJ-fed NR were also most responsive in the 8wk RBG. The 8wk OGTT was more extensive because it measured BG at 30min intervals for 2h. Most significance was between diabetic and non-diabetic NR for the obvious reason that the split-distribution was based on the 30min OGTT values at 8 wks. Figure 1 shows the correlation between the 4wk 30min OGTT and the 8wk 30min OGTT was linear and significant ($r=0.73$, $p<0.001$), emphasizing that the 4wk value was as good a predictor as the 8wk value.

**Fasting Blood Glucose (FBG).** The fasting blood glucose was taken at necropsy, 2-4 days after the 8wk 30min OGTT. The mean FBG for all non-diabetic NR was normal (<60 mg/dl) except for the control diet and PFJ, which were both somewhat elevated. It was noteworthy that the second measure of FBG taken at 8wk OGTT ($t=0$), and several days before the terminal necropsy sample, were all normal for non-diabetic NR, even for the control and PFJ. However, the diabetic NR fed bergamot and PFJ were slightly elevated (>60 mg/dl). Diabetic GCB-fed NR had the highest terminal FBG (Table 3) at necropsy, more than double the value of the 0-time OGTT a few days earlier. Thus, it is noteworthy that FBG at necropsy and the FBG from the 8wk OGTT were different even though they were measured within 2-4 days of each other. The general trend showed that FBG at necropsy tended to be higher than FBG at the 8wk OGTT (Table 2,3).
Organ Analysis

Liver. Although liver size has proved strongly related to T2DM in the NR model, there was no significant difference among livers from these NR. All seemed slightly enlarged because historically the %Liver BW is about 3.0-3.5 range. Nonetheless, a tendency existed for diabetic livers to be larger than non-diabetic livers (Table 3). An independent samples t-test determined that the % Liver BW of non-diabetic (n=12) NR was less than diabetic (n=28) NR fed polyphenol-supplemented diets. Thus, diabetic NR had statistically significantly heavier % Liver BW (4.11 ± 0.63 g) by the end of the study as compared to the non-diabetic NR (3.55 ± 0.21 g, p = 0.005).

Kidney. Kidney weight was not significantly different between diet groups, but again the tendency for the larger kidneys was found in the diabetic NR.

Cecum. PFJ-fed NR had significantly larger cecums compared to all other diet groups for both non-diabetic and diabetic NR.

Adipose Tissue. Table 2 indicates that the PFJ NR had the least total fat and epididymal fat, among all groups, which was significantly less that the Controls. Table 3 indicates that the reduced epididymal fat of the diabetic PFJ NR mostly influenced this finding. Total fat tended to be greater in diabetic NR and was reflected in individual NR pools as well. The exception was control NR where diabetic NR tended to have reduced total fat, suggesting that ketosis and fat breakdown was beginning (Table 3). Diabetic NR revealed expanded BAT pool as well, especially for curcumin-fed NR, suggesting an attempt to divert calories to BAT for oxidation rather than storage (Table 3).

Plasma Lipid Analysis

Total Cholesterol (TC). Plasma cholesterol tended to increase in diabetic NR, and the PFJ-fed NR had significantly higher cholesterol than either Control or curcumin-fed NR (Table 2, Table 3).

Total Triglycerides (TG). TG values were unremarkable, with curcumin-fed diabetic NR exhibiting the highest values.

T2DM Incidence. Table 3 reports the most detailed response of NR to diets based on their OGTT parameters. Anytime the 30min OGTT value exceeded 150 mg/dl, it was considered to have some degree of T2DM in response to the dietary CHO load. OGTT is
applied especially to assess the early incidence of T2DM, whereas RBG is more indicative of chronic glucose elevation and potential tissue damage from circulating glucose (unpublished data, Bolsinger 2014). Because all OGTT are administered by the same standard, for the same amount of time after being fasted, with the gavage of a glucose load proportional to body weight, it is a more accurate representation of their exact acute reaction to glucose intake and metabolism. RBG is a less precise, but useful index of postprandial glycaemia that is more chronic in nature. The 30min OGTT value was selected as ideal because at this point is when the blood glucose usually peaks in the 2h OGTT, and it can be used conveniently to separate the diabetic status between NR. By 60min, the blood glucose normally drops appreciably back down toward baseline. The highest incidence of T2DM on the basis of 30min OGTT was seen in curcumin where 90% (n=9/10) of NR were diabetic after 8wks based on this 30min OGTT parameter. The second highest incidence was seen in Control NR with 70% (n=7/10) developing diabetes. BERG, PFJ, and GCB all had 60% (n=6/10) of the NR developing diabetes. The incidence was also monitored as FBG at necropsy. A FBG that exceeds 60 mg/dl can be considered pre-diabetic and >80 mg/dl as diabetic. The most striking story was that of curcumin, where the incidence of diabetes based on FBG was 20% versus the incidence based on 8wk 30min OGTT of 90%. On the other hand, the PFJ-fed group had an 80% incidence based on FBG, but only 60% incidence based on 30min OGTT. These differences suggest that FBG measures something outside of the OGTT measurement, and the latter measure is best used for determining pre-diabetes.

Correlations

OGTT Consistency. Figure 1 indicates that the 30min OGTT after 4wks was a strong predictor (well correlated) with that same measure after 8wks, suggesting that the results for OGTT were already apparent by 4wks.

Relationship of OGTT and RBG. Figure 2 and Figure 3 revealed that the correlation between 8wk 30min OGTT and 8wk RBG (r=0.631, p<0.0001) was similar to the correlation between 8wk 30+60min OGTT and 8wk RBG (r=0.676, p<0.0001). Both figures show a significant correlation between the OGTT variable and RBG, using data from all the NR and all diets in the study (n=50). This supports the idea that the 30min
value is adequate and not much improved by adding 60min, at least for using 30min OGTT to diagnose early diabetes.

**Relationship of OGTT and % Liver Body Weight.** Figures 4-6 correlate the OGTT value at 30min, 60min, and 30+60min OGTT at 8wks with %Liver BW, since liver weight is the best predictor of terminal pathology. In addition, it was noteworthy that the 0min (FBG) for 8wk OGTT was modestly, but significantly correlated with %Liv BW (r=0.45, p=0.004, data not shown). Figure 4 shows that the 30min for 8wk OGTT was better and significantly correlated with %Liv BW (r=0.67, p<0.0001). Figure 5 shows the 60min for 8wk OGTT was significantly correlated with %Liv BW (r=0.67, p<0.0001) to the same degree as the 30min OGTT, whereas Figure 6 shows that the 8wk 30+60min OGTT had a slightly better correlation to %Liv BW (r=0.71, p<0.0001), but not much better than the 30min or 60min alone. The 90min 8wk OGTT correlation with %Liv BW (r=0.47, p=0.002), as well as the 120min 8wk OGTT correlation with %Liv BW (r=0.54, p<0.0001) were weaker and are not shown.

