OBESITY MANAGEMENT USING DIACYLGLYCEROL AND LOW GLYCEMIC INDEX STARCH IN DOGS

A Dissertation

by

DAISUKE NAGAOKA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Nutrition

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Approved by:

Chair of Committee,	John E. Bauer
Committee Members,	Edward D. Harris
	Stephen B. Smith
	Debra L. Zoran
Chair of Intercollegiate Faculty,	Stephen B. Smith

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ABSTRACT

Obesity Management Using Diacylglycerol and Low Glycemic Index Starch in Dogs.

(May 2008)

Daisuke Nagaoka, B.V.Sc., Nihon Veterinary and Animal Science University;

Ph.D., Nihon University

Chair of Advisory Committee: Dr. John E. Bauer

Obesity is the most common nutritional disorder in small animal medicine and is closely related to the mortality and morbidity of various diseases. Decreasing the incidence of obesity is considered to be the most important way to maintain health, prevent disease, and contribute to longevity. Diet therapy using low glycemic index starch (LGIS) and diacylglycerol (DAG) may thus be a reasonable obesity management tool without unnecessary food restriction, forced physical activity, and impairment of health. Beagles were prepared for a weight loss study by inducing obesity using a high caloric/human snack food combination. These obese dogs were then fed diets containing either LGIS/HGIS and DAG/TAG for a 10 wk weight loss period. The LGIS groups lost more weight than the high glycemic index starch (HGIS) groups (2% vs 1% per wk) due to lower total diet digestibilities. Even though the dogs had consumed similar amounts of the diets on a weight basis, the amounts of metabolizable energy (ME) ingested overall differed between the two starch types. Diet effects were found for plasma triglyceride (TG) at both wk 1 and 8. Post-prandial TG lowering was observed only

with a LGIS/DAG diet combination. LGIS groups showed less decreased post-prandial non esterified fatty acid (NEFA) concentrations compared with HGIS groups at both wk 1 and 8. At both wk 1 and 8, plasma insulin was significantly lower in the LGIS groups although glucose concentrations were similar among all groups. Plasma gastric inhibitory polypeptide (GIP) increased in all groups but tended to be lower in the LGIS groups. Significant time effects were seen in glucagon-like peptide-1 (GLP-1) at both wk 1 and 8; however, diet effects were not observed. Plasma adiponectin concentrations were significantly higher in the LGIS/DAG group vs. all other diet groups. Significantly lower plasma leptin concentrations were found, especially in the LGIS/DAG group. Combinations of LGI starch and oils decreased uncoupling protein-2 (UCP2) mRNA gene expression in the small intestine compared with the combinations of HGI starch and oils. These findings indicate that the LGIS/DAG combination beneficially supports more efficient and healthy weight loss in dogs along with improvement in biochemical and hormonal biomarkers. This combination may be preferred for healthy canine weight loss and to help prevent obesity related diseases.

DEDICATION

To my grandmother, parents, and brother.

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First of all, I would like to thank my committee chair, Dr. John E. Bauer, for his guidance and support throughout the course of this research. It would have been impossible for me to complete this Ph.D. degree without his invaluable and continuous help. I also would like to thank the members of my graduate committee, Dr. Edward D. Harris, Dr. Stephen B. Smith, and Dr. Debra L. Zoran for their guidance and support both as my advisers and as classroom instructors. Thanks also goes to my friends and colleagues, Karen Bigley, Yuka Mitsuhashi, and Luciano Trevizan. Their friendship has kept me going everyday. I also would like to show my gratitude to Dr. Katsumi Ishioka for his advice and help, and also would like to extend my gratitude to the Kao Corporation (Tokyo, Japan) which provided the bioelectrical impedance study instrument and the DAG, TAG and starches used. As to leptin analysis, this work was supported in part by the Morinaga Institute of Biological Science, Inc. (Yokohama, Japan). As to adiponectin analysis, this work was supported in part by 'Academic Frontier' Project for Private Universities: matching funding from Ministry of Education, Culture, Sports, Sciences and Technology of Japan (MEXT), 2005 - 2009.

Finally, I would like to say thanks to my grandmother, parents, brother, and my friends in my country. I'll be back there soon.

NOMENCLATURE

BCS	Body Condition Score
BF	Body Fat
BW	Body Weight
DAG	Diacylglycerol
GIP	Gastric Inhibitory Polypeptide
GLP-1	Glucagon-like peptide-1
HGIS	High Glycemic Index Starch
LGIS	Low Glycemic Index Starch
ME	Metabolizable Energy
NEFA	Non Esterified Fatty Acid
NFE	Nitrogen-Free Extract
TAG (TG)	Triacylglyceride
UCP	Uncoupling Protein

TABLE OF CONTENTS

Page

ABSTRACT iii

DEDICATION v

ACKNOWLEDGEMENTS vi

NOMENCLATURE vii

TABLE OF CONTENTS viii

LIST OF FIGURES x

LIST OF TABLES xi

CHAPTER

I INTRODUCTION 1

Significance of Diacylglycerol Oil 2
Significance of Low Glycemic Index Starch 8
Preliminary Study 11
Objectives and Hypothesis 13

II INDUCTION AND RE-INDUCTION OF OBESITY 14

Introduction 14
Materials and Methods 14
Results 16
Discussion 22

III LIPID METABOLIC AND HORMONAL ALTERATIONS WITH DIACYLGLYCEROL AND LOW GLYCEMIC INDEX STARCH DURING CANINE WEIGHT LOSS 25

Introduction 25
Materials and Methods 28

CHAPTER Page

Results 36
Discussion 55

IV UCP2 GENE EXPRESSION IN CANINE DUODENAL EPITHELIUM 61

Introduction 61
Materials and Methods 63
Results 67
Discussion 69

V SUMMARY AND CONCLUSIONS 73

LITERATURE CITED 79

APPENDIX A-I 88

APPENDIX A-II 89

APPENDIX A-III 90

APPENDIX A-IV 91

APPENDIX A-V 92

VITA 93

LIST OF FIGURES

FIGURE		Page
1	BW, BCS, and kcal/day consumption in obesity induction period	17
2	BW, BCS, and kcal/day consumption in obesity re-induction period	20
3	Food efficiency between obesity induction and re-induction periods	21
4	BW changes during weight loss period	37
5	Comparison of BW changes vs. kcal consumption (A and B)	38
6	Mass and kcal amounts of diets consumed	40
7	Digestibilities of each treatment diet	41
8	Post-prandial plasma glucose concentrations	43
9	Post-prandial plasma TG concentrations	44
10	Post-prandial plasma NEFA concentrations	45
11	Post-prandial plasma insulin concentrations	47
12	Post-prandial plasma GIP concentrations	48
13	Correlations between plasma insulin and GIP	49
14	Post-prandial plasma GLP-1 concentrations	51
15	Fasting plasma adiponectin and leptin concentrations	52
16	Correlations between plasma adiponectin/leptin and BW	53
17	Correlations between plasma adiponectin and leptin	54
18	UCP2 gene expression in the duodenal epithelium	68

LIST OF TABLES

TABLE		Page
1	Summary of BW, BCS, and percent body fat values during obesity induction/re-induction periods	18
2	Compositions of treatment diets	31
3	Dry matter compositions of treatment diets	32

CHAPTER I

INTRODUCTION

Obesity is a well-known, growing global problem in humans (Kopelman, 2000) and, as in humans, the incidence of obesity in the companion animal population is increasing. Obesity is usually defined as an accumulation of excessive amount of adipose tissue in the body. Cats and dogs are considered overweight when their body weight is more than 15% above their optimal body weight, and obese when their body weight exceeds 30% of optimal body weight (Burkholder and Toll, 2000). It is estimated that 40 to 50% of dogs and 20% of cats seen by veterinarians are overweight and that 25% of dogs and 5% of cats are obese (Bauer, 2005). In the United States, it is estimated that 24 to 30% of the pet population seen by veterinarians is overweight or obese (Remillard, 2005). Studies from various parts of the world have estimated the incidence of obesity in the dog population to be between 22 and 40% (McGreevy et al., 2005). In view of these data, a moderate estimate is that 25% of dogs are obese.

Obesity is the most common nutritional disorder in small animal medicine and it can be primary or secondary. Secondary causes of obesity include hypothyroidism, hyperadrenocorticism, or insulinomas (Remillard, 2005). However, the primary cause of obesity is simply defined as a disequilibrium between energy uptake and expenditure.

This dissertation follows the style and format of the Journal of Animal Science.

Obesity is also closely related to the rate of occurrence of various diseases. German (2006) recently summarized numerous diseases reported to be associated with obesity in companion animals. It included metabolic abnormalities, endocrinopathies, orthopedic disorders, cardiorespiratory disease, urogenital disorder, neoplasia, and functional alterations (German, 2006).

In view of the comprehensive nature of obesity related disorders, decreasing the incidence of obesity is considered to be a most important way to maintain health, prevent disease, and contribute to longevity.

In general, recommendations for obesity management are to decrease calorie intake and increase energy expenditure to promote weight loss (Laflamme, 2006). Several exercise strategies in dogs have been suggested including lead walking, swimming, hydrotherapy, and treadmills (German, 2006). However, like humans, dogs and cats are vulnerable to hunger stress and dietary restriction may increase such stress in the animal as well as their owners. One recent focus in companion animal nutrition has been to alter macronutrient composition including protein, carbohydrates, or fat type and amounts and to study the impact such modifications have on animal obesity prevention and therapy (Butterwick and Hawthorne, 1998; Delzenne and Kok, 1999; Hill et al., 2006; Streiff et al., 2002). In addition to increasing physical activities, dietary therapy can be a best solution for obesity management.

Significance of Diacylglycerol Oil

Diet therapy using dietary diacylglycerol containing vegetable oils (**DAG**), especially 1,3-DAG, have been studied in recent years and may be a reasonable obesity management tool for humans (Maki et al., 2002). DAG are naturally occurring

components of edible vegetable oils. They have distinct characteristics from the triacylglycerols (**TAG**) that are typically found in such oils. As such, DAG may produce beneficial effects separate from TAG on post-prandial lipemia, insulin response to feeding, and obesity prevention and therapy. DAG oil is similar in taste, appearance, and fatty acid composition to other oils including rapeseed, soybean, and safflower oil (Takase et al., 2005). In general, DAG appears to have two broad-based health benefits: Suppression of post-prandial serum triglyceride elevations and suppression of body fat accumulation (Yasukawa and Katsuragi, 2004). A potential explanation for these benefits is that the metabolic characteristics of 1,3-DAG are less likely to become stored as body fat due to increased β-oxidation of their component fatty acid and then less efficient re-esterification in tissue cells.

TAG, a primary component of normal edible oil, is absorbed in the small intestine as a form of 2-monoacylglycerol (**MAG**), resynthesized to TAG, and incorporated into chylomicrons, which are released into the intestinal lymph and then enter the blood stream. By pancreatic lipase, fatty acids from the *sn*-1 and *sn*-3 position of TAG are hydrolyzed, resulting in the breakdown of TAG into 1,2-(2,3-) DAG, which can be further hydrolyzed into *sn*-2 MAG and free fatty acid (**FFA**) (Lykidis et al., 1995). In the intestinal epithelial cells, FFAs are bound to the *sn*-1 and *sn*-3 positions of 2-MAG via the 2-MAG pathway (Friedman and Nylund, 1980). TAG have been shown to be resynthesized via the MAG pathway through two steps of enzyme action, the acylation of 2-MAG and the subsequent acylation of 1,2-DAG (Kondo et al., 2003). Monoacylglycerol acyltransferase (**MGAT**) and diacylglycerol acyltransferase (**DGAT**) are the key enzymes for chylomicron TAG resynthesis. The microsomal enzyme,

DGAT, is especially important in that it catalyzes the last step in the synthesis of TAG and has higher substrate specificity for 1,2-DAG compared with 1,3-DAG (Watanabe and Tokimitsu, 2004).

By contrast, 1,3-DAG is digested as 1-MAG and is poorly re-esterified to TAG. It is believed that 1,3-DAG is not utilized extensively by DGAT as a substrate for chylomicron triacylglycerol synthesis because DGAT generally resynthesizes chylomicron triglycerides using 1,2-DAG as the substrate. This factor may be one reason for the suppression of post-prandial triglyceride elevations (Kondo et al., 2003) seen with DAG enriched oils. Therefore, including DAG as a part of a total diet may support decreased re-synthesis of TAG and delay its release as lymph chylomicrons (Yasukawa and Katsuragi, 2004).

Effects of DAG on energy metabolism have also been reported. Dietary DAG intake increased mRNA expression and increased the activity of beta-oxidation-related enzymes in the small intestine and the liver (Murase et al., 2001; Murase et al., 2002). These authors also reported that the mRNA expression of uncoupling proteins increased in the liver and small intestine (Murase et al., 2001; Murase et al., 2002). Energy expenditure enhancement (Kimura et al., 2003) and respiratory quotient decrement (Kimura et al., 2003; Kamphuis et al., 2003) were also noted when dietary DAG was ingested.

Although several beneficial effects of DAG in humans and rodents have been reported, only a few studies regarding DAG in dogs and cats have been reported to date (Umeda et al., 2006; Bauer et al., 2006). Thus additional information is needed in order

to more fully evaluate DAG containing oils as a useful component of companion animal diets.

In the view of safety aspects, several reports exist concerning the long-term consumption of DAG in humans (Yasunaga et al., 2004), rodents (Soni et al., 2001; Kasamatsu et al., 2005), and dogs (Chengelis et al., 2006). A chronic study of diacylglycerol oil in rats showed no compound-related effects on clinical signs, body weights, food consumption, cumulative survival rates, hematology, blood chemistry, urinalysis, organ weights, or on microscopic non-neoplastic changes (Soni et al., 2001). Even though epithelial mammary gland neoplasms were observed in the control groups, these changes were not considered biologically significant and the neoplastic findings were not dose related (Soni et al., 2001). The authors revealed that no toxicologically significant or treatment-related effects of diacylglycerol oil consumption at levels of up to 5.3% in the diet were seen during a two-year chronic rat study.

Studies in humans using double-blind clinically controlled experiments investigated the consumption of high-dose dietary diacylglycerol oil compared with triacylglycerol control oil (Yasunaga et al., 2004). A 12 wk clinical study revealed that there was no significant difference in the occurrence of clinical signs and physical complaints related to test oil consumption. Although some statistically significant effects were reported in hematological and serum chemistry parameters in both DAG and TAG groups, none of these reported changes were considered biologically significant. The authors concluded that no significant or treatment-related adverse effects of DAG oil consumed at a dose of 0.5 g/kg of BW/d as part of a normal diet were seen.

Another safety study was conducted using rats, mice, and cultured Chinese hamster lung cells (Kasamatsu et al., 2005). In their study, DAG oil, both before and after heating did not show any genotoxic effect. They concluded that there are no safety concerns on the genotoxicity of DAG oil under the conditions of normal use.

Chronic dietary toxicity of DAG in beagles has also been studied (Chengelis et al., 2006). The results indicated that the clinical condition of the animals, body weights, body weight gains, food consumption, hematology, urinalysis parameters, and serum chemistry were not affected by DAG. Electrocardiogram, histopathologic findings, and changes in organ weights were also unaffected by DAG diet compared with TAG control diet. The authors concluded that DAG at dietary concentrations up to 9.5% for one year had no effect on normal canine growth and development compared with TAG. From these safety awareness reports, several authors proved that long-term consumption of diacylglycerol oil as part of diet is safe and worth trying.

Studies in mice and humans

Previous studies in obesity prone mice have shown that feeding a diet high in DAG compared to TAG resulted in significant decreases in body weight and lower feed efficiencies even though energy intakes were not different (Murase et al., 2001). Because absorption rates of dietary DAG are not different from TAG (Taguchi et al., 2001; Taguchi et al., 2003), it is unlikely that a difference in energy intake or absorption caused the observed differences. In addition, when DAG was fed, fat pad weights were less after 5 months of feeding and less fat accumulation was found in all four anatomical areas examined; epididymal, mesenteric, retroperitoneal, and perirenal. Other results of this study observed that structural differences between DAG and TAG markedly affected the serum lipid profiles. Also, lower hyperinsulinemic responses and lower elevation of serum leptin concentrations were noted.

Studies in humans using double-blind clinically controlled experiments investigated post-prandial lipemia in healthy men after the ingestion of test emulsions containing either DAG or TAG. Serum TAG concentrations increased significantly relative to fasting in both groups. However, when relative amounts of post-prandial hypertriglyceridemia were compared by calculating the areas under the serum TAG curves over a 6 h period, it was found that mean DAG values were significantly less then that of the TAG group. Post-prandial concentrations of serum cholesterol, insulin, and glucose were unchanged in this study (Taguchi et al., 2000).

Studies in dogs

Studies in dogs to date have included evaluation of DAG feeding in a group of privately owned obese dogs in their homes (personal Kao communication). After a diet run-in period of 4 wk using a common diet, 11 dogs were fed a TAG enriched complete and balanced diet while 10 were fed the same diet supplemented with DAG for an 8 wk period (unpublished). The test diet contained a total fat content of 15% as-fed. At the end of an 8 wk period, it was found that the DAG group lost a significant amount of weight compared to TAG of approximately 0.5% of their starting body weight per week. The owners had been instructed to feed their dogs the same volume of food as usual, the same treats as usual, and exercise as usual. Thus the weight loss appears to have been attributed to the DAG containing diet.

A second study was conducted using obese dogs (BCS > 4/5) in a kennel environment and employed TAG and DAG oil containing diets (Umeda et al., 2006). 26 dogs were evenly divided and were fed either DAG or TAG diet, both at isocaloric amounts based on the amount of energy needed to maintain their overweight condition. Diet compositions incuding fatty acid profiles and digestibilities of the diets were the same with the only difference being oil type; ca. 38% DAG in the DAG diet and ca. 7% DAG in the TAG Diet. Statistically significant reductions in body weight and body fat

mass was observed during a 6 wk dietary period which averaged approximately 0.5% of initial body weight lost per week. There were no significant differences in digestibility and average calorie intake. Serum TG and cholesterol levels were significantly decreased in the DAG group and increases in β-hydroxybutyrate were noted consistent with previous findings in dogs and other species.

Significance of Low Glycemic Index Starch

Compared to studies with DAG, many investigation have been conducted using different types of glycemic index starches in humans, small, and large animals.

Starch is a primary energy source for omnivorous animals (Guilbot and Mercier, 1985) and can be separated into four distinct groups such as absorbable, digestible, fermentable, and nonfermentable, depending on their degree of polymerization and digestibility (Nantel, 1999). Starch is a nonstructural plant storage polysaccharide (Englyst, 1989) and is the main carbohydrate in cereal grains (Bach-Knudsen, 1997). Dietary starch is composed of amylose (linear glucose chain with α-1,4-glucosidic linkages) and amylopectin (branched glucose polymer containing both α-1,4- and α-1,6-linkages).

Starch digestibility is affected not only by processing but also by plant species. High-amylose corn (low glycemic index, **LGI**) starch is a starch from amylomaize corn that has an amylose content higher than 50%. High-amylose corn is grown exclusively for wet-milling. Usually, high amylose corn starch is used in textiles, candies and adhesives (Thomison, 2004). Waxy corn (high glycemic index, **HGI**) is a starch variant of normal corn and usually used by wet-corn millers to produce waxy cornstarch which is utilized by the food industry as a stabilizer/thickener and in the paper industry as an

adhesive. Waxy corn contains 100% amylopectin whereas normal corn contains 75% amylopectin and 25% amylose (Thomison, 2004).

Nguyen et al. (1998) reported that postprandial hyperglycemia and insulin secretion depend on the ratio of amylose to amylopectin consumed. These authors found a positive linear correlation between the starch content of foods and postprandial plasma glucose and insulin response curves, demonstrating that the amount of starch consumed from different complete diets is also a major determinant of the glycemic response of healthy adult beagles (Nguyen et al., 1998).

Studies in normal and diabetic Sprague-Dawley rats found that plasma triglycerides were not significantly affected by the type of starch (Kabir et al., 1998). Also, adipose tissue and liver lipoprotein lipase (**LPL**) activities were not modified by the type of starch in the diet. In diabetic rats, a waxy cornstarch diet induced high basal plasma insulin levels. A second study was conducted by the same authors. To evaluate the role of insulin in this phenomenon, two genes regulated by insulin [GLUT4 and phosphpenolpyruvate carboxykinase (PEPCK)] were investigated. The high glycemic index waxy cornstarch diet compared with the low glycemic index diet resulted in lower hepatic PEPCK mRNA in both normal and diabetic rats. They concluded that high glycemic index starch diet is implicated in stimulating fatty acid synthase (FAS) activity and lipogenesis and might have undesirable long-term metabolic effects (Kabir et al., 1998).

