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Resistant starch is effective in lowering body fat in a rat model of human endocrine obesity

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RESISTANT STARCH IS EFFECTIVE IN LOWERING BODY FAT IN A RAT MODEL OF HUMAN ENDOCRINE OBESITY

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DEDICATION

This thesis is dedicated to my mother, Juliana Chan; my siblings Garrick Robert, Justin Robert, Chanrina Meyer, and Richina Robert; and Andrew Lam. Because of all their support and encouragement while working on my graduate degree, I was able to accomplish so much.

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ABSTRACT

Two studies were performed to determine the effects of resistant starch (RS) on body weight and fat. A 2x2 factorial design was used in both studies, and results were considered significant when $p<0.05$ for both studies. The first study examined the effects of RS in a high fat diet (44.8% of energy) on weight, fat, peptide-YY (PYY) levels, and cecal pH in male Sprague-Dawley rats. Rats were fed a low fat energy control diet for one week prior to diet treatment. On week two, rats were blocked by weight and fed one of the following diets for 12 weeks (n=10): low fat, energy control (LFEC); LF resistant starch (LFRS); high fat, energy control (HFEC); or high fat resistant starch (HFRS). RS did not lower weight or fat with either the HF or LF diets. RS consumption resulted in greater full and empty cecal weights, and a lower pH for the LFRS diet. This data indicate fermentation, even though weight and fat loss did not occur. This is contrary to previous reports with RS, which has been shown to decrease body fat compared to controls. The second study examined the effects of RS on the weight, fat, PYY levels, and glucagon-like peptide-1 (GLP-1) levels in female Sprague-Dawley rats. Ovariectomized (OVX) rats were used to represent rats prone to gaining weight, and sham rats represented normal rats. Rats were assigned to one of four groups (n=10): OVEC, OVRS, SHEC, or SHRS. Rats were fed the EC diet for 6 weeks prior to diet treatment to gain weight after surgery, and then blocked by weight and fat into diet treatment groups, and spent 13 weeks on treatment diets. Energy intake, total gastrointestinal weight, large intestine/cecum weight, and small intestine weight were all higher in RS fed rats relative to EC fed rats. Mesenteric, ovarian, perirenal, retroperitoneal, and total fat pads were lower in RS rats relative to EC rats. Although RS was not effective in lowering body weight or body fat in the first study, the data indicates that resistant starch may lower body weight and fat in postmenopausal women.

CHAPTER 1

INTRODUCTION

Overweight and obesity continue to be a growing problem in the United States among children, adolescents, and adults and across all ethnicities. According to Ogden *et al*, in 2003-2004, the prevalence of obesity among men and women were 31.1% and 33.2%, respectively. Data for female children and adolescents show an increase in the prevalence of overweight from 13.8% in 1999-2000 to 16% in 2003-2004, while male children and adolescents increased from 14% to 18.2% in the prevalence for overweight in these same years. According to the data by Ogden *et al*, approximately 30% of non-Hispanic white adults were classified as obese in 2003-2004, as well as 45% of non-Hispanic black adults and 36.8% of Mexican Americans (Ogden et al., 2006). With this growing prevalence among a variety of ages, races, and genders, overweight, defined as a body mass index (BMI) of 25-29.9, and obesity, defined as a BMI of ≥ 30, are major public health concerns in the United States and many other countries across the globe (Grudy, 2004; Ogden et al., 2006). This concern is due to its association with other life-threatening medical conditions such as cardiovascular disease (Grudy, 2004), diabetes (Mokdad, 2001) hypertension (Montani, Antic, Yang, & Dulloo, 2002), and a variety of cancers (Wellman & Freidberg, 2002). Because of these comorbidities, it is evident that prevention and treatment of overweight and obesity are of extreme importance.

Obesity can be attributed to a plethora of factors, and may also be the result of a multi-factorial environment. In the westernized society, however, overweight and obesity are often credited to the consumption of a high fat diet because of its relatively high energy value (Roy et al., 2003). Diets high in fat are generally considered more palatable and flavorable (Seidell,

1998), which can lead to even more consumption. Another population affected by an increase in body fat is postmenopausal women. Upon cessation of menstruation, the hormone estrogen is no longer produced and released (Danilovich et al., 2000). Estrogen has been well established as a factor in energy metabolism, and its absence is associated with fat accumulation (Shimizu et al., 1997). The postmenopausal woman has 20% more body fat (Ley, Lees, & Stevenson, 1992), and puts her at risk for cardiovascular and diabetic complications (Lean, Han, & Seidell, 1998). Diet therapy is among the possible treatment options for both of these causes of weight gain and has the potential to reduce body fat.

Of the selection of foods targeted for therapeutic use in the treatment and management of obesity, starches and dietary fiber have received much attention or their effects on food intake and weight status. Studies have reported that these foods do so by a variety of mechanisms, such as by promoting satiety sooner, by slowing gastric emptying (Stephen, 1991; Anderson, Smith, & Gustafson, 1994), by diluting energy of the diet (Topping & Clifton, 2001), and by decreasing the absorption of fatty acids (Van Horn, 1997). Resistant starch, in particular, may be a useful tool in combating obesity. Dietary resistant starches are defined as non-digestible fibers that resist, to varying degrees, amylase digestion in the small intestine and are fermented to short-chain fatty acids (SCFA) by microflora in the large intestine (Higgins, 2004). Resistant starch has many similar effects as dietary fiber and may also help in reducing body weight and body fat, as well as protecting against colorectal cancer (Topping & Clifton, 2001) and reducing postprandial glycemia and insulinemia (Raben et al., 1994). The production of SCFAs, mainly acetate, propionate, and butyrate (Topping & Clifton, 2001), when resistant starch is fermented, reduces the pH of the gut and may have an important role in reducing body weight and fat (Silvi, Rumney, Cresci, & Rowland 1999).

The mechanism by which resistant starch may aid in weight loss is through increasing the gene expression and production of peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) in the gut via the production of the SCFAs (Keenan et al., 2006*a*). Both of these gastrointestinal peptides have been shown to reduce food intake (Batterham et al., 2003; Hillebrand, de Wied, & Adan, 2002). If this is the means by which resistant starch reduces food intake and lowers body weight and fat, animals fed diets containing resistant starch will have higher circulating blood levels of PYY and GLP-1. Resistant starch is also known to dilute the energy density of the diet in the same way as fermentable fibers, although not as much as non-fermentable fibers. However, resistant starch as a fermentable fiber fed to animals showed increased gene transcription for PYY and proglucagon in cecal epithelial cells, increased large intestine gene transcription for PYY and proglucagon, and greater plasma levels of PYY and GLP-1 compared to non-fermentable fibers. Acting in the same way as a fermentable fiber, resistant starch has also been shown to help reduce abdominal fat in rats (Keenan et al., 2006*a*). For these reasons, resistant starch is implicated as a promising tool in the management and treatment of obesity.

Study 1 will determine the effects of resistant starch on rats fed high fat (HF) and low fat (LF) diets. Study 2 will determine the effects of resistant starch on the weight and body fat of bilaterally ovariectomized (OVX) rats versus sham-operated rats (SH). OVX female rats will be used because they are known to gain body fat due to the lack of estrogen, and are a good model to test the hypothesis that resistant starch reduces body fat using an endocrine model of obesity (Ainslie, 2001).

OBJECTIVES

The objectives of Study 1 are to determine the effects of resistant starch on weight, body fat, and, cecal weights, and gut signaling of rats on a high fat diet. The objectives of Study 2 are

to determine the effects of resistant starch on the weight, body fat, and gut signaling of OVX rats and SH rats, and also to determine the effects of resistant starch on cecal weights as an indicator of fermentation.

HYPOTHESIS

It is hypothesized that obesity-induced animals fed a diet containing resistant starch will have lower body weight, lower percentage of body fat, lower abdominal fat, greater fermentation, and greater plasma levels of PYY and GLP-1 than those fed a diet without resistant starch.

ASSUMPTIONS

- It is assumed that the rat model will have a digestive physiology and a habitual diet with a composition as close as possible to humans.
- It is assumed that abdominal fat will correlate to total body fat.
- It is assumed that all measurements from the studies will be taken and recorded correctly.
- It is assumed that animal blood and tissue collections are representative of human subjects.

LIMITATIONS

- Not all of the same measurements were taken for both studies.
- NMR was deemed not effective as a measure of total body fat.
- Although the rat model has a similar digestive physiology to that of humans, the results from this study may not directly apply to humans.

CHAPTER 2

REVIEW OF LITERATURE

DIETARY FIBER

Dietary fiber is considered a group of non-starch polysaccharides and lignins of plant origin (Liu et al., 2002; Ajani, Ford, & Mokdad, 2004) that are resistant to digestion by gastrointestinal enzymes in humans and most animals (Burton-Freeman, 2000; Brown, Rosner, Willett, & Sacks, 1999) with complete or partial fermentation in the large intestine, although a universal definition is yet to be established (Nugent, 2005). Sources of fiber often include fruits, vegetables, grain products, legumes, and other sources such as oat and wheat bran (Burton-Freeman, 2000). Fiber can be divided into two groups: soluble fibers that form gels in the presence of water, or insoluble fibers. The soluble fibers include pectins, gums, mucilages, and some hemicelluloses, whereas lignins, celluloses, and the remaining hemicelluloses make up the insoluble fibers (Brown, Rosnar, Willett, & Sacks, 1999). However, the Institute of Medicine (IOM) suggests categorizing dietary fiber in another manner: by its physiochemical properties instead. The terms viscous and fermentable are often now used to describe fibers (Queenan et al., 2007). Other physical properties of dietary fiber include bulk/volume, water-holding capacity, and adsorption/binding (Burton-Freeman, 2000), with different sources of fiber causing different bodily responses (Liu et al., 2002). Because of these characteristics and the resulting physiologic effects, this food group has gained much attention lately from both the public and scientific communities for its numerous health implications. It has the ability to modify physiologic function to benefit one's health, and is thus considered a functional food (Food Nutrition Board, 2001).

Dietary fiber has been shown to be positively associated with cardiovascular health. In a prospective cohort, data showed an inverse relationship between dietary fiber intake and cardiovascular disease (CVD) and myocardial infarction (Liu et al., 2002). Another prospective study showed higher consumption of fiber in the diet was correlated with lower concentrations of C-reactive protein, a marker of inflammation and possible predictor of cardiovascular events. (Ajani, Ford, & Mokdad, 2004). Soluble fiber has been shown to significantly reduce total cholesterol levels, as well as low density lipoprotein (LDL): high density lipoprotein ratio (HDL) (Jenkins et al., 2002). This data plus other scientific evidence further support the reported negative correlation between fiber and CVD, and the benefit of dietary fiber intake on heart health.

Research looking at the relationship between consuming fiber and colon health also seems promising. First of all, fiber normalizes colonic function by increasing fecal weight and bowel frequency (Correa-Matos et al., 2003). In an international correlative study, starch intake was inversely related to both colon and rectal cancer (Cassidy, Bingham, & Cummings, 1994). In a prospective study investigating the relationship between fiber intake and diverticular disease, both soluble and insoluble fibers were inversely associated with risk of developing diverticular disease. Insoluble fiber, however, had a stronger inverse relationship (Aldoori et al., 1998). Doubling fiber intake in populations that consumed very little fiber correlated with up to a 40% decrease in colorectal cancer risk (Bingham et al., 2003). McBurney (1994) reported that consumption of fiber in diets for long-term is associated with several changes in the intestinal milieu, including a change in mass, length, villus appearance, cell proliferation rates, and enterocyte migration along the crypt-villus axis.

Diets high in dietary fiber and complex carbohydrates have also been linked to improved insulinemia. Because of its ability to retard gastric emptying, nutrients in meals consumed with fiber are absorbed relatively distally in the small intestine, which reduces the amount of gastric inhibitory polypeptide (GIP) released. GIP is normally secreted in the proximal small intestine, and is also known to induce insulin release, which can promote hyperinsulinemia (Marshall, Bessesen, & Hamman, 1997). The fact that it also delays gastric emptying may be beneficial regarding blood glucose attenuation because of a delayed release of postprandial glucose when consumed (Nugent, 2005; Queenan et al., 2007)), as well as lowered insulin requirements (Anderson et al., 1995).

Along with the above mentioned health benefits, the role of fiber in the diet has been associated with a lower risk of obesity, and it may do so by multiple mechanisms. First of all, it has been suggested that fiber consumption induces satiety and satiation, mostly due to its bulking effect (Schneeman & Tietyen, 1994). Research suggests that viscous fibers may delay gastric emptying, causing a prolonged sensation of fullness, which would result in less consumption of food (Queenan et al., 2007). Secondly, fiber affects energy density by displacing energy if a person is consuming a specific volume of food versus a certain amount of energy, which has been shown in men in a study by Rolls *et al* (1998). It has also been suggested that dietary fiber disrupts fat and carbohydrate absorption by delayed gastric emptying, and the interference with digestive enzymes and the intestinal surface. By this method, fiber would also affect the amount of calories absorbed (Schneeman & Tietjen, 1994).

The benefits of consuming dietary fiber are quite pronounced in research. In fact, the consequences of rodents fed diets completely lacking in fiber included atrophy of the small intestine and colon, with supplementation of the diet with fiber reversing these effects (Slavin,

Nelson, McNamara, & Cashmere, 1985; Ecknauer, Sircar, & Johnson; 1981). It seems that dietary fiber may be beneficial for those at risk for CVD, diabetes, or even obesity. For these reasons, the National Academy of Sciences recommend 38 and 25 grams of dietary fiber per day for men and women, respectively (Lupton, 2003).

FERMENTABLE FIBERS AND SHORT CHAIN FATTY ACIDS

Carbohydrates in the colon can affect its physiology by being both physically present or being the substrate of fermentation (Topping & Clifton, 2001). The microflora of the gut primarily use carbohydrates as an energy source (Jacobasch et al., 1999). If fiber or starch somehow bypasses the small intestine, anaerobic fermentation of these fibers by bacteria can occur in the large intestine, yielding short chain fatty acids (SCFAs), hydrogen, carbon dioxide, and methane (MacFarlane & MacFarlane, 2003) and metabolizable energy for microbial growth (Topping & Clifton, 2001). Butyrate, acetate, and propionate are the main SCFAs produced, but others can be produced in lesser amounts (MacFarlane & MacFarlane, 2003). Colonocytes use SCFAs as a fuel source, supplying ~60-70% of the colonic energy needs, with butyrate being the preferred fuel even when glucose is available (Topping & Clifton, 2001). Often propionate is favored as well, with both formed into ATP for energy for colonocytes (Jacobasch et al., 1999). The production of SCFAs increases blood flow in the colon, lowers luminal pH, aids in the prevention of abnormal colonic cell population development (Topping & Clifton, 2001), regulates colonocytes' gene expression, cell cycle, and apoptosis, and also exerts trophic effects on the epithelium of the colon (Mentschel & Claus, 2003). Fermentable fibers can also increase the abundance of lactic acid bacteria, which further aid in the fermentation of fibers (Russel & Diez-Gonzales, 1998), and the prevention of potential pathogens that are pH-sensitive, categorizing them as a prebiotic (Topping & Clifton, 2001). For this reason, they have been

added to some oral electrolyte solutions (Oli, Petschow, & Buddington, 1998). Overall, SCFAs improve the digestive and absorptive capabilities of the colon (Rombeau & Kripe, 1990; Tappenden et al., 1997). Levels of SCFAs are highest in the proximal colon, where fermentation is greatest, and levels decrease further down the gut as colonocytes use them, with very little amounts found in the distal colon. Interestingly, most colon cancers and other colonic diseases occur in the distal colon, suggesting the importance of SCFAs in colonic health (Topping & Clifton, 2001). The lowered pH of the lumen upon production of SCFAs is also thought to be protective against colon cancer (Young & Le Leu, 2004).

Research on fermentable fibers and the resulting SCFAs that are produced verify the benefits of maintaining the health of the colon. Fermentable fibers can be used to help recover from diarrhea (Buddington & Weiher, 1999). Correa-Matos et al. (2003) found that consumption of fermentable fibers reduced the recovery time and improved symptoms of induced *S. typhimurium* infection in neonatal piglets indicated by stool consistency and maintenance of physical activity. It is also believed that SCFAs may help prevent foreign bacteria from penetrating the small intestine and colon (Reardon & Tappenden, 1999). In dogs fed a diet with fermentable fibers, it was shown that their intestines were longer, had increased surface area, and greater mucosal mass compared with those fed a poorly fermentable fiber (Buddington, Buddington, & Sunvold, 1999).

Data has also shown that fiber-induced fermentation causes the release of particular gastrointestinal hormones. In canines, glucagon-like peptides (GLP-) 1 and 2, and GIP, which aid in mucosal growth and upregulation of transport processes in the proximal colon, were all increased (McBurney et al., 1998). In rats fed fermentable fibers, there was an increase in intestinal proglucagon (the precursor to glucagon) mRNA and thus greater postprandial GLP-1

was released (Reimer & McBurney, 1996), although this increase in proglucagon was not seen in a later study by Reimer *et al*. (2000). However, increased concentration of proglucagon-derived peptides has been observed by others once fermentable fibers were fed (Gee et al., 1996; Massimino et al., 1998), as well as smaller oscillations in blood glucose concentrations (Massimino et al., 1998). GLP-1 additionally stimulates insulin secretion, inhibits glucagon secretion, and delays gastric emptying, all of which are beneficial in diabetic patients (Holst, 1997). The ability of fermentable fibers to promote GLP-1 secretion makes it a target for treatment in diabetic patients. SCFA production is also associated with an increase in the gastrointestinal hormone peptide YY (PYY), which has been shown to inhibit gastric emptying (Cherbut, 1998) and reduce food intake (Batterham, 2003).