**Comparisons between OGTT vs. % Liver BW and RBG vs. % Liver BW.** In order to shift the analysis to the pathology measured at study end with blood glucose markers, correlations were run using liver weight. Figure 7A demonstrates a significant correlation (r=0.67, p<0.0001) between 30min OGTT and % Liver body weight at 8wks in all 40 NR on test diets i.e. excluding Controls (which were fed a different diet composition). Figure 7B demonstrates these same data but with the diet status indicated. Again, the correlation is strong, but no particular insight comes from the diet examination, except curcumin and bergamot look most consistent, ie. the greater the 30min OGTT the larger the liver. Figure 8A demonstrates that the correlations between RBG at 8wks and % Liver body weight at 8wks in all NR (excluding Controls) were the strongest of all (r=0.80, p<0.0001). Figure 8B shows these same data but with diet status indicated. The correlations between RBG and % Liv BW had slightly stronger correlations than 30min OGTT and %Liver BW, again supporting the idea that RBG is a good predictor of liver enlargement and end-stage pathology.
Discussion

Body weight gain and diabetes induction

It is noteworthy that after 4wks on diet, essentially all the NR that were categorized as "diabetic" at 8wks at study end were already heavier (had gained weight faster) than non-diabetic NR. Thus, even after 4wks one might have predicted the T2DM severity that was to come. An exception was GCB, in which the two subcategories of diabetic NR had similar weights, i.e. the GCB diabetics were not heavier like other diabetic groups. In the extreme cases with curcumin and bergamot, the weight difference was reflected in caloric intake and food efficiency data, which both tended to be greater in diabetics. In fact, as opposed to control NR, all NR given polyphenols ate slightly more calories if they ended up as diabetic, and these extra calories were more efficiently used to gain weight, primarily as body fat except for GCB. This suggests that these three polyphenols (curcumin, bergamot, and PFJ) enhanced weight gain by fat accumulation, at least when weaning pups were primed with hiCHO, before they received the polyphenols. It did not seem to occur when pups were not primed for 2wks, but instead received their polyphenol supplement directly from birth (unpublished Study 107).

For example, the unpublished study from the Hayes Lab supports this idea (Study 107, unpublished data, 2013). In that experiment, 29 NR were fed diets (70:10:20) either as the control (0 g phenolics) or supplemented with curcumin (290 ug GAE/kcal), high-bergamot (290 ug GAE/kcal), low-bergamot (30 ug GAE/kcal) or liquid PFJ (970 ug GAE/kcal) for 7wks directly upon weaning. When comparing the effect of the control diet to the experimental diets, the incidence of diabetes was reduced in the polyphenol-supplemented NRs. Incidence was determined by RBG at 7wks being >75 mg/dl. In the control, 67% of NR developed diabetes using these parameters. In contrast, only 33% of NR fed curcumin developed diabetes; 33% of NR fed PFJ developed diabetes; 50% of NR fed high-BERG developed diabetes; 0% of NR fed low-BERG developed diabetes.

Most striking is the story of bergamot, which revealed promising effects in Study 107. Low-bergamot (30 ug GAE/kcal) was similar to the amount of bergamot in the current experiment (40 ug GAE/kg). In study 107, 100% of the NR fed low-bergamot were still diabetes-free after 7wks on diet, as determined by RBG. By contrast, in the current study, 50% of the NR fed bergamot (40 ug GAE/kcal) had developed diabetes by
8wks (total of 10wks on diet including the 2wk priming period), again based on RBG>75 mg/dl. This emphasizes the destructive impact of the 2wks of priming in the present study. Study 107 found that the hiCHO control diet (70:10:20), which was similar to the current priming diet, caused increased incidence and severity as compared to the other diets. It is possible that early priming pushes the NR “past the point of no return”, so that recovery is somewhat impossible after exposure to hiCHO at weaning. This suggests that post-weaning, especially the first 2wks after weaning, are a formative time for NR metabolism and its disruption by hiCHO may damage their metabolic control mechanism severely. Figure 1 supports this idea because it shows that the correlation between RBG at 4wks and 8wks is significant (p<0.0001). In other words, diabetes is already apparent at 4wks on diet (6wks total exposure to hiCHO) and significantly correlated to the increase in severity at 8wks (10wks total time). It is also evident when noting that the diabetic NR are generally heavier than non-diabetic NR initially even after 2wks of priming at 5wks of age, and finally after 8wks more of supplements (Table 3).

Previous studies from the Hayes Lab have shown that extra weight gain in diabetic NR tends to be as adipose tissue (Bolsinger, 2014). During development of MetS, adipose deposition starts increasing. As T2DM progresses, fat begins to be utilized for energy when insulin resistance becomes severe. This trend can be difficult to detect statistically because different NRs could be in various stages of diabetes at 8wks. Therefore, the degree of adiposity can vary greatly. Nonetheless, PFJ-fed NR had significantly less total fat than controls (Table 2). This could be attributed to the redistribution of calories away from fat, and it supports the earlier data that PFJ is somehow different than the other polyphenols.

**Primbing and the irreversibility of diabetes**

Why might priming have such a severe effect on diabetes incidence and severity in the NR? Studies have shown that the mitochondria, which are essential to aerobic metabolism, could play a major role in the pathophysiology of diabetes (Sivitz & Yorek, 2010). After hopping through the electron transport chain during oxidative phosphorylation, the electrons are generally passed to molecular oxygen. Sometimes a small number of electrons leak during transport, eventually giving rise to reactive oxygen
species (ROS). Although ROS play an essential role in regulatory pathways, they have also been documented for causing tissue and DNA damage, apoptosis, and cancer (Dickinson & Chang, 2011).

A current theory has emerged that links high glucose and fatty acid concentrations in T2DM to increased oxygen use, and therefore increased production of ROS (Sivitz & Yorek, 2010). This increased production has been shown to impair β-cell’s ability to release insulin as well as decrease insulin sensitivity in tissues. It is unlikely that ROS alone can explain the onset of T2DM, but it probably accelerates its development and perpetuates the impairments caused by T2DM. That said, priming with hiCHO could have induced diabetes in the NR by increasing the glucose load on the mitochondria and production of ROS, thereby damaging the mitochondria early on. That hypothesis is currently being explored (Osborne et al, unpublished data Wangh Lab 2015). Because mitochondrial DNA and its susceptibility to damage is inherited from the mother, it is possible that susceptibility to mitochondrial damage plays a role in genetic predisposition to T2DM as seen in the NR. Perhaps the slightest “push” with hiCHO could cause T2DM in one NR (especially males) but not another (particularly females which resist diabetes). These reasons could explain why diabetes seemed not to be helped by polyphenols once early damage had occurred.

