DIETARY FIBER /CARNITINE, DIACYLGLYCEROL, AND LOW GLYCEMIC INDEX STARCH EFFECTS ON OBESITY AND TRIGLYCERIDE RICH LIPOPROTEIN METABOLISM IN DOGS

A Dissertation

by

YUKA MITSUHASHI

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

December 2009

Major Subject: Nutrition

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Approved by:

Chair of Committee,	John E. Bauer
Committee Members,	Edward D. Harris
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ABSTRACT

Dietary Fiber/Carnitine, Diacylglycerol, and Low Glycemic Index Starch Effects on Obesity and Triglyceride Rich Lipoprotein Metabolism in Dogs.

(December 2009)

Yuka Mitsuhashi, B.S., Tokyo University of Agriculture and Technology

Chair of Advisory Committee: Dr. John E. Bauer

Obesity is the most common clinical disorder and is associated with various medical conditions in dogs. Appropriate dietary management potentially provides weight loss in a safe, healthy, and efficacious manner. In order to elucidate whether dietary fiber, carnitine, diacylglycerol (DAG), and low glycemic index (LGI) act on such dietary components, a series of studies was conducted: 1) the combination of dietary fiber/carnitine effect on short term (3 and 7 h) satiety and long term (6 weeks) canine weight loss, 2) the combination of dietary LGI/high glycemic index (HGI) starches and DAG/triacylglycerol (TAG) effect during a 9 week canine weight loss period, and 3) the DAG effect on triglyceride rich lipoprotein (TRL) metabolism isolated from canine plasma 3-4 h postprandially.

The combination of dietary fiber/carnitine supplementation decreased both food and energy intake at 3 h post-feeding, suggesting that this combination diet provided 3 h post-meal satiety. This combination supplement also increased postprandial plasma β-hydroxybutyrate (BHB) at d 42 and body fat and weight loss at d 42 from baseline. This

combination supplement did not alter plasma vitamin A distributions or concentrations although it contained high vitamin A as β-carotene. In the second study, the LGI diets resulted in a more pronounced body weight loss than the HGI diets due to lower diet digestibilities. These data are consistent with LGI diets decreasing metabolizable energy and consequently consuming less energy compared to the HGI diets. The DAG diets lowered postprandial plasma TAG at weeks 1 and 8 in and increased plasma BHB at week 8, suggesting an increase in fat oxidation. The combination of DAG/LGI decreased postprandial total cholesterol at week 8. Lipoprotein concentrations were not altered by diet types. Fasting lipoprotein lipase (LPL) and hepatic lipase (HL) activities were not affected by diets. In the final study, DAG ingestion decreased TRL and plasma TAG concentrations vs. TAG ingestion. The DAG enriched meal increased non-esterified fatty acid, monoacylglycerol, and 1,3-DAG and decreased TAG in TRLs which may be attributed to larger TRL particle size compared to the TAG meal. Consequently, the DAG derived TRLs showed increased affinity of core TAG for LPL and HL *in vitro*. Moreover, the intravenous injection of the DAG derived canine TRLs into mice underwent more rapid blood clearance associated with the greater hepatic uptake compared to the TAG derived TRL injection.

In conclusion, the combination of dietary fiber/carnitine and DAG/LGI preferably reduced body weight and stimulated fat oxidation, which promotes overall weight loss. The postprandial plasma TAG lowering effect of DAG is the result, at least partially, from the efficient clearance of TRLs from blood circulation and their ability to act as a more efficient substrate for plasma lipolytic enzymes.

DEDICATION

To my father

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NOMENCLATURE

BCS	Body Condition Score
BEI	Bioelectrical Impedance
BHB	β-hydroxybutyrate
DAG	Diacylglycerol
D_2O	Deuterium Oxide
HDL	High Density Lipoprotein
HF/C	High Fiber/Carnitine
HGI	High Glycemic Index
HL	Hepatic Lipase
LDL	Low Density Lipoprotein
LF/C	Low Fiber/Carnitine
LGI	Low Glycemic Index
LP	Lipoprotein
LPL	Lipoprotein Lipase
MAG	Monoacylglycerol
ME	Metabolizable Energy
MER	Maintenance Energy Requirement
NEFA	Non-Esterified Fatty Acid
PYY	Peptide YY
TAG	Triacylglycerol

TC	Total Cholesterol
TRL	Triglyceride Rich Lipoprotein
VLDL	Very Low Density Lipoprotein

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CHAPTER I

INTRODUCTION

The World Health Organization defines overweight and obesity as abnormal or excessive fat accumulation that may impair health. Obesity in dogs is also defined as the point at which body weight exceeds 30 % of ideal body weight whereas overweight is considered when body weight is more than 15 % of ideal body weight (1). Obesity has dramatically increased in the United States during the last two decades. Flegal et al. surveyed the prevalence of obesity in 1999-2000 for diverse population of 4115 adult male and female individuals regarded as a nationally representative sample of the United States population. They reported that 64.5 %, 30.5 %, and 4.7% of this population were considered as overweight, obese, and extremely obese, respectively, based on body mass index. The same survey was conducted in 1988-1994, in which the prevalence rate of overweight, obese, and extremely obese was 55.9 %, 22.9 %, and 2.9 % respectively (2). In 2007, the Centers for Disease Control and Prevention revealed that 25.6 % of the United States' populations are obese. In concert with these estimates, it appears that the human obese condition is also associated with canine obesity. For example, in western countries, 22.4 % to 40.0 % of adult dogs are considered overweight and obese (3, 4). In the United States, the prevalence of overweight and obesity is reported as 34.1 % of adult dog as of 1995 (5).

This dissertation follows the style of Journal of Nutrition.

In Australia in 2005, it was reported that 33.5 % of dogs were considered as overweight and 7.5 % as obese (3). In the most recent survey that was conducted in 2008, Okawa et al. found that 28 % of pet dogs in Japan are obese (personal communications). Thus, obesity has become a worldwide problem in dogs as it has in humans.

Obesity is the most common clinical disorder both in humans and dogs. It also appears to be associated with several medical conditions. These include: metabolic syndrome, cardiovascular and musculoskeletal diseases, reduced longevity, osteoarthritis, increased inflammatory mediators, and locomotion problems (6-8). Obesity also increases the risk of fertility failure and surgical risks such as difficulty of anesthetic dose, catheter placement, and decreased stamina (9). Therefore, efforts to manage canine obesity are important to prevent morbidity and mortality associated with these disorders and may provide additional insights into the human condition.

Measurement of obesity

Recognition of obesity is often misunderstood by dog owners. This is because various breeds and size differences make it difficult to evaluate obesity in dogs (10). In humans, body mass index (BMI), which takes into account the ratio of body weight to height is commonly utilized to judge obesity. BMI has strong positive correlation with body fat (11). Such a straight forward and accurate calculation would be difficult for dogs. However, various techniques have been developed to evaluate canine body compositions.

The simplest technique is the body weight measurement. While easy to perform, it requires an ideal body weight as a reference point. Because body weight can vary dependent on dog size and breed, this technique alone has little utility (11).

Morphometric techniques also allow evaluation of body composition (6). They include: tape measurements of body structure, body mass index and body condition score (BCS) in which the most commonly used technique in veterinary practice. BCS is a subjective and semiquantitative method to measure body composition, however, it is easy to measure and not invasive. Therefore, it has the potential for pet owners to utilize this technique to evaluate body composition of their pets. Using a BCS system, palpable and visual assessments are conducted which roughly correlates with subcutaneous fat, abdominal fat, and superficial musculature (7). The most commonly used scoring system in both clinical and research situations are a 9-point scale where BCS of 1 is cachexia, BCS 5 is ideal, and BCS 9 is morbid obesity. With this system, each increase in BCS is approximately equal to a 10 -15 % gain over ideal body weight. Therefore, BCS of 6 and 7 are considered as overweight (10-30% greater than ideal body weight) and BCS of 8 and 9 are considered as obese (more than 30% greater than ideal body weight). This BCS system can be applied to estimate body fat in dogs as shown in Table 1 (7, 12).

Table 1. Estimation of average percent body fat by BCS.

	Dogs	
BCS	Male	Female
	% Body fat	
5	17	20
6	22	26
7	26	31
8	31	37
9	> 35	> 43

Data sited from **7**

Total body water techniques to evaluate obesity include isotope dilution such as deuterium oxide (D_2O), absorptiometry such as dual X-ray absorptiometry (DEXA), and bioelectrical impedance. They are commonly used as research techniques to characterize the obese state. These techniques are more cumbersome, but more precise than morphometric techniques. The principle of these techniques involves a concept based on fat mass and lean body mass (8). For example, D_2O is a non-toxic, non-metabolizable, and non-invasive tracer which freely exchanges with water after intravenous injection. Thus, a post-injection measurement of tracer concentration in plasma allows one to estimate the total body water content (13, 14). In order to calculate fat free mass from total body water, one must rely on the assumption that the water content of lean body tissue is approximately 73%, which may be altered as a function of illness and age (14, 15). However, in most cases, an estimation of body fat can be quite accurately made using the following formula (14):

$$\text{Body fat} = \text{body weight} - \frac{\text{total body water}}{0.73}$$

A strong correlation of body fat mass estimated by D_2O technique and carcass analysis in dogs has been used to validate this method (14).

DEXA methods are capable of estimating body composition accurately (bone mineral content, fat free mass and fat mass) but it requires anesthesia of dogs in order to avoid movement during scans. DEXA uses two low energy X-ray beams (70 and 140 kVp) which differentiate tissue types and quantify tissue density. This is because X-rays at two different levels are impeded differently by bone mineral, lean and fat tissues. Computer algorithms enable conversion of scanned data to tissue density (11, 16). DEXA is considered to be a very precise technique to measure body composition (17). When an algorithm specific for dogs is used, the intra-animal variance is reported to be approximately only 1 % (8, 13, 14).

Finally, bioelectrical impedance (BEI) analysis is a method which conducts a weak electrical current through the body. Because this electrical current can be conducted through fat free mass, it allows total body water, fat free mass, and fat mass to be quantified (11). When more body fat is present in the body, less conductance of electrical current occurs, and thus results in a greater impedance measurement. BEI may be influenced by hydration status, food consumed, ambient temperature, physical activity prior to the measurement, examination table, patient size, shape, age, and posture and electrode positioning (11). Therefore, controlling the above conditions is critical to obtaining reliable results. BEI is noninvasive, safe and not cumbersome. Similar to other total body water techniques, BEI estimates fat free mass based on

assumption of 0.73 mL water /g. It should be noted, recently, a dog-specific portable body fat analyzing tool was developed utilizing the BEI principles (18). It has 4 electrodes which attach 2 cm laterally from the lumbar spine in dogs. This device has been shown to have a strong positive correlation to body fat as assessed by the D_2O technique (γ^2=0.88).

Cause of obesity

Obesity results from an imbalance of energy intake and energy expenditure in which energy intake exceeds energy expenditure. Multiple factors can cause this imbalance in dogs. These include: age, gender, reproductive status, presence of hormonal abnormalities such as hypothyroidism and hyperadrenocorticism (Cushing's syndrome), genetic predisposition, voluntary activity level, diet composition, and palatability (19). However, the most commonly recognized factor in dogs is excessive calorie consumption along with less exercise (20). Therefore, restricting calorie consumption is one of the simplest approaches to counteract obesity in dogs. It should be noted, however, that a loss of more than 2 % body weight in a week increases loss of lean mass as well as restricts essential nutrient consumption; therefore, careful and constant health monitoring by experts are necessary during severe weight loss (21). In addition, excessive calorie restriction generally increases hunger, and in dogs, begging behavior. Such behaviors increase stress of both canines and their owners and thus may decrease the owner's motivation for reducing their pet's body weight. Consequently, appropriate dietary management of obesity must provide both efficient and healthy

weight loss strategies. Such strategies should help conserve lean body mass while reducing metabolic and behavioral stress.

One approach for dietary weight management is to alter lipid metabolism by enhancing fat mobilization, utilization or preventing lipid absorption. Satiety enhancement by dietary supplementation is also an effective strategy in weight management. The extent to which dietary supplements or diet components may help achieve these goals has been the subject of considerable debate (12, 21). Some of these supplements to date include various dietary fiber types, carnitine, diacylglycerol, and low glycemic index starch. All have been studied for weight loss in recent years (21-26). However, which of these nutrients can potentially help achieve efficient and healthy weight loss by modifying lipid metabolism in either dogs or humans is largely unsettled.

Dietary management of obesity

Dietary fiber

Dietary fiber generally refers to those plant components including plant cell wall substances (cellulose, hemicelluloses, pectin, and lignin) as well as intracellular polysaccharides such as gums and mucilage that are resistant to hydrolysis by digestive enzymes (27). Dietary fiber is classified into insoluble and soluble fiber based on water solubility. Insoluble fiber such as cellulose, hemicellulose, and lignin resists fermentation by micro-organisms in the intestine, therefore, it is also called non-fermentable fiber. In contrast, most of the soluble fibers including inulin, pectins, gums

and fructo-oligosaccharides are fermented by intestinal micro-organisms and produce short chain fatty acids and are thus also called fermentable fiber (28).

Dietary fibers possess many different functions and activities as they transit through the gastrointestinal tract. For example, dietary fiber containing diets, in general, contain lower energy density and require longer mastication leading to the secretion of more saliva and gastric acid and, with increased stomach distention may result in increased satiety (29). Moreover, soluble fiber produces a viscous solution in the gastrointestinal tract by absorbing water. This viscous fiber is considered to slow digestion of foods by capturing nutrients including fat, digestive enzymes, and bile acids (30). This phenomenon also delays nutrient absorption. Slowness of gastric emptying also potentially provides satiety (31, 32). Therefore, the longer term intake of dietary fiber potentially reduces body weight by decreasing daily food intake due to increase of satiety. In addition, the delayed process of digestion and absorption by dietary fiber may result in lowering plasma lipid such as triacylglycerol and cholesterol. Furthermore, dietary fiber is known to bind bile acids. These bile acids can thereby be excreted by feces resulting in a large enough loss in some cases to increase bile acid synthesis from cholesterol in the liver (33, 34). Although not fully understood, viscous dietary fiber has been shown to decrease plasma cholesterol concentrations in a dose dependent manner (35).

Human and rat studies

A satiety effect of dietary fiber in humans appears to be conclusive (36-39). For example, Pasman et al. reported decreased energy intake by fiber supplementation using

post-obesity adult women (36). In this study, the subjects were provided 20 g guar gum in orange juice twice a day during the afternoon and evening for one week. Average daily energy intake by fiber supplementation for one week was significantly lower than no fiber supplementation without decreasing satiety and increasing hunger which were measured using a 100 mm visual analogue scale (36). Moreover, when investigators restricted energy intake by 4 MJ per day, the subjects given the fiber supplement significantly felt less hunger than the subjects given no supplement (36). Salas-Salvado et al. also investigated the satiety effect of dietary fiber (38). They provided a fiber mixture (3g of Plantago ovata husk and 1 g of glucomannan) either twice or three times per day for 16 weeks and compared with placebo groups (38). Results included increased satiety using the 100 mm visual analogue scale in fiber mixture groups compared with placebo. Weight loss also tended to be greater in the fiber mixture groups than the placebo group, however, it was not significant (38). The weight loss effect of dietary fiber is likely somewhat inconsistent (40-44) while its satiety effect has been conclusively reported.

Regarding the effect of dietary fiber on lipid metabolism, Salas-Salvado et al. found in the above mentioned study decreased total cholesterol and (low density lipoprotein (LDL)-cholesterol with fiber supplementation for 16 weeks compared with the placebo group (38). However, triacylglycerol in this study was not different among three groups (38). In contrast, Yamamoto et al. evaluated a mixture of two soluble fiber types in normal and diabetic rats for 2-4 weeks. They found that soluble fiber significantly lowered plasma cholesterol, triacylglycerol and food intake (45). Anderson

et al. has also reported cholesterol and triacylglycerol-lowering effects when hypercholesterolemic men were given 21.5 g/1000 kJ of dietary fiber over 24 weeks although they did not specify the fiber type in their study (39). Taken together, dietary fiber (i.e. soluble fiber) indeed appears to decrease plasma lipid concentrations.