The SCFA butyrate particularly has been studied for its role in colonic health. It acts as a signal metabolite, stimulating cell migration and proliferation (Valezquez, Lederer, & Rombeau, 1996). Research has also shown that butyrate has slightly different functions in neoplastic colonic cells than in normal epithelial cells. It affects gene expression and may induce apoptosis, killing off any possible damaged and potentially harmful colonocytes while inhibiting proliferation (Jacobasch et al., 1999; Mentschel & Claus, 2003), and has been shown to reverse neoplastic changes *in vitro* (Ferguson et al., 2000). In normal cells, apoptosis has been observed in the absence of butyrate (Hass et al., 1997). Segain *et al* (2000) has also reported that butyrate inhibits Nuclear Factor-kappa B (NF-κB), a key regulator in inflammatory response. Those suffering from inflammatory bowel disease have increased activity of NF-κB, suggesting that butyrate can potentially reduce the immune response. In fact, patients with ulcerative colitis and colon cancer have been reported to have low levels of butyrate (Chapman et al., 1994).

Concerning weight management, butyrate has been reported to increase gene expression of PYY and proglucagon and may participate in satiety (Zhou et al., 2006)

Research also suggests that other SCFAs can be beneficial for health. Propionate has been shown to suppress fatty acid and cholesterol synthesis *in vitro* (Berggren, Nyman, & Lundquist, 1996) and *in vivo* (Chen et al., 1984), although this is not agreed by all (Queenan et al., 2007). Propionate is also confirmed to kill *E. coli* in an acidic environment (Cherrington et al., 1991). Acetate has been shown to decrease free fatty acid availability in humans and inhibit cholesterolgenesis in rats (Beynen, Buechler, & Van Der Molen, 1982), and Abrahamse *et al* (1999) reported that acetate inhibits DNA oxidative damage due to H_2O_2 in the rat colon. Both propionate and acetate have been observed to induce apoptosis like butyrate in tumorigenic cells. However, they do so to a lesser extent and require higher concentrations (Hague et al., 1995). Butyrate, propionate, and acetate have all been associated with greater colonic blood flow (Mortensen et al., 1991; Kvietys & Granger, 1981), enhancing tissue oxygenation and transport of absorbed nutrients (Topping & Clifton, 2001).

Any fermentable fibers or starches that produce SCFAs, especially butyrate, thus may be targeted for improving gut health and preventing possible pathology from developing. The delivery of butyrate to the distal colon may be particularly important because of its susceptibility to diseases relative to the proximal colon (Topping & Clifton, 2001). The effect of SCFAs on hormone secretion, particularly GLP-1 and PYY, is of great interest regarding weight management.

RESISTANT STARCH

Starch is the polysaccharide storage form of glucose found in plants, and is a major source of carbohydrates in the diet (Nugent, 2005; Sajilata, Singhal, & Kulkarni, 2006). It is

packaged as granules in two forms: the polymer amylose consisting of linear glucose residues connected by α-D-(1-4) linkages, or the branched polymer amylopectin consisting of the same linear α-D-(1-4) linkages plus branched α-D-(1-6) linkages. Starch is usually hydrolyzed by the enzymes α-amylases, glucoamylase, and sucrose-isomaltase in the small intestine (Nugent, 2005); however, this is not the case for all starches. In 1982, Englyst and colleagues found that not all starch was hydrolyzed by enzymes, and this remainder was termed "resistant starch" (Englyst, Wiggins, & Cummings, 1982). Resistant starch, as the name implies, refers to the portion of starch that resists digestion into free glucose and absorption in the small intestine, bypassing it and reaching the large intestine where it is fermented by colonic microflora. This fermentation process, like dietary fiber, results in the production of SCFAs (butyrate, acetate, and propionate), as well as carbon dioxide, methane, hydrogen, and organic acids. However, unlike dietary fiber, fermentation of RS produces more butyrate and less acetate (Nugent, 2005; Sajilata, Singhal, & Kulkarni, 2006).

Resistant starch (RS) is resistant to mammalian enzymes due its structure, and four types exist: RS_1, RS_2, RS_3, and RS_4. RS_1 refers to a physically inaccessible form of starch that is physically protected because the intact plant structure of amyloblasts hinders enzymatic degradation. RS_2 refers to starch in a certain granular form that is tightly packed in a radial pattern, limiting its accessibility to enzymes. RS_3 refers to retrograded amylose formed during cooling of gelatinized starch, and is the most resistant form of RS by mammalian digestive enzymes, but still degradable by microbial fermentation. Finally, RS_4 refers to the formation of new chemical bonds other than the α-D-(1-4) and α-D-(1-6) linkages found in starch (Jacobasch et al., 1999; Sajilata, Singhal, & Kulkarni, 2006), and includes starches that have been etherized, esterified, or cross-bonded with chemicals that reduce their digestibility (Nugent, 2005). Natural

food sources of RS include bananas and legumes (Sajilata, Singhal, & Kulkarni, 2006), and can be found in some amounts in any food containing starch (Nugent, 2005). Most humans tend to consume RS from cooked sources, such as cooked potatoes, breads, and pastas, and these natural sources are usually affected by processing and storage conditions. Commercially available sources of RS, on the other hand, such as Hi-maize® or CrystaLean® (Nugent, 2005), are unlikely to be affected by such conditions (Sajilata, Singhal, & Kulkarni, 2006). The commercial sources have other advantageous properties that make them useful for incorporating into food products, including bland in flavor, fine particle size, increasing the bowl life of breakfast cereals, and being a functional food component. And just as there are different forms of RS, each form may have a slightly different physiological response. Also the amount consumed, what else was consumed at the time, and the cooking conditions all may affect the physiological response as well (Nugent, 2005).

In addition to these forms affecting the resistance of starch, a higher ratio of amylose:amylopectin also lowers the digestibility of starch, with the linear amylose chains forming a packed structure of cross linkages secured by hydrogen bonds upon retrogradation. Amylopectin can interfere with this structure formation during retrogradation (Berry, 1986). Repeated cycles of heating then cooling can increase the retrogradation process (Annison & Topping, 1994). Cooking can also affect the RS content in food, with high moisture and temperature processes disturbing the crystalline structure of RS, thus lowering the content (Sajilata, Singhal, & Kulkarni, 2006). Another factor influencing RS is the addition of endogenous lipids, which can complex with amylose and possibly disrupt the formation of RS (Eerlingen et al., 1994). Finally, fermentation can reduce the content of RS, as is the case in the large intestine when it is broken down by the gut microflora.

Although it is a starch, RS shares similar physiologic properties and health benefits with soluble fiber, as well as benefits unique to its own as an insoluble component of the diet. Both RS and soluble fiber are slow to digest, with RS digestion usually occurring 5-7 hours after consumption, and result in slower, more controlled glycemic response and lowered postprandial insulinemia and potentially increasing satiety (Jenkins et al., 1987; Raben et al., 1994). This response is most likely due to the replacement of digestible starch found in other foods with the indigestible starch (Jenkins, 2002). RS and soluble fiber are both poorly digested in the small intestine, but highly fermented in the large intestine with SCFA production (Bjorck et al., 1987; Goni et al., 1996; Sajilata, Singhal, & Kulkarni, 2006). RS, however, is not viscous like soluble fiber upon arrival in the large intestine (Nugent, 2005), and has been reported to produce a larger proportion of butyric acid than fiber does (Akerberg et al., 1997). It is estimated that about 30-70% of RS is metabolized in the colon with the remainder excreted (Behall & Howe, 1995).

Data supports that RS confers several health benefits. It has been shown to be beneficial to the colonic milieu, including increasing crypt cell production rate, decreasing large intestinal cell atrophy (Sajilata, Singhal, & Kulkarni, 2006), increasing fecal weight and output, reducing fecal and cecal pH (Cassand et al., 1997), decreasing ammonia levels, and altering the activity of bacterial enzymes in a beneficial manner (Silvi et al., 1999) relative to a lack of fiber. Butyrate produced from fermentation of RS is an energy substrate for colonic cells and has been shown to inhibit malignant transformation of colonic cells in vitro (Asp & Bjorck, 1992). RS has also been studied as a treatment option for ulcerative colitis. In a study by Jacobasch *et al* (1999), rats with chemically induced colitis and fed RS showed earlier sign of improvement for markers of inflammation compared to controls. Studies show that RS affects expression of a number of receptors on T- and B- lymphocytes and macrophages, which are all involved in immune

function (Sotnikova et al., 2002), and inhibits NF-κB through butyrate production (Segain et al., 2000). The noted impact on immune response may be of interest regarding inflammatory bowel disease. Because of its tendency to affect the microbial population, RS may also function as a prebiotic stimulating the growth of beneficial bacteria of the gut while suppressing the pathological ones (Young & Le Leu, 2004; Sajilata, Singhal, & Kulkarni, 2006).

Beyond the colon, RS, like fiber, has also been linked to reduced serum cholesterol and triglyceride levels in animal models (Younes et al., 1995; Mathe et al., 1993) and human subjects (Behall, Scholfield, Yuhaniak, & Canary, 1989; Behall & Howe, 1995). Consumption of RS has been associated with decreased incidence of gallstones as well (Malhotra, 1968). Like dietary fiber, it is associated with decreased postprandial glucose and insulin responses, which would be of particular interest in diabetic treatment (Haralampu, 2000).

In addition to its numerous health implications, several studies have supported the use of RS as a treatment option for weight management. Results from several animal studies indicate that consumption of RS may lead to weight loss or reduced weight gain. For example, De Deckere *et al* (1993) reported lowered fat pad weight and decrease adipocyte cell size, and this has been confirmed by other studies (Lerer-Metzger et al., 1996; Kabir et al., 1998*a*) Consumption of RS was associated with a decrease in GLUT-4 expression, an insulin-regulated protein responsible for glucose uptake (Kabir et al., 1998*b*). Younes et al (1995) reported a decrease in the activity of lipogenic enzymes 3-hydroxy-3-methylglutaryl-Co A and fatty acid synthase, which are involved in cholesterol synthesis and fat synthesis, respectively. Another study has shown that RS increases the expression of the LDL receptor in the liver of rats (Fukushima, 2001). Mathe *et al* (1993) demonstrated that RS increased bile acid secretion, which can also affect lipid metabolism. Recently, Keenan *et al* (2006*a*) reported that RS-fed rats had

lower abdominal fat relative to a control group, even though their food intake was not different from the control.

There are a few possible leads as to how RS affects body weight. Most of the research involved in this area focuses on the anaerobic bacterial fermentation of RS into SCFA, and it is through these acids that RS is thought to help in limiting weight gain (Topping, Fukushima, & Bird, 2003). When compared to a non-fermentable fiber, RS had greater amounts of SCFAs produced, along with increased transcription of PYY and proglucagon, and increased plasma levels of PYY and GLP-1 (Keenan et al., 2006). PYY is normally released in the gut in proportion to the amount of calories ingested (Adrian et al., 1985) and is a gut-signaling hormone indicating food intake in the brain (Batterham, 2002). As stated earlier, PYY has been shown to reduce food intake and weight gain when infused in human subjects, and lower endogenous PYY levels have been observed in obese subjects (Batterham et al., 2002). Long-term administration of PYY in murine models has demonstrated decreased weight gain (Batterham, 2003). GLP-1 is also released in response to food ingestion (Deacon, 2005), and acts as a meal termination signal and food regulation (Kreymann et al., 1987; Flint, Raben, Astrup, & Holst, 1998). With the increase of these two peptides, it is suggested that the RS-produced SCFAs are a natural and endogenous method to reduce energy intake and hopefully body weight (Keenan et al., 2006*a*).

Although SCFA production in relation to RS fermentation is highly studied, other putative mechanisms may also be responsible for its role in weight management. First of all, the metabolizable energy value of RS has been calculated to be ~2 kcal/g, whereas digestible carbohydrates have an energy value of ~4 kcal/g (Livesey, 1994). This displacement of kcals by the addition of RS in the diet can displace the total amount of kcals consumed in a day, thus

resulting in a decreased overall caloric intake, just as with dietary fiber. Higgins et al (2004) reported that replacing 5.4% of total carbohydrates with RS in the diet could increase postprandial lipid oxidation. They suggest that this mechanism could help reduce fat accumulation in the long term. Also, foods rich in RS may cause a reduced insulin response due to its lowered glycemic response. Ultimately, this may make fat more accessible for energy use (Tapsell, 2004). It has been suggested that RS may also promote satiety, although this is still controversial (Nugent, 2005). These examples are supplementary ways as to how RS may work in the treatment of obesity and in weight management.

Overall, research agrees that the effects of RS are advantageous to the body with much potential nutritional and commercial value (Asp, Amelsvoort, & Hautvast, 1996). It has a wide range of influence, affecting fermentation in the gut, fecal bulking and transit time, colonic bacterial growth, postprandial glycemia and insulinemia, and energy value of foods (Abia et al., 1993; Annison & Topping, 1994). However, there are some concerns with consumption of RS regarding bloating, flatulence, belching, mild laxative effects and stomach aches when RS is consumed in larger amounts (~30g/day or more) (Heijnen, 1996). Other than these discomforts, the potential physiological benefits of RS are obviously similar to dietary fiber, although it is considered a starch. And it is because of these similarities that deem it worthy of being categorized as a component of dietary fiber to some (De Vries, 2003). Data also confers that RS as part of the diet can be used in weight loss and/or management. And although RS does produce energy through fermentation, this energy value is still less than digestion of starch in the small intestine (Englyst, Kingman, Hudson, & Cummings, 1996). Whether this starch's influence on body weight and body fat is solely due to the production of SCFAs upon fermentation and the subsequent increase in PYY and GLP-1, or other mechanisms is not yet clear. More research is

needed to distinguish its exact manner, but for now its use in the treatment of obesity seems extremely promising.

ENDOCRINE OBESITY

Overweight and obesity are growing problems worldwide, with numerous factors contributing to their cause. Increased energy intake and decreased energy output are often the blame for the sudden epidemic. Increased fat accumulation due to endocrine changes is a lesser known cause of obesity, but may also be responsible for the increased rates due to its resulting metabolic alterations. Particularly, the hormone estrogen has been investigated for its role in energy homeostasis.

It is well understood that the postmenopausal period is related to a striking change in endocrinology (Burger et al., 1995). In postmenopausal women, the ovaries lose their ability to function, including the production and release of estrogen (Danilovich et al., 2000). It has been known for some time that the decrease in estrogen production that results during menopause is followed by a characteristic increase in body fat (Shimizu et al., 1997; Toth, Tchernof, Sites, & Poehlman), with fat accumulation occurring more likely in the upper body, mainly the abdominal area, rather than lower (Tremollieres, Pouilles, & Ribot, 1996; Gambacciani et al., 1996) putting the postmenopausal woman at increased risk for developing CVD and diabetic complications (Lean, Han, & Seidell, 1998). In fact, postmenopausal women have 20% more body fat compared to premenopausal women (Ley, Lees, & Stevenson, 1992).

In the murine model, lack of estrogen production can modify metabolism resulting in obesity, skeletal abnormalities, and infertility (Danilovich et al., 2000). The ovariectomy procedure in rats ceases estrogen production and imitates the symptoms of menopause. This procedure has been shown to increase body weight by many (Kakolewski, Cox, & Valnstein,

1968; Ainslie et al., 2001), and was reported to do so by 22% in just 10 weeks (Kakolewski, Cox, & Valnstein, 1968). In one study that disrupted the follicle-stimulating hormone receptor (FSH-R) in mice by using a knockout model, which also terminates estrogen production, an increase in abdominal fat deposition, curvature in the spinal column, and a decrease in bone weight all occurred. Replacement of estrogen in these knockout mice reversed the adipose accumulation (Danilovich et al., 2000). Mice of both sexes that have the estrogen receptor-alpha knocked-out display increased white adipocyte tissue (WAT) and hyperplasia (Heine et al., 2000). Another study showed that mice deficient in aromatase, the enzyme responsible for the production of estrogen, developed fatty livers and had more abdominal adipose tissue. The study concluded that the fat accumulation was a result of a combination of reduced physical activity and glucose oxidation (Jones et al., 1997). Ainslie *et al* (2001) reported that ovariectomized rats were hyperphagic, had lower levels of spontaneous physical activity, and a similar resting energy expenditure compared to sham-operated rats, all of which resulted in weight gain. Ovariectomized rats treated with estradiol had similar body weights, energy intakes, and physical activity levels to that of sham rats.

Clinical studies in humans also agree that estrogen does have an effect on body weight. A cross-sectional study and a prospective, double blind, placebo-controlled study showed an increase in body mass index (BMI) and body weight, respectively, as menopause transitioned (Burger et al., 1995; Pepi, 1995). Bjorkelund *et al* (1996) also reported an increase in waist-to-hip ratio between the pre- and postmenopausal periods in their longitudinal study. This increase in body weight, and particularly body fat, has been seen by many (Hunter et al., 1996; Zamboni et al., 1992; Kontani et al., 1994). Stevenson *et al* (1994) demonstrated that hormone replacement therapy (HRT) with estradiol in postmenopausal women prevented abdominal

weight gain, and Dellongeville *et al* (1995) described a lowering of BMI once treated with HRT. Another study with newly postmenopausal women found that those on HRT had no significant weight gain, while those on simply a calcium supplement had a significant increase in body fat weight, with most of the weight gain occurring in the trunk and the arms (Gambacciani et al., 1996).