The issue of priming is relevant because it may also represent the global increase of T2DM in children and adolescents (Nesmith, 2001). Studies show that during puberty, there is an increased resistance to insulin in part due to increased levels of growth hormone, which can lead to hyperglycemia. Growth hormone is known to increase gluconeogenesis in the liver and dull the effects of insulin on glucose uptake in the muscle and adipose cells (Widmaier 2011, 565). The “push” from hyperglycemia to full-fledged T2DM, in many of these cases, is paired with genetic factors, obesity, diet, and physical inactivity. Also, evidence suggests that early exposure to certain diets or environments can alter gut microbiota, which in turn can cause immune-mediated or metabolic diseases later in life (Arrieta, 2014). Researchers hypothesize that a “critical window” exists in the formative period of development, which can determine susceptibility to certain diseases such as diabetes. It is noteworthy that T2DM consistently develop in the NR during rapid growth period post-weaning, especially in
males where growth is more rapid than in females, and as seen in the current experiment, in the males that gained the most weight (grew faster).

**Food intake, food efficiency, and T2DM**

As previously discussed, diabetic NR tend to have higher body weights than non-diabetic NR, even when consuming the same diet. Body weight is largely determined by energy intake and energy utilization. Imbalance between these two categories results in weight gain (Dokken & Tsao, 2007). A noteworthy aspect of the data is that although Control NR ate significantly less than all the other NR, their caloric intake was the same (Table 2). This demonstrates that the brain was functioning normally by tracking calories consumed, independent of source, because the caloric intake was more or less uniform between diet groups. It appears that the Control NR somehow accounted for their 60:20:20 diet by eating less volume, but ingested the same calories as the 70:10:20 fed to the test NR. This implies that healthy NR have adequate control over their caloric intake and that the diabetes that developed was not necessarily due to overeating per se, but could reflect imperfect calorie counting by NR prone to T2DM. The diabetic NR within a given diet seemed to lose or lack this control, having greater caloric intake on average.

It is also critical to note that the NR fed any one of the 70:10:20 test diets that led to higher caloric intake also had higher food efficiency, which is the measure of how many calories are actually converted into growth as body mass. In diabetic control NR, the food efficiency did not increase with elevated caloric intake as it did in all the other diabetic groups (Table 3). This suggests that diet energy contribution (70:10:20 for test NR versus 60:20:20 in controls) may have had an effect on food efficiency based on the CHO:fat:protein ratio. Higher food efficiency in diabetic NR may explain their greater body weight. What is it that makes diabetic NR eat more calories than non-diabetic NR? Or is this phenomenon a consequence of another cause, such a genetic predisposition interacting with diet? An essential part of the story may be that of glycemic load (GL), which is an estimate of how much a certain food will raise the blood glucose after ingestion of various sources of glucose. GL is based on the glycemic index (GI), the number assigned to a certain food based on its ability to raise blood glucose when consumed, multiplied by the grams of carbohydrate in the food that the subject has
ingested. An unpublished study at the Hayes lab (Study 100) demonstrated that a hi-fiber, low-GL lentil diet protected NR against T2DM (unpublished data, 2015).

In the current study, the diets fed (minus their polyphenols) mirror some “processed foods” with high GL. In other words, processed foods with increased concentrations of refined CHO, such as sugar, have decreased concentrations of fiber, protein, and water (Schulte et al., 2015). The absence of fiber and protein quickens the absorption of high GL foods into the body. Several studies have pointed to “food addiction” as one of the consequences of consistently eating high GL foods (Schulte et al., 2015). For example, in one study, rats with the tendency to binge-eat exhibited addictive behavior when fed Double Stuffed Oreos or frosting, but not when fed regular chow. Another study revealed that rats fed these types of high GL foods, specifically high in sucrose, have downregulation of dopamine, which is a sign of the addiction profile (Schulte et al., 2015). To put it simply: sugar may be addictive. Therefore, it would make sense that some NRs, that were primed on hiCHO diet, might subsequently increase their food intake and develop diabetes (Table 3). Some NR may be more susceptible to the “addiction” than others, based on other factors such as genetics or the gut microbiome composition (discussed below) or damage to their mitochondrial DNA as mentioned above.

The “addiction” could have also been influenced by the polyphenol supplements. The impact of Bergamot and PFJ were most striking in terms of food efficiency in the NR. No current studies illustrate the possible optimal GAE/kcal intake of PFJ on human subjects in terms of optimal GAE/kcal intake for diabetes prevention; a few papers have approached this topic with bergamot (Leopoldini, 2011). In our NR, when food and caloric intakes in non-diabetic bergamot NR were compared to the other groups, the food efficiency was significantly lower in non-diabetic bergamot NR were compared to diabetic curcumin NR, but surprisingly not compared to the diabetic bergamot NR (Table 3). The diabetic PFJ group had a lower, but not significant, food efficiency, lower even than non-diabetic control and GCB, but these NR had the highest 8wk 30min OGTT on average. Although the n is small, it raises a question because low food efficiency should lead to a lower body weight, which one would assume would be less likely to develop diabetes. However, this was not the case in this instance.
Gut microbiome and polyphenols

Another important aspect of the story is the proposed involvement of the gut flora in the development of diabetes or metabolic syndrome. One of the roles of the microbiota is to regulate body weight and energy homeostasis (Han & Lin, 2014). Studies have shown that microbiota found in obese mice and humans are more effective in harvesting energy from food, thus having increased food efficiency, than non-obese individuals (Lankelma, 2015). It is interesting that in the current study, the NR fed PFJ had significantly larger cecums as percentages of body weight (presumably more bioactive gut flora) than the other groups (Table 2). However, the diabetic and non-diabetic PFJ-fed NR did not have significantly different cecum weights. This suggests that PFJ (perhaps as its polyphenol content) may have had an effect on the cecum size and its gut flora. As previously discussed, PFJ NR also had very low food efficiencies regardless of their glucose responses, suggesting that the enlarged cecum was linked to reduced food efficiency, thereby leaving more food energy to nourish the gut flora.

Previous studies have shown that the NR is protected against T2DM by liquid PFJ fed to unprimed NR (Bolsinger, 2014). The current study utilized NR that were primed for 2wks on hiCHO directly post-weaning, which may have eliminated or reduced the potential for protection against T2DM by such supplements. Spray-dried PFJ, as opposed to liquid PFJ, was used in the current experiment, which may have changed the effectiveness of the PFJ as well. Despite this, PFJ appeared to have an effect on the gut microbiome as shown by the expansion of the cecum and the low food efficiency. Studies have suggested that certain doses of polyphenols can alter gut flora composition by inhibiting certain microbes and enhancing the activity of others (Cardona, 2013).

A connection between PFJ-supplemented diets and changes in metabolism and liver function may also exist. In a previously mentioned study (Study 107) where 3wk old male NR were fed polyphenol supplemented diets for 7wks without priming, PFJ elevated the total cholesterol (TC) level (225±55 mg/dl) significantly compared to curcumin-supplemented NR. This trend was apparent in the current experiment as well. PFJ-fed NR exhibited significantly elevated TC (265±73 mg/dl) compared to curcumin (162±65 mg/dl) and control (186±87 mg/dl) NR (Table 2). The 30min OGTT split-data
revealed that the diabetic PFJ NR had significantly elevated TC (287±86 mg/dl) even compared to diabetic control (192±92 mg/dl), curcumin (163±69 mg/dl), and bergamot-fed NR (186±51 mg/dl), but not GCB NR (261 mg/dl), which were similar (Table 3).