A similar study was conducted in humans investigating long-term consumption of high-amylose starch on insulin and glucose response in hyperinsulinemic subjects compared with normal controls. Areas under the curves for the insulin response was

significantly lower in all subjects after high-amylose consumption. Glucose responses in hyperinsulinemic subjects and control subjects were also similar and did not vary with the type of starch. Fasting plasma triglyceride concentrations were significantly lower in subjects who consumed the high-amylose compared with the standard-starch diet throughout the study. The authors concluded that chronic consumption of high-amylose foods (i.e. low glycemic index) normalized the insulin response of hyperinsulinemic subjects and showed a potential benefit for diabetic subjects (Behall and Howe, 1995).

It has been reported that high glycemic index starches increase body weight and can be a risk factor for diabetes (Pawlak et al., 2004). Studies in several rodents fed different glycemic index starches in addition to other nutritional components indicated that rats given high glycemic index food had more body fat and less lean body mass than those given low glycemic index food. Even though both diet groups consumed the same amount of food and gained the same amount of body weight for 7 wk, the HGI animals had developed hyperinsulinemia. The HGI group also had greater increases, over time, in the areas under the curve for blood glucose and plasma insulin, lower plasma adiponectin concentrations, and higher plasma triglyceride concentrations compared with LGI groups. Also, mice on the HGI diet had almost twice the body fat of those on the LGI diet after 9 wk. The authors concluded that consumption of a HGI diet *per se* adversely affects body composition and risk factors for diabetes and cardiovascular disease in animal models (Pawlak et al., 2004). In addition, several reports are available that indicated a HGI diet has been linked to increased risk of type 2 diabetes independent of body weight in humans (Salmeron et al., 1997a; Salmeron et al., 1997b; Ludwig, 2002).

Preliminary Study

Preliminary studies using non-obese beagles were performed in our laboratory and study results have provided some useful information on the combined effects of DAG and starch type. The protocol for this study was approved by the Texas A&M University Laboratory Animal Care Committee (AUP # 2003-177: Evaluation of dietary diacylglycerol oil on post-prandial response). All dogs were housed at the Laboratory Animal Research and Resources (LARR) facility, Texas A&M University and individually maintained in kennels according to the American Physiological Society Guidelines for Animal Research and according to guidelines set forth by Texas A&M University Care and Use Committee. Prior to entering the study, all dogs had complete blood counts, serum biochemistry profiles tests performed to assure normal clinical status. All dogs used in the study appeared to be clinically and physiologically normal. The study was conducted to evaluate the post-prandial response of a single DAG/starch containing meal in twelve adult female beagles. Each meal contained 60 g of cooked boneless chicken breast (locally purchased) mixed with 20 g of vegetable oil high in either DAG or TAG (Kao Corporation, Tokyo, Japan). Either 25 g of gelatinized waxy-corn starch (high glycemic index starch, **HGIS**) or high amylose corn starch (low glycemic index starch, **LGIS**) were also included (Nihon Shokuhin Kako Co., Ltd., Tokyo, Japan). Four meals were evaluated and their nutrient composition (percent metabolizable energy, **ME**, basis) was 16.5% ME protein, 25.3% ME NFE, and 58.3% ME fat. The post-prandial response of dogs fed each of the diets was evaluated using a crossover design with 2 wk washout periods between meals. Blood samples were collected after withholding food overnight (time 0). The meals were then fed and blood

samples were collected at times 0.5, 1, 2, 3, 4 and 6 h via jugular catheter. All individual dogs received each of the four treatment meals in random order. Plasma triglyceride (**TG**), non-esterified fatty acid (**NEFA**), glucose, and insulin were determined. Significant differences were found for both the peak and extent of hypertriglyceridemia when the DAG diets were consumed. When LGI carbohydorate was included, a peak serum non-esterified fatty acid response was seen after 2 to 3 h. HGIS/TAG resulted in a more drastic decrease of NEFA early on (30 min). Plasma NEFA also varied more widely in both TAG groups compared to DAG. Post-prandial glucose and insulin concentrations demonstrated greater peak elevations with HGIS. The area under the curves of the serum glucose and insulin responses were also greater with HGIS meals.

Based on the preliminary data, the LGIS/DAG combination lowered post-prandial TG, insulin, yielded a more consistent postprandial glucose response, and showed an increased NEFA response compared to the other diet combinations. The physiological significance of these findings remain unknown and additional studies are warranted, but these results are consistent with earlier studies.

In summary, diet therapy using DAG may be effective for the management of obesity, help prevent obesity related disease, and improve quality of life and longevity. In this sense, it is necessary and fruitful to accomplish additional functional investigations to further extend studies of LGIS/DAG combination and its effects on carbohydrate and lipid metabolism during weight loss.

Objectives and Hypothesis

The overall objective of this dissertation is to further evaluate the effect of the combination of dietary diacylglycerol and low glycemic index carbohydrate with respect to its potential to support healthy weight loss in obese dogs. Effects of triacylglycerol and high glycemic index carbohydrate were also be evaluated for comparative purposes. We hypothesized that the combination of DAG and LGI carbohydrate will support more efficient and healthy weight loss in beagles along with the improvement of selected lipid and adipose hormone biomarkers. The effect of these fat and carbohydrate types during canine weight loss on several biochemical (glucose, non-esterified fatty acids, plasma triacylglycerol) and hormonal parameters (insulin, gastric inhibitory polypeptide, glucagons-like peptide-1, adiponectin, leptin) associated with lipid and energy metabolism were thus studied. In addition, uncoupling protein-2 gene expression of small animal intestinal biopsy samples was also determined.

CHAPTER II

INDUCTION AND RE-INDUCTION OF OBESITY

Introduction

Treatment of companion animal obesity is a common nutritional problem in veterinary practice. Most treatment regimens include reduced calorie intake and increased activity, but these are fraught with frustration. A better understanding of obesity management in the pet population may come from modeling how obesity is initially induced under more controlled conditions in a colony setting. Knowledge of key features such as the number of calories, length of time, and food types leading to obesity may provide animal owners with better advice regarding nutrition interventions. In order to study the weight loss effect of LGIS and DAG in dogs, the animals were initially fed a high calorie diet in order to induce similar degrees of obesity. Commercially available dog foods in combination with human food to mimic at home snacking and diet supplementation were used for this purpose. In doing so, it was possible to follow the time course of obesity induction and better understand the occurrence of weight gain in these dogs.

Materials and Methods

Animals

The protocol for this study was approved by the Texas A&M University Laboratory Animal Care Committee (AUP # 2004-201, Evaluation of dietary DAG oil in obesity management). All dogs were housed at the Laboratory Animal Research and Resources (LARR) facility, Texas A&M University and individually maintained in kennels

according to the American Physiological Society Guidelines for Animal Research and according to guidelines set forth by Texas A&M University Care and Use Committee. Prior to entering the study, all dogs had complete blood counts and serum biochemistry profiles tests performed to assure normal clinical status. All dogs used in the study appeared to be clinically and physiologically normal.

Normal to modestly overweight (body condition scores (**BCS**) from 6 to 8 out of 9), sexually intact adult female beagles, ages 2 to 6 yr old, were used in the study (n = 9). All dogs were individually housed in approved sized kennels with 12 h light cycles, allowed free access to water and exercise, and fed free choice during the study.

Diets and feeding

All dogs were initially fed twice their daily calculated number of calories ($2\times125(BW_{kg})^{0.75}$) using a combination of dry extruded commercial pet food (Hill's Science diet, Adult Original, Hill's Pet Nutrition, Inc., Topeka, KS) and locally purchased 50/50 (w/w) blend of canola and soy bean oils (40 g/d). After 3 wk, all dogs were fed this mixture along with 5 Pecan shortbread cookies (Keebler, Inc, Elmhurst, IL) for the next 16 wk (obesity induction period: week 4 to 19). All dogs were then subjected to a weight loss regimen using the treatment diets for the next 20 wk (wk 20 to 39) (See Chapter III). After the weight loss period, obesity was re-induced (obesity re-induction period: week 40 to 56) according to the following regimen: double ration, 40 mL vegetable oil, plus 4 cookies (wk 40 to 49): double ration, 40 mL vegetable oil, plus 2 cookies (wk 49 to 52), 40 mL vegetable oil, plus 1 cookie (wk 52 to 56). During these time periods, kcal consumption, body weight, percent body fat calculated using bioelectrical impedance and BCS were monitored by same person.

Statistical analyses

Data are expressed as means ± SEM. Statistical analyses of all data were performed using repeated measures ANOVA, to test for main effects of time and diet as well as the interaction of time with diet, and significance was set at $P < 0.05$. Tukey test was used as a post-hoc test for multiple comparisons for main effects of diet, time, and diet x time interactions, at a level of significance of $P < 0.05$. All data were found to follow a Gaussian distribution at $P < 0.05$ using the Kolmogorov-Smirnov (KS) test. All statistical tests were performed using GraphPad Prism version 4.00 for Macintosh (GraphPad Software, San Diego, CA, www.graphpad.com).

Results

No effort was made to quantify the amount of exercise of each animal and some variability was observed. Only the amount of total calories offered daily was changed over time based on each animal's starting body weight (10.4 to 14.3 kg range, 12.0 kg median).

Kcal consumed, body weight, and body condition scores during the initial obesity induction period are shown in Figure 1A. A summary of body weights, BCS, percent body fat, and rate of weight gain is presented in Table 1. After the first 3 wk, BCS had decreased somewhat and body weights were not significantly different. The mean daily calorie intake averaged 1228 ± 48 kcal with ca. 2020 kcal/d offered (60% of total kcal offered). Consumption was approximately 50% over calculated maintenance needs although 2.5 times more calories than maintenance calories were offered. Thus some self-regulation of intake was observed during this initial 3 wk period.

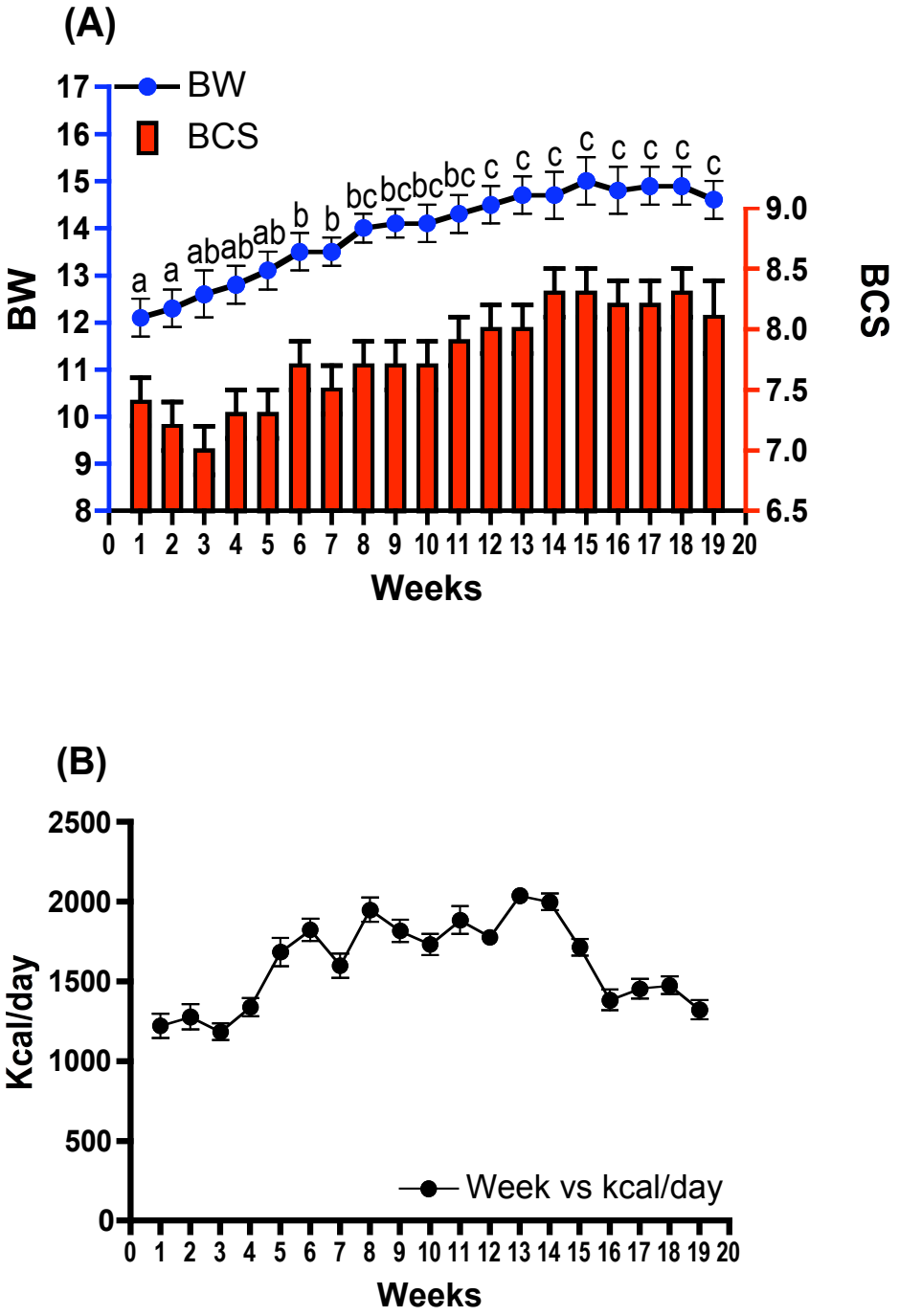

Figure 1. BW, BCS, and kcal/day consumption in obesity induction period. (A) Changes in body weight and BCS during induction period. (B) Changes in kcal/day consumption during induction period. Values are mean ± SEM. Letters not in common for a time point denote significant differences among diets by repeated measures ANOVA, $P < 0.05$.

Table 1. Summary of BW, BCS and percent body fat values during obesity induction/re-induction periods.

		Induction	Re-induction
Body weight (kg)			
	Start	12.17 ± 0.13	12.06 ± 0.33
	End	14.63 ± 0.14*	14.82 ± 0.22*
BCS			
	Start	7.44 ± 0.06	7.00 ± 0.13
	End	8.11 ± 0.10*	8.11 ± 0.07*
% body fat			
	Start	32.10 ± 0.66	34.10 ± 1.35
	End	40.20 ± 0.75*	41.00 ± 0.98**
Rate of gain (kg/wk)		0.205 ± 0.012	0.251 ± 0.006***
Number of weeks to gain BCS = 8		15	10

Percent body fat were determined by bioelectrical impedance.

* Significant difference from start, $P < 0.05$
** Significant difference from start, $P = 0.07$
*** Significant difference from induction, $P < 0.05$ (n=8)

After the cookies were added (weeks 4 to 15), mean daily calorie consumption was increased to 1820 ± 137 kcal with ca. 2470 kcal being offered (74% of total kcal offered) (Figure 1B). At wk 9, mean body weights were statistically significantly increased from those measured during wk 1 to 8. They continued to increase until wk 15 after which a plateau was reached. Similarly, BCS were significantly increased beginning at wk 9 and thereafter reaching a plateau at wk 14, $P < 0.05$. Adding the human snack food (cookies) appeared to stimulate palatability of the entire ration overall.

During weeks 16 to 19, the average daily calorie consumption decreased to 1410 ± 70 kcal (ca. 25% of the amount consumed during week 4 to 15) even though the kcal offered remained constant (ca. 2470 kcal) (Figure 1B). At this stage, the animals had reached an obese set-point (steady state) whereby they were able to maintain their obese body weights at a lower total caloric intake than that needed to achieve their obese state.

In the obesity re-induction period (week 40 to 56), increased rate of weight gain, BCS, and food efficiency became more prominent compared with the obesity induction period. At wk 42, mean body weights were statistically significantly increased from those measured during the initial 2 wk period (week 40 to 41) (Figure 2A). They continued to increase until wk 50 after which a plateau was reached. BCS were significantly increased beginning at wk 41 and thereafter reaching a plateau at wk 49 (Figure 2A). During the entire obesity re-induction period, mean daily calorie consumption was 1022.36 ± 60.62 (mean ± SEM) kcal and was not statistically significant different over all (Figure 2B).

Statistically significant food efficiency differences between induction and re-induction periods were seen (Figure 3A and 3B).

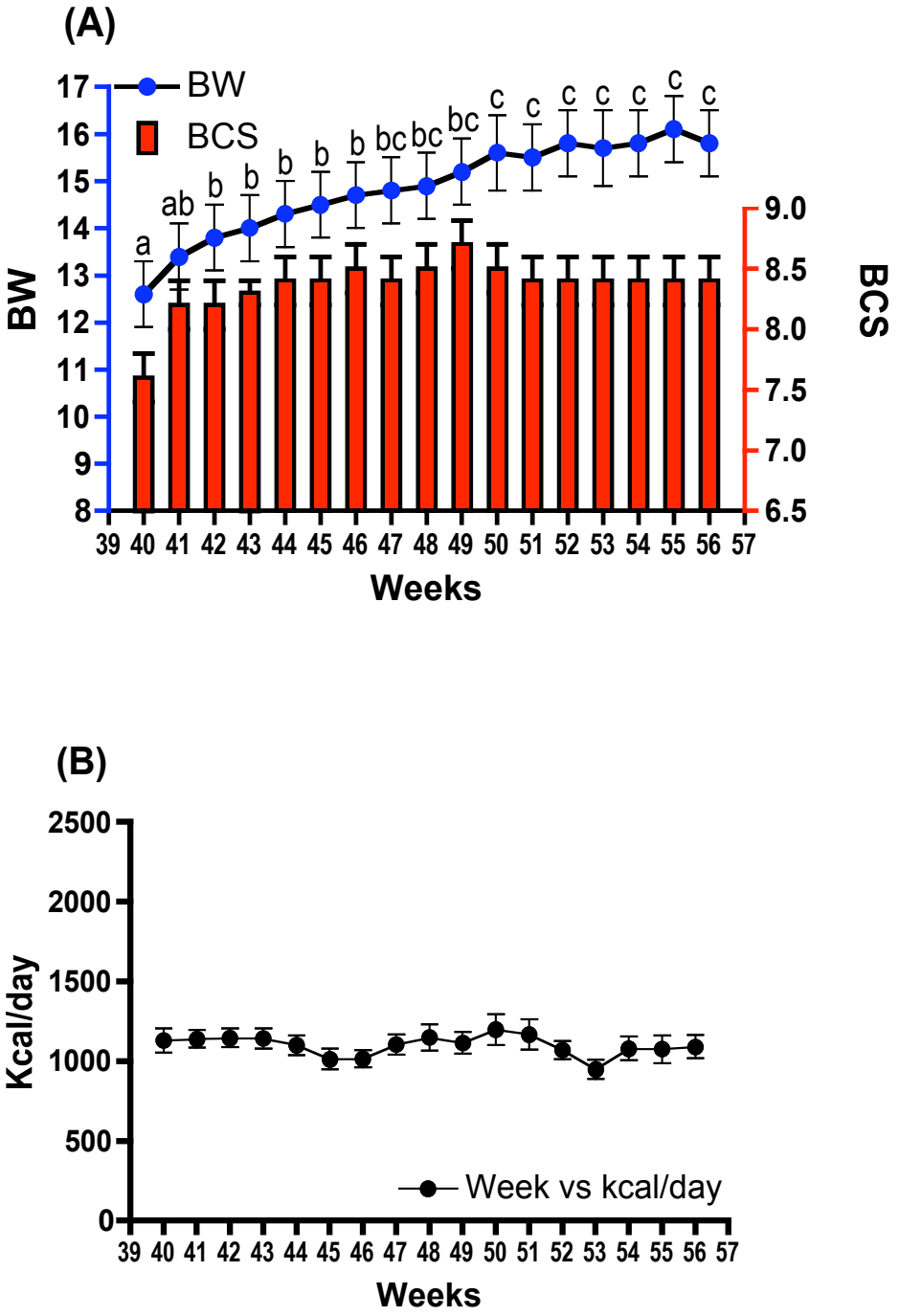

Figure 2. BW, BCS, and kcal/day consumption in obesity re-induction period. (A) Changes in body weight and BCS during re-induction period. (B) Changes in kcal/day consumption during re-induction period. Values are mean ± SEM. Letters not in common for a time point denote significant differences among diets by repeated measures ANOVA, $P < 0.05$.