Not only is there clear evidence of fat accumulation with the onset of menopause, some believe that there is in fact a loss of fat free mass. Poehlman *et al* (1995) noted an average 3 kg loss of fat-free mass in women between their premenopausal and postmenopausal periods in a longitudinal study. Others suggest that there is predominantly a loss in skeletal muscle mass (Poehlman et al., 1993; Aloia et al., 1991). Toth *et al* (2000), however, did not see this similar loss in fat free mass.

Menopause and loss of estrogen production may also be associated with type 2 diabetes, possibly due to weight gain. Considering estrogen's reversal effect on fat accumulation in former studies, research looked at its possible role in treatment of diabetes. Simply knocking out the estrogen receptor resulted in insulin resistance and glucose intolerance (Heine et al., 2000). In one study, estrogen treatment was shown to increase cell surface insulin receptor number in ovariectomized rats, which increased insulin binding and receptor-mediated insulin degradation (Krakower, Meier, & Kissebah, 1993). Geisler *et al* (2002) reported inhibition of both weight gain and hyperglycemia in a male mouse model prone to hyperglycemia when treated with estradiol. In postmenopausal women, estradiol treatment increased secretion of pancreatic insulin and decreased insulin resistance (Stevenson et al., 1994). In men unable to produce estrogen, insulin resistance ensued (Grumbach & Auchus, 1999).

Cardiovascular complications are also a concern with the onset of menopause and the central body obesity that results. Stevenson *et al* (1994) suggest that HRT in postmenopausal women can reduce coronary heart disease by 50%. In another study, hyperlipidemia was shown to result in men with estrogen insufficiency (Grumbach & Auchus, 1999).

The means by which estrogen plays a role in energy homeostasis may work via various hormones. In the rat model, ovariectomy was shown to decrease mRNA levels and serum concentrations of leptin (Shimizu et al., 1997), which signals to the brain decreased fat mass and results in a hyperphagic state as well as decreased energy expenditure (Mantzoros, 1999). Once again, estradiol treatment reversed these effects (Shimizu et al., 1997). Ainslie *et al* (2001), however, did not find decreased secretion of leptin in ovariectomized rats, and Pelleymounter *et al* (1999) also reported no alteration in leptin levels with either ovariectomy or estradiol treatment. Besides leptin, lack of estrogen in the ovariectomized rat model has been shown to increase hypothalamic neuropeptide Y (NPY) mRNA expression, which stimulates food intake and may have a role in excess fat accumulation as well (Shimizu et al., 1996; Ainslie et al., 1999).

Considerable evidence has shown that estrogen indeed plays a role in energy metabolism, and disruption of estrogen synthesis has been shown to result in fat accumulation. Data reports that this weight gain can be blunted or even reversed if estrogen is once again provided, suggesting that it is the deficiency of estrogen that is responsible for the weight gain during the postmenopausal period.

HIGH FAT DIETS

Much attention regarding the amount of fat in the diet has been received lately in relation to weight gain and the prevalence of obesity. High fat diets are often to blame for inducing

obesity. Fat has a higher energy density relative to the other macronutrients, and is considered more palatable, which can easily lead people to consume more. Overall, this combination can lead to a greater energy intake relative to output (Seidell, 1998; Willet, 1998). A "westernized" diet that is highly processed, high in fat, cholesterol, refined sugar, and alcohol, and low in fruits and vegetables has been adopted in the United States and most Western countries (Cordain et al., 2005). Although dietary fat cannot be the sole responsibility of the obesity epidemic, it is a major concern in today's unbalanced diet. And although the body has regulatory systems that attempt to maintain body weight, Woods *et al* (2003) explain, "that when individuals are exposed, on a chronic basis, to a higher mean level of dietary fat, the otherwise incredibly robust negative feedback system that regulates body fat decreases. More fat is stored and the individual moves along the scale toward obesity." Reducing fat intake is a difficult task, especially since fat is often used in foods for its palatability and ability to increase shelf life.

Weight gain and fat accumulation are the most common associations with diets high in fat, but there are certainly other adverse health effects that are linked to high fat diets. Epidemiologic studies have shown that diets high in total and saturated fat have been associated with fasting hyperinsulinemia (Maron, Fair, & Haskell, 1991; Parker et al., 1993; Mayer, Newman, Quesenberry, & Selby, 1993) and have been shown to cause insulin resistance in rats (Storlein et al., 1986). As explained earlier, diets high in dietary fiber control insulin release by reducing the amount of secreted GIP, and RS is likely to have the same effects to counter this with high fat diets.

Epidemiologic data support the relationship between higher fat consumption and weight gain. The observation of migrants who have adopted the western diet and the concomitant weight gain and disease states that are not normally seen in their native countries has further elucidated

the consequences of this eating pattern. The prevalence of obesity was reported to be three times as high in Japanese men living in California relative to their Japan-inhabited counterparts (Kato et al., 1973). The Pima Indians are another prime example of the deleterious effects of a westernized diet that is cheap, palatable, and high in fat (Schrauwen & Westerterp, 2000). Those that still live in Mexico maintain their traditional eating habits and lifestyle, while those living in Arizona are exposed to the convenience of more processed foods high in refined sugars and fat, and less physically-active occupations. The latter group now has a high prevalence of obesity and type 2 diabetes from their exposure to this environment (Esparza et al., 2000). In a longitudinal analysis, Klesges *et al* (1992) reported that women with higher fat intake were related to higher weight gain.

Animal models and clinical trials have associated higher fat diets with weight gain and fat accumulation. When rats were given free access to a high fat diet, they became obese compared to those on a diet with the same composition except for less fat (Woods et al., 2003). In a study by Lissner *et al* (1987), women fed a high fat diet (45-50%) gained weight relative to a medium fat diet (30-35%), and those fed a low fat diet (15-20%) experienced weight loss. In another study by Jeffery *et al* (1995) comparing fat restriction with energy restriction, results concluded that a low fat diet had similar weight loss but better compliance among subjects. Higher fat intake and weight gain or increased adiposity has been supported by others as well (Tremblay, Plourde, Despres, & Bouchard, 1989; Tucker & Kano, 1992).

The relationship between high fat diets and the prevalence of obesity has been observed for quite some time. The extreme conversion into an industrialized society has not permitted our genetics to adjust to all it entails, including lifestyle and eating habits (Cordain et al., 2005). In addition to obesity, high fat diets are related to an assortment of other co-morbidities including

heart disease and type 2 diabetes. The amount of fat alone is not only important to a healthy lifestyle, but the type of fat should also be considered, with more effort in consuming sources of unsaturated fatty acids and less intake of saturated and *trans* fats (Willet, 1998; Cordain et al., 2005). It is imperative to find ways to treat overweight and obesity in an effective method that tailors to the needs and lifestyle of people today.

CHAPTER 3

METHODS

EXPERIMENTAL DESIGN

For both studies, a 2x2 factorial design was used to examine the effects of resistant starch on the body weight, body fat, food intake , peptide-YY (PYY) levels, and glucagon-like peptide-1 (GLP-1) levels in rats.

Study 1. The effects of resistant starch were compared in rats fed a high fat diet (44.8% of energy, 22% fat w/w) to those fed a low fat diet (17% of energy, 7% fat w/w). Half of the rats fed the high fat diet (HF) were assigned to a diet high in resistant starch (RS), and half were assigned to an energy control diet (EC). Half of the rats fed the low fat diet were assigned to a diet high in resistant starch, and half were assigned to an energy control diet (Table 3.1).

Study 2. An endocrine model of obesity, ovariectomy, was used to test the effects of resistant starch. To control for the effects of surgery a sham surgery arm of the 2 X 2 factorial was used. Half of the ovariectomized and sham rats were assigned to a diet high in RS, and half were assigned to an EC diet (Table 3.2).

Table 3.1 2x2 Factorial Design, Study 1 (*n*=40)

	FAT	
STARCH	HF Diet	LF Diet
EC Diet	10	10
RS Diet	10	10

Table 3.2 2x2 Factorial Design, Study 2 (*n*=40)

	SURGERY	
STARCH	OVX Rats	SH Rats
EC Diet	10	10
RS Diet	10	10

ANIMALS

For both studies, rats were maintained on a 12:12 hour light/dark cycle that was illuminated at 7AM. Water and powdered diet were available ad libitum during the experiment. All of the diets were a modification of the American Institute of Nutrition-93 Growing diet (AIN-93G) for growing rats.

Study 1. Forty, 7 week old male Sprague-Dawley rats were used.

Study 2. Forty female Sprague-Dawley rats were used. Twenty of the rats underwent surgery to remove the ovaries, and 20 underwent a sham operation to equate the physiological stress that results from ovariectomy surgery. The surgery was performed on the rats at 10 weeks age by the supplier, Harlan (Indianapolis, IN).

HOUSING

All animals were housed in stainless steel cages with wire mesh bottoms to account for food spillage, and with wire mesh fronts in order to view the animals. The powdered diet was placed in glass food jars by the experimenter, and secured by a wire spring that attached to the wire mesh front of the cage. Water was available via a water nozzle at the back of the cage that released water when it is pushed on. Cardboard sheets were set under each animal's cage to capture any food spillage for later measurement.

PROCEDURES

Study 1.

Diet Preparation. To prepare each diet, macronutrients (without fat) were measured and combined in a large mixing bowl. The macronutrients were mixed for 10 minutes. Micronutrients were then measured, combined in a small mixing bowl with food coloring if necessary, and mixed for 10 minutes. One scoop of the macro mix was then added to the micro mix in the small mixing bowl, and mixed for 10 minutes. Then this micro mix was added to the large mixing bowl and mixed for 10 minutes to evenly distribute the ingredients. Oil or oil/solid fat was then measured and added to the mix, and mixed for 30 minutes. The diet was stored in labeled containers in refrigerators and freezers. The label included diet type, date of preparation, and researcher's name. Ten kilogram batches were made at a time.

The rats were randomly assigned to a low fat control diet, low fat resistant starch diet, high fat control diet, or high fat resistant starch diet. Resistant starch was used in the form of Hi-Maize® cornstarch (National Starch, Bridgewater, NJ). The compositions of the diets are listed in Table 3.3. The energy breakdown of the diets is listed in 3.4.

Table 3.3. Diet Composition Table for LFEC, LFRS, HFEC, HFRS to make a 10 kg Batch

INGREDIENTS	LFEC	LFRS	HFEC	HFRS
Macro Mix				
Amioca (g)	5347	1500	3847	-
Hi-Maize (g)	-	4807	-	4807
Sucrose (g)	500	500	500	500
Casein (g)	2000	2000	2000	2000

(Table Continued)

Cellulose (g)	960	-	960	-
Micro Mix				
Mineral Mix (g)	350	350	350	350
Vitamin Mix (g)	100	100	100	100
Choline Chloride (g)	13	13	13	13
L – Cystine (g)	30	30	30	30
Oil Mix				
Vegetable Oil (g)	700	700	1000	1000
Lard (g)			1200	1200
BHT (g)	0.140	0.140	0.140	0.140
TOTAL	10000 g	10000g	10000g	10000g

Table 3.4. Caloric Composition of the LFEC, LFRS, HFEC, and HFRS diets as % (g/100g)

Nutrient	LFEC	LFRS	HFEC	HFRS
Protein	21	21	21	21
Total Carbohydrates	61.9	61.9	61.9	61.9
Fat	17.1	17.1	44.8	44.8
Kcal/g of Food	3.5	3.5	4.2	4.2

Week 1: Acclimation Period. All animals were fed a low fat control diet for one week to become acclimated to the powdered diet and environment. The weight of the animal, the weight of the empty food jar, the weight of the full food jar, and the food spillage were measured three

times a week (Monday, Wednesday, and Friday) for each animal. After measurements were taken for empty food jar weights, the food jars were refilled and full jar weight was recorded.

Week 2: Group Assignment. After the acclimation period, the animals' weights were used to determine group assignment. The animals' weights were put into ascending order and then ordered into four blocks of 10 replicates. The animals were assigned to one of four treatment groups by choosing one from each block at a time. The result was that the average weight for each group was within one-tenth of a gram of one another.

Weeks 2 through 14: Treatment. Throughout the experiment, the animals' weights, the empty food jar weights, the full food jar weights, and the food spillage were measured every Monday, Wednesday, and Friday beginning between 12PM-2PM. Empty food jars consisted of the leftover food in the food jar. Full food jar consisted of the weight of the food jar once it was refilled with the powdered diet. Every Monday, food jars were switched for clean food jars, and any leftover diet in the food jar was thrown out for fresh diet. Throughout the experimental period, animals were switched to new cages every two weeks for sanitary reasons. The animals were fed on their assigned diets for 12 weeks. Body fat of the animals was measured using the nuclear magnetic resonance (NMR) method at the beginning, middle and end of the treatment period.

Week 15: Sacrifice. The animals were sacrificed via decapitation. Due to the large number of animals, the sacrifice took place over 2 days between days, spaced 2 days apart. Tissues that were collected included blood samples, brain, scrapings of the top cellular layer of the cecum, cecal content samples, peri-renal fat, epididymal fat, and retroperitoneal fat. Measurements that were taken included the entire gastrointestinal tract weight with contents, full cecum weight, empty cecum weight, peri-renal fat weight, epididymal fat weight, and

retroperitoneal fat weight. The ceca were gently rinsed with a saline solution to remove any contents before empty ceca weights were taken. Cecal contents were stored in the freezer. Brains were frozen on dry ice, blood was kept cold on ice before plasma separated by centrifugation and stored in the freezer, and cecal scrapings were stored in foil and frozen in liquid nitrogen during the sacrifice until the tissues could be stored in the freezer.

Cecal contents were used to analyze pH.

Study 2.

Diet Preparation. The same diet preparation procedure used in Study 1 was also used in Study 2.

The rats were randomly assigned to either the energy control diet or resistant starch diet from the ovariectomized or sham surgery groups. The compositions of the diets are listed in Table 3.5. The energy breakdown of the diets is listed in Table 3.6.

Table 3.5. Diet Composition Table for EC and RS diet to make a 10 kg Batch

INGREDIENTS	EC	RS
Macro Mix		
Amioca (g)	4245	-
Hi-Maize (g)	-	5307
Sucrose (g)	1000	1000
Casein (g)	2000	2000
Cellulose (g)	1562	500
Micro Mix		
Mineral Mix (g)	350	350

(Table Continued)

Vitamin Mix (g)	100	100
Choline Chloride (g)	13	13
L – Cystine (g)	30	30
Oil Mix		
Vegetable Oil (g)	700	700
BHT (g)	0.140	0.140
TOTAL	10000g	10000g

Table 3.6. Caloric Composition of the EC and RS diets as % (g/100g)

Nutrient	EC %(g/100g)	RS %(g/100g)
Protein	21	21
Total Carbohydrates	61.9	61.9
Fat	17.1	17.1
Kcal/g of Food	3.3	3.3

Weeks 1 through 6: Acclimation Period. All animals were fed a control diet for four weeks to become acclimated to the powdered diet and environment. The weight of the animal, the weight of the empty food jar, the weight of the full food jar, and the food spillage were measured three times a week (Monday, Wednesday, and Friday) for each animal. After

measurements were taken for empty food jar weights, the food jars were refilled and full jar weight was recorded.

Week 7: Group Assignment. After the acclimation period, the animals' weights were used to determine group assignment. The animals' weights were put into ascending order within ovariectomized and sham surgery groups. The weights were then ordered into two blocks of 10 replicates per surgery type. The animals were assigned to one of four treatment groups by choosing one from each block at a time. The result was that the average weight for each of the two ovariectomized groups was within one-tenth of a gram of one another; and the same for the two sham groups.

Week 7 through 19: Treatment. Throughout the experiment, the animals' weights, the empty food jar weights, the full food jar weights, and the food spillage were measured every Monday, Wednesday, and Friday beginning between 10AM-12PM. Empty food jars consisted of the leftover food in the food jar. Full food jar consisted of the weight of the food jar once it was refilled with the powdered diet. Every Friday, food jars were switched for clean food jars, and any leftover diet in the food jar was thrown out for fresh diet. Throughout the experimental period, animals were switched to new cages every two weeks for sanitary reasons. The animals were fed on their assigned diets for 12 weeks.

Body fat of the animals was measured using the NMR method at the beginning, middle and end of the treatment period.

Week 19: Sacrifice. The animals were sacrificed via decapitation. Due to the large number of animals, the sacrifice took place on two consecutive days. Tissues that were collected include blood samples, brain, cecal scrapings, cecal samples, peri-renal fat, epididymal fat, and retroperitoneal fat. Measurements that were taken included entire gastrointestinal tract weight,

small intestine weight, full cecum weight, empty cecum weight, perirenal fat weight, ovarian fat weight, mesentaeric fat weight, and retroperitoneal fat weight. The ceca were gently rinsed with a saline solution to remove any cecal contents before empty ceca weights were taken. Cecal contents were stored in the freezer. Brains were frozen on dry ice, blood was kept cold on ice before plasma separated by centrifugation and stored in the freezer, and cecal scrapings were stored in foil and frozen in liquid nitrogen during the sacrifice until the tissues could be stored in the freezer.

For analysis of tissues, the animals' brains were removed from the freezer to isolate the arcuate nucleus of the hypothalamus and extract the total RNA from the arcuate. Then the the mRNA expression for proopiomelanocortin in the arcuate nucleus of the hypothalamus was determined releative to the housekeeping gene cyclophilin. The cecal scrapings were used to analyze the mRNA expressions for PYY and GLP-1.