What caused this elevation in TC, specifically in PFJ-fed NR? To explore this question, one should consider what causes the TC elevation in diabetic NR in general. Studies have revealed that high GL diets increase the relative risk of coronary heart disease (CHD), which is associated with high blood cholesterol (Mathews, 2015).

Evidence suggests that chronic intake of high GL diets, such as the hiCHO used in the current experiment, induced chronic hyperglycemia, which in turn predicted an increased relative risk of CHD. Individuals consuming high GL diets tended to have reduced HDL levels and increased LDL and TG levels, which points to interference with liver function. The pathogenesis of CHD and its relation to chronic hyperglycemia is not well understood. The trend of high TC with hiCHO diets and diabetes is evident in previous data published in the Hayes Lab (Bolsinger. 2013). Among 25 4wk old male NR fed 70:10:20 for 4wks, 13 had RBG>400 mg/dl, which is severely diabetic. These NR had significantly higher TC levels than the NR with RBG<150 mg/dl. Since all the NR had the same dietary cholesterol intake, differences in cholesterol can be attributed to cholesterol synthesis in the liver and cells lining the gastrointestinal tract (Widmaier 2011, 556). Because the liver controls cholesterol homeostasis, damage to the liver paired with the presence of elevated TG in the blood stream may impair the enzymes that inhibit cholesterol synthesis. Unfortunately, the physiological effects of PFJ are not completely understood. However, this observation may have something to do with how PFJ expands the cecum and possibly interrupts metabolism. However, in in Bolsinger et al. 2014, where PFJ was fed to 8wk old NR fed chow for up to 17wks, the RBG remained normal and neither TC nor TG were elevated.

PFJ is different from the other polyphenols fed in the present study in that it is not purely a polyphenol. The “juice”, which was spray-dried in this experiment, actually contained 4.7% soluble fiber and 58% CHO. The GAE in the diet was the highest of all the other tested polyphenols (1187ug GAE/kcal). This may account for the significant expansion of the cecum in the PFJ-fed NR. An unpublished study at the Hayes Lab (Study 100, 2015) investigated the effect of low-GL diets on diabetes and MetS in the
NR. That study found that NR fed low-GL Green Lentil diet had enlarged cecums and almost no T2DM, which could be attributed to the enhancement of the activity of “good bacteria” by poorly digested CHO and fiber in lentils in the large intestine. However, NR fed low-GL diets with same amount of fiber as the Green Lentil diet, but a different source (cellulose) did not exhibit enlarged cecum and developed more T2DM, suggesting that the quality of CHO in green lentils was more important than quantity.

To add to the puzzle, NR fed high GL diets also had enlarged cecums, presumably enhancing the activity of “bad bacteria”. Green Lentils in fact are not pure fiber; they also contain 5.4 mg GAE/g of polyphenols. It has been estimated that only 5-10% of polyphenol intake is absorbed in the small intestine and the bulk are thought to collect in the large intestine where it influences gut microbiota (Cardona 2013). Therefore, the polyphenols paired with the fiber may have interacted in an important way to produce these effects. As mentioned, other studies at the Hayes Lab have shown that PFJ protects the NR from T2DM (Bolsinger, 2014). What happened in the current study that led PFJ to exacerbate diabetes NR? Once again, priming with hiCHO at a young age could be the culprit. The priming may have established certain bacterial colonies in the gut, which changed metabolic activity and may have started the process of glucose intolerance early on (Suez, 2014). Once the “bad bacteria” settled in and the diet was switched to PFJ, the hiCHO content paired with the addition of Splenda may have been converted into harmful metabolites in the large intestine. If the PFJ NR were not primed, as is exemplified in the previously discussed Study 107, the effects of PFJ would have likely been beneficial. In Study 107, the cecums of PFJ-fed (2.01±0.51 g) were significantly larger than cecum from the other diet groups (<1.50g). The Study 107 data show that these PFJ-fed NR, though they had enlarged cecums, were also the healthiest group overall with a 7wk 30min OGTT of 96±37 mg/dl, which was significantly lower than control and curcumin-fed NR. This suggests that PFJ has a strong effect on gut microbiota and, by a yet unknown mechanism, enhances their activity under specific dietary circumstances. The hiCHO priming very possibly plays a role in determining which bacteria are present in the gut, and whichever ones exist at the onset of PFJ-consumption will be enhanced. In other words, if a NR starts off with bad bacteria and is given PFJ, those bad bacteria may expand and may promote diabetes, as observed in the
current study. If a NR starts off with relatively good bacteria and is given PFJ, the good bacteria may expand and may protect it from diabetes, as observed in Study 107.

**Polyphenols may have different mechanisms of operation**

One of the goals of this investigation was to compare and contrast the effects of various polyphenols on the development of diabetes in the NR. Initially, the hypothesis was that the polyphenolic supplements would delay or prevent diabetes in the NR based on previous studies and published literature. However, the effect of priming may have been too strong, and it seemed to override the potential beneficial effects of the polyphenols. Nonetheless, certain trends were distinguished that hint that each supplement had a unique mode of action.

As previously discussed, only PFJ significantly enlarged the cecum compared to the other diets (Table 2,3). Curcumin seemed to increase the incidence of diabetes in this study to 90% based on the 30min OGTT split (Table 3). This is curious because curcumin has been shown to have many beneficial effects as an antioxidant in other models (Manjunatha & Srinivasan, 2007). In one study, ob/ob mice were administered curcumin for 6 weeks and it was found to lower plasma fatty acids, TC, and TG concentrations (Ghorbani, 2014). Even in human studies, 100 overweight T2DM patients who consumed 300 mg/d (333 ug GAE/kcal) of curcuminoids for 3 months had decreases in BG and a decrease in plasma fatty acids (Ghorbani, 2014). Although many studies highlight the promise of curcumin, some reports have suggested that the polyphenol may actually be toxic under certain circumstances, including causing DNA damage to both nuclear and mitochondrial DNA (Burgos-Morón, 2010). It could be that the dosage of curcumin for the weanling NR was too high (150ug GAE/kcal) and may have caused DNA damage. In fact, NR given curcumin at an early age were later found to have diabetes and some of the highest levels of mitoDNA mutations ever observed (Osborne, unpublished data 2015). Further work needs to be done with different dosages in respect to age and growth status of the host.

Bergamot is another story: it has shown inconsistent promise in the previous studies at the Hayes Lab. Initially it caused diabetes in 40% of NR at high doses of 290 ug GAE/kcal (Study 107), but also in that study, 100% of the NR fed low-bergamot (30
ug/kcal) did not develop diabetes, based on the 7wk RBG <75 mg/dl split out (unpublished data, 2015). In the current study, which included priming for 2wks with hiCHO, 60% of NR fed low-bergamot (40 ug/kcal) developed diabetes, which was the lowest incidence of T2DM among all the diet groups (Table 3). The low dosage of bergamot in the current study and in Study 107 may have helped prevent diabetes because the high-bergamot diet (290 ug/kcal) appeared to be more damaging than the other diets with TC significantly elevated to 271±85 mg/dl when compared to curcumin with TC 142±35 mg/dl.