The proposed mechanisms for plasma lipid lowering by soluble fiber have been elucidated using a rat model (46-49). For example, when rats were given wheat bran and wheat germ (10 wt% of meal) through a gastric catheter for 4 weeks, gastric and intestinal triacylglycerol lipolysis was significantly reduced (49). Moreover, rats given the wheat bran and wheat germ decreased their mucosal uptake of radiolabled lipid fractions originating from [^{14}C] triolein and [^{3}H] cholesterol which were present in the test meal while increasing the accumulation of these lipids and cholesterol in the cecum (49). As a consequence, it was observed that plasma [^{14}C] lipid fractions and [^{3}H] cholesterol were significantly decreased by both wheat bran and wheat germ (49). Together these results indicate that soluble fiber decreased plasma lipid due to less lipid digestion and absorption in the gastrointestinal tract.

Dog studies

Food intake, weight loss, and hypolipidemic effects of dietary fiber have been intensively researched in humans and rodents; however, their effect in dogs has been somewhat inconclusive to date. For example, one study was performed in which adult female English Pointers were fed 0 to 12.5 % of beet pulp as dry matter in diets for 2 weeks. It was found that incremental addition of beet pulp in the diets did not alter energy intake while dry matter intake was slightly higher when increased amounts of

beet pulp were added to the diets (50). Moreover, Butterwick et al. also reported that 3.1-23.7 g/1000 kJ total dietary fiber supplementation in adult dogs affected neither calorie nor gram intake of food. It should be noted, however, in this study, the food offered was highly restricted (energy offered = approx. $50 \times BW^{0.75}$) for weight reduction purposes (32, 51). Therefore, the result of energy and gram intake may more likely have been affected by food restriction rather than fiber supplementation. By contrast, Jackson et al. found a reduction of calorie intake by including 29 % dietary fiber in a canine diet (52). In support of this finding, Jewell et al. reported that when 12 % to 21 % of crude fiber was added to a diet on an as fed basis, dogs that were fed the diet with fiber consumed less metabolizable energy than the dogs fed a diet containing only 2 % crude fiber (53, 54).

In short term dietary fiber satiety trials, several reports are available. Butterwick et al. measured 3 h postprandial satiety. They administered a restricted amount of food to adult dogs (approx. $50 \times BW^{0.75}$) containing 3.1-23.7 g/1000 kJ dietary fiber, and then provided commercially available foods 3 h afterwards using whose intake and energy consumed as a marker of satiety. They found that the food and energy intake at the 3 h meal feeding was not different due to dietary fiber amount in the diets (32, 51). Weber et al. also evaluated 3 h postprandial satiety using a similar methodology as Butterwick et al. (55). They fed either a high fiber (410 g/MJ total dietary fiber)/high protein diet or only a high protein diet (234 g/MJ total dietary fiber) using restricted feeding ($33 \times BW^{0.75}$) in the morning, followed by offering food *ad libitum* after 3 hours. Their findings indicated that the high fiber containing diet significantly decreased energy

intake at the 3 h food offering (55). In the same study, they conducted 3 and 7 h postprandial satiety after providing the above mentioned diets with no food restriction to dogs. Energy intake at 3 h was significantly lower in the high fiber/protein diet compared with the high protein diet, but the effect was eliminated at 7 h (55). Bosch et al. conducted a 6 h postprandial satiety effect of dietary fiber supplementation in Beagles (56). They compared low fermentable fiber (8.5 % cellulose in a diet) or high fermentable fiber (8.5 % sugar beet pulp and 2 % inulin in a diet) containing diet with a no fiber supplemented diet. Six hours afterwards, they found that the fiber containing diets tended to decrease food intake compared with the unsupplemented fiber diet, but it was not statistically significant (56). Taken together, the effect of dietary fiber on satiety in dogs seems to promise very short term (≈ 3 h) satiety but may not affect satiety longer than 3 h postprandially.

Weight loss occurs due to decreased energy intake as a consequence of dietary fiber supplementation. Because of inconsistent long term satiety, weight loss by dietary fiber is also inconclusive in dogs (32, 51, 54).

Finally, it should be noted that the effect of dietary fiber on lipid metabolism in dogs has been poorly studied. Thus, further research focusing on lipid metabolism is required to understand mechanisms of dietary fiber supplementation for counteracting obesity in dogs.

Carnitine

Carnitine is an amino acid biosynthesized from lysine and methionine in the liver as well as kidney in the presence of ascorbic acid, iron, niacin, and pyridoxine (57, 58). The primary function of carnitine is to transport long chain fatty acids from cytosol into the mitochondrial matrix where fatty acid β-oxidation occurs (57). Therefore, supplementation of dietary carnitine potentially enhances β-oxidation while conserving lean body mass during weight loss.

Human and rat studies

The effect of carnitine supplementation on body fat loss has shown little benefit in human and rat studies (59-63). L-carnitine supplementation to adult ovariectomized rats for 5 weeks did not reduce the weight gain or abdominal fat (60). When overweight women were provided 2 g of L-carnitine twice a day for 8 weeks, body fat mass and lipid utilization were not changed overall compared with the control group (61). Moreover, L-carnitine (5 g/kg in a diet) was supplemented to adult rats with 50 % energy restriction for 23 days (62), yet neither body fat nor 3-hydroxybutyric acid was reduced (62).

Dog studies

Carnitine supplementation has been widely used in formulated dog foods for a weight reduction and maintenance purpose. Although limited studies have evaluated a lean body mass conservation effect of carnitine supplementation in obese dogs, a positive observation was made with carnitine effects on weight loss in dogs. Gross et al. has examined weight loss in obese dogs fed a dry extruded type, low-fat, high-fiber food

with or without L-carnitine supplementation (300 mg/kg of diet) for 12 weeks. Energy intake was restricted so as to maintain a 2.5 % weight loss per week. While food and energy intake were similar with or without L-carnitine supplementation, dogs fed the diet with L-carnitine maintained lean body mass during the same degree of weight loss compared to dogs fed diet without L-carnitine supplementation (64) Therefore, carnitine provided only an effect on body fat reduction to account for the weight loss. Another study has done by Sunvold et al. (65, 66). Obese dogs were fed a low-fat, low-fiber diet with l-carnitine supplementation (50 or 100 mg/kg of diet) for 19 weeks. During the first 7 weeks, dogs were fed *ad libitum*, and then were fed restricted amount of foods to lose 10 % of body weight over an additional 12 weeks. The results revealed that the carnitine supplemented diet groups lost a greater degree of body weight compared with the group with unsupplementated l-carnitine in the diet. In addition, dogs fed the diets with carnitine supplementation had a greater body fat loss independent of dose of carnitine supplementation (65, 66).

Diacylglycerol

Diacylglycerol (DAG) is one of the acylglycerols in which two fatty acids are esterified to either *sn*-1 and 2 (1,2-DAG) or *sn*-1 and 3 (1,3-DAG) positions. Triacylglycerol (TAG), on the other hand, consists of three fatty acids esterified to a glycerol backbone. In general, commercially available edible oils contain mainly TAG. However, these oils also contain DAG as a minor component as shown in Table 2 (67-69).

Table 2. Triacylglycerol and diacylglycerol contents (relative %) in edible oils.

	TAG	DAG		
		1,2-DAG	1,3-DAG	Total
Soy bean oil	97.9	nd	nd	1.0
Palm oil	93.1	nd	nd	5.8
Cotton seed oil	87	nd	nd	9.5
Corn oil	95.8	1.5	2.9	2.8
Safflower oil	96	1.2	2.7	2.1
Olive oil	93.3	nd	nd	5.5
Rapeseed oil	96.8	nd	nd	0.8
Canola oil	97.1	1	1.9	2.9
Sesame seed oil	95.2	1.2	2.9	4.1

nd: not detected
Data sited from 67-69

Recently, vegetable oils enriched in DAG (≥ 80 % DAG, < 20 % TAG, and < 2 % monoacylglycerols (MAG)) were developed and have been commercially available since 1999 in Japan and 2005 in the United States (Enova®, Kao Corporation, Tokyo, Japan) (70). This DAG enriched oil contains 1,2-DAG and 1,3-DAG in approximately a 3:7 ratio. Most of characteristics of DAG including palatability and digestibility are similar to that of TAG. However, due to its unique chemical structure, DAG has been reported to beneficially affect lipid metabolism, plasma lipids, and adiposity. It should be noted that the effects of DAG appear to be a dose dependent, therefore, sufficient amount of DAG may required to be ingested.

Lipid metabolism of DAG

During digestion, fat ingestion stimulates the secretion of cholecystokinin in the duodenum which subsequently stimulates secretion of pancreatic lipase (71). When dietary TAG is ingested, it can be hydrolyzed by pancreatic lipase which selectively hydrolyzes *sn*-1 and 3 positions of fatty acids to yield 2-MAG and two free fatty acids. Because this lipase is highly active, nearly all dietary TAG are hydrolyzed under normal conditions. The free fatty acids and 2-MAG then form micelles with bile acids which are subsequently absorbed into intestinal mucosal cells (71). In the enterocyte, the re-esterification of TAG occurs via the 2 -MAG pathway in which 1,2- (or 2,3-) DAG is formed from 2-MAG and one free fatty acid via monoacylglycerols acyltransferase (MGAT) (72). TAG is then produced from 1,2- (or 2,3-) DAG and a free fatty acid via diacylglycerol acyltransferase (DGAT). The reesterified TAG is incorporated into chylomicrons by microsomal triglyceride transfer protein, and then secreted into the blood stream via the lymphatic system (71).

Compared to TAG, dietary DAG contains 1,2-DAG and 1,3-DAG. Hence, 1,2-DAG is hydrolyzed to 2-MAG and one fatty acid by pancreatic lipase, which will then utilize the 2-MAG pathway in the intestinal mucosa for resynthesis of TAG. By contrast, 1,3-DAG is hydrolyzed to a fatty acid and 1- (or 3-) MAG some of which may be further hydrolyzed to glycerol and a fatty acid by pancreatic lipase (59). In the enterocyte, a small amount of 1- (or 3-) MAG also appears to be converted to 1,3-DAG (73). However, both 1- (or 3-) MAG and 1,3-DAG have little affinity as substrate for MGAT and DGAT and thereby unable to use the 2-MAG pathway (74, 75). Instead, it undergoes the

glycerol 3-phosphate (G3P) pathway. This pathway is considered to be a much slower pathway than the 2-MAG pathway for several reasons: 1) The enzymes catalyzing the 2-MAG pathway have 1/100 times lower Km for the substrates compared with that of the G3P pathway, 2) The G3P pathway is inhibited in the presence of 2-MAG in the intestinal mucosal cell, and 3) TAG formed by the G3P pathway is stored in enterocyte cytosol which is believed to be a slow turn over pool (72, 76). Therefore, a slow process of reesterification of TAG by the G3P pathway is considered to delay TAG secretion into the lymphatic and blood stream.

Yanagita et al. investigated the proposed mechanism that administration of 1,3-DAG containing DAG oil would delay or reduce TAG secretion into the circulation (77). Male Sprague-Dawley rats had cannulae and catheters inserted into the thoracic duct and stomach, respectively, and were administrated either radiolabeled DAG or TAG into the stomach via the catheter. The 24 h radioactivity recovery in the thoracic duct was then monitored. In the first hour, recovery of radioactivity in the DAG administered rats showed nearly half of the radioactivity compared with the TAG administered rats. In addition, plasma TAG was significantly lower in the DAG group than in the TAG group. This reduction in radioactivity by DAG was still observed at 24 h post oil administration. These results suggested that DAG ingestion delayed TAG secretion into the circulation at least until 24 hour postprandially and this reduction would therefore affect plasma TAG concentrations (77).

Effects on chylomicron metabolism

Recently, Yasunaga et al. found a small but significant increase in DAG concentration in chylomicrons of mice that ingested 20 wt % DAG compared with 20 wt % TAG. It was postulated that an increase of DAG incorporation into chylomicrons may have acted to modify the chylomicron oil-water interface and thereby promote acylglycerol hydrolysis. There was also increased lipoprotein lipase (LPL) activity with chylomicrons obtained from DAG fed mice as well as with a lipid emulsion mixed with DAG as substrate (78). Thus, one may conclude that the hypolipidemic effect of DAG is derived from increased LPL mediated lipolysis of acylglycerols in chylomicrons. Another study using Sprague-Dawley rats also supports the lowered plasma TAG concentrations as a consequence of enhanced adipose LPL activity after DAG intake (79) Taken together, DAG-enriched chylomicrons are likely to stimulate LPL activity postprandially. However, it remains to be established whether increased LPL activity is the reason for plasma TAG reduction. For example, a study in cats revealed that LPL deficient male cats fed a 10 wt % DAG containing diet did not decrease plasma postprandial TAG concentrations compared with cats fed a 10 wt % TAG containing diet (80). On the other hand, a human study found when an individual, homozygous for LPL deletion, consumed DAG oil, postprandial plasma TAG was still decreased compared with TAG oil ingestion (81). Moreover, DAG ingestion reduced plasma TAG compared with TAG ingestion in a human deficient in apo CII, which is a cofactor of LPL (73). These results suggest that LPL activity may help reduce plasma TAG, however, the hypolipidemic effect of DAG may occur independently of LPL mediated lipolysis.

Effects on plasma lipids

In support of the above observations Hara et al. found a significant reduction of plasma TAG in rats that were fed more than 5 wt % DAG instead of TAG as a fat source (82). Taguchi et al. also reported a hypolipidemic effect of DAG. In this study, clinically normal men were administered either DAG or TAG oil with different doses (10, 20, and 44 g) for 7 days. Postprandial serum TAG was significantly decreased at 4 and 6 h in the DAG oil group compared with the TAG oil group and the effect was independent of dose given. When plasma lipoprotein fractions (i.e. very low density lipoprotein (VLDL), LDL, high density lipoprotein (HDL)) and chylomicron were separated at 4 and 6 h in individuals administered 20 g of either DAG or TAG, the TAG concentrations in chylomicron, VLDL, and LDL, but not HDL, were also lower in the DAG group than in the TAG group. In addition, the investigators found that HDL cholesterol was increased by DAG ingestion compared with TAG ingestion. Taken together, DAG improved not only postprandial hypertriglyceridemia but also decreased the ratio of LDL/HDL cholesterol which is one of the risk factors of atherosclerosis (83).

Effects on obesity management

DAG fed over a longer time has been shown to have an effect on reducing excessive body fat as well as TAG. Although the mechanism involved is not fully understood, enhancement of genes in the fatty acid oxidation pathway may be a factor to consider in longer term DAG ingestion. For example, Murase et al. found less body weight gain when C57BL/KsJ-db/db mice (i.e. leptin signaling deficient mice) were fed a 4% α-linoleic acid-rich DAG containing diet for 1 month compared with a 4% α-

linoleic acid-rich TAG diet. The DAG containing diet group expressed up-regulation of acyl-CoA oxidase, medium-chain acyl-CoA dehydrogenase, fatty acid binding protein, uncoupling protein-2 mRNA, and β-hydroxybutyrate (BHB) activity in the small intestine and an increased temperature of the rectum. However, those gene expressions which related to fat oxidation in the liver were unchanged due to oil type (84). Meng et al. fed either 20 wt % DAG or TAG in a diet to Sprague-Dawley rats for 8 weeks. Their results showed significant reduction of body weight gain in the DAG group compared to the TAG group at week 8 in spite of the same food intake of the diets. The reduction of weight gain by DAG ingestion was associated with reduction of abdominal body fat (mesenteric, perirenal, and epididymal). At the same time, up-regulation of acyl-CoA carnitine acyltransferase (ACAT) and down-regulation of DGAT were observed in liver as a consequence of DAG ingestion. ACAT is an enzyme providing substrate for fat oxidation while DGAT is a pivotal enzyme for TAG biosynthesis in the liver providing TAG in peripheral tissue via VLDL. Thus, it was concluded that body fat reduction occurs due to the changes in enzyme regulation. Murata et al. also revealed an increase of enzymes related to β-oxidation such as carnitine palmitoyltransferase, acyl-CoA dehydrogenase, acyl-CoA oxidase, 2,4-dienoyl-CoA reductase, and Δ^3, Δ^2-enoyl-CoA isomerase in the liver when 65.8 and 93.9g DAG/kg diet was given to rats for 21 days (85).