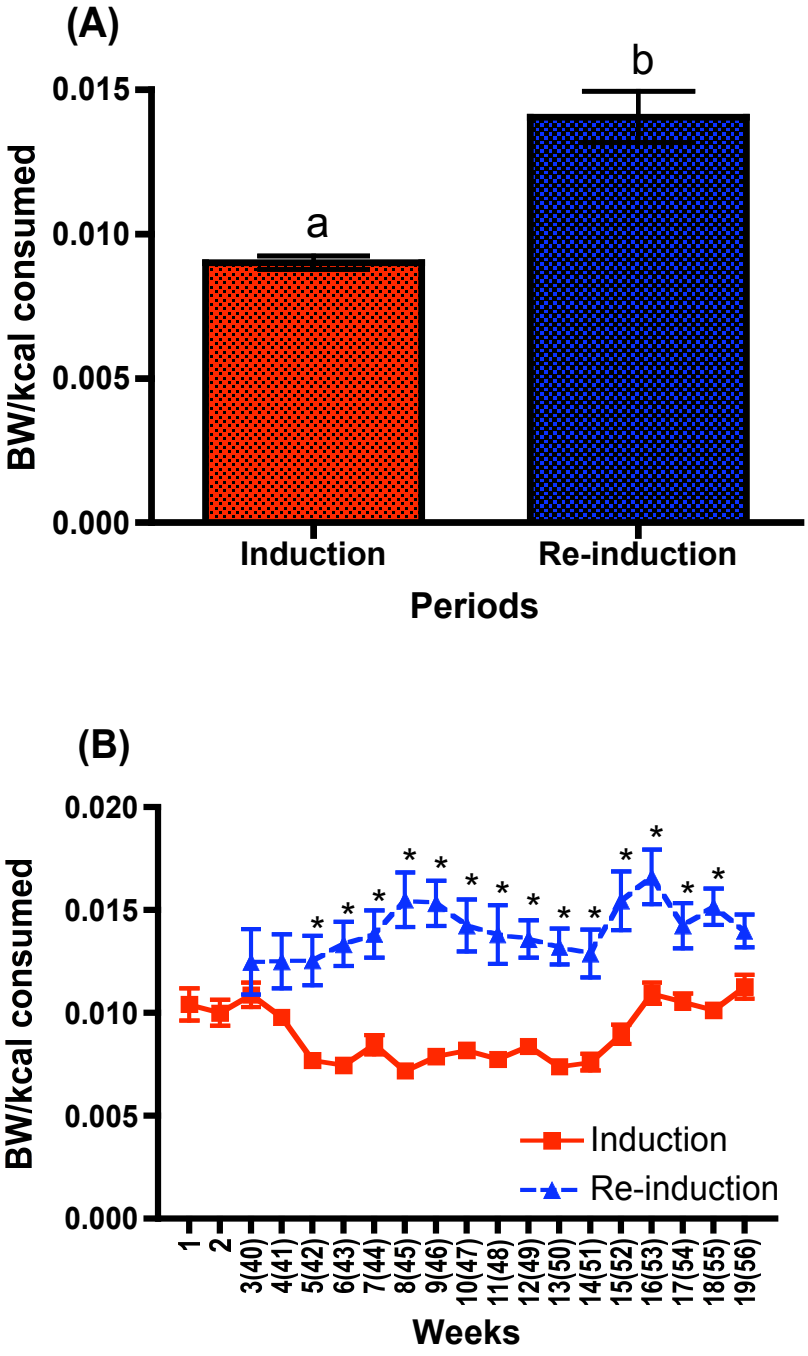

Figure 3. Food efficiency (BW / kcal consumed) between induction and re-induction periods. (A) Total mean food efficiency. Values are mean ± SEM. Letters not in common for a body weight/kcal consumption denote significant differences between period by two-tailed paired t-test, $P < 0.05$. (B) Comparison of food efficiency in each weak. Values are mean ± SEM. Asterisk denote significant differences between periods by repeated measures ANOVA, $P < 0.05$. Week values in parentheses denote the actual week of the entire study period.

Food efficiencies (body weight/kcal consumption) were: induction period, 0.009 ± 0.0002; and re-induction period, 0.014 ± 0.0009 (mean ± SEM).

With respect to re-induction of obesity using the same foods, it should be noted that fewer kcal/day were needed to re-induce the same degree of obesity in the dogs and that less time (15 week vs. 10 week, Table 1) was needed to do so. Rate of weight regain was significantly shorter than for the initial obesity induction (Table 1).

Discussion

This study investigated the relationship between rate of weight gain and calorie intake in a group of adult female beagle dogs. It has also documented the relationship between calorie consumption and regain of body weight after weight loss.

Dieting is often accompanied with repeated bouts of weight loss and regain, a phenomenon called weight cycling, also known as yo-yo dieting (Blackburn et al., 1989; Brownell, 1989; Brownell and Rodin, 1994). Several authors have suggested that weight cycling is a process of clinical importance because increased risks of morbidity and mortality may be associated with fluctuations in body weight (Ernsberger et al., 1996; Jeffery 1996). Effects of weight cycling in humans (Goldbeter, 2006) and obesity induction/re-induction animal models (Brownell et al., 1986; Contreras and Williams, 1989; Jandacek et al., 2004) have been reported. Brownell et al. (1986) reported that the weight cycled animals showed significant increases in food efficiency (weight gain/kcal food intake) during the second restriction and re-feeding periods compared to the first periods. Weight loss occurred at half the rate and regain at three times the rate in the second cycle. Weight cycled animals had a four-fold increase in food efficiency compared to obese animals of the same weight who had not cycled at the end of the

study. The authors concluded that frequent dieting may make subsequent weight loss more difficult.

Studies in Sprague-Dawley rats also revealed that three cycles of weight loss and weight regain resulted in the consumption of increased dietary fat, and increased food efficiency compared with control groups even though their body weights were similar (Contreras and Williams, 1989). Goldbetter (2006) suggested in his study that maintaining weight under a critical value should prevent weight cycling and allow body weight to stabilize.

According to the review article of Prentice et al., the authors concluded that weight cycling suppresses the growth rate of young animals and increases metabolic efficiency. It may also increase the rate of weight gain in the immediate post-restriction phases after repeated cycles (Prentice et al., 1992).

In this study, we also observed a significant increase of food efficiency in the obesity re-induction period compared with the obesity induction period. It was found that once body weight of obese dogs had been reduced, fewer calories were needed to regain the obese steady state. For that matter, the rate of weight gain was greater and at an even lower calorie intake during re-induction of obesity. These results demonstrate the effects of yo-yo dieting on energy metabolism resulting in the rapid regain of body weight after successful weight loss. Our results also suggest that metabolic “down-regulation” of energy needs with weight loss regimens continues after target weights have been achieved.

Finally, it should be noted that in this canine model of obesity, human snack foods appeared to stimulate appetite and increase the overall net calorie consumption of the

total ration. They provide a calorie dense dietary supplement leading to the rapid induction of an obese steady state that requires fewer calories to maintain. This information may help pet owners better understand the need to limit table scraps and human-type food snacks in dogs prone to overweight and obesity.

CHAPTER III

LIPID METABOLIC AND HORMONAL ALTERATIONS WITH DIACYLGLYCEROL AND LOW GLYCEMIC INDEX STARCH DURING CANINE WEIGHT LOSS

Introduction

Obesity is usually defined as an accumulation of excessive amounts of adipose tissue in the body. Cats and dogs are considered overweight when their body weights are more than 15% above their optimal body weight, and obese when their body weight exceed 30% of optimal body weight (Burkholder and Toll, 2000). Obesity, the most common nutritional disorder in small animal medicine, is associated with several metabolic diseases. Therefore, decreasing the incidence of obesity is important for animal health. Diets to help prevent or manage canine obesity should ideally reduce calorie intake while providing satiety. Increased diet fiber has been studied in canine weight loss diet formulas, but the effects of digestible carbohydrate types including high vs. low glycemic index varieties have not. Also, reduced fat formulas have been employed but less is known about the type and form of dietary fats used. Most vegetable oils contain predominately triacylglycerols with small amounts of diacylglycerols. A newly available vegetable oil has been developed containing 80 percent DAG and it has been studied in rodents and humans (Taguchi et al., 2000; Taguchi et al., 2001).

As mentioned in the preliminary study, we indicated that some lipolytic activities, lipids, and carbohydrate metabolism are altered in dogs fed a DAG and low-glycemic index starch combination (Bauer et al., 2006). To further extend this preliminary study,

and to investigate longer term weight loss, the effects of a diacylglycerol/low glycemic index starch combination diet was used to further evaluate several plasma parameters associated with obesity and weight loss.

Among the hormonal changes seen in obese vs. normal weight populations, alterations of insulin, adiponectin, and leptin have been reported and may also include changes in gastric inhibitory polypeptide (**GIP**) and glucagon-like peptide-1 (**GLP-1**) (Carlson et al., 2007; Huypens 2007; McClean et al., 2007).

Glucagon-like peptide-1 is primarily secreted from the distal small intestine and large intestine in response to intraluminal glucose and lipid. GLP-1 not only inhibits gastric acid secretion, but is also involved in the regulation of insulin secretion and glycemic control (Rehfeld, 1998; Furness et al., 1999). Gastric inhibitory polypeptide, also known as glucose-dependent insulinotropic polypeptide, is secreted primarily from the duodenum in response to intraduodenal glucose, fatty acids, and amino acids. Major roles of GIP are inhibition of gastric acid secretion and stimulation of intestinal fluid secretion. Furthermore, GIP stimulates pancreatic insulin release during hyperglycemia (Meier et al., 2002) and dose dependently stimulates the release of GLP-1 (Damholt et al., 1998). Binding of GIP and GLP-1 to its common receptor causes activation, via a stimulatory G-protein, of adenylate cyclase resulting in the formation of cAMP (Holst, 2004). Hormone sensitive lipase (**HSL**) is therefore released and lipolysis may occur. Thus, these incretin hormones are related to not only carbohydrate metabolism, but also affect lipid metabolism and may be involved in body weight regulation.

Adiponectin is an adipocytokine synthesized and secreted exclusively by adipose tissue and found at an almost three-order higher concentration in blood than other

adipocytokines (Chandran et al., 2003). It is also widely recognized that adiponectin is one of the key factors of pathogenesis of atherosclerosis, insulin resistance, and diabetes mellitus (Ouchi et al., 2001; Kondo et al., 2002). The synthesis and secretion of adiponectin is increased by activation of the nuclear receptor, PPARγ, and reduced by caloric excess, presumably associated with leptin deficiency or resistance (Saltiel, 2001). Adiponectin also activates PPARα. Once released, adiponectin increases fatty acid transport directly, stimulates its oxidation and dissipation in skeltal muscle, and reduces the levels of intramyocellular lipids, thus improving insulin signaling (Saltiel, 2001). Therefore, administration of adiponectin results in improved insulin sensitivity and glucose tolerance, and modifies hyperglycemia related to obesity. Under the obese condition, plasma adiponectin concentrations become lower, whereas plasma leptin concentrations become higher.

Leptin is a protein synthesized and secreted primarily by adipose tissue (Zhang et al., 1994). In humans and rodents, blood leptin concentration is known to positively correlate with body fat content and is higher in obesity (Maffei et al., 1995; Tasaka et al., 1997). Leptin, secreted from adipocytes via the blood stream, binds to the leptin receptor in the hypothalamus. It is considered that leptin regulates food intake to decrease appetite in a satiety center. Meanwhile, it also activates the sympathetic nervous system and increases energy expenditure (Friedman and Halaas, 1998). Under the obese condition, plasma leptin concentrations are increased (Maffei et al., 1995). Furthermore, canine leptin is confirmed to have high homology with other animal species (Ishioka et al., 2002a). Based on these data, it is believed that measuring plasma leptin can be a reliable marker to estimate the degree of obesity in dogs.

Materials and Methods

Animals

The protocol for this study was approved by the Texas A&M University Laboratory Animal Care Committee (AUP # 2004-201: Evaluation of dietary DAG oil in obesity management). All dogs were housed at the Laboratory Animal Research and Resources (LARR) facility, Texas A&M University and individually maintained in kennels according to the American Physiological Society Guidelines for Animal Research and according to guidelines set forth by Texas A&M University Care and Use Committee. Prior to entering the study, all dogs had complete blood counts and serum biochemistry profiles tests performed to assure normal clinical status. All dogs used in the study appeared to be clinically and physiologically normal.

Twelve obese, sexually intact adult female beagles, ages 2 to 6 yr old, with body condition scores of 8 to 9 on a 9 point scale, where 5 is ideal, were used in the study. All dogs were individually housed in approved sized kennels with 12 h light cycles, allowed free access to water and exercise, and fed free choice during the study.

Diets and feeding

To conduct this study, three experimental stages were used; obesity induction period (week -8 to -5), acclimation period (week -4 to -1), and experimental period (week 1 to 10). Several different diets were used during these stages; preconditioning diet (week -8 to -5), acclimation diet (week -4 to -1), and four treatment diets (week 1 to 10) suitable for each experimental stage. Each stage and its corresponding diet information is fully described in Appendix A-I and are briefly described below.

During obesity induction (week -8 to -5), all dogs were fed a combination of dry extruded commercial pet food (Hill's Science Diet, Adult Original. Hill's Pet Nutrition, Inc., Topeka, KS), a mixture (50:50 w/w) of canola and soy bean oil (40 g/d) plus 4 to 5 pecan shortbread cookies (Keebler, Inc, Elmhurst, IL) as a preconditioning diet until body condition scores of > 8/9 were attained for all dogs. During this time, calorie intake, body weight, and BCS were monitored weekly by same person to estimate the amount of energy needed to maintain their overweight condition. After this preconditioning period, the dogs were randomly divided into one of four dietary groups (n = 3 per diet group). Percent body fat content was determined using bioelectrical impedance as well as deuterium labeled water dilution techniques to determine a baseline estimate (Okawa et al., 2007). The dogs were assigned to the diet groups to minimize bias with respect to age, BCS, and body fat content.

In the acclimation period (week -4 to -1), all dogs were fed a combination of a 50/50 (w/w) blend of canola and soybean triacylglycerol oils, a 50/50 (w/w) mixture of the HGIS and LGIS starches, and sufficient amounts of water to maintain their obese condition and to accustom them to being fed the treatment diets with the same gruel-like texture. This complete and balanced diet was formulated for canine maintenance. The fatty acid profile of the acclimation diet can be found in Appendix A-II.

During the experimental period (week 1 to week 10), beginning at the end of week -1 (i.e. day 0), one dog from each group was fed its respective treatment diet using a Monday/Wednesday/Friday sequence until all dogs had begun their respective feeding periods (week 1). As noted above, four diets were evaluated: the diets were combinations of: LGIS/DAG; LGIS/TAG; HGIS/DAG; and HGIS/TAG. Ingredients used included

chicken by-product meal (Tyson Foods, Inc., Oklahoma City, OK); either high amylose corn (LGI) or waxy corn (HGI) starches that had been gelatinized (Nihon Shokuhin Kako Co., Ltd., Tokyo, Japan), either TAG or DAG enriched dietary oil (Kao Corporation, Tokyo, Japan), and appropriate mineral and vitamin pre-mixes suitable for canine maintenance (Akey, Lewisburg, OH) (Tables 2 and 3). Additional details and nutrient composition of the treatment diets are also shown in Appendix A-III. On a percent metabolizable energy basis, the diets contained approximately: 25% protein, 38% fat and 36% nitrogen-free extract (**NFE**) (Table 3). The diets were calculated using modified Atwater values to be isocaloric at approximately 4200 kcal/kg (Table 2).

On wk 1 and 8 of the feeding period, prior to collecting post-prandial blood samples, dogs were fed a post-prandial meal which was similar to the treatment diets except that boiled chicken breast meat was substituted for the chicken by-product meal yielding better palatability to assure rapid consumption. The fatty acid profile of the boiled chicken can be found in Appendix A-IV. The contents of this post-prandial meal were 80 g cooked chicken breast meat, 8 g oil (TAG or DAG) and 25 g starch (HGIS or LGIS). Additional details and nutrient composition of the post-prandial diets are shown in Appendix A-V. Food was withheld from the dogs overnight prior to time 0 blood sample collection and the post-prandial meal.

During week 3, a digestibility trial was conducted in which all feces from each dog was collected and frozen (-80°C) for 5 consecutive days, then pooled, for digestibility estimates via proximate analyses performed using a commercial laboratory (Midwest Laboratories, Inc., Omaha, Nebraska).

Table 2. Compositions of treatment diets.

	Diet			
Nutrient (g/kg)	LGIS/DAG	LGIS/TAG	HGIS/DAG	HGIS/TAG
Chicken by-product meal	430	430	430	430
DAG	135	-	135	-
TAG	-	135	-	135
High amylose corn (LGI) starch	430	430	-	-
Waxy corn (HGI) starch	-	-	430	430
Vitamins/mineral pre-mix*	5	5	5	5
Water	1730	1730	2595	2595
ME (kcal/kg)**	4300	4200	4900	4800

*Copper (Cu): 4000 ppm, Iodine (I): 560 ppm, Iron (Fe): 2.40%, Manganese (Mn): 2000 ppm, Selenium (Se): 120 ppm, Zinc (Zn): 4.32%, Vitamin A: 218 mg/kg (1600000 IU/lb), Vitamin D3: 2.95 mg/kg (260000 IU/lb), Vitamin E: 5455 (5443) mg/kg (12000 IU/lb), Vitamin B12: 1.82 mg/kg (4 mg/lb), Riboflavin: 272.7 mg/kg (600 mg/lb), d-Pantothenic acid: 1364 mg/kg (3000 mg/lb), Thiamine: 75 mg/kg (165 mg/lb), Niacin: 3182 mg/kg (7000 mg/lb), Vitamin B6: 90.9 mg/kg (200 mg/lb), Folic acid: 143.6 mg/kg (316 mg/lb), Choline chloride: 41227 mg/kg (90700 mg/lb), Choline: 35811 mg/kg (78784 mg/lb), d-Biotin: 4.5 mg/kg (10 mg/lb). *ppm = μg/g (mg/kg), vitamin A: 1 IU = 0.3 μg, vitamin D3: 40 IU = 1 μg, vitamin E: 1 IU = 1 mg.

**ME were determined from digestibility data.

Table 3. Dry matter compositions of treatment diets.

	Diet			
Diet (g/kg)*	LGIS/DAG	LGIS/TAG	HGIS/DAG	HGIS/TAG
Protein	299.5	299.5	299.5	299.5
Fat	197	197	197	197
Carbohydrate	430	430	430	430
Vitamins/Minerals	5	5	5	5
Ash	4.71	4.71	4.71	4.71
Water	1751.4	1751.4	2616.4	2616.4

*ME were calculated from digestibility data using modified Atwater values. 24.8% ME protein, 39.6% ME fat, and 35.6% ME NFE.

After the 10 wk weight loss regimen was completed (Period I), all dogs were again fed the preconditioning diet (re-induction of obesity). After 16 wk similar degrees of obesity were re-induced and all dogs were again assigned to a treatment diet exactly opposite to the one they had been fed during Period I of the study and the study design repeated (Period II). In this way, an n = 6 sample size was obtained for each diet studied. Start dates for each experimental stage were staggered to make the acquisition of samples manageable.

Post-prandial blood samples

On weeks 1 and 8 of the feeding period, jugular catheters were placed in order to conduct post-prandial blood collection. Food was withheld from the dogs overnight prior to each blood sample collection and a time 0 sample was collected. Blood samples were then collected post-prandially at the following intervals: 15, 30, 60, 120, and 180 min. Samples were placed into EDTA-containing tubes for plasma separation by low speed centrifugation (2500 rpm at 4°C for 20 min). A protease inhibitor was added to all blood samples to prevent proteolysis of small peptide hormones prior to centrifugation. 0.6 TIU/mL of blood of aprotinin (Sigma-Aldrich Co., St. Louis, MO) was added for insulin analysis. For GIP and GLP-1 analyses, 10 μL of dipeptidyl peptidase IV inhibitor (DPP-IV inhibitor: Linco Research, St. Charles, MO) was added per mL of blood because DPP IV is a primary inactivating enzyme of both GIP and GLP-1 (Kieffer et al., 1995). All plasma samples were stored frozen at -80°C until the time of analysis.

Blood samples for adiponectin and leptin analysis

On weeks 1, 4, 8 and 9 of the feeding period, fasting blood samples were collected for adiponectin and leptin analyses. Food was withheld from the dogs

overnight prior to each blood sample collection. Samples were placed into EDTA-containing tubes for plasma separation by low speed centrifugation (2500 rpm at 4°C for 20 min). A protease inhibitor, 0.6 TIU/mL of blood of aprotinin (Sigma-Aldrich Co., St. Louis, MO) was added to all blood samples to prevent proteolysis of small peptide hormones prior to centrifugation. All plasma samples were stored frozen at -80°C until the time of analysis.