CONSTRUCTS AND VARIABLES

Weight of Rats. Animals were measured in a bucket on a balance that was tared to zero before the animal was put in. The weight was measured in grams, and rounded to the nearest gram.

PYY Levels and GLP-1 Levels. Total peptide YY (PYY), total glucagon-like-peptide-1 (GLP-1), and active GLP-1 were assayed with radioimmunoassay kits from Millipore (St. Charles, MO).

Amount of Food Consumption. Food consumption was calculated by subtracting the empty food jar weight and food spillage weight of the current day's measurement from the full food jar weight of the previous day's measurement. Empty food jar weights were measured on a balance, and measured in grams rounded to the nearest gram. The food jars were then refilled

with the appropriate diet. Full food jar weights were measured on the balance in grams, rounding to the nearest gram. Food spillage was accounted for by removing any feces from the cardboard sheet, and weighing the leftover food spillage. The spillage was measured in a weigh boat that was tared to zero before the food was put in. Total food consumed for each rat was measured every Monday, Wednesday, and Friday.

Cecal Content pH. The pH of the cecal contents was determined by homogenizing thawed cecal contents in distilled water (0.5 g wet sample to 5 ml water), and the pH was measured using a combination electrode. The samples were acidified with 1 ml of a 25 % (w/w) solution of meta-phosphoric acid that contained 2g/l of 2-ethyl-butyric acid as an internal standard for the short chain fatty acid contents. Solids in the homogenized samples were separated by centrifugation. Short chain fatty acids in the effluent were analyzed using gas-liquid chromatography.

Body Fat. Total body fat was determined using the NMR method. One at a time, each animal was inserted into Bruker's minispec Lean/Fat Analyzer for measurement. Each animal was measured twice, and the average of the two body fat percentages was used. NMR measurement was taken three times during each study: once before treatment started, once six weeks after treatment began, and once before sacrifice. Upon sacrifice, abdominal fat pad weights were also collected and measured.

STATISTICAL ANALYSIS

Data were analyzed using the Statistical Analysis System (SAS) statistical software package version 9.1. A 2x2 factorial was performed for all measurements. Results were considered statistically significant if $p<0.05$.

CHAPTER 4

RESULTS

STUDY 1

Cumulative food intake was higher for LF rats relative to HF rats (LFEC, 593; LFRS, 612; HFEC, 499; HFRS, 482 ± 14.85 grams, $p<0.0001$) (Fig. 4.1), but energy intake remained the same among all treatment groups (LFEC, 2077; LFRS, 2142; HFEC, 2085; HFRS, 2017 ± 56.3 kJ) (Table 4.1). HF rats did have an increase in body weight compared to LF rats (LFEC, 366; LFRS, 383; HFEC, 397; HFRS, 402 ± 7.84 grams, $p<0.0027$) (Table 2). Disemboweled body weight was higher in the HF rats (LFEC, 351; LFRS, 357; HFEC, 383; HFRS, 387 ± 6.05 grams) compared to LF rats ($p<0.0001$) (Table 2). Full cecum ($p<0.0001$) and empty cecum weights ($p<0.0001$) were higher in RS rats compared to EC rats, and higher in LF rats compared to HF rats (Table 4.1), with LFRS rats having significantly higher full and empty cecum weights relative to the other groups. This resulted in a significant fat x diet interaction. Epididymal fat ($p<0.0001$), abdominal fat ($p<0.0001$), perirenal fat ($p<0.0003$), and the total fat (LFEC, 8.33; LFRS, 8.34; HFEC, 11.43; HFRS, 12.59 ± 0.66 grams, $p<0.0001$) were also higher in the HF rats compared to the LF rats (Table 4.1). Plasma PYY was higher in RS rats compared to EC rats (LFEC, 132.6; LFRS, 302.4; HFEC, 118.2; HFRS, 141.7 ± 15.3) (Fig 4.2). RS rats had a lower cecal contents pH value compared to the EC rats ($p<0.0001$), and LF rats had a lower pH than HF rats ($p<0.0001$). This resulted in a significant fat x diet interaction, with LFRS rats having the lowest pH of cecal contents (LFEC, 8.41; LFRS, 6.67; HFEC 8.23; HFRS, 8.32 ± 0.08) (Fig 4.3).

STUDY 2

In the pre-treatment period, OVX rats consumed more energy (OVX, 2447; SH, 2122 ± 32.14 kJ, $p<0.0001$) and more food than sham rats (OVX, 748; SH, 649 ± 9.82 grams, $p<0.0001$)

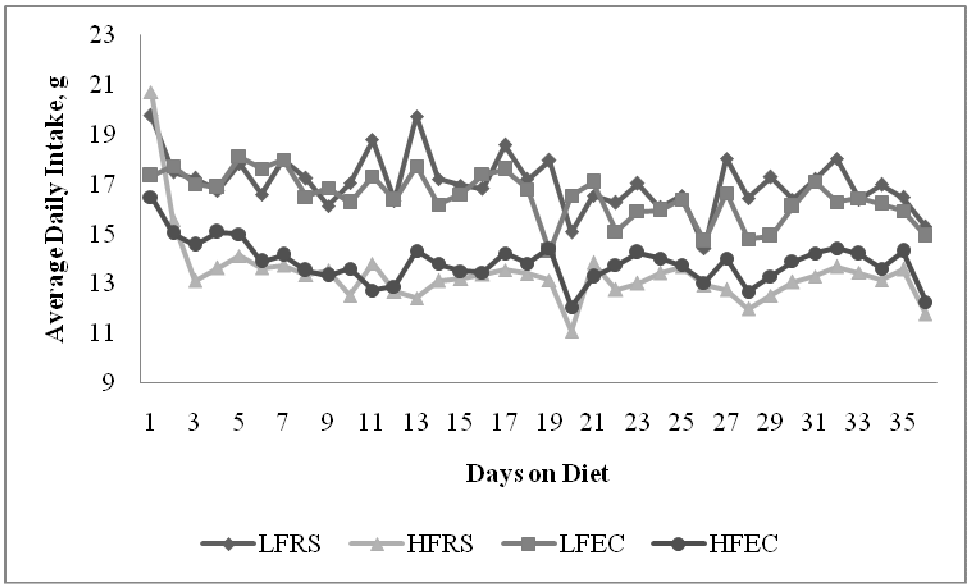

Fig 4.1. Average daily food intake for HF/LF study

Table 4.1. Measurements from HF/LF study according to Fat (low fat [LF] and high fat [HF]) and Diet (energy control [EC] and resistant starch [RS]) as the independent variables

Dependent Variable	LFEC	LFRS	HFEC	HFRS	SEM	Fat	Starch	Interaction
Beginning BW (g)	248	248	248	248	3	NS	NS	NS
Final BW (g)	366	383	397	402	8	0.0027	NS	NS
Disemboweled BW (g)	351	357	383	387	6	0.0001	NS	NS
Full Cecum Weight (g)	3	13.9	2.9	3.5	0.3	0.0001	0.0001	0.0001
Empty Cecum Weight (g)	0.6	2	0.6	0.8	0.05	0.0001	0.0001	0.0001
Epididymal Fat (g)	4.3	4.4	6.1	6.3	0.3	0.0001	NS	NS
Abdominal Fat (g)	2.7	2.8	3.7	4.4	0.3	0.0001	NS	NS
Perirenal Fat (g)	1.3	1.2	1.6	1.9	0.1	0.0003	NS	NS
Total Fat Pads (g)	8.3	8.3	11.4	12.6	0.7	0.0001	NS	NS
Energy Intake (kcal)	2077	2142	2085	2017	56.3	NS	NS	NS

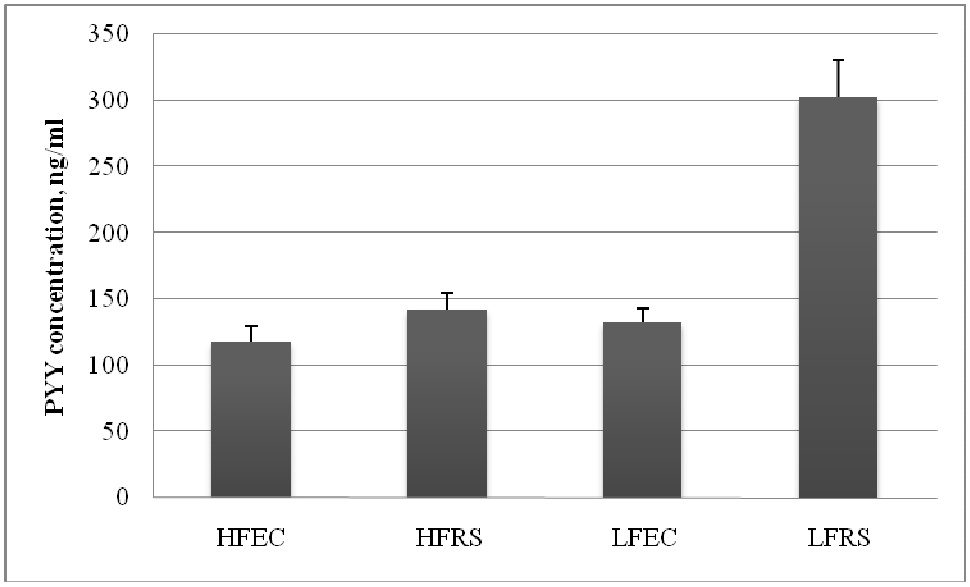

Fig 4.2. Plasma PYY concentration for HF/LF study

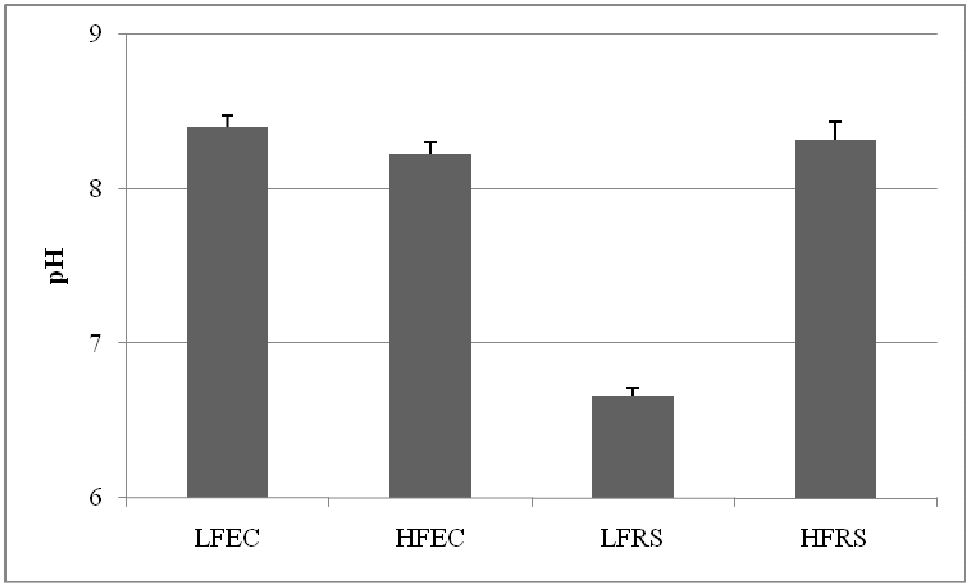

Fig 4.3. pH of cecal contents for HF/LF study

32.14 kJ, $p<0.0001$) and more food than sham rats (OVX, 748; SH, 649 ± 9.82 grams, $p<0.0001$) (Table 3). Body weight change during the pre-treatment period was significantly greater for OVX rats than sham rats (OVX, 112; SH, 44 ± 2.29 grams, $p<0.0001$) (Table 4.2). During the treatment period, energy intake was higher in RS rats compared to EC rats ($p<0.0029$) (OVEC, 4640; OVRS, 4856; SHEC, 4473; SHRS, 4857 ± 93.83) (Table 4.3). Food intake was also higher in RS rats compared to EC rats ($p<0.0029$) (OVEC, 1419; OVRS, 1485; SHEC, 1368; SHRS, 1485 ± 28.7) (Fig 4.4). Body weight was higher only in OVX rats relative to sham rats ($p<0.0001$) (OVEC, 349; OVRS, 342; SHEC, 284; SHRS, 282 ± 5.6), and so was disemboweled body weight ($p<0.0001$). But disemboweled body weight was also higher in EC rats compared to RS rats ($p<0.06$) (OVEC, 332; OVRS, 320; SHEC, 268; SHRS, 260 ± 5.2) (Table 4.3). Full total gastrointestinal weight ($p<0.0001$), full cecum weight ($p<0.0001$), and full small intestine weight ($p<0.0.0349$) were higher in RS rats relative to EC rats (Table 4.3). Mesenteric fat ($p<0.0016$), ovarian fat ($p<0.0405$), perirenal fat ($p<0.0208$), retroperitoneal fat ($p<0.0112$) (Table 4.3), and the total fat pad weights ($p<0.0065$) (Fig 5) were all lower in the RS rats relative to EC rats. Plasma PYY levels were higher in the sham group fed RS (OVEC, 42.4; OVRS, 34; SHEC, 34.7; SHRS, 53.3 ± 3.2). OVX rats fed RS, however, had lower total PYY levels (Fig 4.6). This resulted in a significant diet x surgery interaction. Diet had no effect on plasma GLP-1 levels (OVEC, 5.8; OVRS, 4.9; SHEC, 6.2; SHRS, 5.7 ± 0.5) (Fig 4.7).

Table 4.2. Measurements according to surgery (ovariectomy [OVX] and sham [SH]) after 6 week recovery period and before diet treatments

Dependent Variable	OVX	SH	SEM	Surgery
Beginning BW (g)	208	197	1.8	0.0001
BW after 6 Weeks (g)	346	283	3.9	0.0001
Fat (g)	54.3	39.8	0.7	0.0001
Fat/BW (%)	16.8	16.3	0.2	NS
Energy Intake (kcal)	1452	1427	23	NS
Weight Gain (g)	116	46	2.3	0.0001
Energy efficiency (g/kcal)	8.0	3.3	0.2	0.0001

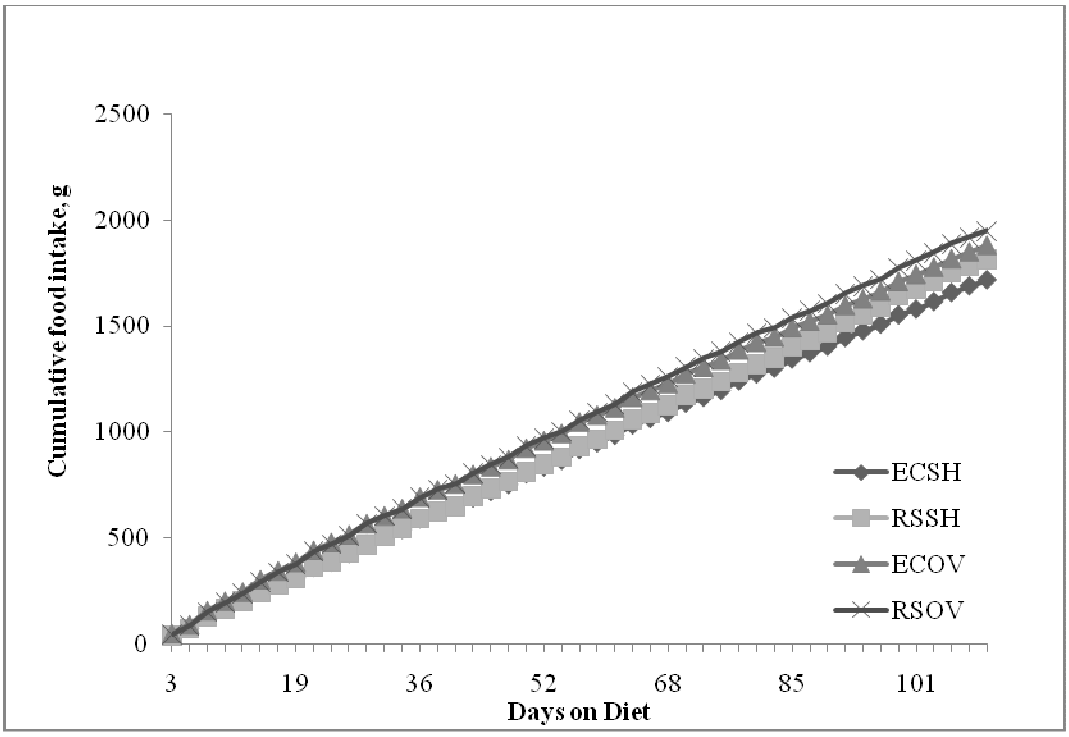

Fig 4.4. Cumulative food intake for OVX/SH study

Table 4.3. Measurements for 13 week study with Diet (energy control [EC] and resistant starch [RS]) and surgery (ovariectomy [OVX] and sham [SH]) as the independent variables