Human studies

In order to determine if the effect of DAG on weight reduction also applies to humans, overweight and obese men and women were given 8-9 g of DAG or TAG oil in a hypocaloric diet for 6 months. The results showed the DAG group significantly decreased body weight and fat mass compared to the TAG group (86). Other human studies also consistently revealed body composition reduction as a consequence of long term ingestion of DAG. One study provided healthy men 10 g/d of either DAG or TAG oil as part of their diet for 16 weeks. At the end of study, both diet groups lost body weight but the DAG group lost a greater degree of body weight than the TAG group. In addition, at week 16, total fat and visceral fat determined by computed tomography was significantly reduced in the DAG group compared with the TAG group (87). Moreover, when C57BL/6J mice (i.e. obesity prone mice) were fed diets containing 30 wt% of either TAG or DAG for 5 months, the DAG diet group showed significantly reduced body weight gain and visceral fat compared with the TAG diet group. Moreover, the reduction was associated with the reduction of circulating leptin which is an indicator of an early period of obesity development (88). A similar mouse study reduced the oil amount to 15 wt % and fed for 8 months also revealed significant less body fat accumulation in the DAG fed mice than in the TAG fed group (84).

Dog studies

Except for the preliminary study reported in this dissertation, only one other study has investigated the hypolipidemic and weight loss effect of DAG in dogs. Overweight mixed gender adult Beagles (BCS of $\geq$ 4/5) were fed either 7 % DAG or

TAG oil coated on an extruded diet for 6 weeks (89). Both diets were isocaloric and had similar nutrient content, fatty acid compositions, and digestibilities. Diets were fed an amount to maintain the dogs' overweight condition during the study. The result showed that both oil groups consumed the same amount of energy; however, only the DAG oil group showed significant deviation in baseline values of body weight and body fat. In addition, the weight loss was significantly greater in the DAG oil group than in the TAG oil group. One possible explanation for these findings is that the DAG oil group significantly increased fasting serum BHB at week 6 compared with week 1. In addition to the weight loss effect of DAG, the investigators found a hypolipidemic effect in dogs including lower fasting serum TAG and total cholesterol concentrations in the DAG group at week 6. Further studies will help better define the effect of DAG in canine weight loss.

Safety

The safety of chronic DAG ingestion has been evaluated in human, rodents, and dogs (90-93). These studies did not find any signs of toxicity after ingesting DAG. In dogs, the chronic dietary toxicity of DAG (up to 9.5 % in a diet) was investigated. After one year DAG ingestion, the clinical conditions which included food consumption, hematology, urinalysis parameters, serum chemistry, electrocardiogram, histopathologic parameters, and changes of organ weights were all within the normal range and were similar to dogs that had ingested TAG (93). Although further research is expected to elucidate longer term (> 1 year) safety of DAG ingestion, current findings support the possible use of DAG as a dog food ingredient for weight management purposes.

Low glycemic index starch

The glycemic index (GI) is defined by the area under a glucose response curve after feeding a carbohydrate food source as compared to a known amount of white bread (94). Thus, the GI concept allows comparison of the glucose response to different carbohydrate-containing foods. The GI value of a food relates to the rate of digestion which is affected by macronutrient interactions, gastric emptying rate, food form, cooking, particle size and dietary fibers, but not soluble fiber (95-97). The GI value also correlates with the different types of starches (98).

Starches have in common linear chains of glucose polymer (i.e. amylose) or highly branched chains of glucose polymer (i.e. amylopectin) (99, 100). Both amylose and amylopectin are hydrolyzed by amylase to release smaller glucose units from the non-reducing ends which are absorbed as monosaccharides into the intestinal enterocyte. However, because it has no branches, amylose is slower to hydrolyze than amylopectin. Consequently, amylose starch results in a lower rate of glucose formation and therefore is considered to have a lower glycemic index (LGI) than amylopectin. LGI foods thus provide a slower and more consistent source of glucose to the blood stream, thereby stimulating less insulin release than high glycemic index (HGI) sugars (101). Because insulin stimulates lipogenesis and inhibits lipolysis, it is theoretically possible that long term weight loss due to a shift to fat oxidation may be result from ingestion of LGI foods compared with HGI foods (102, 103). That concept will be tested in the present study. Although the ingestion of diets containing mixed starch types may minimize a beneficial

effect, this concept of LGI foods for obesity management is well-known in human nutrition.

Human and rodent studies

Feeding normal or diabetic Sprague-Dawley rats with diets (575 g/kg) containing either LGI starch (waxy corn starch) or HGI starch (mung bean starch) for 3 weeks showed no difference in body weight, body fat, plasma TG, and LPL. However, the fatty acid synthase (FAS) activity and gene expression in epididymal adipose tissue was higher in the HGI diet group as compared to the LGI diet group. In addition, animals fed the HGI diet had higher the insulin-regulated glucose transporter, GLUT4, in adipose tissue. Moreover, phosphoenolpyruvate carboxykinase, which is also regulated by insulin and a key enzyme for gluconeogenesis, was significantly decreased in the liver of both normal and diabetic rats with HGI diet ingestion. Thus, these results suggested that LGI starch does not stimulate FAS activity and lipogenesis compared to HGI diet and may thereby provides more fat utilization as long-term metabolic effect. In a rat study, partially pancreatectomised male Sprague-Dawley rats were given diets that contained 542 g/kg of either LGI (60 % amylose and 40 % amylopectin) or HGI (100 % anylopectin) for 18 weeks (105). Food intake was adjusted to maintain the same body weight between the diet groups during the study. It was found that the HGI fed rats needed to have their food intake restricted after 8 weeks in order to maintain their body weight. Consequently, at week 18, the cumulative food intake of the LGI diet group was 13% greater than that of the HGI diet group. The LGI diet group had lower body fat and fasting plasma TG while providing higher lean body mass than the HGI diet group (105).

In humans, the beneficial effect of LGI on obesity management is controversial due to varying selections of GI index/carbohydrate sources in diets. However, at least two studies support a LGI effect (106-110). For example, Bouché et al. evaluated the GI effect on lipid metabolism as well as weight reduction (106). In this study, 11 healthy adult men were offered either a LGI or HGI containing diet twice a day (breakfast and lunch) for 5 weeks in a cross-over design (mean GI; 74% vs. 39.5%). After 5 weeks the LGI diet lowered postprandial plasma glucose and insulin compared with the HGI diet. Moreover, although body weight reduction was not observed as a function of diet, the LGI diet group significantly decreased total body fat compared with the HGI diet and this finding was associated with a decrease in leptin, LPL, and hormone sensitive lipase (HSL) mRNA in the subcutaneous abdominal adipose tissue (106) A second study reported by de Rougemont et al. using 38 overweight, non-diabetic men and women that were offered either LGI or HGI starch (mean GI; <50 for LGI vs > 70 for HGI) *ad libitum* for 5 weeks (107) showed a significant decrease in body weight in the LGI group compared with the HGI group. The LGI diet group also showed improved lipid profiles including decreased total cholesterol, LDL cholesterol, and LDL/HDL cholesterol while these parameters were not altered by the HGI diet.

Dog studies

To my knowledge, not a single study has been evaluated regarding the GI concept on obesity management of dogs. With regard to the glycemic response after LGI administration to dogs, Ngyuen et al. found that the amount of starch consumption in commercially available canine diets is the major determinant of the glucose and insulin

responses of adult healthy dogs (111). This is a consistent finding with human and rodent studies. In addition, in the case of canine weight management where starch type may be more carefully controlled, compared to human condition, the benefits of LGI starch may be more readily demonstrated. Therefore, introduction of the LGI concept to dog foods may help efficient and healthy weight reduction.

Because obesity in dogs as well as humans has become a worldwide problem, a dietary approach for obesity management that is safe, healthy, and efficacious is needed. Although there are many choices of nutrients for obesity management, this dissertation has focused on dietary fiber, carnitine, DAG, and LGI starch. Their use in obesity control has been demonstrated, which justifies the need to determine which of these dietary regimens is more effective for controlling canine and perhaps human obesity.

Preliminary study

DAG and LGI starch have potential to alter lipid metabolism during weight loss as aforementioned. Because these two diet components had not evaluated in dogs, a preliminary study was conducted to evaluate the short term postprandial effect of DAG and LGI (112).

Twelve normal and clinically intact female adult Beagles were used with 4 × 4 Latin square design including 2 weeks as a washout period. Diets were prepared with different types of oil and starch mixture: 20 g of DAG or TAG as fat sources, and 25 g of LGI or HGI as carbohydrate sources. Thus, the four meals were designated: DAG/LGI, DAG/HGI, TAG/LGI, and TAG/HGI. Oils, LGI (High amylose corn starch,

Nihon Shokuhin Kako, Tokyo, Japan), and HGI (Waxy corn starch, Nihon Shokuhin Kako, Tokyo, Japan) were provided by Kao Corporation. In order to enhance the palatability, 60 g of boiled breast meat chicken (locally purchased) was included in the diet as protein source. The nutrient compositions of these meals were 16.5 % protein, 25.3 % carbohydrate, and 58.3% fat calculated on a metabolizable energy basis. Dogs were fasted overnight one day before the study and randomly assigned to a diet group. On the day of the study, dogs were fed one of the 4 meals with 50% of the calculated energy amount needed to maintain their body condition. Blood was collected at 0, 0.5, 1, 2, 3, 4, and 6 h postprandially via jugular catheter into EDTA containing tubes in order to determine postprandial TAG and BHB concentrations. At 6 h, post heparin blood was collected 15 min after intravenous injection of 100 IU Na heparin/kg body weight for LPL and hepatic lipase (HL) activity determinations. Plasma was separated via low speed centrifugation and stored at -20 °C until analysis. An enzyme colorimetric assay was used for the TAG determinations. Two-point enzyme kinetic assay was performed to measure BHB. Finally, LPL and HL activities were measured to determine free fatty acid release by the lipase activities using a radiolabeled artificial emulsion substrate.

A significant reduction of peak values and duration for postprandial plasma TAG was observed after the ingestion of the DAG containing diets. Plasma BHB levels were unchanged among the different diets. The lipase activities were not significantly for the different diets, although the LGI containing diet caused a slight but insignificant increase in HL activity ($P = 0.085$).

This preliminary study examined the postprandial effect of a single meal containing DAG and LGI on lipid metabolism. Plasma TAG lowering effect as a consequence of DAG ingestion was consistently observed as has been reported in other DAG studies. The reason for unchanged BHB may be explained by the study design in which dogs were either given only a single meal or perhaps the amount of DAG given. The lipase activities were unchanged due to DAG but it is unknown whether differences may exist after long term feeding. In conclusion, the DAG and LGI combination diet revealed that it may be possible to improve the post-prandial lipid profile after a single meal. The data also give credence to the possibility that longer term effects of this DAG and LGI combination may provide additional metabolic alterations regarding lipid metabolism and obesity management.

Objectives and hypotheses

The overall objective of this dissertation was to evaluate the effect of dietary fiber/carnitine, LGI, and DAG with respect to their potential to support healthy weight loss in obese dogs. More specified objectives were to 1) investigate the effects of a dietary fiber and carnitine supplement on lipid metabolic and satiety alteration in obese canine during weight loss, 2) evaluate the effect of DAG and LGI on lipid metabolism in obese canine during weight loss, and 3) study the effect of DAG on canine triglyceride rich lipoprotein metabolism.

It was hypothesized that the combination of dietary fiber and carnitine will improve short-term satiety and the combination of DAG and LGI starch will support

more efficient and healthy weight loss in Beagles along with the improvement of selected plasma lipid profiles. It was also hypothesized that DAG will alter TRL lipid composition and provide a modified substrate for lipase activity and tissue uptake.

CHAPTER II

LIPID METABOLIC AND SATIETY ALTERATION WITH THE COMBINATION OF DIETARY FIBER/CARNITINE IN OBESE CANINES

Introduction

Obesity is defined as an abnormal or excessive fat accumulation that may impair health. The common strategy for obesity management in dogs provides a combination of energy restriction and exercise. However, this strategy is not always successful because it often increases metabolic and behavioral stress (113). Therefore, appropriate dietary management of obesity must provide both efficient and healthy weight loss without metabolic or other stress. Here we investigate if weight can be managed by altering lipid metabolism to enhance fat mobilization and utilization or by preventing lipid absorption using dietary fiber and carnitine supplementations.

Pet foods contain various different nutrients and it is impossible to separate each nutrient to be fed. Therefore, the present study aimed to evaluate the combined effects of dietary fiber and carnitine on satiety, weight loss, and lipid metabolism. In order to mimic a practical approach, a commercially available canine dietary supplement was used to provide dietary fiber and carnitine. This supplement also contained increased β-carotene because its main ingredient was pumpkin. Thus, a further objective of this study was to evaluate plasma vitamin A alteration by high dose of β-carotene in dogs.

Materials and methods

Animals

All Beagle dogs used in the studies were housed individually at the Laboratory Animal Research and Resources (LARR) facility, Texas A&M University according to the American Physiological Society Guidelines for Animal Research and guidelines set forth by Texas A&M University Care and Use Committee. Each dog kennel was 8ft long, 9ft high and 4ft wide. Prior to the study, physical examinations, complete blood counts and serum biochemistry profile tests were performed on all dogs to assure their normal clinical status.

Short term satiety study

A commercially available dog food and supplement were fed. Purina ONE® healthy weight formula was selected as a control diet containing lower fiber/carnitine (LF/C). A mixture of Purina ONE® plus a vegetable-based fiber supplement which contained higher fiber and β-carotene from pumpkin, which had been supplemented with carnitine, (Diet DeLite®, Vet Science, LLC) was prepared and designated high fiber/carnitine (HF/C). Both diets were nearly isocaloric (Table 3). The ratio of Purina ONE® and Diet DeLite® (HF/C diet) fed each day followed the feeding instructions provided on the label of the Diet DeLite®. For example, for each 8 oz measuring cup (ca. 96 g) of Purina ONE fed to the LF/C group, 0.75 cup (ca. 72g) of Purina ONE and 0.25 can (15 oz can) (ca. 106g) of Diet DeLite® were mixed as the HF/C diet. Total dietary fiber (TDF) in the LF/C and HF/C diets was 146.5 and 189.7 g/MJ, respectively. In

addition, 19.9 mg/MJ and 1627.2 mg/MJ of carnitine were included in the LF/C and HF/C diet, respectively (Table 3).

Two studies were performed to evaluate a possible satiety effect of the HF/C diet. In each study, 12-14 adult female Beagles ranging in age between 4 to10 years, were fed one of the diets, twice a day, for 2-3 consecutive days using a cross-over design with 7 d wash out periods. To evaluate satiety, dogs were trained to consume foods within 15 min after feeding prior to the studies. Thus, any diet left over 15 min after food presentation during the studies was considered as an index of satiety. For practical reasons, two satiety intervals were used; a 7 h interval and a 3 h interval. The 7 h interval mimicked a pattern in which owners feed their dogs twice a day while the 3 h interval mimicked snack offering between meals or between meal hunger.