Analyses

Blood samples were analyzed for the following biochemical and hormonal parameters by enzymatic, or enzyme linked assays: Plasma triglyceride, non-esterified fatty acids, insulin, gastric inhibitory polypeptide, glucagon-like peptide-1, adiponectin, and leptin. Post-prandial plasma samples were analyzed for glucose, TG, and NEFA using enzymatic and colorimetric assays. Circulating hormones, including insulin, GIP, GLP-1, adiponectin, and leptin were also determined using validated enzyme-linked immunosorbent assay (ELISA) techniques for canine samples. Mercodia Porcine Insulin ELISA (Mercodia AB, Uppsala, Sweden) was used for insulin analysis according to Bennet et al. and Sato et al. (Bennet et al., 2000; Sato et al., 2002). Gastric Inhibitory Peptide (Human) EIA Kit (Phoenix Pharmaceuticals, Inc., Belmont, CA) was appropriately validated by spiking with standard GIP and by serial dilution techniques, and used for GIP analysis. Glucagon-Like Peptide-1 (Active) ELISA Kit (Linco Research, St. Charles, MO) was used for GLP-1 analysis (Nathan et al., 1992). The GLP-1 sequence is highly conserved between species, with no sequence variation occurring at all in mammals. Plasma adiponectin concentrations were measured using the overnight fasting samples and appropriately validated ELISA methodology (Ishioka

et al., 2002a; Ishioka et al., 2006). Adiponectin (Murine/Rat) ELISA kit (Otsuka Pharmaceutical, Co. Ltd., Tokyo, Japan) was used for adiponectin analysis which was performed in Dr. Ishioka's laboratory (Nihon Veterinary and Animal Science University, Tokyo, Japan). Plasma leptin concentrations were measured using the overnight fasting samples and conducted by the procedure of Iwase et al. (2000), and appropriately validated by the procedure of Ishioka et al. (2002c) and was also performed by Dr. Ishioka.

A microplate spectrofluorometer (Gemini EM: Molecular Devices Corporation, Menlo Park, CA) and its software (Softmax Pro ver. 5.0, Molecular Devices Corporation, Sunnyvale, CA) were used to determine GLP-1 concentrations, and a kinetic microplate reader (UV max: Molecular Devices Corporation, Menlo Park, CA) was used for the other parameters.

Statistical analyses

Data are expressed as means ± SEM. Statistical analyses of all data were performed using repeated measures ANOVA, to test for main effects of time and diet as well as the interaction of time with diet, and significance was set at $P < 0.05$. Tukey test was used as a post-hoc test for multiple comparisons for main effects of diet, time, and diet x time interactions, at a level of significance of $P < 0.05$. All data were found to follow a Gaussian distribution at $P < 0.05$ using the Kolmogorov-Smirnov (KS) test. All statistical tests were performed using GraphPad Prism version 4.00 for Macintosh (GraphPad Software, San Diego, CA, www.graphpad.com).

Results

Weight loss, kcal consumption, and diet digestibility

During the acclimation period, all dogs had been fed the chicken by-product meal based, gruel-type diets containing a 50/50 (w/w) blend of canola and soybean oils and a 50/50 (w/w) blend of the HGIS and LGIS starches for 4 weeks. During this time, average body weights of each obese dog were used to estimate the number of calories needed for each dog to maintain its obese body weight (ca. 900 - 1100 kcal range) and this amount was fed during the weight loss periods. However, during the experimental period, the dogs voluntarily consumed only 68% ± 4 (SEM) % of the diet amounts offered on a weight basis. Thus, it was not unexpected that all dogs lost weight under this condition. Nonetheless, when the change in body weight was considered vs. time among the four groups, it was found that dogs fed the LGIS diets lost a larger percentage of their starting obese body weights than those fed the HGIS diets independent of oil type (Figures 4 and 5). For that matter, the LGIS diet groups lost approximately 2.0% body weight per week while the HGIS diet groups lost only 1.0%. Reasons for this difference were ascertained from the results of the digestibility studies of the diets. It was found that the total metabolizable energy of the LGIS diets was considerably lower than that of the HGIS diets (Table 2). Thus even though the dogs had consumed similar amounts of the diets on a weight basis, the amounts of ME ingested overall differed between the two starch types (Figures 6 and 7). Furthermore, the digestibility studies revealed significant differences between the two starch types and a modest, but not significant digestibility difference in protein (Figure 7). Dietary fat digestibility remained high in all diets at approximately 96%.

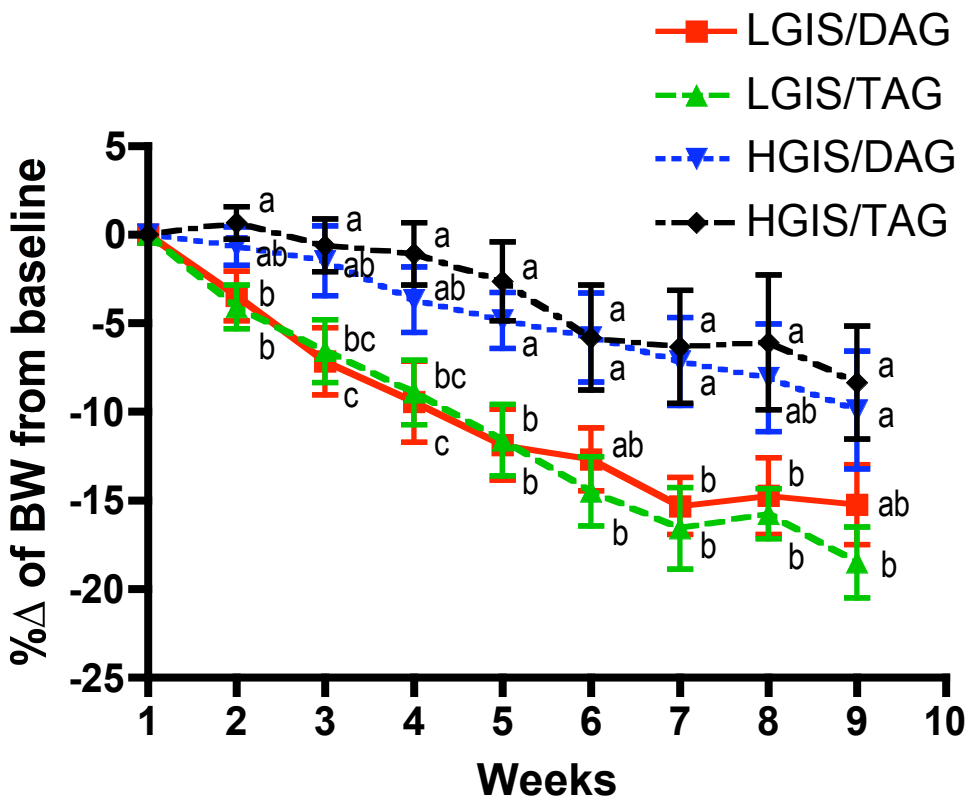

Figure 4. BW changes during weight loss period. Values are mean ± SEM. Letters not in common for a time point denote significant differences among diets by repeated measures ANOVA, $P < 0.05$.

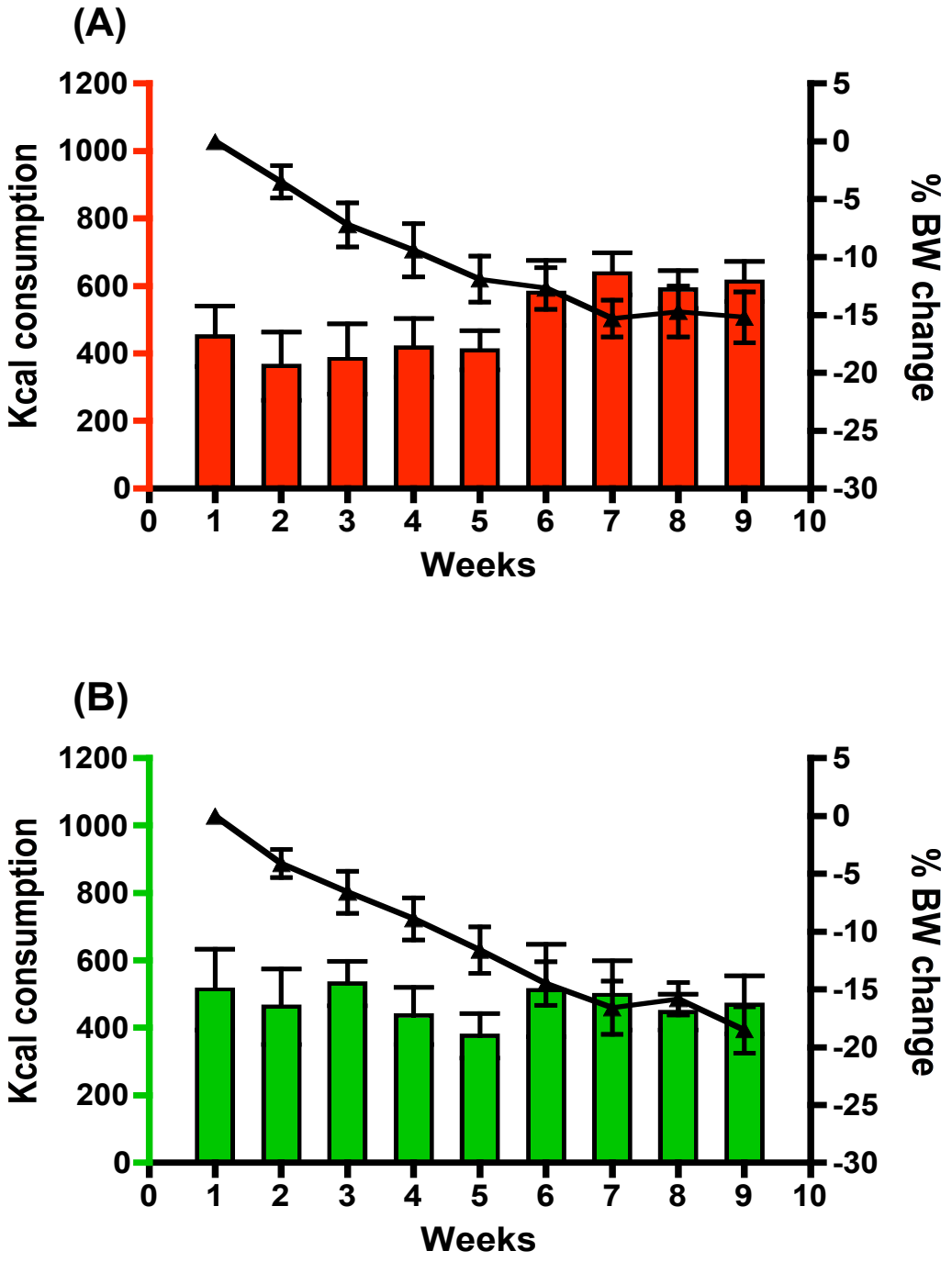

Figure 5. Comparison of BW changes vs. kcal consumption. (A) LGIS/DAG (B) LGIS/TAG. Bars (kcal consumed) and line (percent body weight change) are indicated. Values are mean ± SEM.

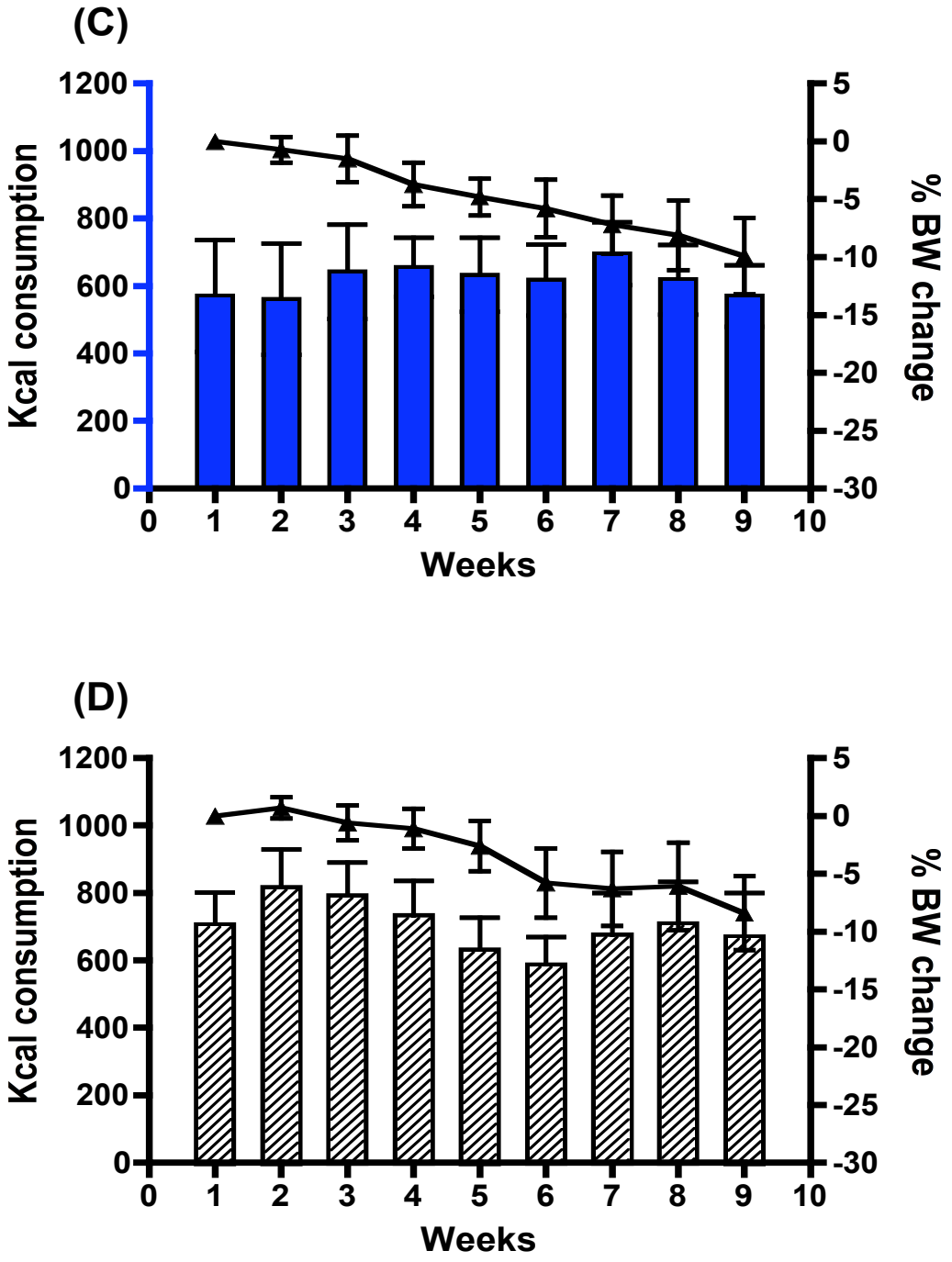

Figure 5. (continued). (C) HGIS/DAG (D) HGIS/TAG. Bars (kcal consumed) and line (percent body weight change) are indicated. Values are mean ± SEM.

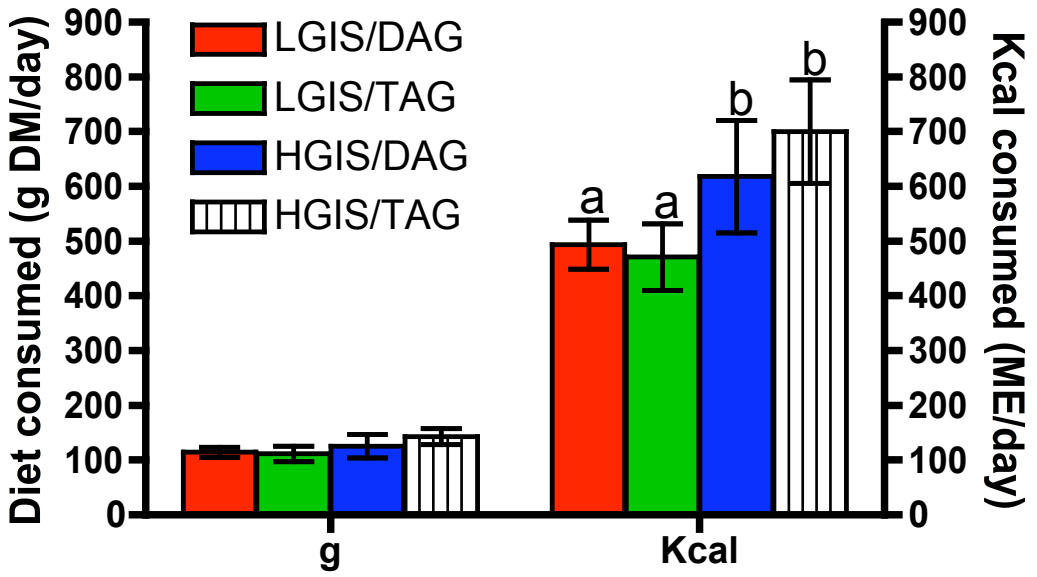

Figure 6. Mass and kcal amounts of diets consumed. Values are mean ± SEM. Letters not in common for a kcal amount denotes significant differences among diets by repeated measures ANOVA, $P < 0.05$.

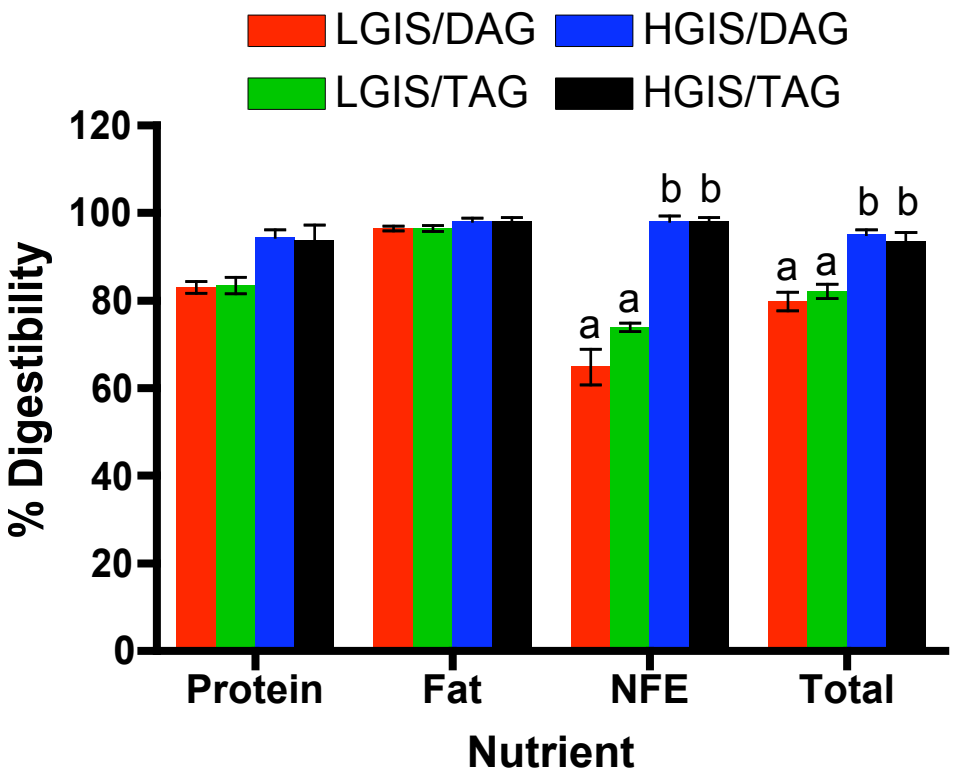

Figure 7. Digestibilities of each treatment diet. Values are mean ± SEM. Letters not in common for a nutrient denote significant differences among diets by repeated measures ANOVA, $P < 0.05$.

Total diet digestibilities were LGIS/DAG, 79.8 ± 5.0%; LGIS/TAG, 82.1 ± 4.3%; HGIS/DAG, 95.0 ± 3.0%; and HGIS/TAG, 93.7 ± 4.5% (mean ± SEM).

Plasma glucose

At both wk 1 and 8, no significant differences due to diet were seen among post-prandial plasma glucose concentrations although the expected time effect ($P < 0.05$) was noted in all groups (Figure 8).

Plasma TG

Diet effects were found for plasma triacylglycerol at both wk 1 and 8 (Figure 9). At wk 1, DAG diet groups decreased post-prandial TG concentrations compared with TAG diet groups. Peak values of TAG diet groups appeared at 180 min, while peak values of DAG diet groups tended earlier. Increased plasma TG were seen only in the LGIS/TAG diet group at 60 min. Post-prandal TG lowering was observed with only the combination of LGIS/DAG diet. The peak values of the TAG diet groups tended earlier in wk 8 than wk 1, whereas the peak values of the DAG diet groups tended later in wk 8 than wk 1.

Plasma NEFA

At both wk 1 and 8, all diet groups showed decreased post-prandial NEFA concentrations. However, the LGIS group decreased NEFA less than the HGIS groups. These differences became less pronounced at wk 8. Generally, plasma NEFA concentrations were initially depressed in all groups at both 15 and 30 min. This lowering effect was statistically significantly different when the LGIS and HGIS diet groups were compared (Figure 10) with significantly less lowering occurring when the LGIS diets were fed.