Dependent Variable	ECOV	RSOV	ECSH	RSSH	SEM	Diet	Surgery	Interaction
Beginning BW (g)	322	326	245	242	4	NS	0.0001	NS
Beginning Fat (g)	54.3	54.4	39.8	39.7	1	NS	0.0001	NS
Beginning Fat/BW (%)	16.8	16.7	16.3	16.4	0.3	NS	NS	NS
Disemboweled BW (g)	332	320	268	260	5	0.06	0.0001	NS
Full GI Tract (g)	17.3	22.3	16.0	20.3	0.9	0.001	0.08	NS
Full Small Intestine (g)	9.8	10.2	9.4	10.7	0.4	0.04	NS	NS
Full Large Intestine (g)	7.5	12.2	6.2	9.6	0.7	0.0001	0.006	NS
Mesenteric Fat (g)	3.1	2.5	2.8	2	0.2	0.0016	0.03	NS
Ovarian Fat (g)	5.1	4.2	4.5	3.4	0.4	0.04	NS	NS
Perirenal Fat (g)	1.9	1.4	1.4	1.1	0.2	0.2	0.02	NS
Retroperitineal Fat (g)	2.2	1.7	1.3	1.1	0.1	0.01	0.0001	NS
Total Fat Pads(g)	12.3	9.9	10	7.6	0.8	0.007	0.009	NS
Energy Intake (kcal)	4640	4856	4473	4856	94	0.003	NS	NS

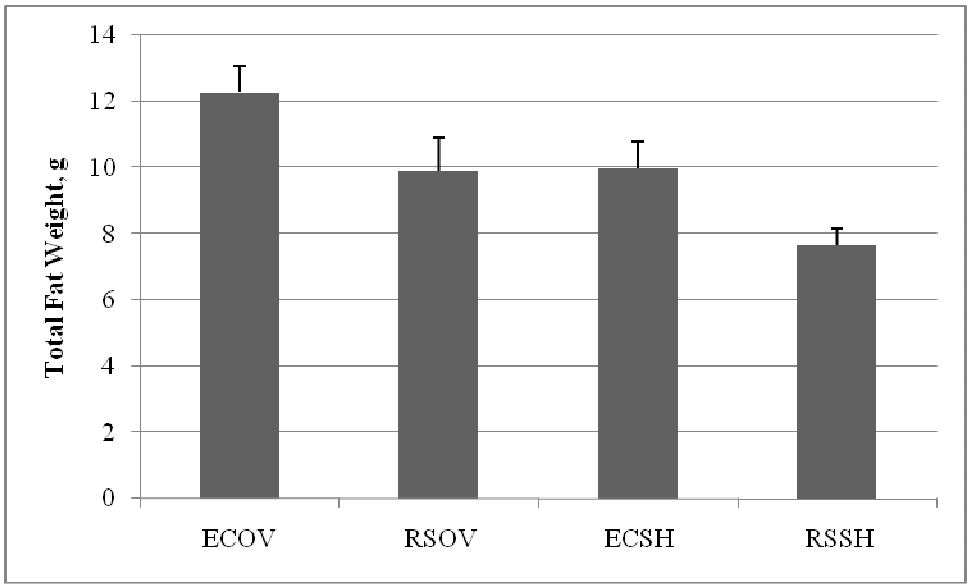

Fig 4.5. Total abdominal fat pad weights for OVX/SH study

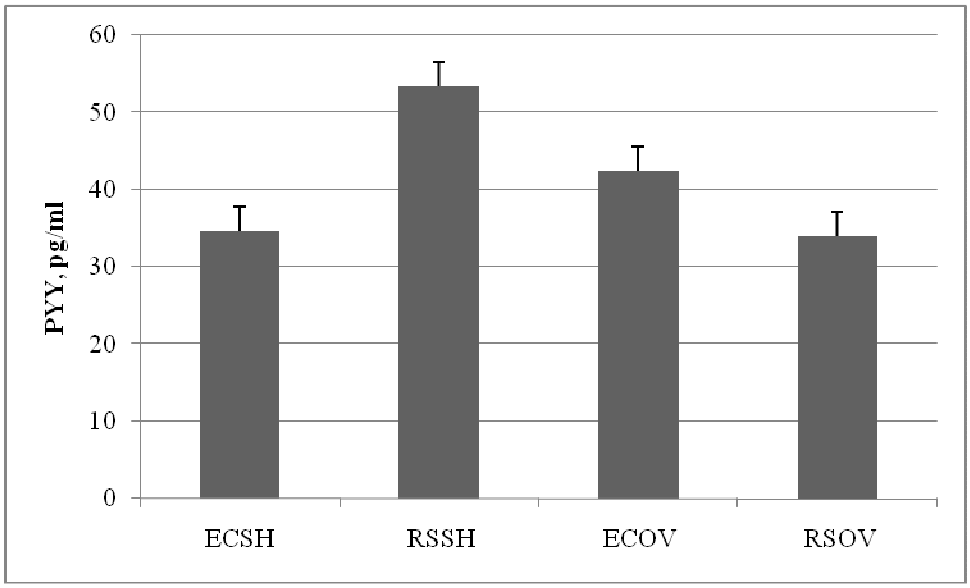

Fig 4.6. Plasma PYY concentration for OVX/SH study

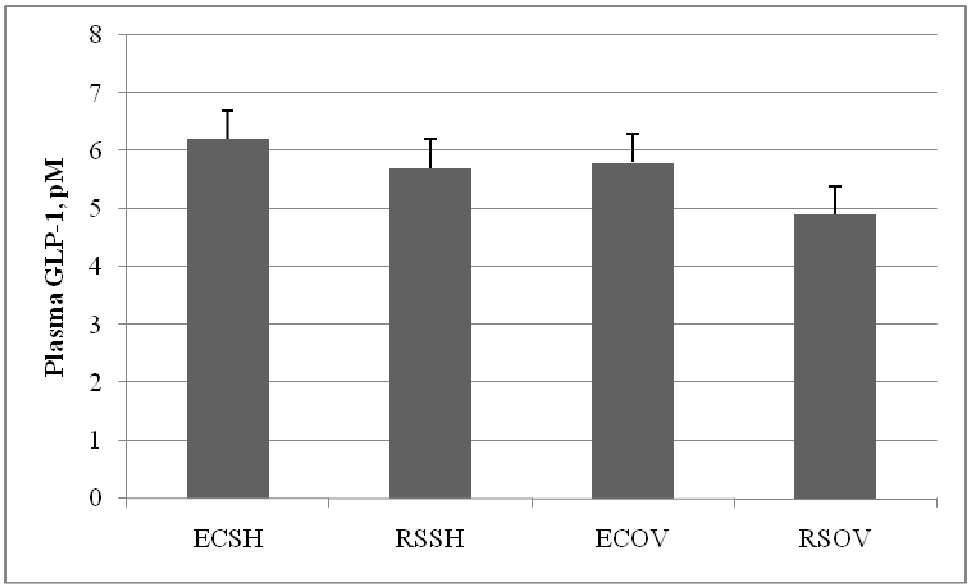

Fig 4.7. Plasma GLP-1 concentration for OVX/SH study

CHAPTER 5

DISCUSSION

Dietary resistant starch is a fermentable fiber that resists digestion in the gut. As a component of the diet, its indigestible quality results in dilution of the metabolizable energy of the diet, fecal bulking comparable to non-fermentable fiber, and fermentation in the colon to SCFAs with a subsequent increase in PYY and GLP-1 expression in the gut (Nugent, 2005; Keenan et al., 2006; Zhou et al., 2006). Energy dilution has been considered a treatment possibility for obesity if a person consumes a certain volume of food as opposed to a certain amount of energy (Rolls et al., 1998; Willet, 1998), thus displacing extra calories with indigestible matter (Howarth, Saltzman, & Roberts, 2001). The gut neuropeptides PYY and GLP-1 have both been reported to decrease food intake (Batterham et al., 2002; Batterham et al., 2003; Strader & Woods, 2005). It is through these mechanisms that RS is proposed to aid in reduction of body fat and weight gain. RS has been reported to reduce fat pad weight (De Deckere et al., 1993; Lerer-Metzger et al., 1996; Kabir et al., 1998*a*) and lower abdominal fat (Keenan et al., 2006*a*).

In the first study we examined the effects of RS in a high fat diet on weight, body fat, fermentation, and gut signaling. In the second study we examined the effects of RS in an ovariectomized rat model of obesity on weight, body fat, fermentation, and gut signaling. RS has been previously demonstrated to decrease body weight and adipose tissue (De Deckere et al., 2003, Keenan et al., 2006*a*) and increase gene expression of PYY and proglucagon, the gene that encodes GLP-1 (Zhou et al., 2006).

In the first study, we found that rats fed the HF diet ad libitum did have an increase in body weight over the treatment period. RS, however, did not reduce body weight or body fat in

either the HF or LF rats. Disemboweled body weight was higher in HF rats relative to LF rats. Cumulative food intake was also higher for LF rats compared to HF rats to compensate for the decreased energy density due to the lowered amount of fat in the diet. Energy intake remained the same for all treatment groups. Epididymal, abdominal, perirenal, and total fat pads were higher in HF rats compared to LF rats. However, RS was not effective in reducing body fat. This is not consistent with the study performed by Keenan *et al* (2006*a*) when fed a LF diet.

Both full and empty cecum weights were, however, higher in RS rats compared to EC rats. The presence of RS in the diet also lowered the pH of cecal contents in the LF diet, resulting in a significant diet x surgery interaction. These results are consistent with previous studies regarding the effects of RS on fermentation (Nugent, 2005; Keenan et al., 2006*a*), although RS was not effective in lowering pH in the HF group. Plasma PYY levels were also found to be higher in the RS rats, which has been attributed to the fermentation of RS in the colon (Keenan et al., 2006*a*; Zhou et al., 2006).

In the second study, both OVX and sham rats were put on a control diet for six weeks to allow OVX rats to gain weight. During this pre-treatment period, OVX consumed more energy and food, and gained more weight than sham rats. After six weeks, the rats were put on their respective diet treatments. During the treatment period, both energy and food intake were higher in RS rats compared to EC rats. By the end of the study, body weight was still higher in OVX rats relative to sham rats. RS rats did have lower disemboweled body weights compared to EC rats, although this only approached statistical significance ($p<0.06$). However, mesenteric, ovarian, perirenal, retroperitineal, and total fat pads were all lower in RS rats compared to EC rats. Small intestine weight, cecum weight, and total gastrointestinal weight were all higher in RS rats relative to EC rats. Lowered disemboweled body weight, lowered fat pads, and greater

intestinal weights in RS rats were also seen in a previous study by Keenan *et al* (2006*a*). This once again reinforces the fact that RS increases fermentation and lowers body fat in the endocrine obese model and those fed a low-fat diet.

Unlike the previous study, plasma PYY levels were only increased in sham rats fed RS and not OVX rats fed RS. The presence of RS also had no effect on GLP-1. Both Zhou *et al* and Keenan *et al* reported an increase in GLP-1 and PYY levels, and increased proglucagon and PYY gene expression, attributing these gut peptides as to at least one mechanism of body fat reduction.

Both studies performed here were to examine the effects of RS on obese models. The rats in the first study were fed a high fat diet to model a more "westernized diet" to induce obesity, and the rats in the second study were ovariectomized to induce obesity. RS was not effective in lowering either weight or body fat in the high fat diet or low fat diet. The effect of RS on a high fat diet (44.8% of energy, 22% fat w/w) has not been tested before, but RS has been shown to reduce body fat in a low fat diet (17.1% of energy, 7% fat w/w). We did not observe this even in our low fat diet. A possibility for its ineffectiveness on the low fat diet in this study may be attributed to the lowered amount of sucrose in the diet. In the study by Keenan *et al* (2006*a*) and another by Shen *et al* (2008), 100g of sucrose/kg of diet was used, whereas only 50g of sucrose/kg of diet was used in this study. This lowered amount may have affected the palatability of the low fat diet, which is already low in fat, and rats may not have consumed enough RS to be effective. We compared body weight, disemboweled body weight, and body fat between our rats and Shen's rats to evaluate how the difference in the amount of sugar effected these measurements. Our rats and Shen's rats were ordered from the same company, were the age and type of rat, and arrived on the same day. Upon comparison, Shen's rats had a greater body

weight and disemboweled body weight for both the energy control and resistant starch diets (Figures 5.1 and 5.2). Body fat was also higher in Shen's rats relative to our rats (Figure 5.3). Both our low fat diet and Shen's diet had the same amount of fat, but Shen's diet was prepared with 100g sucrose and fed for only 65 days whereas ours was prepared with 50g of sucrose and fed for 85 days. The combination of a low fat diet and a lowered amount of sugar may have compromised food consumption, which ultimately could have affected RS consumption and effectiveness or possible fat gain by EC rats.

In this study, we also noted that full and empty cecum weights were significantly lower in the high fat diet than the low fat diet, and pH was significantly higher in the high fat diet compared to the low fat diet. These results indicate that fermentation was eliminated with the high fat diet. Dietary fat has previously been reported to reduce fermentation of fiber in

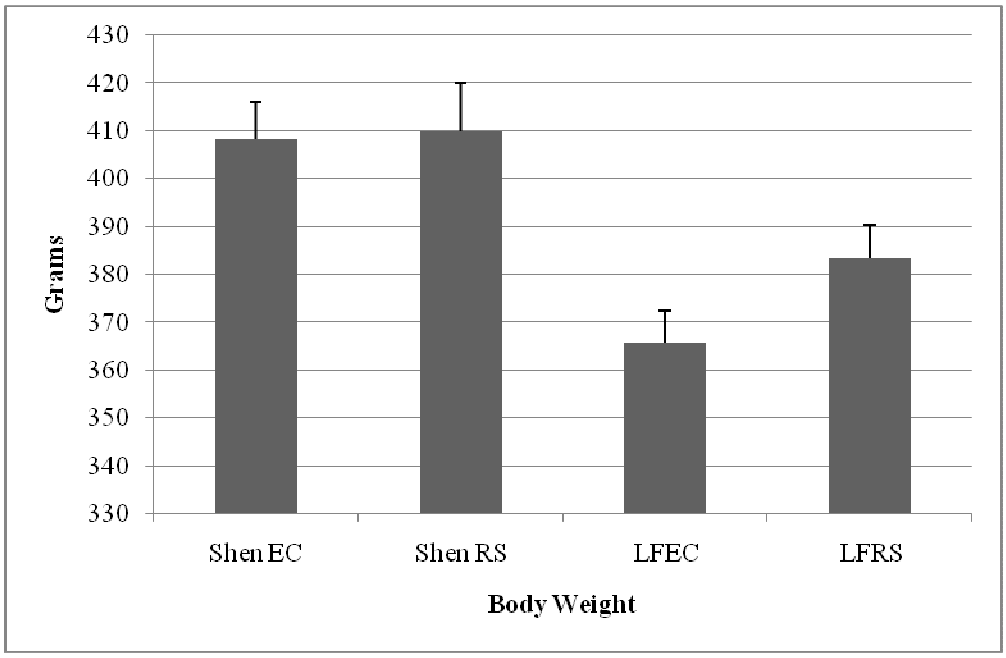

Fig 5.1. Body weight comparison of rats from Shen's low fat diet with 100g of sucrose and our low fat diet with 50g of sucrose

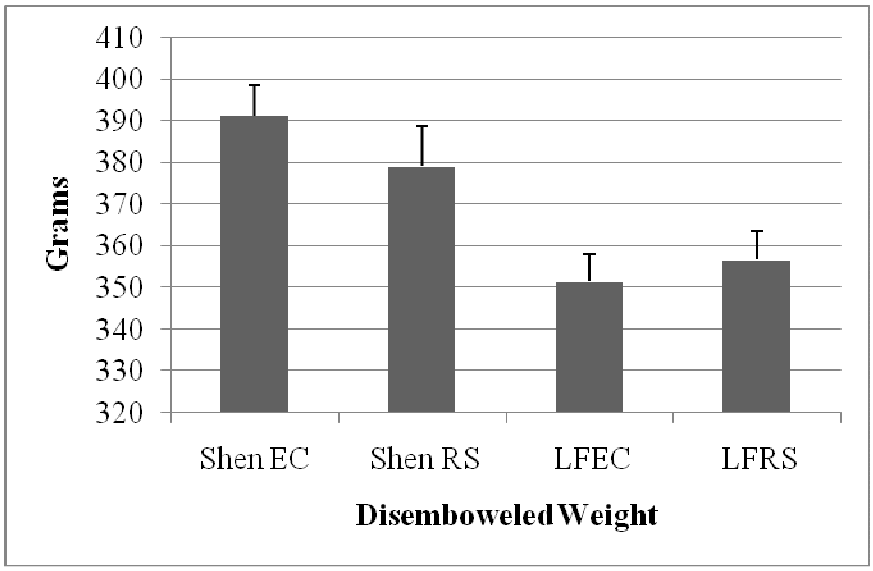

Fig 5.2. Disemeboweled body weight comparison of rats from Shen's low fat diet with 100g of sucrose and our low fat diet with 50g of sucrose

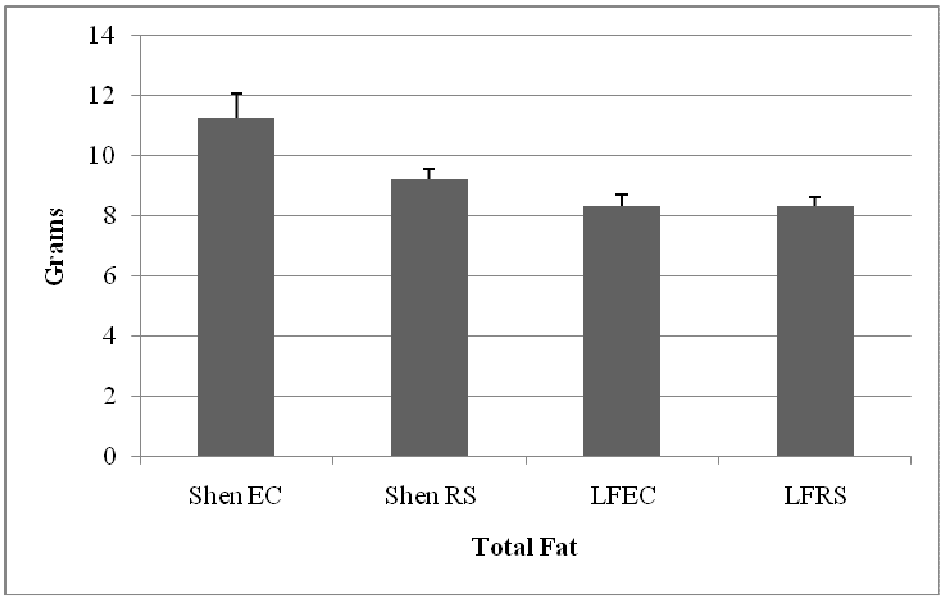

Fig 5.3. Total fat comparison of rats from Shen's low fat diet with 100g of sucrose and our low fat diet with 50g of sucrose

ruminants (Macleod & Buchanan Smith, 1972; Devendra & Lewis, 1974; Kowalczyk et al., 1977). If fat can affect fiber fermentation, then the high fat diet used in this study may also be blunting the fermentation of RS. Endogenous lipids have also been shown to complex with the amylose chains in RS, which can disrupt RS formation (Eerlingen et al., 1994). If RS is not formed, it is most likely being digested just as a starch, fermentation is not allowed to take place, and the subsequent physiological effects of RS are being hindered.