In the 7 h interval study diets were prepared at 8:00 am and 3:00 pm using 1.2 times the amounts of calculated maintenance energy requirement (MER, $125 \times BW^{0.75}$) for each dog. Although the HF/C diet contained a greater amount of moisture than the LF/C diet (56 % vs. 5 %), the moisture content was not considered to be a factor and therefore was not adjusted between the two diets. In the 3 h interval study dogs were fed at 8:00 am and 11:00 am, again at 1.2 times MER. In this trial, the moisture content of the diets was adjusted to be the same for the two diets. In both trials, energy and gram food intake of each feeding period and daily total energy consumption were recorded. Water was offered *ad libitum* and dogs were allowed to freely exercise.

Table 3. Nutrient compositions of diets.

Nutrient Compositions	Diet			
	LF/C		HF/C	
	g/100g as is	*g/MJ*	*g/100g as is*	*g/MJ*
Protein - Combustion	12.7	327.1	12.3	330.0
Fiber, Crude	1.1	28.6	2.3	63.0
Total dietary fiber	5.7	146.5	7.0	189.7
Insoluble fiber	4.8	122.7	5.9	157.8
Soluble fiber	0.9	23.9	1.2	31.9
Crude Fat	4.7	121.6	4.3	115.6
Ash	3.0	76.9	3.0	81.6
Carbohydrates, Calculated	22.2	573.6	21.7	585.4
Retinol [a]	563.5	14,543.9	844.2	22,723.9
β-carotene [b]	0.02	0.56	4.02	108.19
Total Vitamin A [a]	600.5	15,500.1	7,542.8	203,035.3
Carnitine [b]	0.8	19.9	60.5	1627.2
Moisture	56.3	1453.1	56.3	1515.4
Metabolizable energy, Calculated [c]	678.9		651.0	

[a] Retinol and vitamin A are used an unit of IU/100g as is, or IU/MJ, 1IU=0.3 µg retinol, and 0.6µg β-carotene, respectively. Total vitamin A was calculated by sum of retinol and β-carotene and converting values to IU. [b] β-carotene and carnitine are used an unit of mg/100g as is, or mg/MJ. [c] Metabolizable energy is used an unit of kJ/100g as is.

Peptide YY (PYY), one of the satiety hormones which is secreted by the gastrointestinal tract, was determined as an endogenous satiety marker. Its peak level has been reported to appear in the circulation 1-2 h after food, fat, and fermentable fiber ingestion (116, 117). Therefore, blood samples were collected 0, 45, and 120 min after the foods were offered at 8:00 am during the 3 h interval study from a cephalic vein into heparin containing tubes. Three hundreds and sixty KIU/mL whole blood of aprotenin (protease inhibitor) was added into the tubes immediately before the blood collection. Plasma were separated by slow speed centrifugation and stored at -80 °C until analyses.

Plasma PYY determination

Plasma PYY was determined by an enzyme immunoassay kit (Phoenix Pharmaceuticals, Inc, Burlingame, CA) after protein extraction.

Protein extraction. Plasma was acidified with an equal amount of a buffer containing 1 % trifluoroacetic acid in 99% distilled water (DW) (Buffer A, Phoenix Pharmaceuticals, Inc, Burlingame, CA). The plasma was mixed and centrifuged at 5000 ×g for 30 min at 4 °C. A C18 SEP-COLUMN (200 mg, Varian, Inc, Palo Alto, CA) was equilibrated with 1 mL buffer which contained 60 % acetonitrile and 1 % trifluoroacetic acid in 39 % distilled water (Buffer B, Phoenix Pharmaceuticals, Inc, Burlingame, CA) followed by 3 mL buffer A three times under a 1-2 in Hg vacuum. The acidified plasma was loaded onto the pre-treated C18 SEP-COLUMN and the column was slowly washed twice with 3 mL buffer A. After discarding the wash waste, a clean tube was prepared and the peptide was slowly eluted with 3 mL of buffer B. The eluent was dried under nitrogen gas and stored at -20 °C until analyses.

Immunoenzyme assay. The extracted protein was reconstituted with an assay buffer provided in the EIA kit. Standard peptides (0.01, 0.1, 1, 10, 100, and 1000 ng/mL) were prepared from a stock peptide solution. Fifty µL of blank, samples and standards were placed into immunoplate wells which were pre-coated with secondary antibody. Fifty µL of primary antiserum were added to each well except the blank permitting binding of secondary antibody to the Fc fragment of the primary antibody. Fifty µL of biotinylated peptide were also added in each well. The immunoplate was then sealed with an acetate plate sealer (APS) and incubated for 2 h at room temperature with orbital shaking (Lab-line Junior Orbit Shaker, Lab-line Instruments, Inc, Melrose Park, IN). The APS was removed and the plate was washed 4 times using 350 µL of the assay buffer, then 100 µL of streptavidin-horseradish peroxidase were added to each well. The plate was resealed with APS and incubated using the orbital shaker for 1 h at room temperature. The plate was again washed 4 times using 350 µL of the assay buffer and 100 µL of TMB substrate solution were added. The plate was resealed with APS, covered with aluminum foil in order to protect it from light and incubated with the orbital shaker for 1 h at room temperature. Two mol/L of hydrochloric acid were added into each well to stop the reaction. The plate was read using a microplate reader (Molecular Devices Corporation, Menlo Park, CA) and its software (SoftMax® Pro, Molecular Devices Corporation, Sunnyvale, CA). The absorbance was set at 450 nm. All samples were tested in duplicate.

Six week weight loss study

Seven intact adult female obese Beagles ranging in age between 4 and 10 years were used employing a completely randomized design. The recommended amount of food restriction without losing excessive lean body mass loss is generally considered to be 60 % MER (21). Therefore, each dog was fed either the LF/C (n=3) or HF/C (n=4) diets at 60 % MER once daily in the morning for 42 d. Body weights were monitored weekly. Percentage body fat was measured using a body fat analyzer employing bioelectrical impedance (Kao Corporation, Tokyo, Japan) at d 1, 28, and 42. At d 1 and 28, 60 min postprandial blood was collected. On the final day of the study (d 42), 0 and 60 min postprandial blood was also collected. Plasma was separated by low speed centrifugation and stored at -80 °C until analyzed.

Plasma triacylglycerol determination

Plasma triacylglycerol (TAG) concentrations were measured by an enzymatic colorimetric assay. Six µL of samples were placed into microplate wells. Two hundred µL of triacylglycerol GPO reagent (Bayer HealthCare AG, Leverkusen, Germany) were pipetted into the wells and mixed. The plate was incubated at 37 °C for 10 min and left at room temperature for another 10 min to cool. The absorbance of each well was read at 490 nm with a microplate reader (Molecular Devices Corporation, Menlo Park, CA) and its software (SoftMax® Pro, Molecular Devices Corporation, Sunnyvale, CA). A standard curve was generated using 6 µL of 0, 50, 100, 200, 300, and 500 mg/dL of calibrator standards (NEAL Diagnostics, East Providence, RI). Distilled water served as a blank. Samples were tested in triplicate.

The triacylglycerol GPO reagent contained 2.5 mmol/L of ATP, 2.5 mmol/L of Mg acetate, 0.8 mmol/L of 4-aminoantipyrine, 1.0 mmol/L of DHBS, > 3000U/L of GPO, > 100 U/L of glycerol kinase, > 2000 U/L of lipoprotein lipase, > 300 U/L of peroxidase, and 53 mmol/L of buffer (pH 7.0). This reagent catalyzes the following sequenced reactions after mixing with plasma samples: lipoprotein lipase hydrolyzes TG to glycerol and non-esterified fatty acid; glycerol kinase catalyzes glycerol 3 phosphate in the presence of ATP: G3P and oxygen produce dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2) by GPO: Peroxidase catalyzes H_2O_2, 4-aminoantipyridine, and 3, 5-dichloro-2-hydroxybenzene sulfonate to quinoneimine dye. This dye is red in color which can be measured at 490 nm.

Plasma total cholesterol determination

Total cholesterol (TC) was measured using an enzymatic colorimetric assay described by Warnick (118). One L of 50 mmol/L PIPES buffer (pH 6.9) was prepared with 17.30 g of disodium salt (Sigma-Aldrich, St. Louis, MO) to form the buffer. To this was added 1.292g of sodium cholate (Sigma-Aldrich, St. Louis, MO) and 1 mL of Triton X-100 (Sigma-Aldrich, St. Louis, MO) to extend its shelf life. The PIPES buffer was warmed to 37 ºC and pH adjusted to 6.9 with 1.0 mol/L hydrochloric acid. Reagent A: 0.102 g of 4-aminoantipyrine and 1.492 g of potassium chloride (Sigma-Aldrich, St. Louis, MO) in 100 mL the PIPES buffer; Reagent B: 0.08 g of 2-hydroxy-3,5-dichlorobenzene sulfonic acid (Sigma-Aldrich, St. Louis, MO) in 100 mL PIPES buffer. Reagent C was prepared by mixing 1:1 volumes of reagent A and B with 10 U/mL of horseradish peroxidase, 0.5 U/mL of cholesterol esterase, and 0.5 U/mL of cholesterol

oxidase (Sigma-Aldrich, St. Louis, MO). Horseradish peroxidase was dissolved in saline while cholesterol esterase and cholesterol oxidase were dissolved in 3 mol/L sodium chloride.

Enzyme reaction. Six µL of plasma were placed into microplate wells. Two hundred µL of reagent C were pipetted into the wells and mixed. The plate was incubated at 37 °C for 30 min and left at room temperature for another 10 min. Absorbance of each well was read at 490 nm with a microplate reader (Molecular Devices Corporation, Menlo Park, CA) and its software (SoftMax® Pro, Molecular Devices Corporation, Sunnyvale, CA). A standard curve was generated using 6 µL each of calibration standards (0, 50, 100, 200, 300, and 400 mg/dL) (NEAL Diagnostics, East Providence, RI) with 200 µL of the same reagent. All samples, blank, and standards were tested in triplicate.

The enzyme reaction includes the following catalytic sequences: Esterified cholesterol is hydrolyzed to free cholesterol and non esterified fatty acid by cholesterol esterase: cholesterol oxidase catalyzes free cholesterol to cholest-4-en-3-one and H_2O_2; and quinoneimine dye is produced by horseradish peroxidase in the presence of 4-aminoantipyrine, 2-hydroxy-3,5-dichlorobenzene sulfonic acid, and H_2O_2. The quinoneimine dye has high absorbance at 490nm, and is proportional to the concentration of total cholesterol present in the sample.

Lipoprotein determination

Lipoprotein (LP) fractions (β, pre-β, α1, and α2 -LPs) were separated by electrophoresis using 1 % agarose gel (TITAN GEL Lipoprotein Electrophoresis

System, Helena Laboratories, Beaumont, TX). The procedure followed company instructions with some modifications. Briefly, 2 μL of sample were placed on an agarose gel after blotting the application area using Titan Gel Blotter A, allowing 7 min to diffuse into agarose. Twenty five mL of TITAN GEL lipoprotein buffer were placed into a TITAN GEL chamber system. This buffer is a barbital-sodium barbital buffer with thimerosal and 0.1 % sodium azide as preservatives. The plate was placed into the chamber in the appropriate direction. The gel was electrophoresed at 90 volts for 40 min, removed from the chamber, and dried at 70 ºC for approximately 40 min. Visualization used 0.05 g of Fat Red 7B dissolved into 200 mL of methanol as a working solution. The dried gel was immersed in a solution containing 5 mL of distilled water and 25 mL of the working solution for 2.5 min, then destained in methanol-distilled water (1:1, v/v) for 20 sec. After removing the destaining solution, the gel was placed in 2 % glycerol in distilled water for 20 sec to allow stain fixation then dried at room temperature overnight. Quantitative measurements of the bands were performed using a Model GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA) and its software (Quantity One, Bio-Rad, Hercules, CA). The absolute amounts of each LP fraction were calculated based on TC concentrations and results were expressed as lipoprotein cholesterol in mmol/L.

Plasma β-hydroxybutyrate determination

β-hydroxybutyrate (BHB) is one of the end products of fatty acid oxidation in the liver. Because BHB is not utilized in the liver, it is mobilized to peripheral tissues via the

circulation and used as one form of energy. Therefore, plasma BHB was measured as a marker of fatty acid oxidation *in vivo* using a two point kinetic method.

In preparation for the analysis, 0.1 mol/L of tris (hydroxymethyl) methylamine (Sigma-Aldrich, St. Louis, MO) with adjusted to pH to 9.5 using small volumes of 0.1 mol/L of hydrochloric acid. A working reagent was formed by mixing 19.2 mL buffer, 0.4 mL of 15 U/mL BHB dehydrogenase (Roche Diagnostics, Indianapolis, IL), and 0.4 mL of 117 mmol/L β-NAD^+ (Sigma-Aldrich, St. Louis, MO). The working solution was stable for 2 h. Fifty μL of samples were placed into microplate wells, and 200 μL of working reagent were pipetted into the wells promptly. The microplate was placed into the microplate reader (Multiskan Ascent, Labsystem, Waltham, MA), and scanning was started for 30 min at 30 sec interval and 340 nm. This microplate placement in the microplate reader was carried out quickly because catalytic reactions by the reagent started immediately after the working reagent was pipetted. A standard curve was generated by 0, 0.05, 0.1, 0.2, and 0.4 mmol/L of β-hydroxybutyrate from 20 mmol/L of stock BHB solution (Sigma-Aldrich, St. Louis, MO). Distilled water served as a blank. The data was transferred to Microsoft Excel and the concentrations of samples were calculated by subtracting the 0 min optical density (OD) from 30 min OD. The subtracted OD was fitted into the standard curve and converted to mmol/L.

The basis of the analysis is to oxidize BHB in the sample to acetoacetone using β-NAD^+ and BHB dehydrogenase. The kinetics of NADH formation measured spectrophotometrically at 340 nm is directly proportional to the quantity of BHB present in the sample.

Plasma vitamin A determination

The HF/C diet contained a higher amount of retinol (22,723.9 IU/MJ) than the LF/C diet (14,543.9 IU/MJ) and very high amounts of β-carotene (108.2 mg/MJ) compared with the LF/C diet (0.6 mg/MJ) because the major ingredient of Diet DeLite® was pumpkin. The National Research Council (NRC) reports 5,279 IU/MJ as a recommended allowance and 222,932IU/MJ as a safe upper limit (SUL) of vitamin A in a diet (119). In dogs, β-carotene is considered as provitamin A; therefore, if all the β-carotene in the diet is converted to retinal which can then further convert to retinol and retinyl esters, the total vitamin A (β-carotene + retinol) in the HF/C diet would be 203,035.3 IU/MJ. This amount exceeds the recommended allowance and is close to the SUL concentration. Although conversion of all dietary β-carotene to retinal probably does not physiologically occur in dogs, it was of interest to determine whether plasma vitamin A concentrations (retinol and retinyl esters) of dogs fed the HF/C diet would alter circulating vitamin A concentrations and its ester distributions after 6 weeks of feeding. Therefore, plasma vitamin A concentrations and distributions were also measured in the study.

Vitamin A extraction. Two hundred μL of plasma and 200 μL of ethanol were placed into a culture tube and mixed. One thousand μL of hexane were added to each tube and mixed followed by centrifugation at 1500 ×g for 5 min. Nine hundred μL of the upper layer were transferred into a clean culture tube and dried under nitrogen gas. One hundred μL of ethanol:acetonitrile (1:1, v/v) were then added to each tube and mixed.

The solution was filtered through using GHP Arcodisc® 25 mm syringe filter with 0.45 µm GHP membrane (PALL Life Sciences, Ann Arbor, MI).