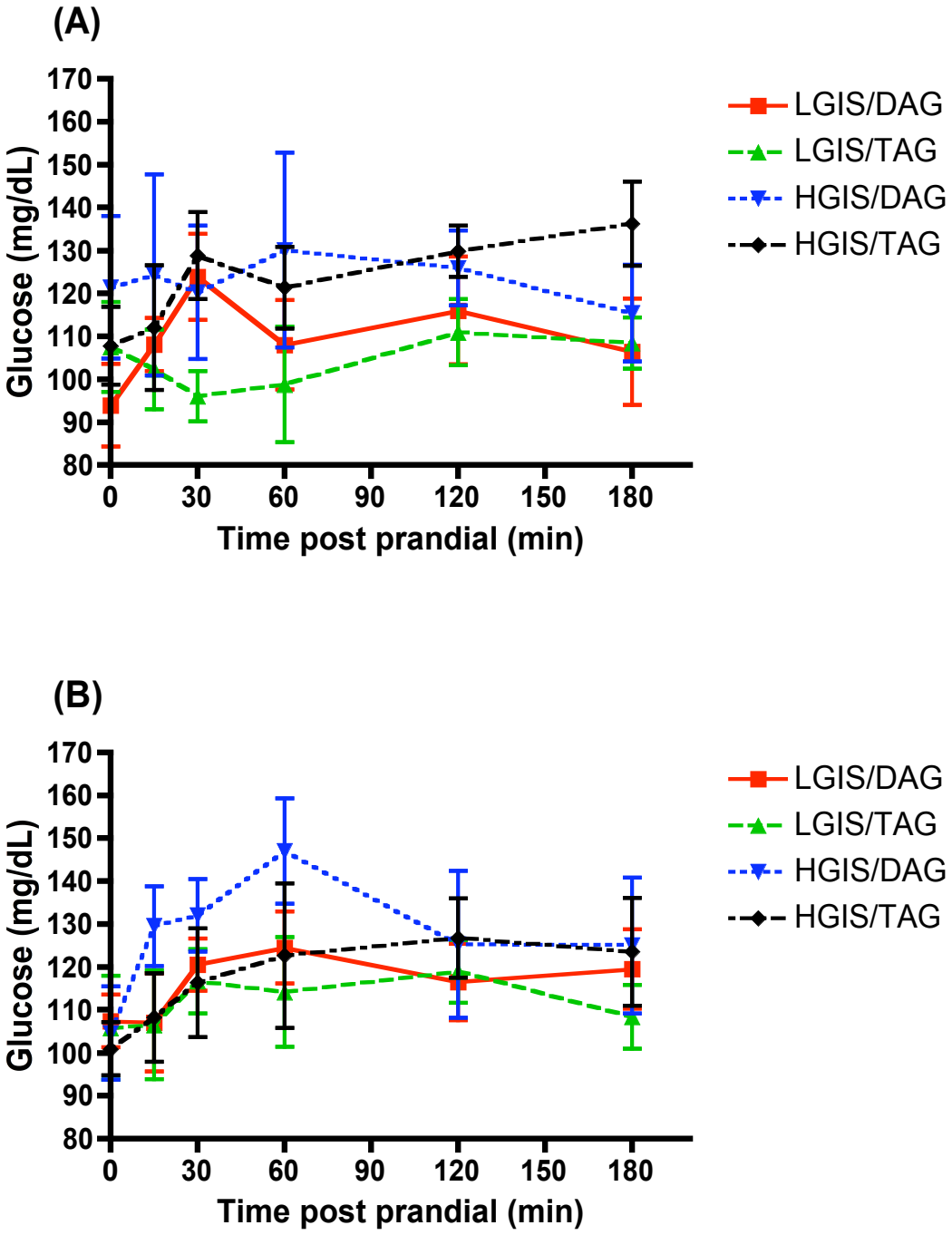

Figure 8. Post-prandial plasma glucose concentrations. (A) week 1 and (B) week 8. A significant time effect was observed but no significant diet differences. Values are mean ± SEM.

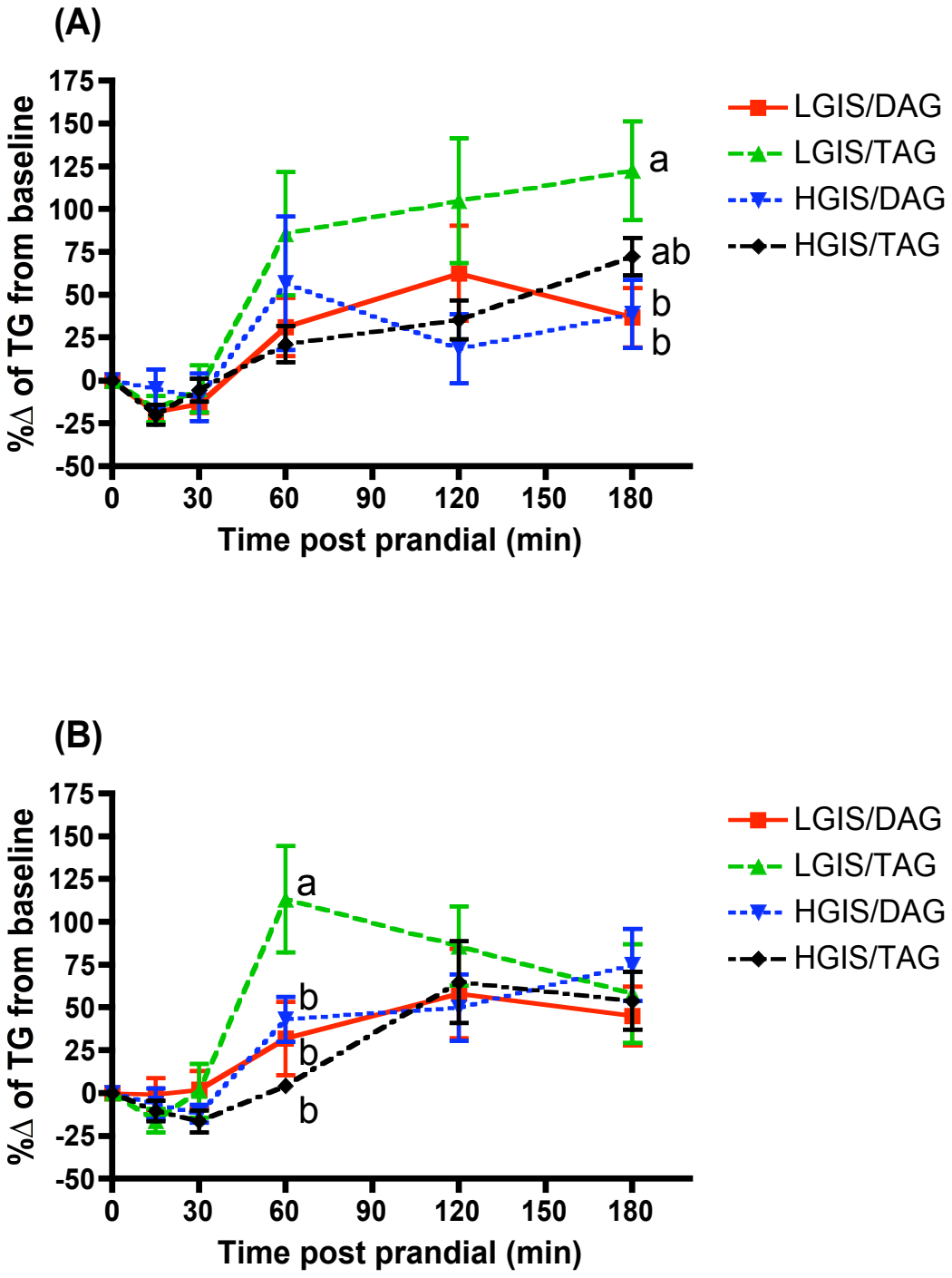

Figure 9. Post-prandial plasma TG concentrations. (A) week 1 and (B) week 8. Values are mean ± SEM. Letters not in common for a time point denote significant differences among diets by repeated measures ANOVA, $P < 0.05$.

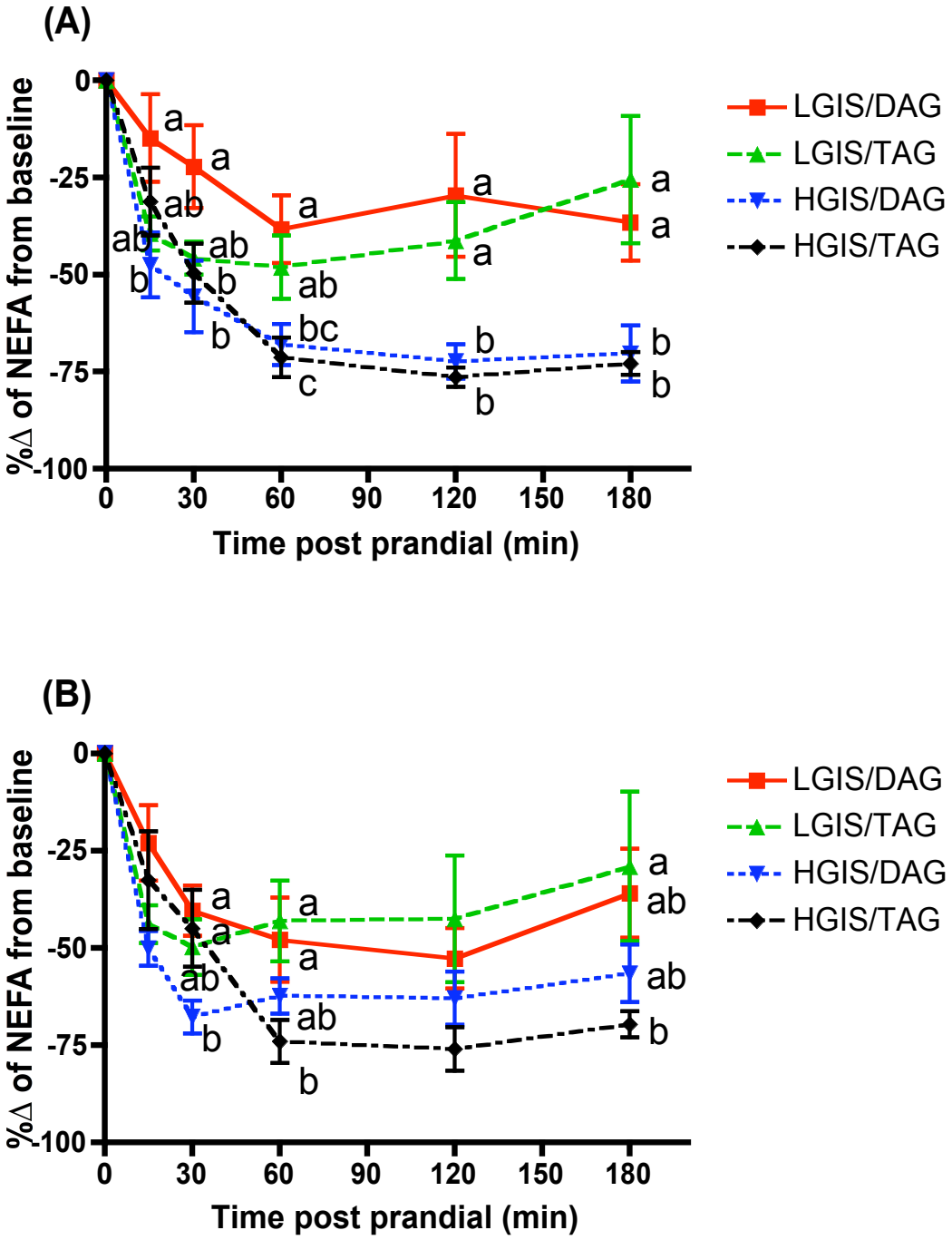

Figure 10. Post-prandial plasma NEFA concentrations. (A) week 1 and (B) week 8. Values are mean ± SEM. Letters not in common for a time point denote significant differences among diets by repeated measures ANOVA, $P < 0.05$.

Plasma insulin

Post-prandial changes in insulin concentrations included expected time effects at both wk 1 and wk 8 but diet effects were also observed (Figure 11). At wk 1, statistically significant increases of plasma insulin were observed with HGIS diet groups compared with LGIS diet groups. At wk 8, insulin peak values of all diets were observed at 120 min but some blunting of this response had occurred such that the diet effect was less marked compared with wk 1. At both wk 1 and 8, plasma insulin was significantly lower in the LGIS diet groups even though glucose concentrations were similar among all groups (Figure 8).

Plasma GIP

At both wk 1 and 8, plasma GIP concentrations increased in all diet groups and statistically significant time effects ($P < 0.05$) were seen at 60 min post-prandially (Figure 12). At wk 1, LGIS diet groups tended toward lower GIP concentrations compared with HGIS diet groups but these differences were modest. Peak values appeared at 30 min, leveling off at 60 min. At wk 8 differences among diets, especially starch effects, became more prominent. Peak GIP values with LGIS groups again appeared at 30 min then leveled off while the HGIS diet group values continued to increase at 60 min. A statistically significant diet effect was also noted at 60 min with increased GIP concentrations in the HGIS/DAG diet group compared to the other 3 diets (Figure 12). Finally, a positive linear correlation between insulin and GIP was observed (week 1) which was statistically significant at 15 and 30 min post-prandially (Figure 13).

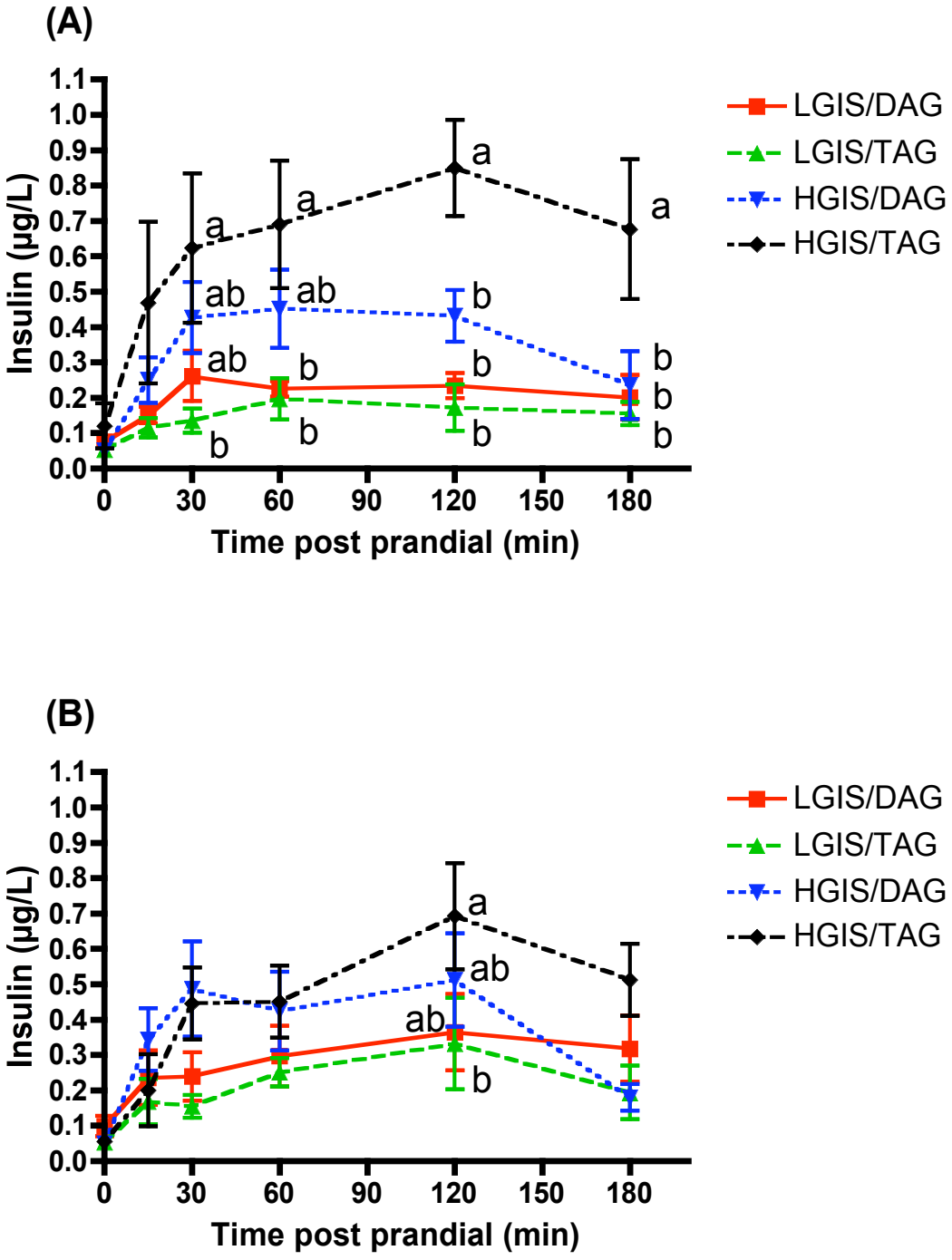

Figure 11. Post-prandial plasma insulin concentrations. (A) week 1 and (B) week 8. Values are mean ± SEM. Letters not in common for a time point denote significant differences among diets by repeated measures ANOVA, $P < 0.05$.

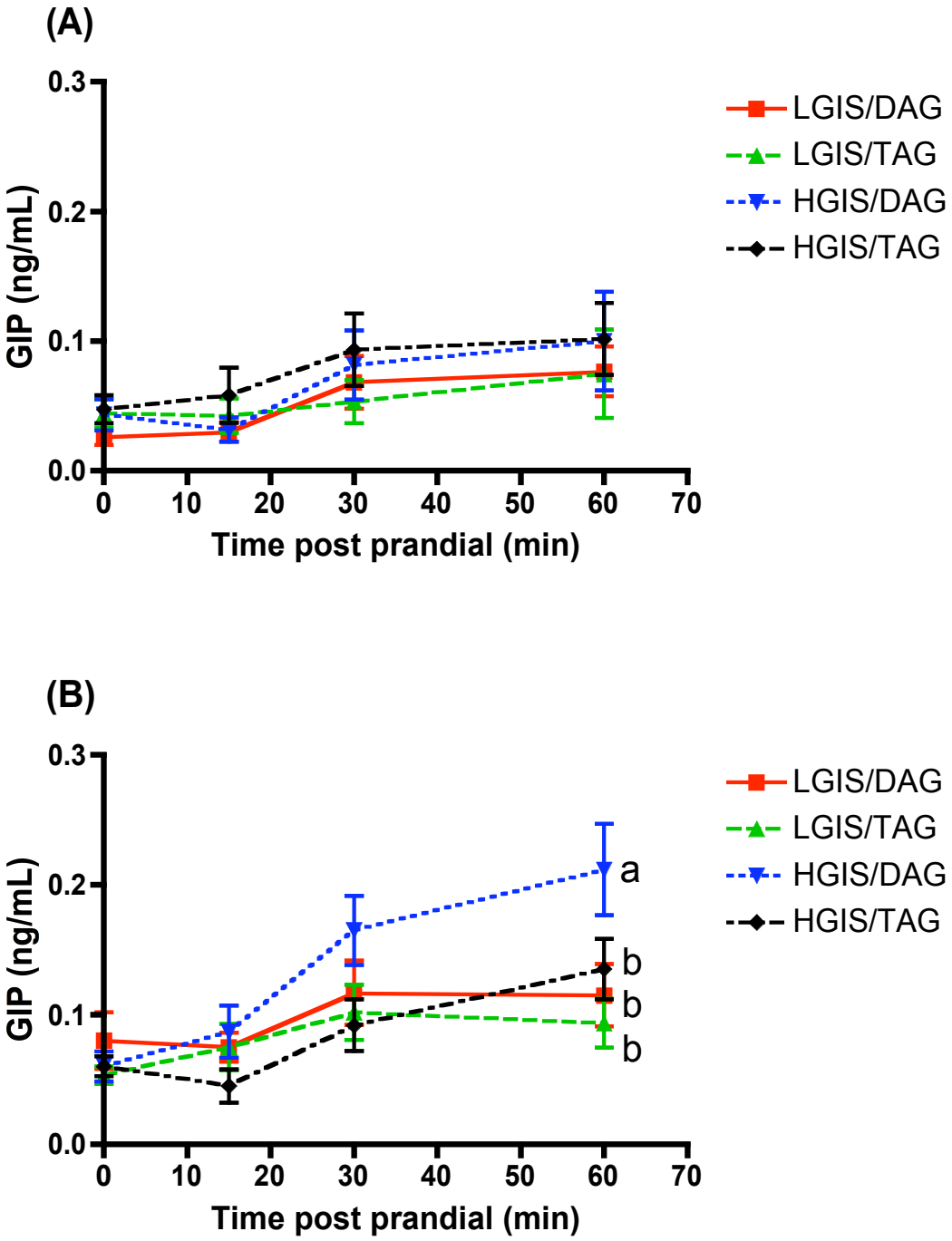

Figure 12. Post-prandial plasma GIP concentrations. (A) week 1 and (B) week 8. Values are mean ± SEM. Letters not in common for a time point denote significant differences among diets by repeated measures ANOVA, $P < 0.05$.