GCB may also operate by a unique mechanism. GCB GAE has been shown to suppress weight gain in mice (Shimoda, 2006). Interestingly, the current data shows that diabetic and non-diabetic NR fed GCB had normal, similar body weights at 4wks and 8wks, as compared to the other diet groups in which diabetic NR body weights were greater (Table 3). Shimoda et al concluded that the caffeine found in GCB suppresses adipose formation. Recently, GCB extract (without caffeine) has been marketed as a popular weight-loss drug, though its effects have not been documented extensively. The mechanism of GCB is still a mystery, but the current study demonstrated that a dose of 210 ug GAE/kcal failed to perform well, as diabetes was consistently high by any measure, especially FBG, which suggests liver damage.

**30min OGTT, RBG, FBG, as T2DM indicators**

*Relevance to humans.* In general, blood glucose is the key diagnostic indicator of T2DM; therefore it is essential to determine the various BG parameters, which could be informative of the progression of the disease. In the current study, FBG, 2h OGTT, and RBG were examined. For the sake of comparison, it is helpful to compare the levels that humans would exhibit under certain conditions (Table 4, below). These values are important for clinically diagnosing diabetes when it is already established; however, they do not target early diabetes, which is part of our objective with the NR so various interventions can be tested at specific point in the disease process. By the time a human has a FBG exceeding 126, or even 100, it may be relatively late to reverse or protect against T2DM further progression by beginning dietary or lifestyle intervention.
One of the goals of this study was to find early cut-off points in blood glucose as an indicator that are more sensitive to altered internal metabolic conditions. The NR is a valuable model because it is highly sensitive to dietary glucose as a function of the whole diet, very similar to humans; it is also unique because of the wide range of BG that it exhibits (from approximately FBG of 20 mg/dl (normal NR) to >600 mg/dl in uncontrolled T2DM). Ultimately, it is imperative to ascertain when a critical time point presents itself to intervene in protecting NR and humans from T2DM and what defect is being targeted for restoration. In other words, how and why does T2DM begin in terms of diet glucose load? As previously discussed, early childhood and puberty are thought to be key times. Appropriate blood glucose levels can monitor this progression most effectively.

**BG Sensitivity of the NR in T2DM.** This was the first study in the Hayes Lab to actually measure all 3 categories (FBG, OGTT, RBG) of glucose levels at least twice during the course of study in the same NR, which is helpful in setting a *time course* and *severity* of diabetes progress. Data compiled from more than a thousand NR from the Hayes Lab suggest that their normal FBG is 40-60 mg/dl, and FBG is the least likely BG to be altered by diet, at least early on. By definition then, diabetes onset in the NR can be considered a non-fasting problem and it must have something to do with how the NR metabolizes glucose after eating. This scenario is also evident in the current study when the FBG at 0-time for OGTT is observed to average about 50 mg/dl, but less than 60

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<th>Table 4. Comparison of BG parameters in Humans and NR</th>
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<td><strong>FBG (mg/dl)</strong></td>
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<td>Healthy</td>
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<td>Pre-diabetic</td>
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<td><strong>RBG (mg/dl)</strong></td>
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<td><strong>OGTT (30') (mg/dl)</strong></td>
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</table>
mg/dl. Thus, pre-diabetes can be characterized in the NR as FBG 60-80 mg/dl. Compared to the human cut-offs previously mentioned, (FBG 100-125 mg/dl) the NR appears to be a more a more sensitive model. The goal was to describe diabetes with at least 2 clinical markers, one indicating the “incidence”, including early diabetes, was found to be the 30min OGTT; the second focused more on diabetes “severity”, and was found to be RBG, with FBG a distant third.

In a related set of data with more than 500 male and female weanling NR (NR Study 129, Appendix Table A and B), NR were fed a hiCHO challenge for 2wk or 4wk then split into quintiles based on 30min OGTT responses to gain an overview of their postprandial responsiveness during early diabetes induction by diet. The design is such that it separated the genetic variation present in the NR in both males and females. The results indicated that the FBG obtained from 0-time in that assay for all 5 quintiles of 30in OGTT for males after 4wks on hiCHO was very stable (41-67 mg/dl), even as the 30min OGTT showed significant differences between quintiles. For instance, the average FBG for the lowest quintile at 4wks on hiCHO was 41±10 mg/dl, while the 30min OGTT glucose was 82±15 mg/dl. At the other extreme, the highest quintile for 30min OGTT of 336±95 mg/dl still had a relatively low FBG of 67±34 mg/dl, but it is rather clear that the 30min OGTT identified the relative risk for NR prone to T2DM in those 255 males. This measurement is only slightly above the “normal” FBG of 40-60 mg/dl, even though the NR in this highest quintile are well on their way to diabetes. The pre-diabetic NR in Study 129 also exhibited FBG between the range of 41-67 mg/dl. This suggests that 30min OGTT is a more sensitive measurement of blood glucose than FBG for separating the various degrees of T2DM progression, especially early on in the disease. This is a very important finding because it is generally assumed by most diabetologists that FBG is all one needs clinically when monitoring FBG in patients being screened for diabetes. It misses the early stages of diabetes. In the current study, FBG was a less sensitive screen for T2DM than the 30-min OGTT. For early or “pre-diabetic” NRs, the FBG dd not exceed the normal 60 mg/dl in most cases, even when glucose metabolism appeared to be poorly controlled after an acute dose of glucose. Most NR with advanced T2DM have high FBG >100 mg/dl, which suggests that FBG is useful only for marking “severe” T2DM, but not the total “incidence” that includes early diabetes (Table 3).
OGTT for T2DM. According to our interpretation of the overall data, each BG indicator has a unique contribution in determining the stage of T2DM. The 30min OGTT is clearly the most sensitive test for early “incidence”. This is not surprising given that the procedure is highly controlled in terms of time, a large amount of glucose dosed in proportion to body weight (about 10% of total daily calories in one dose into a fasting system), and the methodical consistency of the procedure. The 30min OGTT value helps answer the question: what is the glucose doing to the whole system and how quickly? This test provides information about glucose absorption rate as well. This is why it is generally important not only to measure the 30min value, but also the 60, 90, and 120min so one can see the entire time course of glucose added to the system. In a healthy individual, the peak should be at 30min because the glucose is absorbed into the blood, and then by 60min insulin should be functioning to clear it out of the blood stream. But in a pre-diabetic individual, not only would the 30min peak be high, but the 60min peak may also be elevated because of the quantity of glucose still in the blood due to whole body insulin resistance or poor insulin secretion needed to clear it (insulin deficiency).