High performance liquid chromatography. High performance liquid chromatography (HPLC) was employed using the following conditions: 1.0 mL/min isocratic flow of acetonitrile:ethanol:diethylamine (50:50:0.001, v/v/v) (Waters™ 510, Waters Corporation, Milford, MA), UV detection (Waters™ 486 Tunable absorbance detector, Waters Corporation, Milford, MA) at 325 nm, Nova-Pak® C18 4 µm pore sized column (3.9 diameter × 300 nm length) (Waters Corporation, Milford, MA). The samples were placed in a Waters™ 717 plus autosampler and 30 µL of the sample were injected into the system. Preliminary studies found the absence of retinyl arachidonate in dog plasma; it was thus synthesized in the lab and used as an internal standard. Moreover, retinyl esters (retinyl linoleate, retinyl oleate, retinyl palmitate, and retinyl stearate) were also synthesized for generating standard peaks.

Preparation of vitamin A standard. *Column preparation.* A column was prepared using the methodology described by Bridge et al. (120). Thirty g of alumina (Alumina Neutral, Analtech, Newark, DE) were mixed with 50 mL of hexane. Next, 1.5 mL of alumina were added slowly in the alumina-hexane mixture, and stirred to deactivate the alumina. The alumina was left at least 2 h at room temperature. An empty column with 1 cm diameter was prepared and a 1 cm diameter frit was placed in the bottom of the column. The deactivated alumina was packed in the column to fill until the alumina bed reached a 4.5-5 cm height. The bed was shielded with another frit with 1 cm diameter.

Preparation of vitamin A standard. *Retinol esterification.* Ten μmol of retinol (Sigma-Aldrich, St. Louis, MO), 0.1 mL of acyl chloride solution, and 0.1 mL of triethylamine solution were pipetted into a clean tube and mixed. The tube was incubated at room temperature for at least 10 min using an orbital shaker (Lab Rotator, Lab-line Instruments, Inc, Melrose Park, IL). In order to visualize the progress of the reaction, high performance thin layer chromatography (HPTLC) followed by visualization (described below) was used. After the reaction was complete, 1 mL of distilled water and 4 mL of diethyl ether were added to the tube. The upper layer was removed and 1 mL of distilled water was again added to wash the products. Five mL of petroleum ether were then added followed by anhydrous sodium sulfate until the solution became clear. The solution was transferred to a clean tube, dried in a stream of nitrogen, and then rehydrated with 1 mL of hexane. The alumina column mentioned above was washed with 12 mL of hexane under 1 in Hg vacuum (Vac-Elut^{TM}, Varian, Harbor City, CA). The sample was loaded onto the alumina followed by 15 mL of 1 % dioxane in hexane, which elute only retinyl esters. The eluent was dried under nitrogen gas and rehydrated with 7.5 mL of hexane. Purity was checked using HPLC in above mentioned conditions. Under these condition, peaks of retinyl arachidonate, retinyl linoleate, retinyl oleate, retinyl palmitate and retinyl stearate were shown at approximately 8, 9.5, 12.5, 13.5, and 18 min retention time, respectively (Appendix A).

Visualization of the progress of reaction for retinol esterification. A glass chamber (29.5 cm wide × 15 cm high × 10 cm deep) was equilibrated with 100 mL of cyclohexane: ether (4:1, v:v) mixed solvent. One μL of sample was taken during the incubation and spotted on a 4 × 8 cm silica gel sheet (Polygram® Sil G/UV_{254}, Alltech, Deerfield, IL) and developed in the equilibrated chamber. The migrated spots were charred using a charring solution which contains a mixture of 10 % $CuSO_4$ and 8 % phosphoric acid. Rf value of retinol and retinyl ester were approximately 0.15 and 0.80, respectively. When the retinyl ester spot became a major spot on the sheet and the retinol spot became barely visible, the reaction producing retinyl esters was considered to be complete.

Statistical analyses

Data were expressed as means ± SEM. All data were analyzed by SPSS 15.0 for Windows. Repeated measures ANOVA using a general linear model was performed using all data. Diet and time (feeding time periods or postprandial times) were included as a within-subject factor for the satiety studies. For the data obtained from the weight loss study, diet was considered as a between-subject factor and week as a within-subject factor. Follow up tests using pairwise comparisons were performed where statistically significance was found with Bonferroni adjustment. The paired t-test was assessed to check for a diet effect in the satiety studies between each feeding time period. For the weight loss study, in order to assess the independent effects of diet on d 42 of plasma lipids independent of body weight and fat loss, an analysis of covariance (ANCOVA) model was used controlling for body weight and fat change from baseline (d 1).

Normality and homogeneity of variances of all data were analyzed before all tests were conducted. Nonnormally distributed data were assessed by appropriate non parametric tests. Where variances were not homogeneous, data were transformed as logarithms. Differences were considered significant at $P < 0.05$.

Results

Food intake in the satiety studies

The first purpose of this study was to measure the satiety effect of the combination of dietary fiber/carnitine in short term. To do this we conducted the 7 and 3 hour interval feeding trials and monitored food intake. The results of the study are shown in Table 4.

In the 7 h feeding interval the HF/C diet contained 56.3% moisture while that in the LF/C diet was 5.4%. Because there was no correction applied for the moisture content, a diet effect was observed with a higher weight of food intake in the HF/C diet group ($P < 0.001$). This higher intake by the HF/C diet was consistently found at both the 1st feeding (8:00 am) and 2nd feeding (3:00 pm) periods in the same day compared with the LF/C diet ($P < 0.001$). Although the LF/C diet group consistently consumed less food than the HF/C diet group, the food intake of the LF/C diet at the 2nd feeding was significantly greater than the 1st feeding (Table 4, $P = 0.001$). It should be noted that both diet groups consumed similar energy intake in daily and the 2nd feeding period. However, energy intake at the 1st feeding period of the HF/C diet group was greater than

Table 4. Food and energy intake of 7 and 3 h interval trials in the satiety studies.

	7 h interval			3 h interval		
Food intake, *g/d*	LF/C	HF/C	*P* -value	LF/C	HF/C	*P* -value
1st fed	103.6 ± 12.1	278.9 ± 34.4	<0.001	490.3 ± 49.1	420.9 ± 45.0	ns
2nd fed	141.6 ± 13.6*	329.5 ± 34.1	<0.001	281.4 ± 61.1*	149.8 ± 36.7*	0.040
total	245.2 ± 38.5	608.3 ± 66.1	<0.001	771.6 ± 93.1	570.7 ± 66.6	0.014
Energy intake, *kJ//d*						
1st fed	1445.4 ± 168.4	1840.2 ± 206.7	0.030	3116.6 ± 345.4	2896.5 ± 312.2	ns
2nd fed	1975.7 ± 189.3*	2089.2 ± 216.7	ns	1865.5 ± 405.2*	1032.7 ± 253.2*	0.049
total	3421.2 ± 537.4	3929.4 ± 397.7	ns	4982.1 ± 635.5	3929.2 ± 460.3	0.039

Values are mean ± SEM. Moisure contents in the HF/C diet and the LF/C diet in the 7 h interval trial was 56.3 % and 5.4 %, respectively. In the 3 h interval, the moisture contents in the diets were equilibrated. Asterisks denote significant difference from 1st fed. *P*-values are for Student's t-test between LF/C and HF/C diet. $P < 0.05$ is considered significant.

that of the LF/C diet ($P = 0.03$) while the LF/C diet significantly increased the energy intake of the 2nd feeding period (Table 4, $P = 0.001$).

In the 3 hour feeding interval, although dogs fed both diets consumed less food and energy at the 2nd feeding period, the degree of suppression of intake was higher with the HF/C diet. This lower intake at the 2nd feeding period with the HF/C diet consequently resulted in a decreased total daily weight of food and energy intake (Table 4, $P < 0.05$).

Plasma PYY concentrations in the satiety studies

PYY concentrations in the LF/C diet were numerically greater at each postprandial time including 0 min (Table 5). Therefore, a significant main diet effect was observed in postprandial plasma PYY with the greater PYY concentrations in the LF/C diet ($P = 0.039$). However, when PYY concentrations were converted to relative change based on time 0, the main diet effect was eliminated (data not shown). Moreover, no statistically significant diet effect was observed at each postprandial period (Table 5).

Table 5. Postprandial plasma peptide YY (PYY) concentrations taken in response to food consumed in the 1st feeding period in the satiety study (3 h interval trial).

Postprandial time	Diet		*P* -value
	LF/C	HF/C	
Plasma PYY, *nmol/L*			
0 min	0.028 ± 0.006	0.018 ± 0.004	ns
45 min	0.029 ± 0.005	0.019 ± 0.003	ns
120 min	0.048 ± 0.027	0.024 ± 0.005	ns

Values are mean ± SEM. *P*-values are for Student's t-test between diets: ns represents non significant. $P < 0.05$ is considered.

Body weight and body fat in the weight loss study

All dogs lost body weight independent of diet ($P = 0.003$). Follow up repeated measures ANOVA within each diet group revealed only the HF/C diet group had a significant ($P = 0.018$) decrease in body weight at d 42 compared with d 1 and 28. This same effect was observed in body fat content. While all dogs lost their body fat, the HF/C diet significantly decreased the body fat at d 42 compared with d 1 and 28 ($P = 0.013$, Table 6).

Plasma lipid profiles in the weight loss study

The HF/C diet demonstrated significantly elevated BHB concentrations at d 42 postprandially compared with the LF/C diet ($P = 0.049$, Table 7) whereas the BHB concentrations at 0 min of d 42 were the same independent of diet. However, after adjusting the BHB concentrations at d 42 with % body weight and body fat with a separate ANCOVA model, the significance at 60 min of d 42 was blunted, yet still moderately significant (ANCOVA with % body weight: $P = 0.084$, ANCOVA with % body fat: $P = 0.061$).

Neither time nor diet effects were observed for plasma TG, TC, and PYY (Table 7). In addition, these findings were unchanged when loss of body weight and body fat were included as a covariate at d 42.

Table 6. Body weight and body fat during weight loss study.

		Day			*P*-value		
		d 1	d 28	d 42	time	diet	time × diet
Body weight, *kg*							
	LF/C	14.6 ± 1.3	13.5 ± 1.3	13.2 ± 1.3	0.003	ns	ns
	HF/C	15.8 ± 1.6 [a]	14.3 ± 1.4 [a]	13.8 ± 1.4 [b]			
Body fat, *%*							
	LF/C	44.3 ± 2.0	36.6 ± 6.6	34.6 ± 4.1	0.030	ns	ns
	HF/C	45.8 ± 2.5 [a]	38 ± 3.8 [a]	35.6 ± 3.8 [b]			

Values are mean ± SEM. Letters not in common denote significant differences among days within the diet, $P<0.05$. *P*-value is for repeated measures ANOVA using GLM model; ns represents non significant.

Table 7. Postprandial plasma lipid, lipoproteins, and peptide YY concentrations.

			Diet		*P* -value	
		Postprandial time	LF/C	HF/C	Repeated measures ANOVA	Student's t-test
BHB			*mmol/L*			
	d 1	60 min	0.16 ± 0.03	0.20 ± 0.06	ns	ns
	d 28	60 min	0.16 ± 0.02	0.19 ± 0.01	ns	ns
	d 42	0 min	0.19 ± 0.03	0.21 ± 0.02	ns	ns
		60 min	0.11 ± 0.02	0.18 ± 0.03	ns	0.049
TG						
	d 1	60 min	0.57 ± 0.17	0.81 ± 0.14	ns	ns
	d 28	60 min	0.32 ± 0.09	0.52 ± 0.15	ns	ns
	d 42	0 min	0.41 ± 0.00	0.47 ± 0.09	ns	ns
		60 min	0.39 ± 0.03	0.48 ± 0.09	ns	ns
TC						
	d 1	60 min	6.31 ± 0.99	5.86 ± 0.58	ns	ns
	d 28	60 min	6.01 ± 0.92	6.06 ± 0.95	ns	ns
	d 42	0 min	2.55 ± 0.42	2.15 ± 0.55	ns	ns
		60 min	6.15 ± 0.83	5.39 ± 1.27	ns	ns
pre-β+β LP-cholesterol						
	d 1	60 min	1.24 ± 0.20	1.78 ± 0.03	ns	ns
	d 28	60 min	1.79 ± 0.39	1.90 ± 0.36	ns	ns
	d 42	0 min	1.45 ± 0.18	1.40 ± 0.39	ns	ns
		60 min	1.34 ± 0.11	1.33 ± 0.32	ns	ns
α2 LP-cholesterol						
	d 1	60 min	2.06 ± 0.39	1.48 ± 0.29	ns	ns
	d 28	60 min	1.49 ± 0.32	1.38 ± 0.24	ns	ns
	d 42	0 min	1.96 ± 0.62	1.42 ± 0.51	ns	ns
		60 min	1.93 ± 0.63	1.28 ± 0.36	ns	ns
α1 LP-cholesterol						
	d 1	60 min	2.93 ± 0.42	2.47 ± 0.27	ns	ns
	d 28	60 min	2.70 ± 0.25	2.66 ± 0.42	ns	ns
	d 42	0 min	3.18 ± 0.44	2.65 ± 0.57	ns	ns
		60 min	2.87 ± 0.31	2.65 ± 0.64	ns	ns
PYY			*nmol/L*			
	d 1	60 min	0.008 ± 0.001	0.023 ± 0.009	ns	ns
	d 28	60 min	0.015 ± 0.005	0.022 ± 0.010	ns	ns
	d 42	0 min	0.007 ± 0.005	0.016 ± 0.011	ns	ns
		60 min	0.012 ± 0.002	0.017 ± 0.008	ns	ns

Values are mean ± SEM. D 1, 28, and 42 indicate sample taking in days.
P-value of repeated measures includes diet, time, and diet × time effect. No such significances were observed in plasma lipid profiles. *P*-value of Student's t-test indicates the difference between diet at each day or each postprandial period; ns represents non significant. $P < 0.05$ is considered significant.

A pre-β LP fraction was not clearly observed after electrophoresis of plasma samples. Therefore, the pre-β fraction was combined with the β fraction and data were presented as pre- β+ β LP-cholesterol. Statistical analyses did not find any significant time or/and diet effects in any of LP cholesterol fractions including pre- β+ β LP, α2, and α1 LP-cholesterols (Table 7). Moreover, the loss of body weight and body fat included as covariates at d 42 of LP-cholesterol fractions also did not show any significant diet effect.

Plasma vitamin A concentrations in the weight loss study

The major vitamin A found in the dogs' plasma at 60 min postprandially was retinol and retinyl stearate with relative percentages of approximately 26% and 35%, respectively (Table 8). Retinyl palmitate (16%), retinyl oleate (12%), and retinyl linoleate (3%) were also observed (Table 8). The distributions of those vitamin A were consistent regardless of diet, and conditions such as fasting or 60 min postprandial period. At d 42, absolute concentrations of plasma vitamin A were measured in addition to the relative % of plasma vitamin A in order to evaluate the effect of dietary vitamin A supplementation on absolute concentrations of plasma vitamin A. It was found that plasma vitamin A concentrations were not different between diets. Additionally, the HF/C diet group showed numerically increased total retinyl ester (retinyl stearate, retinyl palmitate, retinyl oleate, and retinyl linoliate) concentrations compared with the LF/C diet group but this difference was not significant (Table 8). Result obtained at d 42 were unchanged when the loss of body weight and body fat were included as covariates at d 42.

Table 8. Relative and absolute concentrations of plasma vitamin A distributions.