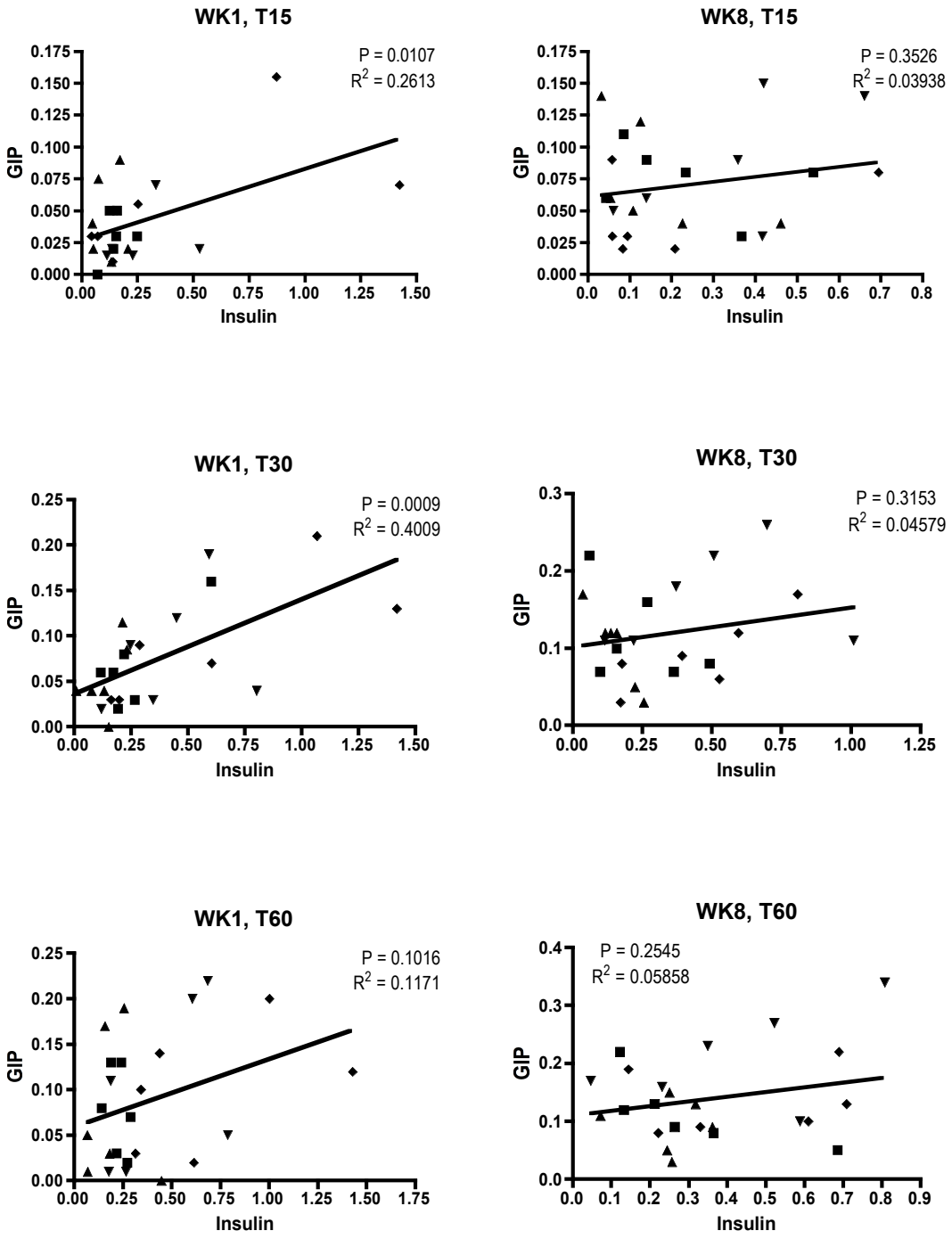

Figure 13. Correlations between plasma insulin and GIP. 15, 30, and 60 min. R-squared and P-values are indicated.

Plasma GLP-1

At wk 1, plasma GLP-1 concentrations increased in all diet groups but differences among the diet groups were not seen. At wk 8, differences among the diet groups became varied. Peak values of LGIS/TAG diet group appeared at 15 and 60 min while the HGIS/DAG diet group had significantly higher concentrations than the other diet groups at these times (Figure 14). Statistically significant time effects were seen at both wk 1 and 8 at the 60 min post-prandial time periods. However, main effects of diet on GLP-1 were not observed in this study.

Plasma adiponectin

Fasting plasma adiponectin concentrations were significantly higher in the LGIS/DAG diet group compared with all other diet groups (Figure 15). LGIS diet groups increased plasma adiponectin concentrations with time (i.e., weight loss), whereas HGIS diet groups tended lower over time. However, correlations of adiponectin and body weight were not observed during the experimental period (Figure 16)

Plasma leptin

Significantly lower plasma leptin concentrations were found, consistent with weight loss, in all groups, especially the LGIS/DAG diet group (Figure 15). Positive correlations of leptin and body weight were also found during the experimental period (Figure 16). Correlations between leptin and adiponectin were not observed (Figure 17).

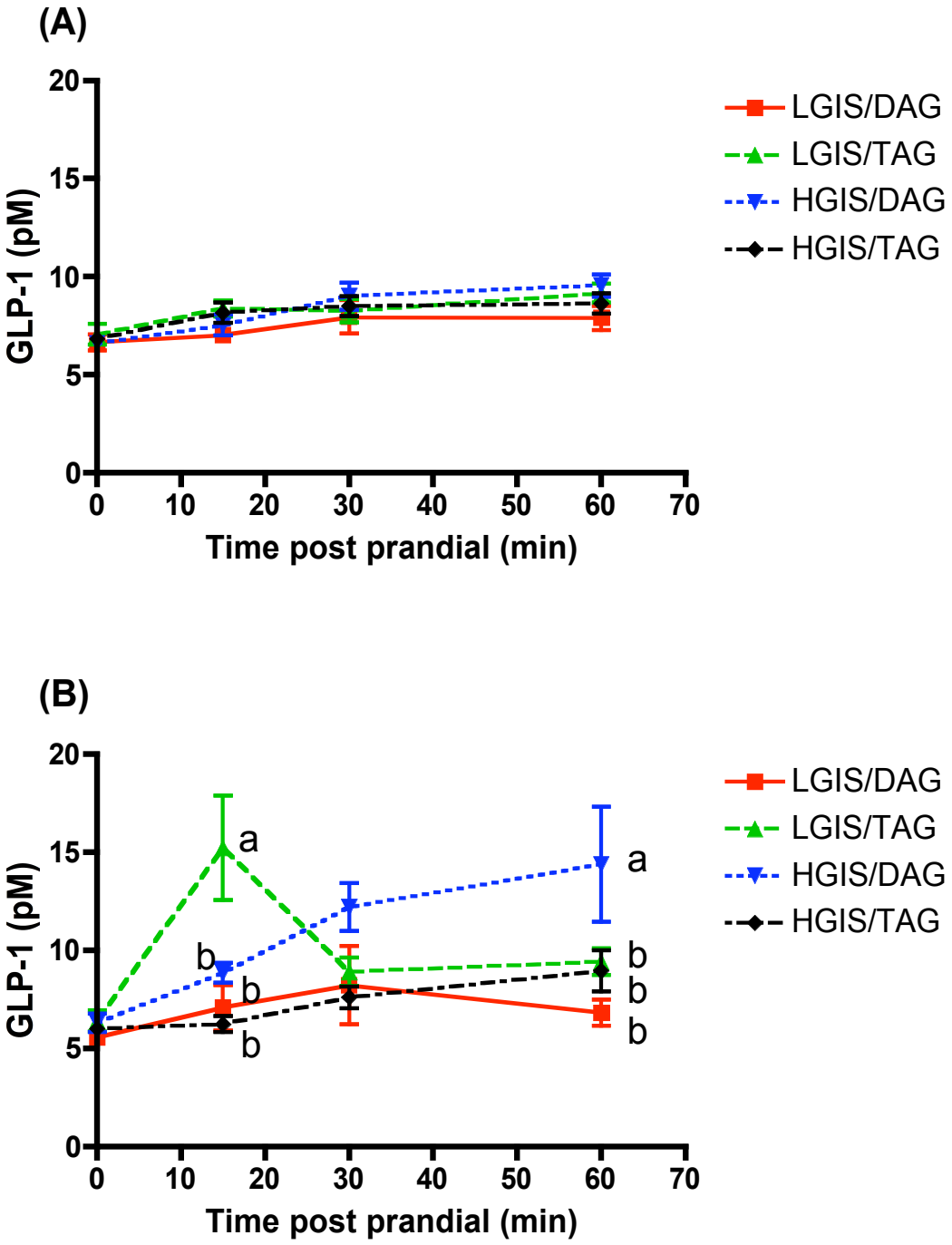

Figure 14. Post-prandial plasma GLP-1 concentrations. (A) week 1 and (B) week 8. Values are mean ± SEM. Letters not in common for a time point denote significant differences among diets by repeated measures ANOVA, $P < 0.05$.

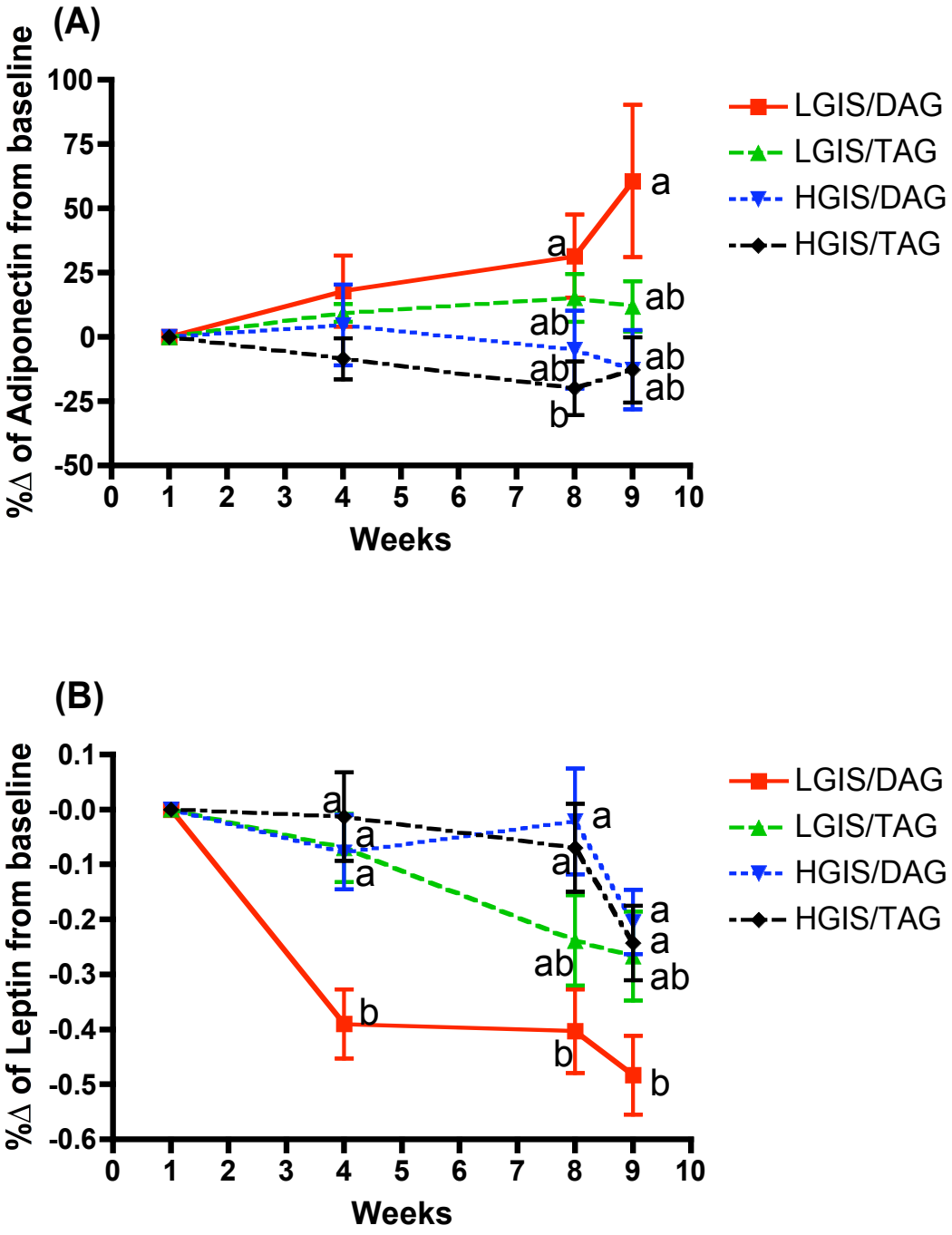

Figure 15. Fasting plasma (A) adiponectin and (B) leptin concentrations. Values are mean ± SEM. Letters not in common for a time point denote significant differences among diets by repeated measures ANOVA, $P < 0.05$.

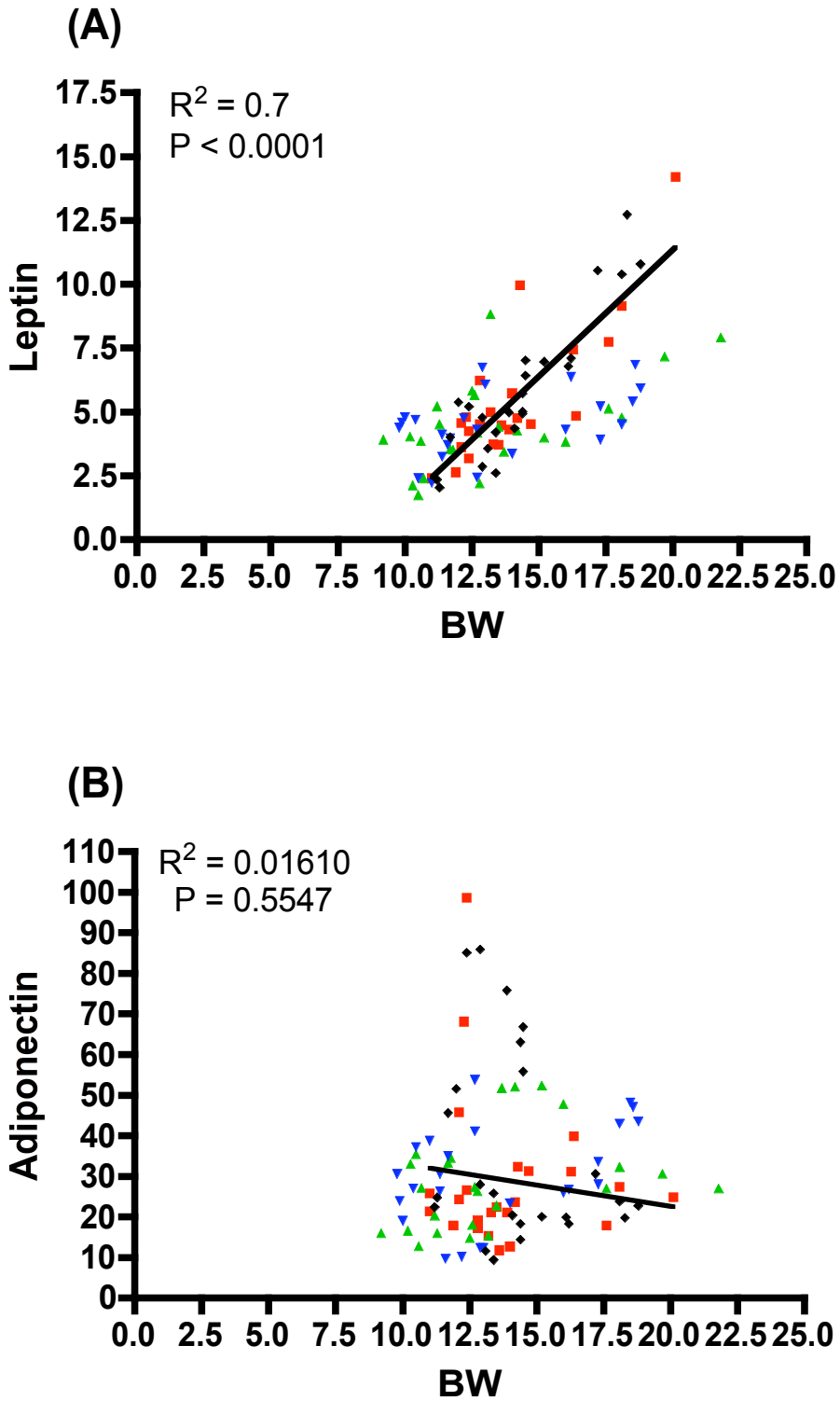

Figure 16. Correlations between plasma (A) adiponectin / (B) leptin and BW. R-squared and P-values are indicated.

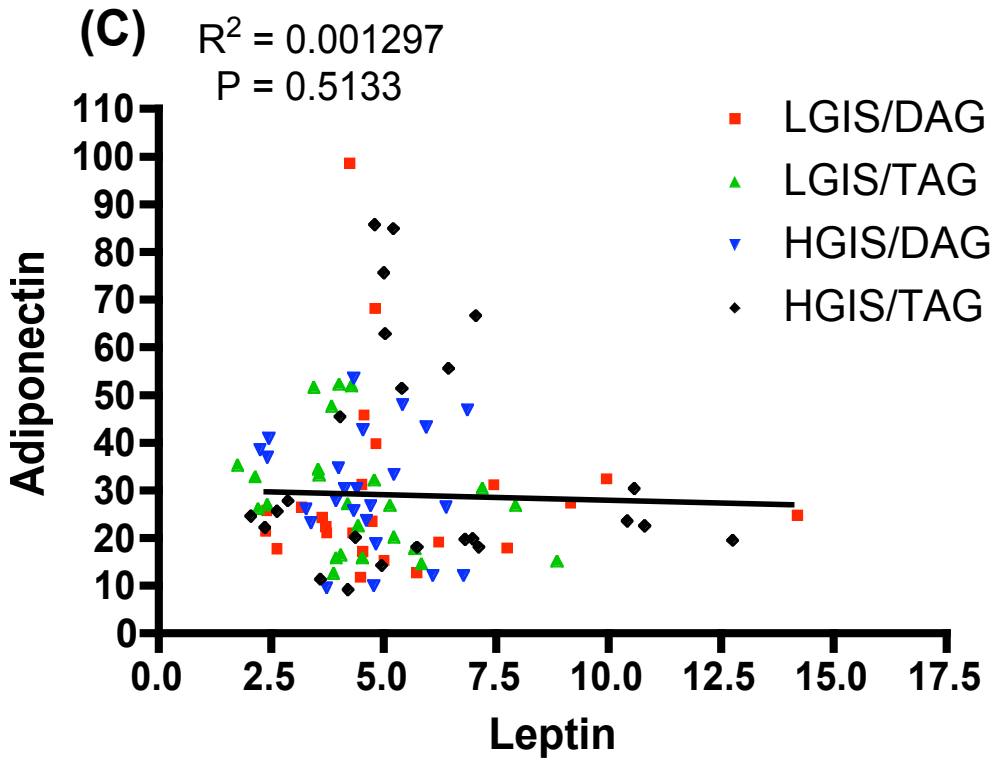

Figure 17. Correlations between plasma adiponectin and leptin. R-squared and P-values are indicated.

Discussion

All dogs lost body weight during this study. The primary reason for this effect is that, on average, the dogs voluntarily consumed approximately 68% of the total calories offered them on a daily basis. In veterinary practice, canine obesity is treated using diet management techniques designed to reduce daily calorie intake by approximately 35 to 40% of the amount that would maintain that particular animal's ideal body weight. In the present setting, dogs were offered the number of calories calculated to maintain their obese weights (ca. 950 kcal/d). However, as noted, only 68% of these calories were ingested by the dogs (ca. 500 to 700 kcal range). This would compare to about 550 kcal which is approximately 65% of the number of calories to maintain the animals' average ideal weights were they presented in their obese state to a veterinary practice (i.e., 65% of ME = (0.65) 130 (12 kg)$^{0.75}$ = 544 kcal). Because the dogs lost from 1.0 to 2.0% body weight per week (a typical clinical weight loss target), these data help validate the approach used in veterinary practice as all dogs remained healthy and active during the experiment while successfully losing weight.