In Table 3, all NR assessed with the 2h OGTT at 8wks, including the diabetic NR, peaked at 30min; this value was significantly higher in diabetics than their non-diabetic counterparts. One aspect of Table 3 was ultimately to divide diabetic and non-diabetic NR and identify differences in pathology. The cut-off for diabetes was chosen to be 150 mg/dl because previous experiments have shown that when young male NR at 2-4wks post-weaning had an OGTT, diabetes incidence was found to be 60-80% when split at 30-min BG > 150 mg/dl. This observation paired with proportionality with human sensitivity to glucose gave rise to this value as the cutoff determinant.

In Table 3, the 30-min peak was chosen to represent the entire 2h OGTT because in correlations with terminal data (%Liver BW) it was best suited to predict the disease found at the end of the study. In Figures 4-6, it was evident that FBG, 90min OGTT, and 120-min OGTT had weak correlations with %Liver BW (the strongest simple disease marker, see section 7 below) than 30min, 60min, or 30+60min OGTT, all of which were about equal. That is, the correlation between 30min OGTT or 60min OGTT versus %Liver BW were nearly identical and both significant. In Figure 6, the correlation of 8wk 30+60min OGTT with %Liver BW showed a slightly stronger correlation (r=0.71,
p<0.0001) than with 30min (r=0.67, p<0.0001) but was still considered equal. This finding is consistent with other unpublished experiments from the Hayes Lab. For instance, in Study 83, where OGTT was run on 51 male 9wk old NR, the 30+60min OGTT correlated significantly to % Liver BW (r=0.77, p<0.0001). The 30min OGTT correlation with %Liver BW was a close second-best (r=0.76, p<0.0001). Because the two correlations proved comparable, the 30min was chosen for splitting data in Table 3 for the sake of clarity and simplification. Total area under the curve (AUC) may be the best index but it was not determined in this experiment (Tschritter, 2003). Other Hayes Lab studies have determined that <150 mg/dl at 30min was a reliable indicator of semi-advanced T2DM in the NR (Study 129, Appendix). If the peak is > 150 mg/dl, a lack of physiological control over glucose metabolism is operating. But the 60min OGTT value may better indicate severity because the higher the 60min value, the more insulin resistant they are. This could explain why 30+60min had a slightly better correlation with %Liver BW: it may be more reflective of severity and had a higher correlation with RBG (Figure 3) than the 30min OGTT, whereas elevated FBG > 60 mg/dl was a sign that the liver cannot cope well or perform its function anymore.

Furthermore, the second indicator, RBG, is less sensitive to early diabetes diagnosis than the OGTT, but is more revealing about pathophysiology of chronic diabetes. This is because the chronic blood level all day long that exposes the tissue to abnormal elevated glucose and gives rise to the increase in glycosylated proteins such as HbA1c (Herman, 2010). In addition, the 8wk RBG and 30min OGTT are significantly correlated, so they are interrelated (Figure 2). It is interesting that the correlation between 8wk 30+60min OGTT is a slightly stronger correlation with 8wk RBG than the 8wk 30min OGTT because of the added severity information that the 60min OGTT provides (Figure 3). Table 3 demonstrates that the RBG, even after splitting diet groups into non-diabetic and diabetic subgroups, had large standard deviations (Table 3). This already implies that the 8wk 30min OGTT split captures a wide range of RBG.

If RBG is not as sensitive to early diabetes as the 30min OGTT, what is the purpose of the RBG? Our data reveal that it is best linked to end stage pathophysiology and tissue damage. This may be the reason why the 30min OGTT distribution in Table 3 did not show any correlation with %Liver BW or %Kidney BW, that is, it was too
sensitive an index for early T2DM. But in previously mentioned Study 107, where the NR were not primed before intervention with polyphenols, the diabetes incidence table was based on the 7wk RBG where >75 mg/dl was considered diabetic. Those data showed significance for RBG and %Liver, and in some cases %Kidney among diabetic NR. This suggests that the best way to analyze “severity” may be to split the data by RBG >75 mg/dl for diabetics and <75 mg/dl for non-diabetics.

To explore this possibility in the current study, Figure 7A and Figure 8A compared %Liver BW to 8wk 30min OGTT and 8wk RBG, respectively. The %Liver BW was significantly related to 8wk 30min OGTT with r=0.67 (p<0.0001), indicating that liver weight increases in NR that are developing diabetes, both early on or in the later stages. The %Liver BW was also significantly correlated to 8wk RBG with a stronger r=0.80, indicating that RBG was a better measurement of severity. The reason for this could be precisely because RBG is not as sensitive as OGTT, and once elevated, it implies the tissues have been exposed to elevated glucose for a longer time.

If a healthy individual recorded his RBG throughout the day, the blood glucose levels should generally be <120 mg/dl at any given test time, even under stress or after some alteration in diet. An individual with diabetes, on the other hand, would exhibit swings in blood glucose, or have a consistently high value greater than 125-140 mg/dl. Therefore, NR with high RBG beginning at 75-100 mg/dl (50% to 100% above normal FBG of about 50 mg/dl) will generally already have chronic hyperglycemia and will have been accumulating damage from excess glucose and fatty acids for longer periods than the NR that show elevated 30min OGTT but are not yet seriously insulin resistant with a high RBG. This damage is likely to cause inflammation of the liver and kidneys, which accounted for the enlarged organs recorded in Study 107 and other unpublished studies from the Hayes Lab.

The least sensitive BG marker is the FBG since it does not detect early onset of diabetes; but its elevation indicates seriously advanced T2DM is present. After a 16h fast, which allows energy metabolism to rest, the NR utilizes all its reserves, including insulin, and tries to revamp the system by lowering the elevated BG. If not severely diabetic, the NR may be able to clear excess glucose from the blood stream slowly over those 16 hours. Most rats in this study accomplished this task, so either they produced extra insulin
overnight or their insulin resistance slowly declined. The 9/50 NR in the present study that could not accomplish this task were more or less equally distributed across diets, except for GCB, which surprisingly had no NR with FBG>60 mg/dl. The reason for that is unclear, but GCB might induce a higher metabolism rate that causes energy burn out overnight and lower body weight and liver weight.

There were two values for FBG at 8wks: one at 8wk 0-time for the 2h OGTT and one at necropsy. These measurements were taken a few days apart. The FBG at the 8wk 0-time in the 2h OGTT shows that most of the diabetic groups on average have FBG < 60mg/dl, whereas all groups had 2-3 NR in the diabetic category based on 30min OGTT, except for GCB. Moreover, there was no significant difference in % incidence between or within groups, except for GCB with 0% incidence based on FBG. This reflects the data previously discussed from Study 129, where FBG did not reflect the sensitivity of the 30min OGTT. The RBG, on the other hand, is more sensitive than FBG but less than 30min OGTT because it does not have the overnight “metabolic rest” to clear out the system. If hyperglycemia is already present, an increased glucose load is inevitable when more food is added to the system. This aspect of RBG is evident in Table 3.