	Postprandial time		Diet		*P* -value
			LF/C	HF/C	
Relative amount			*relative %*		
Retinol					
	d 1	60 min	30.62 ± 9.10	21.27 ± 3.79	ns
	d 28	60 min	39.65 ± 7.77	30.06 ± 6.06	ns
	d 42	0 min	31.90 ± 4.01	27.95 ± 4.39	ns
		60 min	37.79 ± 9.17	27.29 ± 3.59	ns
Retinyl linoleate					
	d 1	60 min	3.69 ± 0.32	3.37 ± 0.39	ns
	d 28	60 min	3.33 ± 0.86	2.85 ± 0.25	ns
	d 42	0 min	3.54 ± 0.65	3.07 ± 0.20	ns
		60 min	3.16 ± 0.93	2.84 ± 0.22	ns
Retinyl oleate					
	d 1	60 min	12.95 ± 1.39	11.87 ± 0.76	ns
	d 28	60 min	11.78 ± 1.99	13.21 ± 0.91	ns
	d 42	0 min	12.98 ± 1.35	14.21 ± 0.74	ns
		60 min	12.13 ± 2.21	14.43 ± 1.13	ns
Retinyl palmitate					
	d 1	60 min	14.15 ± 2.56	16.52 ± 0.31	ns
	d 28	60 min	13.00 ± 2.50	16.00 ± 2.27	ns
	d 42	0 min	14.01 ± 1.49	16.37 ± 2.45	ns
		60 min	12.95 ± 2.30	16.50 ± 1.95	ns
Retinyl stearate					
	d 1	60 min	35.94 ± 5.32	40.60 ± 3.01	ns
	d 28	60 min	30.77 ± 3.68	32.05 ± 3.36	ns
	d 42	0 min	33.76 ± 0.54	31.96 ± 2.15	ns
		60 min	31.53 ± 3.25	32.42 ± 1.66	ns
Absolute amount			*mmol/L*		
Retinol	d 42	60 min	5.44 ± 1.95	4.82 ± 0.79	ns
Retinyl linoleate	d 42	60 min	0.28 ± 0.08	0.30 ± 0.06	ns
Retinyl oleate	d 42	60 min	0.95 ± 0.24	1.43 ± 0.60	ns
Retinyl palmitate	d 42	60 min	1.08 ± 0.37	1.74 ± 1.03	ns
Retinyl stearate	d 42	60 min	2.47 ± 0.53	3.15 ± 1.21	ns

Values are mean ± SEM. *P*-value is for *P*-value of repeated measures includes diet, time, and diet × time effect. No such significances were observed in plasma lipid profiles. Student's t-test between diet at each day or postprandial period were also not different: ns represents non significant. $P < 0.05$ is considered significant.

Discussion

The present study aimed to evaluate the effect of carnitine and dietary fiber supplementation on satiety as well as weight loss using commercially available dog foods and supplements in order to address a practical question in obesity management. Diet DeLite® was selected as a supplement to enrich dietary fiber including both soluble and insoluble fiber types, carnitine, and total vitamin A. In order to provide similar nutrient compositions and calories, Purina ONE® healthy weight formula was selected. The combination of the Purina ONE® plus Diet DeLite® supplement diet provided 30 % more dietary fiber with the same ratio of soluble and insoluble fiber, 82 times more carnitine, and 27 times more total vitamin A compared to Purina ONE® alone.

In the satiety studies, a significantly greater amount of food intake was observed in the HF/C diet during the 7 h interval trial. This finding was apparently not related to a dietary fiber effect on satiety that has been previously reported (31, 32, 51-53). This is because in the 7 h interval study, moisture contents differed between diets but were not considered. Therefore, increased moisture content in the HF/C diet resulted in a greater weight of food intake while providing similar daily energy intake compared with the LF/C diet. Because moisture has been reported to increase palatability in dogs, the higher moisture contents of the HF/C diet vs. the LF/C diet probably resulted in greater energy intake in the 1st feeding period (12, 121). In spite of the moisture difference, this increased energy consumption at the 1st feeding period of the HF/C diet appeared to have provided more 7 h post-satiety compared to the LF/C diet. The reason for this is because the energy intake of the HF/C diet at the 2nd feeding period was not increased over that

of the first period, but it was increased with the LF/C diet. Overall, it was concluded that this moisture unequivalence between diets was a more dominant satiety factor than dietary fiber and therefore failed to determine the satiety effect of dietary fiber at the 7 h interval.

The food intake of the 7 h feeding interval trial was affected by moisture content of diets. Therefore, in the 3 h interval trial, an equivalent amount of moisture in the HF/C diet was added to the LF/C diet to control for any unwanted palatability and satiety differences due to moisture content. The results indicated the HF/C diet provided better satiety by decreasing both food (gram basis) and energy intake at the 3 h post food administration period. Weber et al. reported lower food consumption in the 2nd feeding period after 3 h interval when dogs were fed a high fiber/high protein containing diet (410 g/MJ total dietary fiber) compared with a high protein diet only (234 g/MJ total dietary fiber). Jackson et al. also found a satiety effect on dietary fiber in dogs (52). Therefore, the satiety effect observed in this 3 h interval study appeared to be the result of higher dietary fiber contents in the HF/C diet compared to the LF/C diet. Whether or not carnitine also provides an additive effect on satiety is unknown. Several researchers have reported that fat oxidation is an important regulatory stimulus for the regulation of food (122-124). Carnitine is considered to increase β-oxidation postprandially, therefore, the possibility exists that carnitine may affect food intake. However, food intake regulation by fat oxidation more likely occurs due to subsequent high fat intake (122-124). If so, in the present study, because the fat content in both diets was similar, the different carnitine contents in the diets probably do not regulate food intake.

Furthermore, a study using intraperitoneal injection of lipolysis stimulator (i.e. beta-3-receptor agonist: CGP 12177) found approximately 40% decreased food intake while increasing BHB up to 1.0 mmol/L (122). This result appears to show some relationship between food intake and fat oxidation, however, beta-3 receptor agonist also alter gastrointestinal transit time and thus other mechanisms to reduce food intake beyond fat oxidation may be present (125, 126).

In the present study, PYY was selected as an endogenous satiety marker and was measured. It was because fermentable fiber has been found to increase PYY secretion in the large intestine as well as blood circulation in rat (127-129). Moreover, PYY is produced by the L-cells which are predominantly observed in the distal gastrointestinal tract in dogs (130). In the present study, the fermentable fiber contents of the diets were unknown, however, the soluble fiber is generally considered to largely reflect fermentable fiber (28). The HF/C diet contained 30 % more soluble fiber than the LF/C diet, therefore, it was expected to increase plasma PYY postprandially in the HF/C diet vs. the LF/C diet. Nevertheless, plasma PYY concentrations were not different between diets which are consistent with several canine and human reports. For example, Bosch et al. measured canine plasma PYY 0-495 min after high or low fermentable fiber fed to healthy mixed gender Beagle groups yet they did not find any different plasma PYY due to diets (56). Moreover, human studies measuring plasma PYY after fermentable fiber ingestion found blunted PYY concentrations compared to a rat study with similar study design (28, 29). Furthermore, it has been reported that insoluble fiber intake eliminated postprandial PYY elevation in adult women (132). Taken together, these reports indicate

that PYY in dogs and humans may not be as sensitive to fermentable fiber as in rats. In addition, higher insoluble fiber contents in the HF/C diet may attenuate the possible fermentable fiber effect on plasma PYY concentrations. Another factor to consider is that dogs used in the present study were either overweight or obese. Obese subjects usually show a delayed onset of satiety after consuming a meal (133). Therefore, the overweight/obese condition of dogs in the present study may also result in less sensitivity to 2 hours postprandial PYY secretion. In order to clarify these speculations, further investigations will be needed.

During the 6 week weight loss study, all dogs consumed all foods that were offered. The amount of food offered aimed to achieve a 1.0-2.0 % rate of weight loss per week. Therefore, as expected, all dogs lost weight. However, only the HF/C diet group significantly decreased their body weight at d 42 compared with days 1 and 28. The rate of weight loss was 2.1 % for the HF/C diet which is close to the range of recommended safe weight loss (21). On d 42, significant difference in BHB concentrations were observed with the HF/C diet vs. the LF/C diet. However, this effect was eliminated after correcting the BHB concentrations using body fat or body weight loss as covariates. It is noteworthy that body weight and body fat are also influenced by diets and they are intuitively linked with BHB concentrations. Thus, using an ANCOVA including these two factors as covariates may be inappropriate for this data and Student's t-test is likely to be more appropriate. Consequently, our finding of less of a decrease in BHB concentration with the HF/C diet suggested greater β-oxidation at d 42. This finding is consistent with more body fat loss with the HF/C diet on this same day and is reflected

by greater β-oxidation. The higher amount of β-carotene in the HF/C diet may also help provide more body fat loss because retinoic acid, the metabolite of retinol, suppresses the expression of peroxisome proliferator-activated receptor γ which can result in the suppression of adipocyte differentiation in rat (134). However, we are unable to address this possibility separating due to the carnitine supplementation which was also present in this study. Overall, however, the HF/C diet appeared to preferentially reduce body weight while conserving lean mass loss by enhancing fat utilization in dogs.

Although soluble fibers have been reported to have a hypocholesterolemic effect, the 31.9 g/MJ amount of soluble fiber used in this study did not influence the plasma lipid profiles including cholesterol, TAG, and LPs during the 6 week feeding period (45, 39).

As noted earlier, the HF/C diet contained a high amount of β-carotene. Dogs are considered to be able to utilize β-carotene as a vitamin A source due to the presence of enzymes (i.e. β-carotene 15, 15'-dioxigenase) which cleave β-carotene to 2 retinals as do humans, rodents, chickens, and pigs (135). Therefore, when all β-carotene in the diet is theoretically converted to vitamin A, the total vitamin A (retinol + β-carotene) in the HF/C diet amount was close to the SUL recommended by the NRC. One concern with the HF/C diet was that this higher total vitamin A concentration may result in intoxication after 6 weeks feeding. NRC does not regulate any dietary dose recommendation of daily β-carotene intake. It should be noted, however, β-carotene is considered to have low toxicity for dogs compared with dietary retinol supplementation.

Therefore, the β-carotene amount contained in the HF/C diet (i.e. 90% of the dietary vitamin A) was not expected to have harmful consequences (119).

Both in humans and dogs, the liver is the main storage reservoir for vitamin A (136) and in humans, fasting plasma vitamin A does not reflect overall vitamin A status except in chronic vitamin A intoxication (137, 138). However, it has been found that supplementation of β-carotene to children and adults for more than 6 weeks increased both fasting plasma β-carotene and retinol which is the main form of vitamin A in blood circulation in this species (139). By contrast, in dogs, to our knowledge, no study has been conducted to measure plasma vitamin A concentrations including retinol and retinyl esters after feeding β-carotene. It has been reported that vitamin A supplementation in dogs alters plasma retinyl ester concentrations dose-dependently, and these are the major plasma vitamin A components in dogs (140, 141). Therefore it was of interest whether plasma vitamin A was altered by longer term ingestion of a high amount of retinol and very high amount of β-carotene in the diet. In order to address this possibility, we evaluated plasma vitamin A concentrations and retinyl ester distributions in the present study. Our finding indicates that high amount of dietary vitamin A supplementation, mainly as β-carotene, showed no effect on either the 60 min postprandial or fasting plasma vitamin A distributions. The absolute concentrations of plasma vitamin A at d 42 were also not different between the diets. These results suggest that both fasting plasma and early postprandial vitamin A concentrations do not reflect a high dietary dose of β-carotene supplementation as a source of vitamin A after 6 weeks in dogs. There are several possible reasons for this observation. First, the HF/C diet

contained 56% more retinol than the LF/C diet (22,724 g/MJ vs. 14,544 g/MJ); however, the retinol amount in the HF/C diet is only 3 times more than allowable retinol concentration according to the NRC recommendation (119). Goldy et al. found increased plasma retinol when 2.3 times more than SUL amount of retinol was administered to dogs. However, the increase of plasma retinol concentrations was not found when they fed less than 1.2 SUL amount of retinol to dogs in the same study (142). Therefore, the dietary retinol in the present study was not high enough to alter plasma retinol concentrations. Second, another study investigated a single oral administration of 0, 50, 100, and 200 mg of β-carotene to dogs and found an increase of plasma β-carotene in a dose dependent fashion (143). In that study, peak plasma β-carotene concentrations occurred at 6 h postprandially which is in contrast to a human study that found peak postprandial plasma β-carotene concentrations at approximately 24-48 h (144, 145). Moreover, plasma β-carotene in dogs decreased rapidly to its baseline concentrations within 24 h post-feeding which is much faster than in humans. In humans, the blood clearance of β-carotene was an apparent half-life of 6-11 days (141, 146). These results suggest that dogs absorb and clear β-carotene more rapidly than human and possibly cleave more of it in the liver or peripheral tissues carried by lipoproteins. This more rapid absorption and clearance of β-carotene in dogs may therefore affect fasting and early postprandial plasma vitamin A concentrations differently after its supplementation compared with that in humans.

In conclusion, the combination of dietary fiber and carnitine supplementation using a commercially available dog food and supplement in obese canines was

evaluated. This supplement combination improved satiety at 3 h postprandially while increasing fat oxidation and was successful in decreasing a greater degree of body fat loss associated with body weight loss. Plasma lipids were unchanged in this supplement combination. Concentrations and distributions of plasma vitamin A were not affected in spite of a dietary amount close to the NRC SUL vitamin A supplementation for dogs. Obesity is a problem that has reached epidemic proportions in dogs as well as humans. These findings may help provide better choices for both owners and professionals to achieve healthy weight loss of their pets.

CHAPTER III

THE EFFECT OF DIACYLGLYCEROL AND LOW GLYCEMIC INDEX STARCH ADMINISTRATION ON LIPID METABOLISM DURING CANINE WEIGHT LOSS

Introduction

Obesity in dogs is considered multifactorial, however, the most widely recognized factor is excessive calorie consumption along with less exercise. Therefore, reducing calorie consumption is one of the simplest approaches to counteract obesity. It should be noted, however, that excessive calorie restriction generally increases hunger, and in dogs, begging behavior. Such behaviors increase stress on both canines and their owners and therefore may affect the pet owner's motivation for reducing their pet's body weight. Consequently, prevention as well as appropriate dietary management of obesity must provide both efficient and healthy weight loss strategies to achieve weight loss while conserving lean body mass and reducing metabolic and behavioral stress. In Chapter I in this dissertation, it was found that the combination of dietary fiber and carnitine acts such dietary components to provide beneficial canine weight loss. Diacylglycerol enriched oil and low glycemic index starches may be also help achieve efficient and healthy weight loss and manage the obese state.

DAG is one of acylglycerols in which two fatty acids are esterified to either *sn*-1 and 2 (1,2-DAG) or *sn*-1 and 3 (1,3-DAG) positions. TAG, on the other hand, comprises the major acylglycerol type in edible oils and consists of three fatty acids esterified to a

glycerol backbone. DAG, specifically the 1,3-DAG isomer, has been reported to enhance fat oxidation as an adjunct to a hypolipidemic effect in both humans and rats (70 ,73, 85, 147-149). In dogs, we previously evaluated postprandial effects of a single meal containing 20 g DAG oil mixed with 60 g of boiled boneless chicken breast, and 25 g of high- or low-glycemic index starches to adult female Beagles (112). The results indicated that DAG containing meals significantly decreased 2 and 3 h postprandial plasma TAG (112). Umeda et al. also found a plasma TAG lowering effect along with plasma cholesterol reduction and plasma β hydroxybutyrate enhancement when 7 % DAG was included in diets fed to Beagles for 6 weeks (89). Thus, hypolipidemic effects have also been shown in canine studies. However, longer term effects of DAG specifically with respect to postprandial lipid metabolic alteration as well as lipoprotein metabolism have not been studied in the canine model.