When the change in body weight was considered vs. time among the four groups, dogs fed the LGIS diets lost a larger percentage of their starting obese body weights than those fed the HGIS diets. As noted, the total ME of the LGIS diets were considerably lower than that of the HGIS diets even though dogs consumed the same amounts of diet on a dry weight basis. Thus dogs had consumed lower amounts of ME overall yet were not denied having a larger volume of food in their daily meal, thereby potentially contributing to satiety. The reason for this difference was primarily because digestibility of the LGIS was significantly lower than the HGIS, while fat types were not

different, and protein was only modestly different. Thus total digestibilities of the LGIS diets were less than the HGIS diets.

In addition to more weight loss, a second benefit of LGIS diets is that they resulted in a significant reduction of circulating insulin when measured over time in response to a mixed meal while glucose concentrations were similar in all groups. This insulin effect was most prominent early on during this diet study (wk 1) and not as marked at wk 8. Consequently there appeared to be some adaptation to this dietary component with time. Even though these effects were independent of dietary oil type, other distinct advantages of the DAG oil, when combined with the LGI starch, in a mixed diet were observed. As mentioned, the insulin effect appeared to occur more prominently early during the weight loss period. By contrast, DAG oil effects appeared to be more pronounced later in the experiment (i.e., wk 8). Thus, significant post-prandial triacylglycerol lowering was observed with the DAG/LGIS compared to the TAG/LGIS combination especially at wk 8. And also, both at wk 1 and 8, increased post-prandial TG concentrations was observed with LGIS/TAG diet especially early on. It is well known that insulin enhances LPL activity in adipocytes. Less insulin environment with LGIS diet also cause less LPL activity so that relatively higher plasma concentrations of TG was observed compared with HGIS diet groups. Post-prandial NEFA concentrations were decreased less with the LGI starch containing diets at week 8. It has reported that decreased insulin levels promote HSL activity and lipolysis (Van Harmelen et al., 1999). With the LGI starch, if glucose flux into cells had been decreased as a result of an attenuated insulin response, then relatively more tissue lipolysis from storage sites would be favored, resulting in fatty acid mobilization and the NEFA elevations observed. An

attenuated release of insulin with LGI starch may also result in less active utilization of circulating NEFA. Taken together, these lipid metabolite changes support the possibility of lower TAG entering the bloodstream after eating (DAG effect) while increased relative amounts of NEFA were available for energy in a lower insulin environment (LGIS effect) and while maintaining glucose homeostasis. Thus, the findings of lower digestibility, increased but safe rate of weight loss, effective loss of percent body fat, improved insulin response and lower post-prandial TG concentrations demonstrates that the DAG oil and LGI starch combination is preferred for weight loss in this canine model.

Although no diet differences were observed in circulating GIP concentrations at week 1, the positive linear correlation of all diet group data between circulating insulin and GIP at 15 and 30 min post-prandially is consistent with the known stimulating effect of GIP on insulin secretion.

Because GIP secretary cells are more abundant in the upper part of the small intestine (Damholt et al., 1999), it follows that there would be an early post-prandial insulin stimulatory effect as seen in the present study (i.e., at 15 and 30 min).

When the diets were fed for a longer period (i.e., 8 wk), it appeared that the intestinal cells of animals fed the HGIS diets continued to secrete amounts of GIP post-prandially further adding to a more sustained insulinotropic effect. Indeed circulating insulin levels were significantly higher in the HGIS diets compared with the LGIS diet groups. It remains to be seen whether a longer post-prandial sampling period would additionally verify this latter possibility.

Effects of another insulin secretagogue, GLP-1 was less notable in this study. No effects of diet on GLP-1 were noted yet time effects, post-prandially, were found for

this polypeptide hormone in the circulation. It should be noted that the determination of circulating levels of GLP-1 does not allow the direct assessment of the actions of GLP-1 in which numerous interactions among paracrine, endocrine, neural, and luminal influences may exist at the cellular level. Nonetheless, it is noteworthy that while a relationship between GIP and insulin was observed, this was not the case for GLP-1. GIP has insulinotropic activity and is believed to be released from the upper gut while GLP-1 is released from the lower gut and has a glucagon-lowering effect. An immunocytochemical study revealed the exact locations of GIP and GLP-1 producing cells in the dog (Damholt et al., 1999). GIP - secreting K cells were equally distributed in duodenum and jejunum, with the GLP-1 - secreting L cells concentrated in the jejunum (5% duodenum, 73% jejunum and 22% ileum). This study also found that the middle section of the small intestine containing 69% of the K cells also contained 51% of the L cells, and over 30% of the L cells in this region were found adjacent to K cells.

It has been reported that glucose does not directly stimulate canine L cells (Damholt et al., 1998). It is more probable that glucose releases GIP from the upper intestine that, in turn, stimulates GLP-1 secretion. Damholt et al. concluded that the ability of GIP to stimulate GLP-1 secretion is probably mediated through activation of protein kinase A. In the present study, the sampling schedule used (0 to 60 min) may not have been long enough to see any substantial changes in GLP-1 although a significant time effect was found during the post-prandial period studied.

Also, species differences have been reported using isolated perfused ileal segments from dog and rat (Manaka et al., 1996). It was found that GLP-1 release occurred when rat segments were perfused with GIP but not when dog segments were

studied. They concluded that GLP-1 release in rat, but not in dog, is modulated by GIP, the upper GI tract factor, and that the discrepancy in GLP-1 response to GIP might be due to species differences. Obviously numerous other factors may influence the increase of GLP-1 post-prandially in dogs; however, in the present study, changes in canine post-prandial plasma insulin and GIP do not appear be associated with changes in GLP-1 concentration during the first 60 min post-prandially.

Finally, plasma adiponectin concentrations observed in the present study were significantly higher in the LGIS/DAG diet group compared with all other diet groups. Even though we did not observe a significant inverse correlation between adiponectin and body weight, the changes seen were consistent with weight loss. In addition, we did not observe an inverse correlation between adiponectin and leptin. By contrast in humans, blood adiponectin concentration negatively correlates with the degree of obesity and is lower in obese individuals (Havel, 2004; Matsuzawa et al., 2004). In dogs, it has reported that blood leptin concentrations were dramatically altered by fasting compared to those seen a few hours after feeding (Ishioka et al., 2005), whereas blood adiponectin concentration was nearly unchanged (Peake et al., 2003). Ishioka et al. (2006) also noted that while plasma adiponectin can be an additional marker of adiposity in dogs, it is a less sensitive obesity index compared with leptin. Brunson et al. (2007) reported that adiponectin gene expression and protein secretion are unaffected in a high-fat fed, obese dog model. They concluded that plasma adiponectin concentrations in dogs are differently regulated compared to obese humans. Even though the dogs lost an appreciable amount of weight, body fat, and decreased their BCS from 8.4 to 7, either the relative amounts of weight lost or the short term of the present experiment may help

explain the lack of correlation of adiponectin with weight loss or with leptin seen in the present study.

In this study, we observed significantly lower plasma leptin concentrations in all diet groups, especially the DAG/LGIS diet group. Also a positive correlation between leptin and body weight was observed. These findings are similar to these of other investigations (Ishioka et al, 2002a; Ishioka et al., 2007), and it is concluded that leptin concentration is consistent with weight loss and may be a more reliable marker than adiponectin in dogs as in humans and rodents.

CHAPTER IV

UCP2 GENE EXPRESSION IN CANINE DUODENAL EPITHELIUM

Introduction

The uncoupling proteins (**UCPs**) are members of the mitochondrial transporter family that dissipate the mitochondrial proton gradient as heat more than via ATP synthesis (Cadrin et al., 1985; Cannon et al., 1982). They are roughly divided into 3 subtypes and show high homologies with each other. UCP1, a so called mitochondrial carrier protein, is seen exclusively in brown adipose tissue (BAT) which is the major site of regulatory thermogenesis in small rodents. UCP2 is widely located in most tissues studied in human and rodents including white adipose tissue (WAT) and skeletal muscle, which is thought to be the major thermogenic organ in large animals including humans (Boss et al., 1998). UCP3 is expressed mainly in skeletal muscle in humans, and BAT and skeletal muscle in rodents (Boss et al., 1998). The UCPs reduce the mitochondrial membrane potential via a characteristic uncoupling activity and may be involved in the regulation of thermogenesis and energy expenditure to greater or lesser degrees (Klaus et al., 1991; Cannon and Nedergaard, 1985). Although all subtypes are considered to have mitochondrial uncoupling activity, it has been reported that the main and prominent function of UCP1 is thermoregulation. By comparison, UCP2 is involved in the control of reactive oxygen species (ROS) generation and UCP3 in the handling of lipids as fuels (Ledesma et al., 2002).

In rats, both short and long term studies have shown that high protein diets up-regulate hepatic UCP2 mRNA gene expression. Thus, UCP2 mRNA expression is related to the level of dietary protein intake (Petzke et al., 2005; Petzke et al., 2007).

According to Murase et al. (2002), a study in obesity-prone C57BL/6J mice fed a diet high in DAG compared with TAG resulted in significant decreases in body weight and lower feed efficiencies. In addition, UCP2 mRNA expression in the small intestine also increased. Several studies of the UCPs have been reported in small animals and humans, but only a few canine reports exist (Ishioka et al., 2002b). Thus, it is of interest to determine whether dietary DAG results in increased UCP2 gene expression during canine weight loss consistent with the observations of Murase et al. (2002) in mice.

Preliminary study

Our preliminary study provided some useful information on the above question. Three normal, sexually intact adult female beagles, all 5 yr old, with body condition scores of 5 on a 9 point scale, where 5 is ideal, were used in the study. The dogs were individually housed in approved sized kennels with 12 h light cycles, allowed free access to water and exercise, and fed free choice during the entire 6 weeks of study.

A 6-wk experimental period was used; divided into three stages (two weeks each). For the first 2 wk, all dogs were fed a premium quality pet food (Hill's Science Diet, Adult Original. Hill's Pet Nutrition, Inc., Topeka, KS) to establish a baseline (stage 1). After this period, dogs were fed the same diet containing TAG for 2 weeks (stage 2). In the last 2 weeks, dogs were fed the same diet containing DAG (stage 3). At the end of each stage, tissue samples from duodenal epithelium were obtained via endoscopic

biopsy under general anesthesia (see below for techniques used) and used for UCP2 analysis.

Our results showed that the TAG containing diet tended to lower UCP2 gene expression in the small intestine compared with DAG containing diet, and DAG containing diet seemed to increase UCP2 gene expression, but no statistically significant difference was observed.

To further extended this observation, we investigated how longer-term feeding of different DAG and TAG containing diets affect UCP2 mRNA expression in canine small intestine. For this purpose, diets containing either DAG or TAG and either LGIS or HGIS were compared during weight loss. The number of the dogs we used in this study was also increased (n = 6 per diet group) compared with our preliminary study (n = 3).

Materials and Methods

Animals

The protocol for this study was approved by the Texas A&M University Laboratory Animal Care Committee (AUP # 2004-202: Molecular biology of uncoupling protein). All dogs were housed at the Laboratory Animal Research and Resources (LARR) facility, Texas A&M University and individually maintained in kennels according to the American Physiological Society Guidelines for Animal Research and according to guidelines set forth by Texas A&M University Care and Use Committee. Prior to entering the study, all dogs had complete blood counts and serum biochemistry profiles tests performed to assure normal clinical status. All dogs used in the study appeared to be clinically and physiologically normal.

Twelve obese, sexually intact adult female beagles, ages 2 to 6 yr old, with body condition scores of 8 to 9 on a 9 point scale, where 5 is ideal, were used. All dogs were individually housed in approved sized kennels with 12 h light cycles, allowed free access to water and exercise, and fed free choice during the study. All dogs were randomly divided into one of the four dietary groups assigned (n = 3 per diet group). The dogs were assigned to the diet groups to minimize bias with respect to age, BCS, and body fat content.

Diets and feeding

Four diets were evaluated. The diets were combinations of: LGIS/DAG; LGIS/TAG; HGIS/DAG; and HGIS/TAG. Ingredients of these diets included chicken by-product meal; either high amylose corn (LGI) or waxy corn (HGI) starches that had been gelatinized, either TAG or DAG enriched dietary oil, and appropriate mineral and vitamin pre-mixes suitable for canine maintenance. On a percent metabolizable energy basis, the diets contained: 25% protein, 38% fat and 36% nitrogen-free extract (NFE), approximately. The diets were isocaloric at approximately 4200 kcal/kg. All diets are described in Appendix A and also in the previous chapter. An experimental weight loss period of 10 weeks was used. During weeks 9 and 10, biopsies were taken from one dog from each diet group using a Monday/Wednesday/Friday sequence until all dogs had been biopsied. All dogs were fasted overnight, anesthetized, and tissue was collected via endoscopic biopsy (Olympus America Inc., Center Valley, PA). All dogs were administered the following medication to conduct these procedures: glycopyrrolate as pre-anesthetic medication, propofol for induction, and isoflurane to maintain general

anesthesia. Approximately 1 g of tissue specimens were collected immediately from a duodenal epithelial site, snap-frozen in liquid nitrogen and stored at -80°C for subsequent RNA isolation.

After the 10 wk weight loss regimen was completed, all dogs were again fed the commercial diet, oil, and cookies for 16 weeks in order to attain their initial levels of obesity (re-induction of obesity). All dogs were then assigned to a treatment diet exactly opposite to the one they had been fed during the first period of the study and fed for 10 weeks to repeat the study design. In this way, an n = 6 sample size was obtained for each diet group. Tissue samples collected were used for UCP2 mRNA gene expression analysis as described below.

Preparation and analysis of RNA

Each tissue sample was thawed and homogenized using an ultrasonic homogenizer (Model 300 V/T, BioLogics Inc., Manassas, Virginia) and total RNA was extracted using TRIzol (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions. Extracted total RNA was purified by appropriate procedures as follows; serial extractions with chloroform, isopropyl alcohol, and 75% and absolute ethanols. Yield and purity were quantified by measurement of absorbance at 260 and 280 using a spectrophotometer (DU7400 diode array spectrophotometer, Beckman Coulter, Inc., Fullerton, CA). Purified total RNA (5 μg) extracted from the duodenal epithelium were blotted onto positive charged nylon membrane, Hybond-N+ (GE Healthcare UK Limited, Buckinghamshire, UK), using a Minifold II Slot Blot System (Schleicher &

Schuell, Inc., Keene, NH), baked in an incubator for 20 min at 80°C, sealed, and stored in the freezer until the time of the analysis.

Extracted total RNA were reverse transcribed (RT) using AMV Reverse Transcriptase (USB Corporation, Cleveland, Ohio) and oligo-dT primer linked to an adapter sequence (Adapter + T15). Polymerase chain reaction (PCR) was performed with a cDNA template and primers designed from the reported sequences (Ishioka et al., 2002b). UCP2 and 36B4 primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and were as follows: UCP2 (Forward primer (murine 1-) 5'- ATG GTT GGT TTC AAG GCC AC -3', Reverse primer (canine - 640) 5'- GGT CAT CTG TCA TGA GGT TG -3'), 36B4 (Forward primer (canine 1-): 5'- TGG AAA TCC AAC TAC TTC CTT AAG -3', Reverse primer (canine - 787): 5'- CAG CAA GTG GAA AGG TGT AAT CAG T -3'). PCR was conducted for 35 cycles at 94°C for 1 min, at 55°C for 1 min and at 72°C for 1 min in a solution (50 μL) of 10 μmol of each primer and 2 units of rTaq DNA Polymerase (GE Healthcare UK Limited, Buckinghamshire, UK). The PCR products were sequenced using Progene (Techne (Cambridge) LTD., Cambridge, UK). For probes, cDNA of UCP2 and 36B4 was electrophoresed in a 2.0 % agarose gel, purified using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare UK Limited, Buckinghamshire, UK).

Purified RNA was labeled using AlkPhos Direct Labeling and Detection System with CDP-Star (GE Healthcare UK Limited, Buckinghamshire, UK) according to the manufacturer's instructions. The probes were hybridized to the blotted membranes for 18 h at 55°C. Rinsed membrane for all probes were placed and exposed to KODAK

BioMax MS Film (KODAK Industrie, Cedex, France) for 2 to 8 h to obtain optimal images. To determine the intensities of the bands, films were scanned and analyzed with an imaging densitometer (Model GS-700, Bio-Rad, Hercules, CA) and ImageJ 1.38x (NIH, National Institute of Health, USA). For normalization, 36B4 (acidic ribosomal phosphoprotein) mRNA was used and the UCP2 mRNA levels were calculated relative to the 36B4 mRNA levels.

Statistical analyses

Data are expressed as means ± SEM. Statistical analyses of all data were performed using repeated measures ANOVA, to test for main effects of time and diet as well as the interaction of time with diet, and significance was set at $P < 0.05$. Tukey test was used as a post-hoc test for multiple comparisons for main effects of diet, time, and diet x time interactions, at a level of significance of $P < 0.05$. All data were found to follow a Gaussian distribution at $P < 0.05$ using the Kolmogorov-Smirnov (KS) test. All statistical tests were performed using GraphPad Prism version 4.00 for Macintosh (GraphPad Software, San Diego, CA, www.graphpad.com).

Results

Although there were no statistically significant differences, UCP2 mRNA levels of the LGIS diet groups tended to be lower compared with the dogs in the HGIS diet groups (Figure 18). This starch effect was unexpected. By contrast, differences between DAG and TAG diet groups were not observed in either of the LGIS and HGIS diet groups as was hypothesized. Our results indicated that combinations of LGI starch and

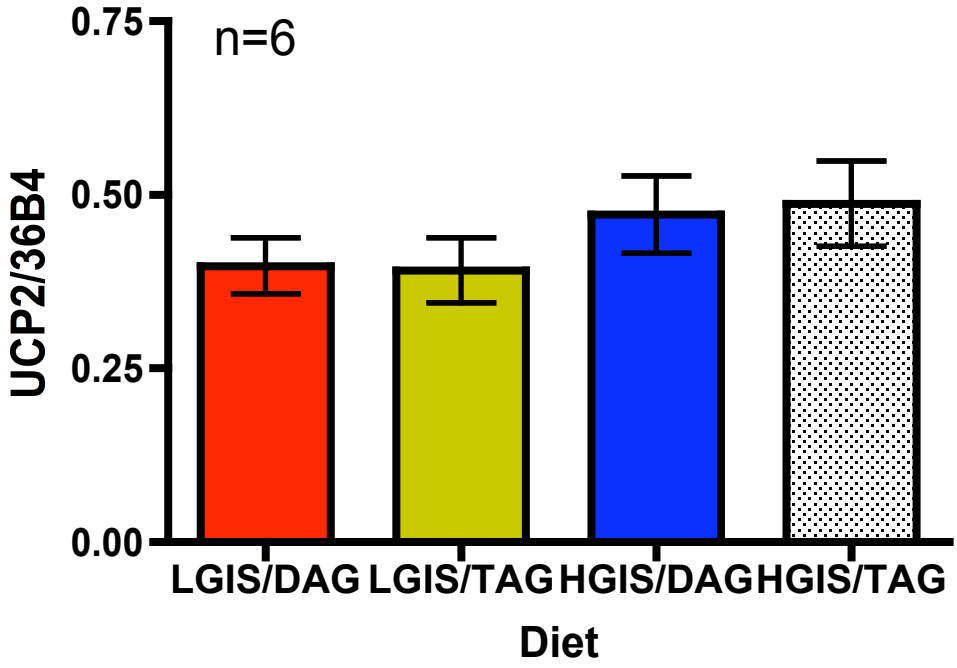

Figure 18. UCP2 gene expression in the duodenal epithelium. The amounts of UCP2 mRNA were calculated relative to the 36B4 mRNA levels. Values are the means ± SEM of six animals.

oils decrease the UCP2 mRNA expression in the canine small intestine compared with the diet combinations of HGI starch and oils.

Discussion

Although numerous UCP studies in humans and animals have been reported, only a few canine reports exist (Ishioka et al., 2002b). In general, UCP2 is ubiquitously expressed in most tissues in humans and rodents (Dullo and Samec, 2001). In dogs, Ishioka et al. (2002b) reported that UCP2 mRNA was expressed in most tissues as well, and is particularly abundant in adipose tissue, spleen and lung. In this study, we used duodenum epithelium. According to Murase et al. (2002), dietary DAG stimulates UCP2 gene expression in the small intestine in mice. It is reasonable to examine the small intestine because it is the first and most susceptible organ exposed to dietary fat.

GAPDH has been used primarily to normalize gene expression. In this study, we used 36B4 (acidic ribosomal phosphoprotein) to calculate the UCP2 mRNA levels. This was because we adjusted the conditions to Murase's study (Murase et al., 2002).