In the second study, food and energy intake were higher in the RS rats compared to EC rats. Interestingly though, EC rats still had a greater disemboweled body weight. This effect appears to be driven by increased energy expenditure because energy intake was lower in EC rats. In an unpublished study by Zhou *et al* (2008), mice fed an RS diet also had a higher cumulative food intake and lower body fat, and with a reported lower respiratory exchange ratio (RER). This method of measuring respiratory gases is an indication of fat burning. The lower RER in RS mice compared to controls indicates greater fat burning. Higgins *et al* (2004) also reported that replacement of 5.4% of total carbohydrate with RS significantly lowered the respiratory quotient (RQ, a measurement of respiratory gases at the cellular level), and increased fat oxidation. This moderate level of RS fed in humans also indicates greater fat burning. This data suggests that in our studies RS does not decrease food intake, but its role is to increase fat burning, which appears to promote its ability to reduce body weight and body fat.

Also in the second experiment, lowered disemboweled body weights, lowered fat pads, and greater intestinal weights were all observed in the RS rats relative to EC rats, even though there was no difference observed in GLP-1 between the diet treatments, and PYY appeared to be effective only in sham rats fed RS. As stated before, it has been previously reported by Keenan *et*

al and Zhou *et al* that increased gene and protein levels of PYY and GLP-1 are associated with consumption of RS. The fact that these two gut hormones were not associated with RS consumption, but larger intestinal weights and reduced body fat were both still seen with RS consumption suggests that RS may work through other hormones or another mechanism beyond gut signaling. Previous work has presented gene array data demonstrating vast differences in expression of the cecal cell genome in rats (Keenan et al., 2006*a*; Keenan et al., 2006*b*). Other factors possibly involved include other gut hormones, neural, and immune factors.

In conclusion, the data from the current study suggests that RS is ineffective in reducing body weight and body fat in rats when fed concomitantly with a high fat and low fat diet. These results are contrary to previous reports of the association between RS consumption and reduced body fat and body weight. It is possible that the high fat diet may blunt the effects of RS fermentation. However, RS consumption did indicate that fermentation took place in rats fed the low fat diet, although weight gain was too low in rats fed the low fat, RS diet due to due decreased sucrose levels. In our second study, adding resistant starch to the diets of OVX rats was effective in reducing accretion of abdominal body fat, even though the data did not support it was done through gut signaling with PYY and GLP-1 as previously demonstrated. The mechanism for this remains to be determined. However, this suggests that dietary resistant starch may improve health by reducing body fat in postmenopausal women. Further research investigating other possible means by which dietary resistant starch is effective in reducing body weight and body fat is warranted.

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APPENDIX A:

STUDY PROTOCOL

PENNINGTON BIOMEDICAL RESEARCH CENTER

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

GUIDELINES FOR SUBMISSION OF NEW PROTOCOL FORMS AND EXISTING PROTOCOL AMENDMENTS FOR IACUC REVIEW

NEW PROTOCOL

A new protocol form must be filled out and submitted to the IACUC:

1) to receive approval to begin experiments/procedures using laboratory animals;

2) if there are changes in major operative procedures in **previously approved** protocol (the protocol is not assigned a new number); or

3) if there are changes in species in a **previously approved** protocol (the protocol is not assigned a new number).

PROTOCOL RENEWAL

As required by the Public Health Service Policy all protocols must be reviewed by the IACUC every three years. The term *renewal* refers to IACUC action on a previously approved, but EXPIRING protocol. A new protocol form must be submitted for a renewal at least every three years. Once renewed and approved by the IACUC, the protocol is assigned a new number.

PROTOCOL AMENDMENTS

A protocol may be amended if the **original intent** of the protocol does not change. A new protocol form is generally not necessary; instead, simply submit a letter to the IACUC, citing the protocol number and title, and outlining the change(s) requested. For questions concerning protocol amendments contact the IACUC chairman or attending veterinarian. Examples of changes that may be made by a protocol amendment include:

1) addition of personnel to a protocol;
2) changes in drugs or drug dosages;
3) changes in animal numbers;
4) changes in group designations; or
5) changes in time length of experiments.

INSTRUCTIONS FOR USING ANIMAL CARE AND USE PROTOCOL FORM:

1. **The most recent version of the IACUC Animal Care and Use Protocol form is available on PINE under IACUC.**

2. **Retrieve the form and save the file in another location on your computer or network space: In the toolbar, select [File] and select [Save as…] . Give the file any name you prefer.**

3. **Gray boxes are shown for inserting responses to the questions. Text may be inserted by placing the cursor at the return and typing or pasting text. Color has been used to highlight especially important areas. The form will print in black if the usual LaserJet printer is used.**

4. **The IACUC encourages the use of tables, flow diagrams etc. for some responses. However, Word® Forms does not allow these functions. Tables and diagrams may be created and attached to the protocol where appropriate.**

5. **After completing the form, you may want to remove excess hard returns between sections in order to limit the finished protocol form to a minimum number of pages.**

6. **Should you encounter any problems while using this form, please contact Nancy Pease at ext. 3-2577.**

7. **Submission instructions: Form must be typed. (Use additional sheets if necessary and attach to this form). SUBMIT ORIGINAL to the IACUC Administrator's Office, Rm. B1022.**

8. **Delete the first two pages of instructions prior to saving your final version.**

PROTOCOL FOR ANIMAL CARE AND USE

SECTION 1: Investigators

Principal Investigator	Office Phone:	Home Phone:	Email Address
Roy J. Martin	578-2284	766-2684	martinrj@pbrc.edu
Co-Investigator:	Office Phone:	Home Phone:	Email Address
Jun Zhou	763-2531	924-6308	zhouj@pbrc.edu

SECTION 2. Project Title:

The role of PYY and GLP-1 in energy balance with resistant starch fed animals

SECTION 3. Funding Source: Is this protocol associated with a grant or contract?

☒ Yes ☐ No

Funding agency: **NIH**

Grant or contract title: Dietary Resistant Starch: The role of PYY and GLP-1 in energy balance

☒ Pending ☐ Funded

SECTION 4. Investigator's Statement regarding the assurance for the Humane Care and Use of Vertebrate Animals.

By signing this form, I agree to abide by Pennington Biomedical Research Center's Policy for the Care and Use of Animals. This project will be in accordance with the NIH "Guide for the Care and Use of Laboratory Animals" (except as explained in the accompanying protocol), and the PBRC Animal Welfare Assurance on file with the U.S. Public Health Service. I further assure the Committee that:

1) **I will abide by all federal, state, and local laws and regulations governing the use of animals in teaching and research.**
2) **The investigators and technicians are or will be adequately trained to perform the research techniques required in these studies.**
3) **I will use the fewest number of animals required to produce the appropriate statistical power for this study.**
4) **The research proposed herein is not unnecessarily duplicative of previously reported**

research.

5) **For those completing Section 14.2 and 14.3: I have reviewed the pertinent scientific literature and the sources and/or databases as noted in Section 14, and have found no valid alternative to any procedures described herein which may cause more than momentary pain or distress, whether it is relieved or not.**

Roy Martin	Professor	
Type Name of Principal Investigator	Title/Rank	Date

Principal Investigator Signature

Jun Zhou	Post-Doc	
Type Name of Co-Investigator	Title/Rank	Date

Co-Principal Investigator Signature

Animal housing and veterinary care must be coordinated with Comparative Biology.

Signature of Comparative Biology Representative (required)

SECTION 5. Animal Species:

Species: ☒ Mouse ☐ Rat ☐ Other	Strain: C57BL/6J
Sex: ☐ Male ☐ Female ☐ Either ☒ Both	Weight (or age for rodents): six weeks old
Source: ☒ Commercial Vendor ☒ PBRC Breeding Colony ☒ Collaborator	

Species: ☐ Mouse ☒ Rat ☐ Other	Strain: Sprague Dawley
Sex: ☒ Male ☐ Female ☐ Either ☐ Both	Weight (or age for rodents): eight weeks old
Source: ☒ Commercial Vendor ☐ PBRC Breeding Colony ☐ Collaborator	

Species:	Mice	Rats	Other
Number of animals needed:			
Year 1: →	200	340	
Year 2: →	580		
Year 3: →			
TOTAL: →	780	340	
Maximum # needed at one time:	200	100	

SECTION 6. Abstract Plan of Research/Teaching: In the space below, using non-technical terms provide a brief (1 – 2 paragraphs) layman's description of the project. Include the aim of the study and how this study may benefit human or animal health or advance scientific understanding of biological processes.

Resistant starch is a dietary carbohydrate that resists digestion in the small intestine and is fermented in the large intestine. Our preliminary studies showed that feeding resistant starch significantly altered energy balance and decreased body fat in rodents. Additionally, resistant starch fed rodents had higher gene expressions for peptide YY (PYY) and proglucagon (a precursor of glucagon-like peptide-1, GLP-1) in the gut, and higher serum levels of PYY and GLP-1. PYY and GLP-1 are satiety peptides secreted from the gut. Administration of either PYY or GLP-1 reduces food intake. Thus, we hypothesize that the decreased body fat is due to increased PYY and/or GLP-1 (PYY/GLP-1) in resistant starch fed animals. The hypothesis will be tested in three specific aims. First, we will block PYY/GLP-1 action to determine if PYY and GLP-1 are required for resistant starch to decrease body fat. This will be achieved by using PYY/GLP-1 receptor antagonists or by using the PYY/GLP-1 receptors knock out mice. Second, we will investigate if PYY/GLP-1 decrease body fat via visceral nerves or the brain. The peripheral sites of action for PYY/GLP-1 will be tested by destroying visceral afferent neurons to block signals to the brain. Finally, we will test if resistant starch decreases body fat in robust obese animal models. A diet-induced obesity model and a genetic obese model will be used to see if resistant starch can prevent obesity. These experiments will reveal that PYY and GLP-1 are part of the mechanism of resistant starch on reducing body fat. Thus, a simple dietary intervention can increase levels of these peptides and reduce body fat naturally without surgery or pharmaceutical means. This dietary approach is potentially of great therapeutic importance in the prevention of human obesity.

SECTION 7. Special Husbandry Requirements: Do your animals have special needs to be addressed by Comparative Biology?

☒ Yes ☐ No

If yes, please complete each section that is different from the standard of care for rodents. The standard of care is: plastic shoebox, corn cob bedding, rodent chow, ≅21-22°C room temperature, ≅55% humidity, and tap water.

TEMPERATURE RANGE:	21-22°C room temperature		**HUMIDITY:**	55 (%)
LIGHT CYCLE (hours light/hours dark)			12/12	
CAGING	Type: 1) Shoe boxes for breeding mice		Size: single /multi	Filter tops required? ☐ Y ☒ N
BEDDING/LITTER	Type: corncob and nesting material		Autoclaved? ☐ Y ☒ N	Changes/week: 3
CAGING	Type: 2) appropriate SS hanging wire cages for experimental mice and rats		Size: Single	Filter tops required? ☐ Y ☒ N
BEDDING/LITTER	Type: pan liners and weigh backs		Autoclaved? ☐ Y ☒ N	Changes/week: 1 By investigator only*
WATER	Sterile: ☐ Y ☒ N	De-ionized: ☐ Y ☒ N	Acidified: ☐ Y ☒ N	Tap: ☒ Y ☐ N
Other:	* weigh backs will be changed 3 to 7 times per week by investigator when measuring spillage. Pan liners will be changed 1 time per week by investigator			
DIET	Special Feeding Requirements: Investigator will mix custom diets (see table below) and feed			

	experimental animals 3-7 times per week. Breeder mice will be provided breeder chow by DCB.

IF USING WIRE BOTTOM CAGES, PLEASE JUSTIFY:

We will measure food intake in our animals. Hanging wire cages are required so that we can accurately account for food spillage. As experiment period could last as long as 12 weeks, a PVC tube will be placed in each mouse cage to reduce the stress caused by wire cage. Breeder pairs will be multi-housed in shoeboxes. Prior to due date, males will be removed and either place with an available female or single housed in shoe boxes. Females will be singled housed with neonates until weaned. Weanlings will remain in shoe boxes until placed in experiments.

OTHER SPECIAL NEEDS:

Table 1. Experimental Diet Composition

Ingredient	Control (g/kg)	30% RS(g/kg)	High Fat Control (g/kg)	High Fat 27% RS (g/kg)
Casein	200.0	200.0	200.0	200.0
Sucrose	100.0	100.0	50.0	50.0
Cornstarch - Amioca® (100% amylopectin)	424.5		384.5	
Cornstarch - Hi-Maize® (high amylose)		530.7		480.7
Cellulose fiber	156.2	50.0	96.2	
Soybean oil	70.0	70.0	100.0	100.0
Vegetable shortening			120.0	120.0
Mineral Mix (AIN-93G)	35.0	35.0	35.0	35.0
Vitamin Mix (AIN-93)	10.0	10.0	10.0	10.0
Choline Chloride	1.3	1.3	1.3	1.3
L-Cystine	3.0	3.0	3.0	3.0
Total	(3.27 kcal/g)	(3.27 kcal/g)	(4.2 kcal/g)	(4.2 kcal/g)
Protein	**22.2% kcal**	**22.2% kcal**	**17.2% kcal**	**17.2% kcal**

Carbohydrates	**59.7% kcal**	**59.7% kcal**	**38.1.7% kcal**	**38.1% kcal**
Fat	**18.1% kcal**	**18.1% kcal**	**44.8% kcal**	**44.8% kcal**

SECTION 8. Hazardous Materials: Will zoonotic/recombinant agents or hazardous chemical agents be PRESENT IN THE ANIMAL ROOM?

☐ YES ☒ NO

If "yes" complete the appropriate section(s) below.

8.1 **Zoonotic/Recombinant Agents:** If zoonotic (infectious to humans) or recombinant agents will be used, this protocol request must be submitted to the LSU Institutional Biological Recombinant DNA Safety (IBRDS) Committee for approval PRIOR TO CONSIDERATION by the IACUC. Submit copy of the IBRDS approval letter to the IACUC Administrator with the animal protocol.

List Agent(s): _____

IBRDS Approval Date: _____ PBRC Safety Officer: _____

8.2 **Hazardous Chemicals:** If hazardous chemical are to be used in the animal room, submit the proposal with Appendix A completed, to the Biosafety Committee for prior approval. SOPs for some commonly used materials are available on PINE.

List Chemical(s): _____

Biosafety Committee Approval Date: _____

8.3 **Radioisotopes:** Use of radioisotopes is not allowed in Comparative Biology.

Will radioisotopes be used in animals? ☐ YES ☒ NO

List Radioisotopes: _____

Are you certified by the Radiation Safety Committee? ☐ YES ☐ NO

Where (Room #'s) will radioisotopes be used in animal experiments? _____

Is this a terminal experiment? ☐ YES ☐ NO

If you have any questions regarding approval of the use of zoonotic/recombinant agents, hazardous chemicals, or radioisotopes see the PBRC Safety Office @ 3-2667.

SECTION 9. Type of Project: (check the appropriate box)

☐ **TYPE A -** Pain or distress will not be induced; animals will only be used for injections, collections, or procedures causing nothing more than minor discomfort; or will be humanely euthanized prior to induction of pain or distress.

☒ **TYPE B -** Pain or distress will be relieved by appropriate therapy.

☐ **TYPE C -** Drug intervention for pain or distress would interfere with the protocol. Specific scientific justification MUST be provided below for Type C protocols.

Justification for Type C protocol:

none

SECTION 10. Summary of Procedures: Answer each of the following. If a section is not applicable, indicate so by "N/A". Your target audience is a faculty member from a discipline unrelated to yours.

10.1. **Rationale**: Explain your rationale for animal use. Briefly explain why should this study be done and what hypothesis/es will be tested?