Because insulin stimulates lipogenesis and inhibits lipolysis, more weight loss may be possible with longer term ingestion of LGI foods compared with HGI foods (101-103). Although the ingestion of diets containing mixed starch types may minimize such a beneficial effect, the concept of LGI foods for obesity management is well-known in human nutrition. In the case of canine weight management where starch type may be more carefully controlled, the benefits of LGI starch may be more readily demonstrated. Therefore, the objective of this study was to determine the effects of DAG, LGI, or combination of DAG and LGI on plasma postprandial and fasting lipid metabolism as well as lipoprotein metabolism in adult Beagles during a 9 week weight loss period.

Materials and methods

Animals

This study was approved by the Texas A&M University Animal Care and Use Committee under animal use protocol # 2004-201 entitled "Evaluation of dietary DAG oil in obesity management". All Beagle dogs were housed individually at the LARR facility, Texas A&M University according to the American Physiological Society Guidelines for Animal Research and guidelines set forth by Texas A&M University Care and Use Committee. Each kennel for dogs was 8ft long, 9ft high and 4ft wide. Prior to the study, physical examinations, complete blood counts, and serum biochemistry profile tests were performed on all dogs to assure their normal clinical status.

Twelve obese, intact female adult Beagles were selected from the colony in the LARR facility. The age of the dogs ranged from 2 to 6 years old at the beginning of the study. In order to assure an obese starting point, a 9 scale BCS system in combination with body fat measurements was determined for each dog. BCS assesses each dog's obese condition by palpation and observation, therefore, it is a subjective evaluation. Generally, a BCS more than 8 out of 9 and % body fat > 35% indicate canine obesity and these values were therefore used as indices of obesity for this study. Dogs were given water *ad libitum* and allowed exercise freely inside their kennels during the study.

Diets and experimental design

Four experimental diets were prepared using a mixture of starch (LGI vs. HGI) and oil (DAG vs. TAG) types: LGI/DAG (LD), LGI/TAG (LT), HGI/DAG (HD), and HGI/TAG (HT). The diets were formulated using chicken by-product meal (Tyson Foods, Inc., Oklahoma City, OK), DAG or TAG enriched dietary oil with similar fatty acid compositions (Appendix B) (Kao Corporation, Tokyo, Japan), and LGI or HGI starch to provide the same amount of macronutrients in each diet (24.8 % ME protein, 39.6 % ME fat and 35.6 % ME carbohydrate). Vitamin/mineral premix for dogs (Akey, Lewisburg, OH) was added to satisfy canine health maintenance requirement. Gelatinized high amylose corn starch and waxy corn starch was used as the LGI and HGI starch sources, respectively (Nihon Shokuhin Kako, Tokyo, Japan) (Table 9). In order to eliminate composition alterations by batch differences, all ingredients except oils were homogenized separately with the starch types and stored at room temperature before the study started and were used throughout the study. The homogenized ingredients had a powder texture, to which 2-3 volumes of water were added along with 13.5 wt % oils before feeding. After mixing these homogenized powders with oil and water, all diets had a gruel appearance due to the presence of the gelatinized starches. Obesity had been induced in the dogs prior to the study. During induction of obesity, the dogs were fed dry foods (Science Diet® Adult Original), a mixture of canola and soy bean oil (40 g), and pecan shortbread cookies (Keebler, Inc) as an appetite stimulant. Once they reached an obese body weight based on BCS, the pecan shortbread cookies were removed and their obese body weights were maintained for an additional 2 months.

Table 9. Composition of experimental diets.

Nutrients	Diet			
	LD	LT	HD	HT
	g/Kg			
Poultry byproduct meal	430	430	430	430
DAG	135	-	135	-
TAG	-	135	-	135
LGI (High amylose corn starch)	430	430	-	-
HGI (Waxy corn starch)	-	-	430	430
Vitamin/mineral pre-mix [1]	5	5	5	5
Water	1730	1730	2595	2595
Compositions[2]	% dry matter			
CrudeProtein (Kjeldahl)	32.4	31.9	33.3	34.4
Fat (Acid hydrolysis)	22.0	22.8	23.9	23.3
Carbohydrate	39.9	38.8	38.5	37.6
Crude Fiber	<2.0	<0.2	<0.2	<0.2
Ash	5.7	6.5	4.3	4.7
	kJ/g			
Gross energy	23.7	22.7	23.1	22.5
Metabolizable energy[3]	17.96	17.61	20.58	20.22

[1] Mixture contains following nutrients; Copper, 4000mg/kg; Iodine, 560mg/kg; Iron, 2.40%; Manganese, 2000mg/kg; Selenium, 120mg/kg; Zinc, 4.32%; Vitamin A, 218mg/kg; Vitamin D3, 2.95mg/kg; Vitamin E, 5455mg/kg; Vitamin B12, 1.82mg/kg; Riboflavin, 272.7mg/kg; d-Pantothenic Acid, 1364mg/kg; Thiamine, 75mg/kg; Niacin, 3182mg/kg; Vitamin B6, 90.9mg/kg; Folic Acid, 143.6mg/kg; Choline Chloride, 41227mg/kg; Choline, 35811mg/kg; d-Biotin, 4.5mg/kg, ppm=µg/g, vitamin A: 1 IU=0.3µg, vitamin D3: 40 IU=1µg, vitamin E; 1 IU=1 mg.
[2] Composition of each diet were measured by Midwest Laboratories, Inc.
[3] Metabolizable energy was determined from digestibility data.

The reason for this extension of time was to model a more metabolically stable form of obesity because the animals' metabolism may be dynamically different with long-standing obesity. Next, an acclimation diet was formulated using a 50/50 (w/w) blend of LGI and HGI starches, a 50/50 (v/v) mixture of canola and soy bean oils, the vitamin premix, and chicken by-product meal. This diet was then fed for 4 weeks prior to the weight reduction period (wk -4 to wk -1). This diet was formulated to provide the same macronutrient and fatty acid compositions as the experimental diets (Appendix C). At week 1, the dogs were assigned into 4 groups, blocked by age, body weight, and body condition scores to minimize bias and fed one of the experimental diets (LD, LT, HD, or HT) using an obese maintenance energy requirement (MER) ($125 \times (\text{obese body weight})_{kg}^{0.75}$) amount. Diets were prepared each morning and fed for 9 weeks. Five h after food presentation, food was removed from the kennels and weighed. Body weight was monitored weekly. BCS was checked at week 1 by the same individual due to its subjective nature. At weeks -1, 4, and 9, body fat was measured.

This study was designed as a partial cross-over. That is, each dog was fed two of the four diets after an appropriate wash out period. Briefly, after the first 9 week regimen (period 1), obesity was re-induced. This process took 10 weeks and was maintained as mentioned above. Complete details regarding the obesity induction and re-induction periods have been previously published (150). Dogs were then again fed the acclimation diet for four weeks followed by assignment to a treatment diet exactly opposite to the one they had been fed during period 1 of the study for 9 weeks (e.g. if fed LD in period 1, they were then fed HT in period 2).

Fecal collection

Twenty-four h fecal samples were collected for 5 consecutive days during week 3. These feces were pooled for each dog, frozen at -80 °C, and macronutrient digestibilities were analyzed using proximate analyses by an outside laboratory (Midwest Laboratories, Inc., Omaha, Nebraska).

Blood collection

Blood was collected at several periods in various ways as follows: At weeks 1 and 8, postprandial blood was collected at 0, 15, 30, 60, 120, and 180 min. After withholding food overnight, in order to enhance rapid consumption, diets for postprandial samples were prepared with a mixture of 80 g of boiled chicken breast meat, 8 g of either TAG or DAG oil, 25 g of LGI or HGI starch and water. These diets were formulated to obtain a similar degree of nutrient composition as the experimental diets (23.7 % ME protein, 37.9 % ME fat and 38.4 % ME carbohydrate). Dogs had catheters placed into their jugular veins before the postprandial diets were fed. Blood was collected via the catheters into EDTA containing tubes.

At weeks 1, 4 and 8, food was withheld overnight and 7 mL of fasting blood was taken via a jugular vein into EDTA tubes. During weeks 1 and 8 on days when postprandial samples were not taken, dogs were fasted overnight and 3 mL of post-heparin blood was collected from via a cephalic vein 10 min after intravenous injection of 100 I.U. Na heparin/kg-body weight into the opposite cephalic vein.

Finally, at weeks -1, and 4, dogs were fasted overnight and 0.2 g deuterium oxide (D_2O) /kg-body weight ($\geq$ 99.75% purity) was administered via a cephalic vein and 3

mL blood was collected before and 90 min after D_2O injection from the opposite cephalic vein into EDTA tubes.

Plasma was separated by low speed centrifugation for 15 min and stored at -20 ºC until analyses. Less than 50 µL of plasma was stored at 4 °C for no more than 2 d to determine LP fractions.

Plasma lipid determination

Plasma TAG was measured by an enzymatic colorimetric assay. TC was measured using an enzymatic colorimetric assay. LP fractions (β, pre-β, and α-LPs) were separated by electrophoresis using 1 % agarose gel (TITAN GEL lipoprotein Electrophoresis System, Helena Laboratories, Beaumont, TX). Relative amounts of each fraction were determined by densitometry (Model GS-700 Imaging Densitometer, Bio-Rad Laboratories, Hercules, California). Absolute amounts of the LP fractions were calculated based on TC concentrations and results presented as lipoprotein cholesterol in mmol/L. BHB was measured as a marker of *in vivo* fat oxidation using a two point kinetic method.

Plasma LPL activity measurement

LPL determination *in vitro* followed the methods of Nilsson-Ehle and Ekman with some modifications (151). First, a radiolabeled lipid emulsion was prepared as a substrate of LPL by mixing 7.92 µmol of triolein (Nu-Chek Prep, Inc., Elysian, MN), 0.6 µmol of lysophosphatidyl choline (Sigma-Aldrich Co., St. Louis, MO), and 1×10^7 cpm/µmol amount of labeled triolein ([9,10-^{3}H(N)]-triolein, PerkinElmer, Inc, Waltham, MA). Because triolein was dissolved in organic solvents such as toluene and ethanol, the

mixture was first evaporated under nitrogen gas. 2.4 mL of 0.2 mol/L tris-HCl buffer (pH 8.0) was then added to the dried mixture and sonicated (Model 300 V/T Ultrasonic homogenizer, Biologics, Inc., Manassas, Virginia) in an ice bath for 4 min with 30 sec interval with 40 W setting in order to homogenize and introduce triolein into the lipid core. After the radiolabeled lipid emulsion was prepared, 0.3 mL dog plasma was heated at 56 °C for 15 min and added as a source of apo-CII which is a cofactor of LPL activity. Because albumin is the protein that binds fatty acids released by lipase activity, 0.3 mL of 4 % (w/v) bovine serum albumin (BSA) in 0.2 mol/L tris-HCl (pH 8.0) was also added to the substrate mixture. This substrate was stable at least 6 h at 4 °C.

Second, the substrate mixtures were reacted with an LPL source. Post-heparin dog plasma was diluted 25-fold (4 µL sample, 96 µL 0.15 mol/L NaCl) as LPL enzyme source. One hundred µL of diluted plasma were placed in duplicate in glass tubes. Two clean tubes were prepared and 100 µL of 0.15 mol/L NaCl was added as blank. One hundred µL of the substrate mixture were added to each tube and incubated for 30 min at 37 °C with constant shaking. In order to stop the reaction, 3.25 mL methanol: chloroform: heptane (1.41:1.25:1 (v/v/v)) was added to the tubes and 1.05 mL of 0.1 mol/L potassium carbonate buffer (pH 10.5) were then added to each tube and mixed on a vortex-type mixer for 15 sec followed by centrifugation for 15 min at 4000 ×g (Allegra® X-15R Centrifuge, Beckman Coulter, Fullerton, CA). One mL of the upper layer containing methanol: water was placed into Pony Vial™ (PerkinElmer, Inc, Waltham, MA). Thirty µL of substrate mixture were also prepared in a separate Pony Vial™. Finally, 5 mL of liquid scintillation counting cocktail (Ultima Gold™ ,

PerkinElmer, Inc, Waltham, MA) were added to each tube and labeled fatty acid released was measured by a scintillation counter (1900TR Liquid Scintillation Analyzer, Packard Instrument Company, Downers Grove, IL).

Plasma HL activity measurement

A similar methodology used for the LPL determination was employed to measure HL activity. The differences were 1) timing of albumin introduction into substrate mixtures, 2) concentration of sodium chloride, 3) buffer pH, and 4) dilution amount of post heparin plasma. Briefly, after the mixture of triolien, radiolabeled triolein, and lysophosphatidyl choline was dried under nitrogen, 1.8 mL of 0.2 mol/L tris-HCl buffer (pH 9.0) were added which is the optimal pH for HL activity. Because Nilson et al. found that HL activity was inhibited when BSA was added after sonication (151), therefore, 1.2 mL 1 % (w/v) BSA in 0.2 mol/L tris-HCl buffer (pH 9.0) were added to the substrate mixture before sonication. This mixture was then sonicated and stored at 4 °C until use. Fifty µL of post-heparin plasma were placed into a tube which was then adjusted to 1 mol/L NaCl by adding 50 µL 2 mol/L NaCl which inhibits LPL activity. Substrate was added, incubated for 15 min at 37 °C, the reaction was stopped, and radiolabeled fatty acids released were measured via scintillation counting.

The counts obtained from both LPL and HL were converted to nmol FA/min/mL using a formula in Appendix D.

Body fat determination

Two techniques were used for determining body fat percentage. First, one of three body fat analyzers (Kao Corporation, Japan) was used. These analyzers contained two probes which enable body fat mass to be measured by sending a weak electrical current through the body and determining bioelectrical impedance (BEI). Briefly, before the measurement, dogs were gently restrained on an insulated material without any stress. In order to increase the electric conductance, axillas were cleaned with 70 % ethanol solution. Each probe was then placed on the axilla and BEI was measured five consecutive times. The average value was used for the calculation obtained for body fat using the following formula.

$$\text{Body fat} = 10.02 \times \frac{\text{AC}}{\text{BL}} - 11.792 + 0.07 \times \text{BW} \times \frac{\text{AC}}{\text{BL}} + 0.166 \times \text{BEI}$$

where AC, BL, and BW denote abdominal circumference (cm), body length (cm), and body weight (kg), respectively. The unit for BEI is ohm.

Second, D_2O dilution technique was used as the standard body fat percentage method which is known for its high accuracy and reliability. The body fat by D_2O dilution technique was determined by measuring the concentrations of deuterium in pre- and post- D_2O diluted plasma with isotope ratio mass spectrometry at an outside laboratory (Kao Corporation, Japan). In order to address the most accurate body fat analyzer among the three analyzers tested correlations of body fat obtained from each analyzer vs. D_2O dilution were determined.

Statistical analyses

Data was expressed as means ± SEM. Postprandial TAG, BHB, and TC concentrations were converted to the % change from the baseline concentration (0 min) before analyses since some of these concentrations at 0 min showed wide variance. All data was analyzed by SPSS 15.0 for Windows. Data obtained during the postprandial period was analyzed by repeated measures ANOVA using a general linear model (GLM) with feeding periods, oil types (DAG vs TAG), and starch types (HGI vs LGI) as between-subjects factor, and postprandial time as a within-subject factor. Fasting samples were also analyzed by repeated measures ANOVA using a GLM model with feeding periods, oil types, and starch types as between-subject factors and week as a within-subject factor. When a main feeding period effect was observed, the data obtained from period 1 and 2 was separately analyzed by repeated measures ANOVA. When main oil, starch, time, or interaction effects were found, pairwise comparisons using a Bonferroni correction were assessed at each level for follow up tests. To assess diet effects within each time or week, two-way ANOVA was conducted as oil and starch types as a fixed factor. Because body weight was reduced during the study, % body weight loss (based on week 1 body weight) was included in an ANCOVA model as a covariate to determine starch, oil or/and oil × starch interaction effects independent of body weight. Where significant main oil or starch effects were observed, follow up multiple comparisons were assessed. When oil and starch interaction was observed, multiple comparisons at each level by Bonferroni adjustment were performed. Linear regression analysis was conducted for body fat data obtained between the D_2O dilution

technique vs. the three body fat analyzers. In addition, correlation of weekly TC and lipoprotein fractions was also determined using linear regression analysis. Normality of dependent variables and homogeneity of population variance were analyzed before all tests were conducted. If data was nonnormally distributed, appropriate non parametric tests were performed. Where variances were not homogeneous, data was transformed using appropriate algebraic calculations. Differences were considered significant at $P \leq 0.05$.