Shifting gears to the FBG at necropsy, it is evident that these values are generally greater than those from the 8wk OGTT 0-time. How could the FBG increase so much within a few days? It is possible that the stress of fasting before the 8wk OGTT several days prior, the actual 2h OGTT procedure itself, and another 16h fast a few days later before necropsy may have stressed the NR to the point where their FBG at necropsy became elevated. This serves as an alert that the terminal measures of FBG should be reconsidered, and perhaps the FBG at necropsy discontinued. Those few stressed NR could have ingested more food than usual after the initial 8wk OGTT testing, or increased cortisol release may have been counteracting insulin, contributing to hyperglycemia.

**Diabetic NR have Liver damage, potentiating NAFLD**

As previously discussed, declining health of the liver can be inferred from a rising RBG. However, there are other methods for determining what happens to the liver during diabetes. A t-test on individual liver weight for all normal NR versus all individual diabetic NR revealed that diabetic NR as a whole had significantly heavier livers than
non-diabetic NR. This is a very important point because it explains the correlation with %Liver BW. Also, the liver is crucial in metabolic processes and unpublished histopathology from the Hayes Lab and Tufts Veterinarian Pathology Lab reveal that hepatic fatty deposits accumulate as diabetes progresses, leading to NAFLD.

Because of the involvement of the liver in most metabolic processes, many elements can indicate malfunctioning of the liver. For instance, the present data show that the plasma triglyceride levels of diabetic NR tend to be more elevated than those in non-diabetic NR (Table 3), and previous studies on severe diabetes in this species show marked increases in both TC and TG levels (Bolsinger, 2013). Insulin resistance and rising glucagon increases the breakdown of adipose tissue, which increases the amount of fatty acids in the blood that recycle to the liver and are resecreted or stored abnormally (Papandreou & Andreou, 2015). This eventually leads to steatosis because the liver retains the fat. Non-alcoholic fatty liver disease (NAFLD) has been shown to be associated with Metabolic Syndrome and diabetes. Insulin resistance has been linked with lipolysis, which leads to the increased quantity of fatty acids in the plasma and increased deposition in the liver (Westphal, 2008). Studies have shown that humans who consumed a hiCHO diet not only had increased hepatic fat, but also had higher efficiency for metabolizing fructose into fat (which is a component of sucrose) (Papandreou & Andreou, 2015). This pattern is supported by the present data and previously published NR data: diabetic NR tend to have higher food efficiency, high plasma TG, and larger livers than their non-diabetic NR counterparts (Chaabo et al., 2010; Bolsinger, 2013, 2014).

The pathogenesis of NAFLD is still unclear. It is thought that the cause and consequence form a vicious cycle that leads to the progression of diabetes and the worsening of steatosis to NASH or cirrhosis. Insulin signaling may be inhibited at the insulin receptors by hepatic steatosis; this impending insulin resistance induces the hepatic steatosis by effecting lipolysis and increasing plasma TG. The inflammation and infiltration of fat in the liver is also apparent by observation at necropsy. All of the livers of the NR in this study had speckled livers, in which the white specks are fat.
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References


Suez, J. et al., 2014. Artificial sweeteners induce glucose intolerance by altering the gut microbiota. *Nature*, 514(7521), pp.181–186. Available at: [http://dx.doi.org/10.1038/nature13793](http://dx.doi.org/10.1038/nature13793).


# Data

## Tables 1-3.

<table>
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<th>INGREDIENT</th>
<th>73 MBS</th>
<th>133</th>
<th>145</th>
<th>146</th>
<th>147</th>
<th>148</th>
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<tbody>
<tr>
<td>ug GAE/kcal</td>
<td>Priming (0g)</td>
<td>Control (0g)</td>
<td>73MBS + 1g Curcumin 150ug GAE/kcal</td>
<td>73MBS + 1.1g Bergamot 40ug GAE/kcal</td>
<td>73MBS+96.3 g PFJ (49,300GAE)</td>
<td>73MBS+12g GCB 210 ug GAE/kcal</td>
</tr>
<tr>
<td>kcal/g</td>
<td>4.0</td>
<td>5.0</td>
<td>4.0</td>
<td>4.0</td>
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<td>4.0</td>
</tr>
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<td>g/Kg</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Casein</td>
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<td>106</td>
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<td>0</td>
<td>0</td>
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<td>0</td>
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<td>186</td>
<td>186</td>
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</tr>
<tr>
<td>Cornstarch*</td>
<td>200 (+60 gel) 201 (+60 gel)</td>
<td>200 (+60 gel)</td>
<td>200 (+60 gel)</td>
<td>180 (+60 gel)</td>
<td>189 (+60 gel)</td>
<td></td>
</tr>
</tbody>
</table>

### Fat:
- Margarine B (80% fat) | 54 (44 fat) | 118 (94 fat) | 55 (44 fat) | 55 (44 fat) | 55 (44 fat) | 54 (44 fat) |
- Splenda | 0 | 0 | 0 | 0 | 0 | 0 |
- Mineral mix (Ausman - Hayes) | 46 | 46 | 46 | 46 | 46 | 46 |
- Vitamin mix (Hayes - Cathcart) | 12 | 12 | 12 | 12 | 12 | 12 |
- Choline chloride | 3 | 3 | 3 | 3 | 3 | 3 |
- Curcumin (Indian 61%) | 0 | 0 | 1 | 0 | 0 | 0 |
- Bergamot (HP Ingredients) | 0 | 0 | 0 | 1.1 | 0 | 0 |
- PFJ powder | 0 | 0 | 0 | 0 | 96.3 | 0 |
- Coffee Bean | 12 | 0 | 0 | 0 | 0 | 12 |
- Cholesterol 0.06% | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 |

*All diet gels made with 60g cornstarch added to 800ml water*
Table 2. Diabetes assessment parameters of 3 wk old NR primed for 2 wks with hiCHO 73-MBS and fed diet containing no phenolics (control), or phenolics (curcumin, bergamot, PFJ, or GCB) for 8 wks.