Resistant starch has been used as a dietary carbohydrate in humans. However, the effects of reducing body fat by resistant starch are equivocal due to the complexity of human dietary studies. Using rodents to study the effects of resistant starch will allow control of the genetic verities, daily activity and dietary components. Using knock out mice could allow us to study the mechanism of resistant starch on reducing body fat. The results of our study will provide the important mechanistic information on how resistant starch reduces body fat. More specifically, if resistant starch reduce body fat through increase in secretion of PYY and GLP-1 in the gut.

10.2. **Species Selection**: How and/or why you selected the animal species indicated?

Mice and rats are selected because these animals are commonly used for mechanistic studies on body weight regulation and energy balance.

10.3. Experimental Design: Provide a concise description of the proposed use of animals. This description should allow the IACUC to understand the experimental course of each group of animals from its entry into, until the endpoint of the experiment. A flow chart (timeline/sequence), diagram, and/or a table (see note below) indicating **animal numbers and group assignments** are STRONGLY recommended to help the IACUC understand what is proposed. Include experimental procedures and any physical, chemical, or biological agents **(name, dose, volume, route, and frequency**) that may be administered. Details of specific nonsurgical and surgical procedures should be provided in Sections 10.5 and 10.6 respectively. **Form Note:** Due to the difficulty of inserting tables and flow charts/diagrams directly into the form consider creating these in Word and attaching them to the end of protocol.

.

Animal number and timeline for proposed studies is listed below.

Experiment	Animal number	Year	Length
Expt. 1.	140 SD rats	1	10 days
Expt. 2	100 SD rats	1	Six weeks
Expt. 3	100 SD rats	1	Six weeks
Expt. 4	80 mice	2	12 weeks
Expt. 5	100 mice	1	12 weeks
Breeding mice for Expt. 6	100 mice	1	
Breeding mice for Expt. 7	100 mice	2	
Expt. 6	200 mice	2	12 weeks
Expt. 7	200 mice	2	12 weeks

Experiment 1. Determine the timing of sample collection.

We will feed two groups of SD male rats with either control or resistant starch diet for ten days. At the end of Day 10, the rats will be killed by decapitation at 0, 2, 4, 8, 12, 16, and 20 hours after the beginning of the dark cycle (ten rats for each time points for each group). The blood, brain and tissues from the gut will be collected from each rat. PYY/GLP-1 expression levels in the blood and gut will be measured for each rat.

Experiment 2. Will injecting both Y_2R and GLP-1R antagonists simultaneously in rats block the action of both PYY and GLP-1?

BIIE0246 is a well-established PYY receptor antagonist and des-His1 Glu9-exendin-4 is a GLP-1R antagonist. Both antagonists have been used safely in mice and rats at the dosage described below for PYY and GLP-1 functional studies. (1-7)

Protocol: One hundred male SD rats will be divided into four weight matched groups: (1) saline, (2) BIIE0246 (2.0ug/g body weight, dissolved in 10% DMSO of saline i.p.), (3) des-His1 Glu9-exendin-4 (0.23ug/g body weight, dissolved in saline, i.p.) and (4) BIIE0246 plus des-His1 Glu9-exendin-4. The injection dosages and route will be the same as group (2) and (3). The injection volume for all groups will be 0.2ml/100g body weight and the injection frequency will be once per day. Each injection group will be subdivided into resistant starch fed group and control diet fed group (12 rats for each sub-group). Food intake and body weight for each rat will be measured three times per week. At the Week 5 after the first injection, all rats will be subjected to measured body fat by NMR. The experiment will be terminated six weeks after the start of injections. All rats will be killed by decapitation and their blood, gut, fat depots will be collected.

Experiment 3. Test if PYY & GLP-1 decrease body fat via visceral nerves or the brain.

Visceral afferent nerves from the gastrointestinal tract carry important signals to the brain to control energy balance and body fat. Thus, we will treat rats with capsaicin to destroy visceral afferent neuron and determine if the effects of resistant starch can be abolished. Capsaicin has been used traditionally in study the involvement of visceral sensory neurons (1, 8-10).

Protocol: One hundred male SD rats, weighing 150-180g, will be fed the control diet a week before sub-grouped into capsaicin or vehicle treatment. Capsaicin-treatment consists of 3 injections of progressively increasing doses over a period of 3 days, each under gaseous anesthesia. Rats will be anesthetized with isoflurane and doses of 12.5, 30, and 75 mg/kg of capsaicin will be injected ip. The first and second injections typically result in temporary respiratory arrest, but breathing will restart spontaneously or with the help of gentle chest massage. The entire procedure is carried out under deep Isoflurane anesthesia. Ten minutes after resumption of spontaneous breathing, anesthesia is discontinued and animals allowed to recover. At the highest dose on the third day, there is usually no respiratory arrest. Capsaicin will be dissolved in 10% ethanol/10% Tween 80 and sterile saline, and delivered at 0.6 ml/100g for the highest dose. For control

treatment, only the vehicle is injected. Two days after the last capsaicin or vehicle treatment, a CCK feeding suppression test will be conducted to confirm that capsaicin-sensitive visceral afferents have been destroyed by capsaicin treatment. One day after the last CCK feeding suppression test, both vehicle treated and capsaicin treated rats will be stratified by weight and assigned to either the control diet, or the resistant starch diet for a six-week study. Body weight and food intake will be measured three times per week. Four days before the end of the study, gut transit time will be measured. At the end of the study, the all rats will be killed by decapitation. The blood, fat depots, gut and brain will be collected.

CCK *feeding suppression test to confirm destroying capsaicin sensitive visceral afferents*

CCK suppresses food intake via visceral afferents. Thus, administration of exogenous CCK will decrease food intake in control rats but not in capsaicin treated rats. All rats will be fasted overnight. In the morning, CCK-8 (6ug/kg body weight, dissolved in saline) or saline will be injected (i.p. 0.2ml/100g body weight) into rats 30 minutes before feeding. One-hour food intake will be measured for all rats. Half the rats of each treatment group (capsaicin or vehicle) will receive CCK first and the other half will receive saline first, with tests 3 days apart. Saline treatment will be used to measure normal food intake after an overnight fast. CCK treatment should not decrease food intake in capsaicin treated rats but should decrease more than 50% food intake in control rats. It there are problems with CCK feeding suppression test, a sucrose solutions test will be used as an alternative to confirm the destroying capsaicin sensitive visceral afferents.

A sucrose solution test to confirm the destroying capsaicin sensitive visceral afferents(9)

Three days before the first exposure to sucrose, all rats will be offered daily the 10% sucrose solution and allowed to lick for 10 seconds (<0.2ml). The purpose of this training is to eliminate any neophobia toward the new food and source but without allowing significant ingestion and its metabolic consequences. By the fourth day, all rats immediately approached the spout and stared drinking. The 10% test sucrose solution will be given in the morning between 8:30 and 11:00am in the bottle attached to the cage front. Intake levels for each rats will be measured at 30 minutes and 60 minutes after offering the test sucrose solution. Capsaicin treated rats should over consumption sucrose solution when compared with controls.

Measurement of gut transit time(11, 12):

Each rat will be fed a bolus (2.5 g) of the appropriate experimental diet mixed with glass beads (10 mg; diameter range 150-170um) at midnight. The rats will be fasted (with free access to water) for 12 h before feeding the boli and all the boli should be eaten completely. One hour later, rats will be given free access to food again. Six hours after feeding the boli, fecal samples will be collected in glass scintillation vials every 2 h up to 30 h after feeding and then every 6 h up to 48 h after feeding. The gut transit time will be measured by counting the rate of glass beads in the fecal sample.

Experiment 4: Determine if resistant starch decreases body fat in a genetically obese mice model

Intracerebroventricular injection of PYY or GLP-1 have decreased food intake in lean and obese mice and their action is additive. Therefore we will determine if the increased plasma levels of both PYY and GLP-1 in resistant starch fed mice are sufficient to reduce body fat in ob/ob mice.

Protocol: Forty ob/ob (B6.V-Lepob/J) mice and forty of their lean (Lepob heterozygote) littermates will be obtained from Jackson Lab and fed the resistant starch or control diet for 12 weeks. Body weight and food intake will be measured three times per week. Body fat will be measured at the week 0, 6, 11, when diet treatment starts. At the end of 12 weeks, all mice will be decapitated and blood, fat depots will be collected.

Experiment 5: Test if resistant starch decreases body fat in a diet induced obesity mice model

C57BL/6J mice is susceptible to long-term high fat induced obesity. Furthermore, it has been shown that injections of PYY and GLP-1 reduce food intake in this model of obesity.

Protocol: one hundred male C57BL/6J mice will be divided into four groups and fed one of following four diets. 1) Control diet, 2) resistant starch diet, 3) high fat control diet , and 4) high fat resistant starch diet for 12 weeks. Body weight and food intake will be measured three times per week. Body fat will be measured at the week 0, 6, 11, when diet treatment starts. At the end of 12 weeks, all mice will be decapitated and blood, fat depots will be collected.

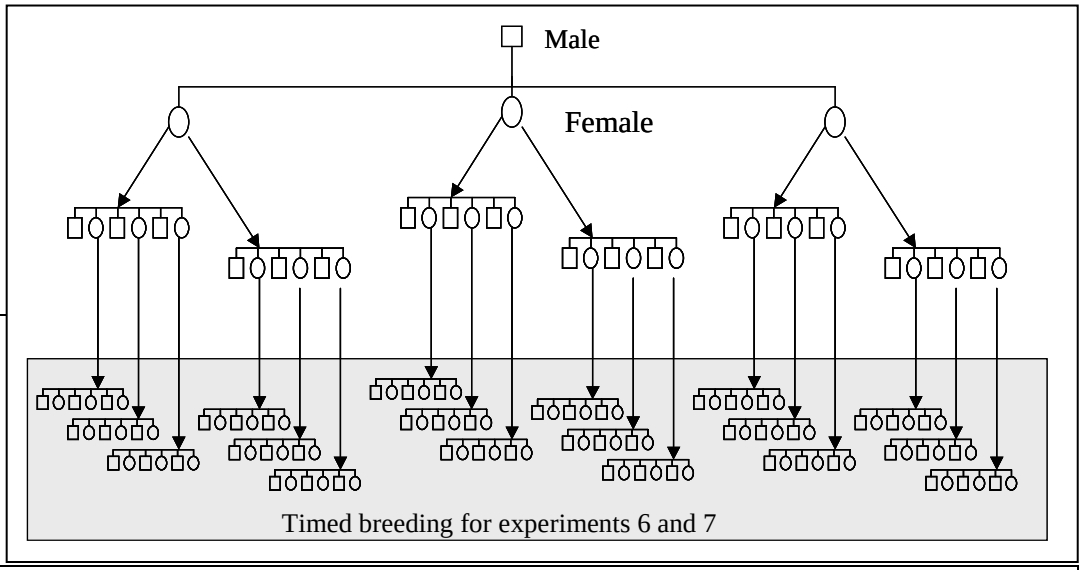
Experiment 6: and 7 will determine if PYY/GLP-1 receptors are required for resistant starch to decrease body fat. We will use Y_2R knock out mice and GLP-1R knock out mice to test our hypothesis. GLP-1R knock out mice are currently available in PBRC comparative biology facility and we have obtained the permission to breed them from Dr. Drucker. The Y_2R knock out mice will be obtained from Dr. Herbert Herzog. As Y_2R knock out and GLP-1R knock out mice are homozygoteous, all progeny will be used to replenish the breeding colony or the experiments. . However, we will genotyping retired breeding mice to confirm the correct genotype. We will also breeding C57Bl/6J mice at the same condition and use these mice as inbreed control for the experiment 6: and 7.

Breeding strategy.

Initial breeding will be set up with one male for 3 females in shoebox cages. Once pregnancy is evident, female mice will be are single housed in shoe box cages with corncob bedding and nesting material until pups are weaned at 21 days. Male and female progeny mice will be used to replenish the breeding colony. We expect 6 to 10 mice per litter, thus allowing for 2 breedings of Generation I to produce a minimum of 18 females and 6 males. The synchronized timed breeding of these progeny should produce adequate numbers of each type for experiments 6 and 7. If enough Generation III progeny are not produced for experiments 6 and 7, then a second breeding of Generation II will be initiated. Both male and female mice will be used in these

experiments to limit the time needed achieve adequate numbers for all experiments. A small number of the unneeded weanlings will be genotyped to determine if cross breeding to produce $Y_2R^{-/-}$ / GLP-$1R^{-/-}$ double knockout mice is possible. Any correct genotyped mice will be held for cross breeding. A separate protocol would be written for this.

Figure: Breeding strategy for each breeding set:



Generation I: Initial breeding group 3 females / one male

Generation IIa: First breeding -groups set up with all progeny

IIb: Second breeding -groups set up with all progeny

Generation III: breeding from IIa and IIb progeny timed to coincide

Number of animals: 200

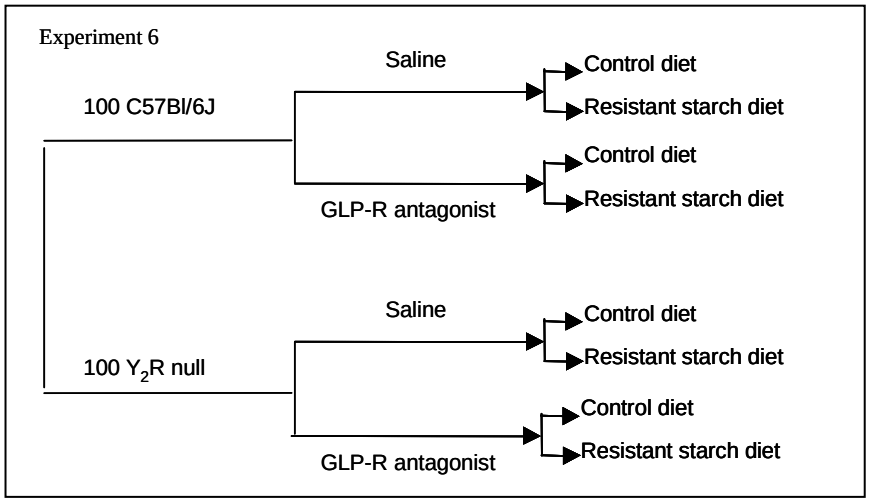
100 C57Bl/6J controls: 2 breeding sets (to produce 300 male or female for Expt. 6, 7)

50 $Y_2R^{-/-}$ knock out: 1 breeding set (to produce 150 male or female for Expt. 6)

50 GLP-1R$^{-/-}$ knock out: 1 breeding set (to produce 150 male or female for Expt 7)

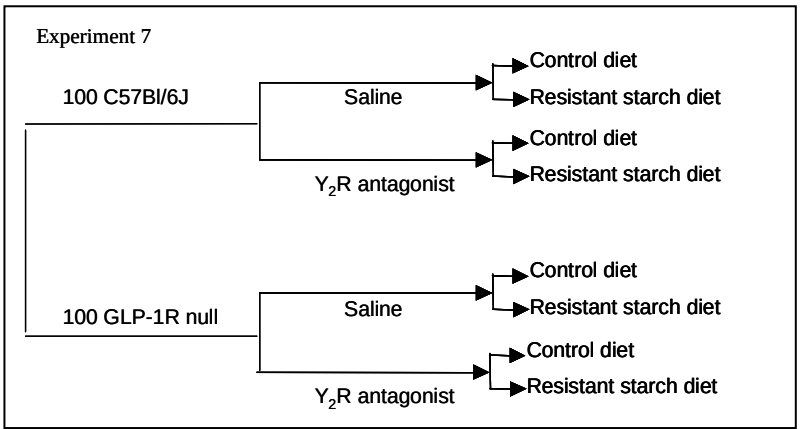
Retired breeders and extra weanlings will be used to determine optimum dosages and for genotyping

Experiment 6 Will injecting the GLP-1R antagonist into Y2R null mice block the action of both GLP-1 and PYY?



Protocol: Y2R null mice and their wild type littermates will be obtained from Dr. Herbert Herzog. Forty Y2R null mice and forty wild type littermates (6-week old males and females) will have free access to the control diet and water for a week. Body weight and food intake will be measured daily. At the end of one week of baseline measurements, the mice in each genotype group will be divided into two subgroups: Saline-injected and GLP-1R antagonist-injected (des-His1 Glu9-exendin-4, 2.33ug/0.1ml/10g body weight, dissolved in saline). The injection (i.p.) will be performed daily. Each injection group will be further subdivided into two subgroups: one subgroup will be fed the resistant starch diet and the other group will remain on the control diet. Body weight and food intake will be measured three times per week and body fat will be measured every ten days. The experiment will end one week after the body fat becomes significantly lower in the saline-injected wild type mice fed resistant starch as compared with the same genotype mice but fed the control diet. This significantly lower body fat in resistant starch fed mice will occurred within 12 weeks from our previous experiment results. At the end of study, all mice will be killed by decapitation and their blood, brain, fat depots, and gut will be collected.

Experiment 7: Will injecting the Y2R antagonist into the GLP-1R null mice block the action of both PYY and GLP-1?



Protocol: The same protocol will be used as in experiment 6, except we will use forty GLP-1R null mice and forty wild type littermates (6-week old males and females), and the antagonist for Y2R (BIIE0246, 20ug/0.1ml/10g body weight, dissolved in 10% DMSO of saline, i.p.) will be injected into these mice. The GLP-1R knockout mice and their wild type littermates will be obtained from Dr. J. Drucker.

10.4. **Justification of Animal Numbers**: Justify the method used to determine the number of animals used for the experiments described in Section 10.3. This could include statistical power analysis, numbers necessary as a continual source of tissue harvest for ongoing work, prior experience with similar experiments or a limited number for pilot or feasibility studies. Numbers described here should coincide with Sections 5 and 10.3.