Several factors influencing UCP2 gene expressions have been reported (Boss et al., 1998; Diehl and Hoek, 1999). UCP2 expression is generally increased in situations of oxidative stress, in which circumstance UCP2 would protect cells by limiting mitochondrial ROS production therefore preventing the onset of apoptosis (Diehl and Hoek, 1999). Significant positive correlations have also been reported between UCP2 mRNA levels in WAT and body mass index (BMI) in humans (Millet, et al., 1997).

Boss et al. (1998) suggested in a review article that UCP2 is expressed in pancreatic islets and might, by decreasing cellular ATP levels, blunt glucose-induced

insulin secretion. And leptin, which decreases the insulin response to glucose and other fuels, was found to increase UCP2 mRNA expression in pancreatic islets (Koyama et al., 1997; Zhou et al., 1997). Thus, Boss et al. hypothesized that a high level of UCP2 could decrease insulin secretion (Boss et al., 1998).

In our study, lower plasma insulin was observed with the LGIS diet groups compared with HGIS diet groups while glucose concentrations were similar among all groups. Our results also show that higher plasma leptin concentrations were observed in HGIS diet groups compared with LGIS groups. These findings are similar to the hypothesis of Boss et al. (1998).

It has also been reported that UCP2 mRNA expression is enhanced by PPARγ agonists in rats pancreatic islets (Shimabukuro et al., 1997). Boss et al. (1998) also hypothesized that increased levels of NEFA in pancreatic islet cells may enhance UCP2 expression, and in turn, impair insulin secretion. The effect of NEFA on UCP2 gene expression might be mediated by PPARγ (Boss et al., 1998). This potential NEFA effect was not supported in the present study. Our results indicated that lower plasma NEFA concentrations were observed in HGIS diet groups compared with LGIS groups which is opposite to the speculation of Boss et al. (1998). However, differences in NEFA concentrations may exist in plasma vs. pancreatic islet cells during feeding. Our NEFA changes were observed post-prandially while the intestinal biopsies were obtained in the fasted state.

In addition, our results show that the 1,3-DAG diets did not stimulate UCP2 gene expression in the canine small intestine. We observed a more prominent starch effect

than oil effect. Although there was no significant difference among the diet groups, the combinations of LGI starch and DAG/TAG tended to lower UCP2 gene expression in the small intestine compared with the diet combinations of HGI starch and DAG/TAG. This effect was unexpected. There are some reports in rodents regarding protein and fat on UCP2 gene expressions (Petzke et al., 2005; Petzke et al., 2007), but no information is available as to the relationship with carbohydrate and UCP2 gene expression in rodents or, for that matter, in dogs.

From the above information, we speculate that the long term consumption of a LGIS diet mediated decrease of UCP2 mRNA expression may be a metabolic down regulation. First of all, the LGIS diet groups had lower digestibilities. The associated lower metabolizable energy also resulted in more body weight loss compared with the HGIS diets. In this case, in the face of lowered energy intake from the LGIS diets during weight loss, UCP2 may reduce its uncoupling activity and thus favor relatively more ATP production than heat generation. Less expression of UCP2 in canine small intestine may thus protect the non-obese state under conditions of moderate and/or insufficient energy intake and may also play an adaptive role during down regulation of energy metabolism with weight loss. In this way, intestinal UCP2 upregulation may be involved in the well known thermal effect of feeding and possibly be down regulated during starvation to conserve energy via favoring ATP production. A study utilizing simple caloric restriction in UCP2 mRNA expression may further clarify this possibility. Alternatively, the possibility also exists that decreased UCP2 mRNA expression may be due, at least in part, to the decrease leptin concentrations seen with the LGIS diets.

Indeed, Koyama et al. found that leptin increased UCP2 mRNA expression in pancreatic islet cells. Thus it can be hypothesized that the HGIS diet associated increase in leptin may be associated with increased small intestinal UCP2 mRNA expression as well.

Although unknown at this time, species differences regarding UCP2 expression may also exist as may be the case in the GLP-1 data between dogs and mice (Manaka et al., 1996). Finally any potential oil effect in canine intestine may be less sensitive or not strong enough to surpass the starch or total calorie restriction effects seen on UCP2 gene expressions compared with mouse intestine.

Because there are no previous canine reports examining the effects of DAG and LGI starch on UCP2 mRNA expression, this study is the first to do so. Although further investigation is required, it has provided interesting data in the dog and may lead to additional hypothesis regarding UCP effects on energy metabolism.

CHAPTER V

SUMMARY AND CONCLUSIONS

Obesity is the most common nutritional disorder in small animal medicine closely related to the mortality and morbidity of various diseases. Decreasing the incidence of obesity is considered to be a most important way to maintain health, prevent disease, and contribute to longevity. Diet therapy using low glycemic index starch (LGIS) and diacylglycerol (DAG) may be a reasonable obesity management tool without food restriction, forced physical activity, and impairment of health.

The overall objectives of this dissertation were to investigate lipid and carbohydrate modification with the combination of diacylglycerol (DAG) and low glycemic index starch (LGIS) in obese canine during weight loss. We hypothesized that the combination of DAG and LGI carbohydrate may support more efficient and healthy weight loss in beagles along with the improvement of biochemical and hormonal biomarkers. Also, UCP2 gene expressions in small intestine was also evaluated for this purpose.

To conduct this study, obesity was initially induced/re-induced in all beagles. All dogs were initially fed twice their daily calculated number of calories ($2\times125(BW_{kg})^{0.75}$) using a combination of dry extruded commercial pet food and 50/50 (w/w) blend of canola and soy bean oils (40 g/d). After 3 wk, all dogs were fed this mixture along with 5 Pecan shortbread cookies for the next 16 wk (obesity induction

period: wk 4 to 19). The major findings of this initial study can be summarized as follows:

A significant increase of food efficiency was observed during the obesity re-induction period compared with the obesity induction period. It was found that once body weights of obese dogs had been reduced, fewer calories were needed to regain the obese steady state. For that matter, the rate of weight gain was greater at an even lower calorie intake during re-induction of obesity. These results demonstrate the effects of yo-yo dieting on energy metabolism resulting in the rapid regain of body weight after successful weight loss. Results also suggest that metabolic "down-regulation" of energy needs with weight loss regimens continues after target weights have been achieved.

Human snack foods appeared to stimulate appetite and increase the overall net calorie consumption of the total ration. They provide a calorie dense dietary supplement leading to the rapid induction of an obese steady state that requires fewer calories to maintain. This information may help pet owners better understand the need to limit table scraps and human-type food snacks in dogs prone to overweight and obesity.

In the canine weight loss study, obese dogs were fed diets containing either LGI/HGI starch and DAG/TAG for a 10 wk weight loss period. When the change in body weight was considered vs. time among the four diet groups, dogs fed the LGIS diets lost a larger percentage of their starting obese body weights than those fed the HGIS diets. The total metabolizable energies of the LGIS diets were considerably lower than those of the HGIS diets even though dogs consumed the same amounts of diet on a dry weight basis. Thus, the LGIS dogs had consumed lower amounts of ME overall yet

were not denied having a larger volume of food in their daily meal, thereby potentially contributing to satiety. The reason for this difference was primarily because digestibility of the LGIS was significantly lower than the HGIS, while differences due to fat types were not seen, and protein was only modestly different. Thus total digestibilities of the LGIS diets were less than the HGIS diets overall.

In addition to more weight loss, a second benefit of the LGIS diets is that they resulted in a significant reduction of circulating insulin when measured over time in response to a mixed meal while glucose concentrations were similar in all groups. Even though these effects were independent of dietary oil type, other distinct advantages of the DAG oil, when combined with the LGI starch, in a mixed diet were observed. The insulin effect appeared to occur more prominently early on during the weight loss period of this study. By contrast, DAG oil effects appeared to be more pronounced later on in the experiment (i.e., wk 8). Thus, significant post-prandial triacylglycerol lowering was observed with the DAG/LGIS compared to the TAG/LGIS combination especially at wk 8. Also, post-prandial NEFA concentrations were decreased less with the LGI starch containing diets at wk 8. Taken together, these lipid metabolite changes support the possibility of lower triacylglycerol entering the bloodstream after eating (DAG effect) while increased relative amounts of NEFA were available for energy in a lower insulin environment and while maintaining glucose homeostasis (LGIS effect).

Although no diet differences were observed in circulating GIP concentrations at wk 1, the positive linear correlation of all diet group data between circulating insulin and GIP at 15 and 30 min post-prandially is consistent with the known stimulating effect of

GIP on insulin secretion. Because GIP secretory cells are more abundant in the upper part of the small intestine, it follows that there would be an early post-prandial insulin stimulatory effect as seen in the present study (i.e., at 15 and 30 min). When the diets were fed for a longer period (i.e., 8 wk), it appeared that the intestinal cells of animals fed the HGIS diets continued to secrete amounts of GIP post-prandially further adding to a more sustained insulinotropic effect. Indeed circulating insulin levels were significantly higher in the HGIS diets compared with the LGIS diet groups. It remains to be seen whether a longer post-prandial sampling period would further confirm this latter possibility.

Effects of another insulin secretagogue, GLP-1 was less notable in this study. No effects of diet on GLP-1 were seen yet time effects, post-prandially, were found for this polypeptide hormone in the circulation. It should be noted that the determination of circulating levels of GLP-1 does not allow the direct assessment of the actions of GLP-1 in which numerous interactions among paracrine, endocrine, neural, and luminal influences may exist at the cellular level. In the present study, the sampling schedule used (0 to 60 min) may not have been long enough to see any substantial changes in GLP-1 although a significant time effect was found during the post-prandial period studied. Obviously numerous other factors including species differences may influence the increase of GLP-1 post-prandially in dogs compared with other animals.

Plasma adiponectin concentrations observed in the present study were significantly higher in the LGIS/DAG diet group compared with all other diet groups. Higher

concentrations of adiponectin compared with the other diets may activate more PPARα thus induction of more lipolysis may occur which may also improve insulin resistance.

Significantly lower plasma leptin concentrations were observed in all diet groups, especially the DAG/LGIS diet group. Also a positive correlation between leptin and body weight was seen. These findings are similar to those of other investigations, and it is concluded that leptin concentration is consistent with weight loss and may thus be a reliable marker in dogs as in humans and rodents.

In addition to lower digestibility, and an increased, but safe, rate of weight loss, these data suggest that the LGIS/DAG diet combination beneficially altered biochemical and hormonal parameters such as lower post-prandial TG, lower insulin, GIP, and increased adiponectin and decreased plasma leptin concentrations while maintaining normal glucose concentrations. These alterations may help prevent post-prandial hyperlipidemia and hyperinsulinemia which are related to obesity and may induce obesity related disease such as diabetes mellitus and cardiovascular diseases.

Our results demonstrated that the 1,3-DAG diets did not stimulate UCP2 gene expression in the canine small intestine during weight reduction. We observed a more prominent starch effect than oil effect. Although there was no significant difference among the diet groups, the combinations of LGI starch and DAG/TAG tended to lower UCP2 gene expression in the small intestine compared with the diet combinations of HGI starch and DAG/TAG. From the data obtained, we speculate that the long term consumption of a LGIS diet mediates a decrease in UCP2 mRNA expression and is a marker of metabolic down regulation associated with lower diet digestibility. The

associated lower metabolizable energy of the LGIS diet resulted in more body weight loss compared with the HGIS diets. Thus, in the face of lowered energy intake from LGIS during weight loss, UCP2 may have reduced its uncoupling activity and thereby favoring relatively more ATP production than heat generation. Less expression of UCP2 in canine small intestine may thus help protect the non-obese state under conditions of moderate and/or insufficient energy intake. It may also may play an adaptive role during down regulation of energy metabolism with weight loss. Although unknown at this time, species differences regarding UCP2 expression may also exist. Finally any potential oil effect in canine intestine may be less sensitive or not strong enough to surpass the starch or total calorie restriction effects seen on UCP2 gene expression compared with mouse intestine where a DAG oil effect was seen.

Finally, it should be noted that the diet combination of LGIS/DAG supports healthy weight loss and may help prevent obesity related diseases compared with the other diet combinations studied. Although further investigations may be needed, these data help characterize the complex nature of obesity and weight management in the canine model.

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APPENDIX A-I

Diet descriptions

Preconditioning Diet (Obesity induction/re-induction period, 4 weeks; wk -8 to wk -5)

- Premium quality pet food (Hill's Science Diet, Adult Original)
- TAG (50/50 (w/w) mix of commercially available canola and soybean oil)
- Cookie (Sandies Pecan Shortbread, Keebler, MI)

  80kcal/cookie (calories from fat: 45 kcal)

Acclimation Diet (Acclimation period, 4 weeks; wk -4 to wk -1)

- Chicken by-product meal with vitamin/mineral premix
- Starch (50/50 (w/w) mix of LGI and HGI starch)
- TAG (50/50 (w/w) mix of commercially available canola and soybean oil)

Treatment Diet (Experimental period, 10 weeks; wk 1 to wk 8)

LGIS/DAG; LGIS/TAG; HGIS/DAG; HGIS/TAG

Post-prandial Diet (Post-prandial experiment: wk 1 and wk 8)

- Cooked chicken breast meat (80g)
- Combinations of LGIS/DAG; LGIS/TAG; HGIS/DAG; HGIS/TAG*

  *Containing either TAG or DAG (8g) and either LGIS or HGIS (25g)

APPENDIX A-II

Fatty acid composition of acclimation diet*

Fatty Acid	Relative %
14:0	0.3
14:1 (n-5)	ND
16:0	13.77
16:1 (n-7)	2.37
17:0	ND
17:1	ND
18:0	5.11
18:1 (n-9)	38.15
18:1 (n-7)	3.17
18:2 (n-6)	28.31
18:3 (n-3)	5.45
20:0	0.56
20:1 (n-9)	0.62
20:2 (n-6)	ND
20:3 (n-3)	ND
20:3 (n-6)	ND
20:4 (n-6)	0.41
20:5 (n-3)	ND
22:0	ND
22:1 (n-9)	ND
22:4 (n-6)	ND
22:5 (n-3)	0.28
22:6 (n-3)	0.41
24:0	0.33
24:1 (n-9)	0.26
Unidentified	0.23
SFA	20.07
MUFA	44.57
PUFA	35.13
n-3	6.14
n-6	28.99

*19.7% fat (as fed) and 4228 kcal/kg as estimated by calculation from nutrient composition using modified Atwater factors. Values are averages of two representative samples. ND = not detected.; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

APPENDIX A-III

Description of treatment diet

Protein source:

• Low ash chicken by-product meal N/TX 1200 (Tyson Foods, Inc., USA)

Protein 70% (Minimum), Fat 12-17% (Target 14.5%)

Ash 11% (Maximum), Moisture 3.5-8% (Target 5%)

Carbohydrate source:

• Alpha-corn starch and alpha-waxy starch

(Nihon Shokuhin Kako Co., Ltd., Tokyo, Japan)

1. Low glycemic index (LGI) starch: Alpha-corn starch

(70% amylose, 30% amylopectin)

2. High glycemic index (HGI) starch: Alpha-waxy starch

(100% amylopectin)

Lipid source:

• Dietary Diacylglycerol (DAG) and Triacylglycerol (TAG)

(Kao Corporation, Tokyo, Japan)

*Dietary DAG generally contain < 20% of TAG, > 80% of DAG, and < 3% of MAG.

The ratio of the isoforms of dietary DAG: 1,2-DAG: 30%, 1,3-DAG: 70%)

*Fat contents is also available from chicken by-product meal.

Vitamin and Mineral source:

• Vitamin/Mineral Mix (Premium Adog premix)

(North American Nutrition Companies, Inc., Ohio, USA)

Guaranteed analysis:

Copper (Cu): 4000 ppm, Iodine (I): 560 ppm, Iron (Fe): 2.40%, Manganese (Mn): 2000 ppm, Selenium (Se): 120 ppm, Zinc (Zn): 4.32%, Vitamin A: 218 mg/kg (1600000 IU/lb), Vitamin D3: 2.95 mg/kg (260000 IU/lb), Vitamin E: 5455 (5443) mg/kg (12000 IU/lb), Vitamin B12: 1.82 mg/kg (4 mg/lb), Riboflavin: 272.7 mg/kg (600 mg/lb), d-Pantothenic acid: 1364 mg/kg (3000 mg/lb), Thiamine: 75 mg/kg (165 mg/lb), Niacin: 3182 mg/kg (7000 mg/lb), Vitamin B6: 90.9 mg/kg (200 mg/lb), Folic acid: 143.6 mg/kg (316 mg/lb), Choline chloride: 41227 mg/kg (90700 mg/lb), Choline: 35811 mg/kg (78784 mg/lb), d-Biotin: 4.5 mg/kg (10 mg/lb). *ppm = μg/g (mg/kg), vitamin A: 1 IU = 0.3 μg, vitamin D3: 40 IU = 1 μg, vitamin E: 1 IU = 1 mg.

APPENDIX A-IV

Fatty acid composition of oil containing food components (relative %)

Fatty Acid	TAG	DAG	Chicken
14:0	0.07	0.03	0.46
16:0	4.94	2.76	28.64
16:1(n-7)	0.14	0.11	4.35
17:0	0.09	0.09	0.12
18:0	1.89	1.33	12.48
18:1 (n-9)	32.09	37.34	27.07
18:1 (n-7)	1.79	2.17	6.01
18:2 (n-6)	50.31	47.6	11.73
18:3 (n-3)	7.49	6.57	0.38
20:0	0.36	0.33	0.1
20:1 (n-9)	0.63	0.7	0.33
20:2 (n-6)	0.06	0.07	0.3
20:3 (n-6)	ND	ND	0.62
20:4 (n-6)	ND	ND	2.49
22:0	0.26	0.29	ND
22:1	0.14	0.05	ND
24:0/22:6 (n-3)	0.09	0.1	0.4
24:1 (n-9)	0.1	0.1	0.32
Unidentified	1.34	2.53	4.22

Values are average of two representative samples (g/100 g total fatty acid).
ND = not detected.

APPENDIX A-V

Fatty acid composition of treatment diets* (relative %)

	Diet			
Fatty Acid	LGIS/DAG	LGIS/TAG	HGIS/DAG	HGIS/TAG
14:0	0.23	0.19	0.25	0.38
14:1 (n-5)	ND	ND	TR	ND
16:0	11.55	12.54	10.3	16.4
16:1 (n-7)	2.15	1.89	2.26	3.52
17:0	TR	0.1	TR	0.12
17:1	0.11	ND	0.11	ND
18:0	4.11	5.36	3.89	5.66
18:1 (n-9)	39.67	37.55	38.74	36.57
18:1 (n-7)	3.89	1.68	2.89	3.31
18:2 (n-6)	30.1	29.86	32.91	27.08
18:3 (n-3)	2.76	3.28	3.33	2.93
20:0	0.41	0.65	0.72	0.42
20:1 (n-9)	0.93	0.94	0.65	0.58
20:2 (n-6)	ND	0.17	ND	ND
20:3 (n-3)	ND	ND	ND	ND
20:3 (n-6)	ND	ND	ND	ND
20:4 (n-6)	0.37	0.28	0.43	0.37
20:5 (n-3)	ND	ND	ND	ND
22:0	TR	0.6	0.39	0.41
22:1 (n-9)	ND	0.19	TR	0.13
22:4 (n-6)	TR	ND	TR	ND
22:5 (n-3)	ND	ND	ND	ND
22:6 (n-3)	0.36	0.78	0.42	0.68
24:0	ND	ND	ND	ND
24:1 (n-9)	0.6	0.55	0.49	0.63
Unidentified	2.53	1.38	1.92	1.2
SFA	16.46	19.44	15.64	23.39
MUFA	47.35	44.8	45.27	44.74
PUFA	33.67	34.37	37.18	31.05
n-3	3.12	4.06	3.75	3.6
n-6	30.55	30.31	33.43	27.45

*All diets 19.7% fat (as fed) and 4228 kcal/kg as estimated by calculation from nutrient composition using modified Atwater factors. Values are averages of two representative samples. ND = not detected; TR = trace (< 0.1%); SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

VITA

Daisuke Nagaoka received his Bachelor of Veterinary Science degree in veterinary medicine from The Nihon Veterinary and Animal Science University, Tokyo, Japan in 1997. Meanwhile, he passed the board exam and received his veterinary license from the Government of Japan, Ministry of Agriculture, Forestry and Fisheries. After that, he entered the graduate program at Nihon University, College of Bioresource Sciences in April 1997 and received his Doctor of Philosophy degree in March 2000.

He entered the graduate program at Texas A&M University in September 2004 and received his second Doctor of Philosophy degree in May 2008. His research interests include veterinary internal medicine (nutrition) and surgery in small animals. He plans to work as a veterinary clinician in Japan.

Dr. Daisuke Nagaoka may be reached at Nagaoka Animal Medical Research, 2-10-20 Mori, Isogo-ku, Yokohama, Kanagawa 235-0023, Japan. His email is daisukenagaoka@mac.com.