<table>
<thead>
<tr>
<th>Diet:</th>
<th>133 Control</th>
<th>145</th>
<th>146</th>
<th>147</th>
<th>148</th>
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<tbody>
<tr>
<td>ug GAE/kcal CHO:Fat:Protein (% en)</td>
<td>0 (60:20:20)</td>
<td>150ug GAE/kcal (70:10:20)</td>
<td>40ug GAE/kcal (70:10:20)</td>
<td>1187ug GAE/kcal (70:10:20)</td>
<td>210 ug GAE/kcal (70:10:20)</td>
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<td>kcal/g</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial (5wks of age)</td>
<td>52 ± 11a</td>
<td>57 ± 13</td>
<td>54 ± 7</td>
<td>64 ± 7a</td>
<td>54 ± 13</td>
</tr>
<tr>
<td>After 4 wks</td>
<td>86 ± 10</td>
<td>88 ± 11</td>
<td>81 ± 10</td>
<td>89 ± 9</td>
<td>87 ± 9</td>
</tr>
<tr>
<td>After 8wks</td>
<td>99 ± 11</td>
<td>111 ± 31a</td>
<td>95 ± 11a</td>
<td>99 ± 9</td>
<td>98 ± 9</td>
</tr>
<tr>
<td>Food intake</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/d</td>
<td>7 ± 1abc,d</td>
<td>9 ± 1a</td>
<td>9 ± 1b</td>
<td>9 ± 1c</td>
<td>9 ± 1d</td>
</tr>
<tr>
<td>kcal/d</td>
<td>36 ± 6</td>
<td>37 ± 3</td>
<td>35 ± 5</td>
<td>37 ± 5</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>Food efficiency (g BW gain/1,000 kcal)</td>
<td>23 ± 5</td>
<td>26 ± 17a</td>
<td>21 ± 7</td>
<td>17 ± 6a</td>
<td>23 ± 5</td>
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<tr>
<td>Random blood glucose (mg/dl)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After 4wks*</td>
<td>95 ± 76a</td>
<td>66 ± 20ab</td>
<td>91 ± 87b</td>
<td>84 ± 35</td>
<td>104 ± 111</td>
</tr>
<tr>
<td>After 8wks</td>
<td>132 ± 98</td>
<td>165 ± 158</td>
<td>151 ± 161</td>
<td>182± 202</td>
<td>217 ± 54</td>
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<tr>
<td>Fasting blood glucose (mg/dl)</td>
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<tr>
<td>After 8wks</td>
<td>80 ± 33a</td>
<td>52 ± 13abc</td>
<td>66 ± 22</td>
<td>75 ± 21b</td>
<td>89 ± 33c</td>
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<td>OGTT Initial† (mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t=0</td>
<td>59 ± 23</td>
<td>52 ± 20</td>
<td>52 ± 24</td>
<td>53 ± 30</td>
<td>55 ± 17</td>
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<tr>
<td>t=30</td>
<td>217 ± 66</td>
<td>212 ± 71</td>
<td>213 ± 50</td>
<td>219 ± 55</td>
<td>194 ± 17</td>
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<tr>
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<tr>
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<td>255 ± 101</td>
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<tr>
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<td>162 ± 125</td>
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<td>103 ± 69</td>
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<tr>
<td>t=120</td>
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<td>117 ± 92</td>
<td>87 ± 61</td>
<td>109 ± 69</td>
<td>76 ± 33</td>
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<td>Liver</td>
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<td>4.04 ± 0.6</td>
<td>3.99 ± 0.6</td>
<td>3.92 ± 0.7</td>
<td>3.90 ± 0.6</td>
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<td>Kidney</td>
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<td>0.72 ± 0.1</td>
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<td>5.31 ± 1.03a</td>
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<td>73 ± 7</td>
<td>68 ± 8</td>
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<td>14 ± 1</td>
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<td>186 ± 87a</td>
<td>162 ± 65bc</td>
<td>203 ± 86</td>
<td>265 ± 73b</td>
<td>238 ± 62c</td>
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<td>100 ± 48a</td>
<td>75 ± 34</td>
<td>71 ± 58</td>
<td>69 ± 28</td>
</tr>
</tbody>
</table>

Values are mean ± SD (n=7-10)

a,b,c... Means in a row sharing common superscripts are significantly different (p<0.05) using one-way ANOVA and Fisher's PLSD test.
†OGTT determined initially after priming, after 4 weeks on diet, and after 8 weeks on diet.
* RBG at 4 weeks was analyzed with decreased n: n=9 (133,145), n=8 (146,147), n=7 (148)
Figures 1-8B.

**Figure 1. 30min OGTT: 4wk vs. 8wk**

![Graph showing correlation between 4wk and 8wk 30min OGTT.](image)

- $R^2 = 0.44$
- $r = 0.73$
- $p < 0.001$

**Figure 2. Correlation of 8wk 30min OGTT versus 8wk RBG**

![Graph showing correlation between 8wk 30min OGTT and RBG.](image)

- $R^2 = 0.40$
- $r = 0.63$
- $p < 0.0001$
Figure 3. Correlation of 8wk 30+60min OGTT versus 8wk RBG

\[ R^2 = 0.46 \]
\[ r = 0.68 \]
\[ p < 0.0001 \]

Figure 4. Correlation of 8wk 30min OGTT versus %Liver BW

\[ R^2 = 0.45 \]
\[ r = 0.67 \]
\[ p < 0.0001 \]
Figure 5. Correlation of 8wk 60min OGTT versus %Liver BW

$R^2 = 0.45$
$r = 0.67$
$p < 0.0001$

Figure 6. Correlation of 8wk 30+60min OGTT versus %Liver BW

$R^2 = 0.50$
$r = 0.71$
$p < 0.0001$
Figure 7A. Correlation of 30min 8wk OGTT and % Liver BW

Figure 7B. Comparison of Diets: 30min OGTT vs. % Liver BW

$R^2 = 0.45$

$r = 0.67$

$p < 0.0001$
Figure 8A. Correlation of RBG at 8wks and % Liver BW

\[ R^2 = 0.62 \]
\[ r = 0.80 \]
\[ p < 0.0001 \]

Figure 8B. Comparison of Diets: RBG vs. %Liver BW

- Curcumin
- Bergamot
- PFJ
- Green Coffee Bean
Appendix

### Table A: FBG from the OGTT (30 min) in male Nile Rats (WT and DP) at 2 and 4 weeks split into quintiles (NR Study 129)

<table>
<thead>
<tr>
<th>Quintile</th>
<th>2 wks (n=165)</th>
<th>4 wks (n=90)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FBW</td>
<td>0 min (FBG)</td>
</tr>
<tr>
<td>1</td>
<td>56±9</td>
<td>55±20</td>
</tr>
<tr>
<td>2</td>
<td>57±7</td>
<td>54±19</td>
</tr>
<tr>
<td>3</td>
<td>60±10</td>
<td>55±16</td>
</tr>
<tr>
<td>4</td>
<td>61±7</td>
<td>62±23</td>
</tr>
<tr>
<td>5</td>
<td>61±7</td>
<td>65±21</td>
</tr>
</tbody>
</table>

### Table B: FBG from the OGTT (30 min) in female Nile Rats (WT and DP) at 2 and 4 weeks split into quintiles (NR Study 129)

<table>
<thead>
<tr>
<th>Quintile</th>
<th>primed 2 wks (n=138)</th>
<th>primed 4 wks (n=109)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FBW</td>
<td>0 min (FBG)</td>
</tr>
<tr>
<td>1</td>
<td>44±7</td>
<td>56±18</td>
</tr>
<tr>
<td>2</td>
<td>47±7</td>
<td>60±17</td>
</tr>
<tr>
<td>3</td>
<td>47±7</td>
<td>60±18</td>
</tr>
<tr>
<td>4</td>
<td>51±9</td>
<td>68±21</td>
</tr>
<tr>
<td>5</td>
<td>48±7</td>
<td>75±34</td>
</tr>
</tbody>
</table>
Figure A. FBG and 30' BG for 2 wk old male NR split into quintiles based on 30' OGTT response (n=165) (NR Study 129)

Figure B. FBG and 30' BG for 4 wk old male NR split into quintiles based on 30' OGTT response (n=90) (NR Study 129)