The number of animals used in each experiment is based on a power analysis and on the investigator's experience. The standard deviation of the different measurements determines the number of animals to be used in the above experiments and the standard deviation varies with different measurements. To provide adequate tissue and blood samples for measurements of food intake, blood hormone, and gut peptide/brain neuropeptide gene expression, 10-25 rats and 20 to 25 mice are required. Additionally, the number of animals needed may be reduced as adequate information from preliminary experiments and breeding numbers are achieved.

10.5. **Non-Surgical Procedures**: List and describe each non-surgical procedure to which any animal or group of animals may be subjected. Indicate, where appropriate, analgesic, anesthetic, and/or tranquilizing agents that will be used to minimize discomfort and pain.

1. Food intake and body weight measurements: During the defined experimental periods within the study protocol, food intakes and body weight will be measured either daily or every 2-3 days. Food and tap water will be provided ad libitum. Food cups with experimental diets (table 1) will be prepared and provided by investigator. We anticipate very little stress under these situations.

2. Over night fasting: For CCK feeding suppression and gut transit time, the rats will be fasted overnight. Food cups will be removed at the start of the dark cycle and returned on the following morning and water will be available at all times.

3. Measurement of gut transit time: Each rat will be placed in a metabolism cage with free access to water and fasted for 12 hours before feeding a bolus (2.5 g) of the appropriate experimental diet mixed with glass beads (10 mg; diameter range 150-

170um). After one hour and rats will be given free access to food again and will remain in the metabolism cages for 48 h after feeding for collection of fecal samples. The size of the glass beads should not interfere with digestion or cause stress. The rats will be returned to their normal cages after the procedure.

4. Intraperitoneal injection:

Y_2R/GLP-1R antagonists, saline, and CCK will be intraperitoneally injected into animals. The injection will be carried by an experienced investigator to reduce the transitional discomfort of each animal.

5. Visceral afferent lesion by capsaicin injections: The neurotoxin capsaicin will be i.p. injected to rats in experiment 3 to lesion their visceral afferent. The entire procedure is carried out under deep Isoflurane anesthesia (induction concentration of 4 -5% isoflurane with 20% oxygen and a 1 L/min flow rate, maintenance concentration of 2% isoflurane). The capsaicin will be injected consecutively for three days with increased dosages as described in Experiment 3. The first and second injections typically result in temporary respiratory arrest, but breathing will restart spontaneously or with the help of gentle chest massage. Ten minutes after resumption of spontaneous breathing, anesthesia is discontinued and animal is allowed to recover.

6. Body fat measurement by NMR: We observed difference in body weight for resistant starch fed and control fed groups in mice studies. Body fat will be measured by NMR for all animals before the end of experiments. The mice will be handled daily before this measurement to reduce stress effects during this procedure. We anticipate little stress effects under such situation.

7. Breeding, Weaning and genotyping: One male and 3 females will be initially obtained for each of the three homozygote mouse models ($Y_2R^{-/-}$, GLP-1R $^{-/-}$, and C57Bl/6 $^{+/+}$ control). We anticipate 2 breedings for the initial females and 1 second generation intercross breeding to produce the number of animals required for preliminary testing and experiments. Breeding animals will be placed in shoe box cages with corncob bedding and nesting material in each cage. One male will be placed with up to three

females per shoe box. Once a female is visibly pregnant, she will be single housed until after all pups are weaned. Pups will be weaned at 21 days. Male and females will separated and multi-housed in shoeboxes until reaching maturity (six weeks of age). At that time they will either be placed in shoeboxes for intercross breeding, or placed in stainless steel cages for experimental procedures. The initial breeding mice will be ear-punched for identification and tail-clipped for genotyping. For tail tip collection for genotyping, the mouse is restrained briefly and 2-3 mm of the distal tip of the tail is rapidly excised with a sterile scalpel blade or sharp scissors. Before the mouse is returned to the cage, the end of tail is touched to a silver nitrate cauterizing stick to minimize bleeding and dipped in bupivicaine/lidocaine mixture as a local anesthetic.

10.6. **Surgical Procedures:**

Does this protocol contain survival surgical procedures?

☐ YES (Complete 10.6.a-h) ☒ NO

Does this protocol contain non-survival surgical procedures?

☐ YES (Complete 10.6.a-f) ☒ NO

Does this protocol contain multiple major survival surgeries to occur on an individual animal?

☐ YES (Must provide scientific justification below.) ☒ NO

Justification for multiple major surgical procedures:

10.6.a **Surgical description:** Describe in detail each surgical procedure. Include surgical prep of animal, aseptic techniques, surgical approach, wound closure and suture removal plan.

10.6.b. **Location:** Where will surgeries be performed?

10.6.c. **Personnel:** List all personnel involved in the appropriate row below.

	Name	Lab Phone	Home Phone	Email
Surgery				

Anesthesia				
Post-op care				

10.6.d. **Anesthesia:** Complete for each anesthetic drug used.

Drug	Dose	Freq	Route of Administration
Isoflurane	4-5%	1ml/min	Inhalation

10.6.e. **Anesthetic monitoring:** Check method(s) used for anesthetic monitoring.

☐ Palpebral reflex ☒ Toe pinch withdrawal

☐ Other _____

Frequency of monitoring: Procedure last about 10 minutes, we will constant monitoring animals for 30 minutes after procedure started.

10.6.f. **Paralytic agents:** Will paralytics be used?

☐ Yes If "yes" complete table below. ☒ No

Agent	Dose	Method of Monitoring

10.6.g. **Postoperative care:** Describe postoperative monitoring and care provided.

First 24 hours:

Second 24 hours:

Thereafter:

10.6.h. **Postoperative analgesia:** Investigators should assume that procedures that cause pain in humans also cause pain in animals. Please complete table below for analgesics.

Drug	Dose	Route of Administration	Freq/Duration
bupivicaine/lidocaine mixture	once	At site of tail clip	Locally

10.7 **Euthanasia:** What method or agent will be used to euthanize the animals? Include dosage and route of administration. Also include a secondary method of euthanasia to ensure death. Methods of euthanasia that are not considered "acceptable" by the latest version of the AVMA Panel on Euthanasia require scientific justification.

CO2 for retired breeders and incorrect genotyped weanlings. Decapitation for experimental mice and rats in order to collect brain samples for measurement of neuropeptides NPY and AGRP as well as their gene transcription. Anesthesia interferes with these brain neuropeptides measurements.

Method: _____

Drug: _____

Route of administration: _____

Secondary method: _____

Who will perform euthanasia? Jun Zhou, Kathleen L McCutcheon

Justification for use of "conditionally acceptable" methods of euthanasia:

Animals will be euthanized at the end of the experiments by rapid decapitation. Due to the nature of the biochemical measurements to be performed, euthanasia of some animals must be carried out by decapitation without anesthesia. Stress and anesthesia interferes with the brain neuropeptides measurements of neuropeptides NPY and AGRP as well as their gene transcription. Individuals who have had previous training and experience with the procedure will carry out decapitation in order to minimize stress.

SECTION 11. Procedure Checklist: Check "Yes" or "No" to each of the following questions. The information you provide in this section is very important in highlighting specific points of your study that are important considerations for the IACUC in their review process.

11.1. **Restraint:** Will animals be restrained? (Restraint refers to immobilization or other restrictions to normal movement beyond momentary holding for injections, etc.)

☒ YES ☐ NO

If "yes" how? Body fat will be measured by NMR without anesthesia

For tail tip collection for genotyping, the mouse is restrained briefly and 2-3 mm of the distal tip of the tail is rapidly excised with a sterile scalpel blade or sharp scissors

How long? Less than 1 minute

Describe monitoring during restraint period. Respiratory rate

Who is responsible? Jun Zhou, Kathleen McCutcheon

11.2. **Animal Transport:** Will it be necessary to take live animals outside of Comparative Biology? If animals are removed from Comparative Biology they **cannot** return.

☐ YES ☒ NO

If "yes", justify and answer questions below.

Justification:

List rooms where animals will be taken:

How long will they be kept in laboratory? Animals cannot be held outside of Comparative

Biology for greater than 12 hours without IACUC approval.

List procedures performed on animals:

11.3. **Food and water restriction:** Will food and/or water be withheld? (Please refer to the IACUC Policy Statement on food and water restriction posted on PINE under IACUC)

☒ YES ☐ NO

If "yes" for how long? Overnight – up to 12 hours

11.4. **Blood collection:** Will blood be collected?*

☒ YES ☐ NO

If "yes":

How often? Once , at the end of experiment after decapitation

What is the maximum number of collections on a given animal? 1

What is the maximum volume per collection?	All
What collection technique will be used?	Truck blood
Who will perform collection?	Jun Zhou and Kathleen L McCutcheon

* Guidelines for blood collection. The **single** blood collection limit is 10% of the animal's estimated blood volume (6ml/100g bw), which for a mouse is 60 µl/10g of body weight and for a rat is 600 µl/100g of body weight. The **repeat** blood-sampling limit is the volume equal to 1.5% of the animal's body weight per 2 weeks. Collections exceeding these limits require scientific justification.

11.5. **Animal Breeding:** Will breeding of animals be part of this protocol?

☒ YES ☐ NO

If "yes":

What breeding scheme (pairs, trios, harem) will be used?	Pair, trio and harem
Who will be responsible for breeding animals?	Jun Zhou and Kathleen L McCutcheon

Who will be responsible for maintaining records and reporting use of animals generated

in breeding colony to Comparative Biology office?	Kathleen L McCutcheon and Jun Zhou

What will the disposition of excess offspring or animals of incorrect genotype?

Culled – CO2 if necessary. Excess mice will be used to determine dosage for experiments

11.6. **Genotyping:** Will collection of tail tips or other tissue be required for genotyping?

☒ YES ☐ NO

If "yes" describe technique and what anesthetics/analgesics will be used.

Mice will be restrained briefly and 2-3 mm of the distal tip of the tail is rapidly excised with a sterile scalpel blade or sharp scissors. Before the mouse is returned to the cage, the end of tail is touched to a silver nitrate cauterizing stick to minimize bleeding and dipped in bupivicaine/lidocaine mixture as a local anesthetic.

11.7. **Identification:** Will individual animals be identified?

☒ YES ☐ NO

If "yes" how? (e.g. ear punch, ear tag, tattoos)

Breeder mice will be ear punched for identification. Weanlings will be ear punched at weaning before genotyping

11.8. **Adverse effects:** Do you anticipate any adverse effects of the experimental procedures on the animals?

☒ YES ☐ NO

If "yes", list the possible effects (e.g., pain, discomfort, % weight loss, maximum tumor size, fever, minimum packed cell volume, etc) and how they will be monitored and addressed.

- Minimum weight loss due to overnight fasting.
- Prolonged housing in stainless steel cage, a PVC tube will be provided for resting surface and animals will be monitored when they are weighted.
- Transit pain due to tail tips and intraperitoneal injection
- Temporary respiratory arrest due to capsaicin injection. The breathing will restart spontaneously or with the help of gentle chest massage. The entire procedure is carried out under deep Isoflurane anesthesia. Ten minutes after resumption of spontaneous breathing, anesthesia is discontinued and animals are allowed to recover.

11.9. **Death as an endpoint:** Is death an endpoint in your experimental procedure? Note: *Death as an endpoint refers to experiments in which animals die as a direct result of the experimental manipulation, not due to euthanasia at the end of a study, e.g. acute toxicity testing, assessment of virulence of pathogens, neutralization tests for toxins.*

☐ YES ☒ NO

If "yes" provide scientific justification.

11.10. **Emergency treatments:** Are there emergency treatments by the Comparative Biology veterinary staff that would not be allowed?

☒ YES ☐ NO

If "yes", list treatments not allowed.

Contact investigators. Antibiotics, stress, and some medications will interfere with experiments

11.11. **Antibody production:** Will animals be used for antibody production?

☐ YES ☒ NO

If "yes", list adjuvants to be used.

For use of Complete Freund's Adjuvant provide scientific justification.

11.12. **Exotic species:** Are you using wild or exotic species for which permits are necessary?

☐ YES ☒ NO

ATTACH COPY of permits. (*Permits are required for protocol approval.)*

SECTION 12. **Animal Management:**

Check all applicable below:

CARE OF SICK ANIMALS	DISPOSAL OF DEAD ANIMALS
☒ Call Investigator	☒ Call Investigator
☐ Clinician to Treat	☐ Necropsy
☐ Euthanasia	☐ Disposal
	List any special requirements for disposal?

SECTION 13. **Disposition of Animals:** (What will be done with animals at the conclusion of the project? Check appropriate boxes.)

☒ Animals will be euthanized.
☐ Animals may or will be **TRANSFERRED** to another IACUC-approved protocol(s).

Protocol Number:

Appropriate transfer forms must be completed and submitted to the Comparative Biology Office when animals are transferred.

☐

SECTION 14. Narrative Statements: Federal regulations mandate that you provide a written narrative statement regarding whether the experiments described are unnecessarily duplicative, and whether you have considered alternatives for those procedures causing pain and distress.

14.1. Provide a narrative statement in the box below indicating that the proposed experiments do not unnecessarily duplicate previous experiments. In this statement, include sources used to make such a determination (e.g., Databases, workshops, expertise in the field, etc).

After search electronic database, we confirm that the proposed studies are novel and will not unnecessarily duplicate previous experiments.

If your source is an electronic database(s), complete the following boxes.

Date Search Completed:	04-01-2006
Database(s) Searched:	Medline
Keywords:	Resistant starch, PYY, GLP-1, knock out, receptor
Years covered in search:	1966-present

For Type B or C protocols answer 14.2 and 14.3 if you indicated Type B or C in Section 8.

14.2. Provide a narrative statement, in the box below, indicating whether or not you have considered alternatives to procedures producing more than momentary or slight pain or distress. Indicate what those alternatives were and why they are not appropriate.

We have considered alternative procedures, but none that are appropriate for test our hypothesis.

14.3 List the sources used to make the determination in 14.2. If your source is an electronic database complete the boxes below.

Sources: Electronic database

Date Search Completed:	04-01-2006
Database(s) Searched:	Medline
Keywords:	Resistant starch, PYY, GLP-1, knock out, receptor
Years covered in search:	1966-present

SECTION 15. Investigator Training: In accordance with IACUC policy, all personnel conducting animal-based research must attend a Comparative Biology/IACUC Orientation Course. The principle investigator is responsible for training for skills specific to their research project. Indicate individual responsible for this training below.

List all persons involved in animal care and use for this study below.

Name	Training and Experience?	If no, who will do training?
Jun Zhou	☒ Y ☐ N	
Kathleen L McCutcheon	☒ Y ☐ N	
Anne Raggio	☒ Y ☐ N	

NOTE: All personnel must complete Comparative Biology's training course in order to have access to Comparative Biology.

APPENDIX B:

ABBREVIATIONS

AIN-93G	American Institute of Nutrition- 93 Growing diet
BMI	Body mass index
CVD	Cardiovascular disease
EC	Energy control
GIP	Gastric inhibitory peptide
GLP-1	Glucagon-like peptide-1
HDL	High-density lipoprotein
HF	High fat
HRT	Hormone replacement therapy
IOM	Institute of Medicine
LDL	Low-density lipoprotein
LF	Low fat
NF- κB	Nuclear factor-kappa B
NMR	Nuclear magnetic resonance
NPY	Neuropeptide Y
OVX (OV)	Ovariectomized
PYY	Peptide YY
RER	Respiratory exchange ratio
RS	Resistant starch

RQ	Respiratory quotient
SCFA	Short-chain fatty acid
SH	Sham operation
WAT	White adipose tissue

APPENDIX C:

DEFINITIONS

Body Mass Index (BMI)	mass (kg)/height^2 (m^2)
Cecum	the beginning part of the large intestine that receives waste material from the small intestine
Glucagon-like Peptide-1 (GLP-1)	gastrointestinal peptide and hormone that acts in inhibiting food intake
Obesity	having a BMI of ≥30
Overweight	having a BMI of 25-29.9
Peptide YY (PYY)	gastrointestinal peptide and hormone known for its anorectic properties
Respiratory Exchange Ratio (RER)	ratio between CO2/%O2 used to indicate which fuel is being oxidized for energy production in the body
Resistant starch (RS)	a type of starch that is resistant to the effects of digestive enzymes and is not digested in the small intestine, but fermented by microflora in the large intestine
Respiratory Quotient (RQ)	an indicator of which fuel (carbohydrate or fat) is being metabolized to supply the body with energy at the cellular level
Short-Chain Fatty Acids (SCFA)	end-product produced by fermentation of resistant starch in the large intestine

VITA

Julina Robert was born in Metairie, Louisiana, September, 1983 to parents Juliana Chan and Richard Robert. She graduated from Ponchatoula High School in Ponchatoula, Louisiana, as a valedictorian in May of 2001. Julina received her Bachelor of Science degree in human ecology with a concentration in dietetics from Louisiana State University Agricultural and Mechanical College, Baton Rouge, Louisiana, in December of 2005. She began graduate school in January of 2006 from Louisiana State University Agricultural and Mechanical College. During her career as a graduate student, she worked as a Graduate Teaching Assistant for two years, presented her thesis research as a poster at Experimental Biology 2008 Conference in San Diego, California, and has become a member of the American Dietetic Association. Julina plans to graduate with her master's in human ecology with a concentration in molecular nutrition. Upon completing her master's program, Julina plans to enter a dietetic internship program and pursue a career as a Registered Dietitian.