Results

Food consumed, metabolizable energy, and digestibility

Each dog was fed an amount of diet needed to maintain its obese body weight ranging from 3000 to 5500 kJ/d during the study which is the amount of obese MER. Nevertheless, all dogs voluntarily consumed only 68 ± 4 % (mean ± SEM) of food per a day that was offered on a weight basis. When food intake was considered on a per group basis, the LGI containing diet group consumed significantly less energy than the HGI diet group (Table 10, $P = 0.036$). It should be noted that this starch effect was not observed for the amount of food consumed on a dry matter weight basis. Macronutrient digestibilities showed significantly lower carbohydrate digestibility with the LGI containing diets as well as protein and total digestibility (Table 10, $P<0.005$). In comparison, fat digestibility remained high independent of diets (97.4 ± 0.4%) (Table 10). Lowered macronutrient digestibility in the LGI diets consequently resulted in lowered metabolizable energy (ME) (Table 10, $P < 0.001$). Energy consumed was

Table 10. Food consumed, digestibility, and metabolizable energy in experimental diets.

	Diet				*P* -value		
	LD	**LT**	**HD**	**HT**	**Oil**	**Starch**	**O × S**
Energy consumed, *kJ/d*	**2065.4 ± 187.8 [a]**	**1973.5 ± 254.0 [a]**	**2587.9 ± 429.8 [b]**	**2929.2 ± 396.0 [b]**	**ns**	**0.036**	**ns**
Food consumed, *g DM/d*	**115.0 ± 9.4**	**111.8 ± 13.8**	**125.8 ± 21.4**	**143.1 ± 14.7**	**ns**	**ns**	**ns**
Digestibility, *%*							
Starch	**64.9 ± 4.1 [a]**	**73.9 ± 0.9 [a]**	**98.2 ± 1.2 [b]**	**98.1 ± 0.9 [b]**	**ns**	**<0.001**	**ns**
Protein	**83.1 ± 1.3 [a]**	**83.5 ± 1.8 [a]**	**94.4 ± 1.9 [b]**	**93.9 ± 3.5 [b]**	**ns**	**0.004**	**ns**
Fat	**96.5 ± 0.6**	**96.5 ± 0.7**	**98.2 ± 0.7**	**98.1 ± 1.0**	**ns**	**ns**	**ns**
Total	**79.8 ± 2.1 [a]**	**82.1 ± 1.7 [a]**	**95 ± 1.2 [b]**	**93.7 ± 1.8 [b]**	**ns**	**<0.001**	**ns**
Metabolizable Energy, *kJ/g*	**18.0 ± 0.6 [a]**	**17.6 ± 0.3 [a]**	**20.6 ± 0.3 [b]**	**20.2 ± 0.8 [b]**	**ns**	**<0.001**	**ns**

Mean ±SEM, n=6.; ns denotes no statistical difference. Values in a row without a common letter differ, $P < 0.05$.
P-values are for ANOVA with starch and oil as fixed factors. O × S represents an interaction effect of oil and starch.
DM = dry matter.

calculated based on ME, therefore, it was not surprising that less energy but not less amount of food was consumed when the LGI diets were fed.

Body fat analyzer validation

Three preproduction prototype body fat analyzers (analyzers 1, 2, and 3) were supplied by the Kao Corporation. All were neither invasive nor cumbersome to use and may have had potential for use as a routine body fat measurement tool in the laboratory as well as in veterinary practice. In order to validate these analyzers' accuracy, % body fat was measured and compared using the D_2O dilution technique on the same day that body fat was measured with each analyzer (week -1 and 4). The strongest correlation coefficient between % body fat and D_2O dilution was obtained using analyzer 2 which resulted in a significant positive linear relationship (Appendix E; $\gamma^2 = 0.518$, $P < 0.001$). The correlation coefficient of analyzer 1 and D_2O injection was 0.461 (Appendix E; $P < 0.001$). Although analyzer 3 was the simplest analyzer with respect to its ease of operation, its correlation coefficient was 0.291 (Appendix E; $P = 0.001$). Based on these results, analyzer 2 was selected as the most accurate body fat analyzer among the three analyzers tested and used in the present study.

Body weight and body fat

Prior to the study, the dogs' body weight, % body fat, and BCS were 14.8 ± 0.2 (SEM) kg, 48.9 ± 3.3 %, and 8.4 ± 0.1, respectively. Except for one dog during period 1 eating the HT diet, all dogs lost body weight during the study. When body weight was converted to % change of body weight relative to week 1, a statistically significant main starch effect was seen ($P = 0.001$) with greater % body weight loss in the LGI diet

groups compared with the HGI diet groups. Two-way ANOVA revealed that this body weight lowering effect by the LGI diets started at the 2nd week of the study and remained significant thereafter (Figure 1; $P < 0.01$).

All dogs significantly decreased their % body fat averaging a 9.3 % loss at the end of the study (Figure 2; $P = 0.001$). However, the loss of body fat occurred independent of starch and oil types. Body weight and % body fat were positively correlated but the correlation coefficient was less than 0.5 ($\gamma^2 = 0.489$, $P < 0.001$).

Plasma TAG

Fasting plasma TAG concentrations were significantly elevated at week 8 compared with weeks 1 and 4 independent of oil and starch types (Table 11, $P < 0.001$). When the TAG concentrations at weeks 4 and 8 were controlled by % body weight lost using ANCOVA model, the TAG elevations at week 4 and 8 were no longer significant.

At week 1, the postprandial TG concentrations were statistically increased at 60 min, and remained higher than 0 min independent of diets (Figure 3, $P < 0.001$). When diet effects were considered vs. postprandial time, the DAG containing diets showed significantly lower TAG concentrations at 180 min (Figure 3, $P = 0.009$) while at the same time, those of the TAG containing diets appeared to reach peak concentrations. By contrast, at week 8, not only was there a significant elevation of TAG concentrations after 60 min independent of diet but also a time × oil × starch interaction was observed (Figure 3, $P < 0.02$) . Further analysis within each postprandial time revealed

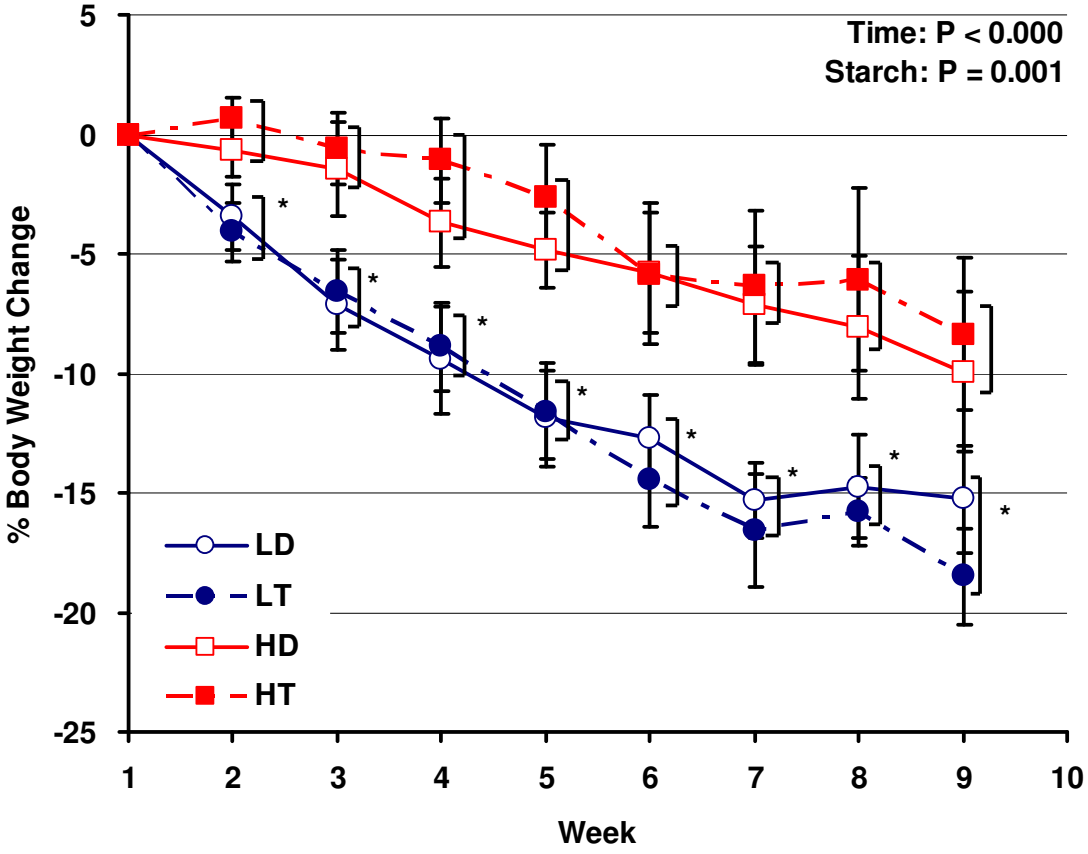


Figure 1. Body weight changes based on week 1. Values are mean ± SEM. *P*-values are for GLM repeated measures ANOVA. Asterisks represent significant differences between starch types by ANOVA within week, $P < 0.01$. Slopes of the LGI and HGI containing diet groups were -1.9 ± 0.2 and -1.0 ± 0.4, respectively.

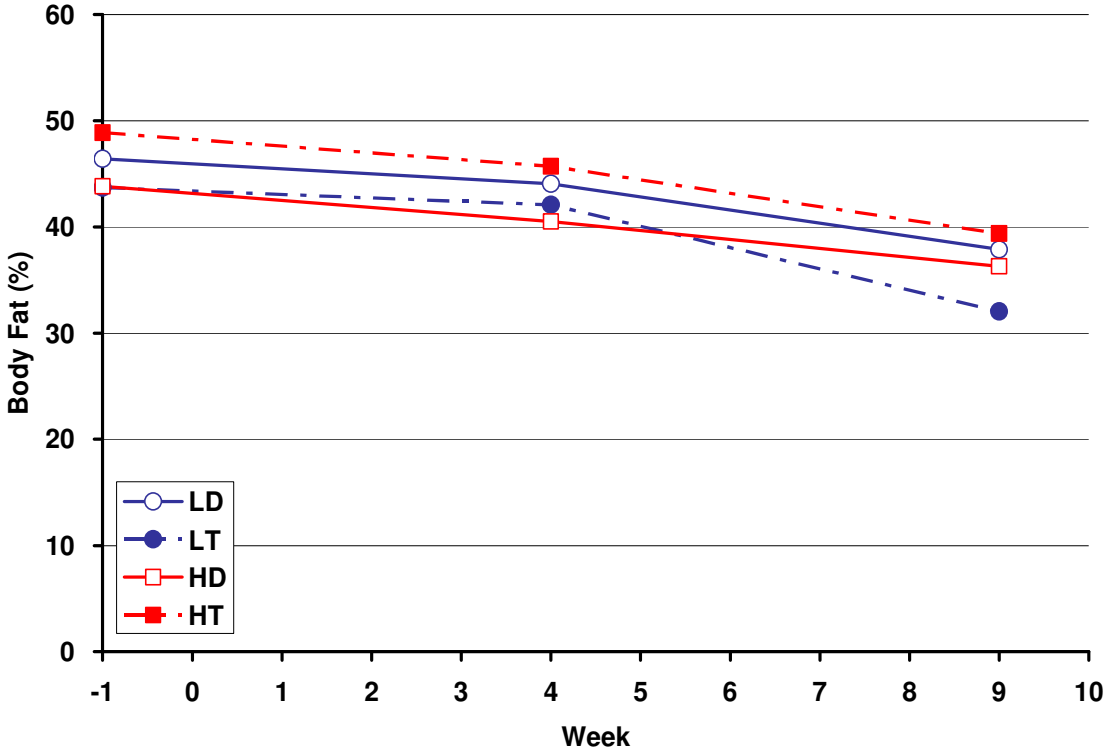


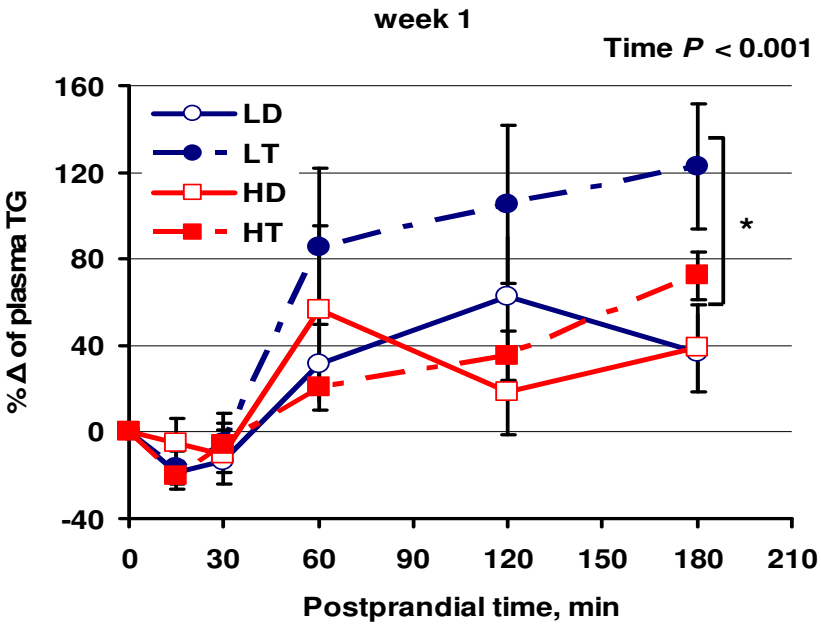
Figure 2. Weekly % body fat in the experimental diets.
P-values are for two-way ANOVA with General Linear Model repeated measures. No significant difference was observed among diets. Significance was considered as $P < 0.05$.

Table 11. Fasting plasma lipid profiles.[1]

		Diet				*P*-value			
		LD	LT	HD	HT	Repeated measures	ANOVA Oil	ANOVA Starch	ANOVA O × S
Triglyceride				*mmol/L*					
	WK 1	0.33 ± 0.03	0.27 ± 0.03	0.29 ± 0.04	0.30 ± 0.03	<0.001 [2]	ns	ns	ns
	WK 4	0.35 ± 0.04	0.27 ± 0.04	0.35 ± 0.02	0.36 ± 0.03		ns	ns	ns
	WK 8	0.47 ± 0.06	0.39 ± 0.04	0.47 ± 0.08	0.53 ± 0.03		ns	ns	ns
BHB									
	WK 1	0.20 ± 0.04	0.20 ± 0.03	0.20 ± 0.02	0.16 ± 0.02	ns	ns	ns	ns
	WK 8	0.14 ± 0.01	0.20 ± 0.02	0.17 ± 0.01	0.21 ± 0.04		ns	ns	ns
Total Cholesterol									
	WK 1	5.21 ± 0.49	4.52 ± 0.53	4.87 ± 0.72	6.82 ± 0.74	0.011 [3]	ns	ns	ns
	WK 4	4.23 ± 0.36 [a]	5.03 ± 0.89 [a]	5.75 ± 0.97 [b]	7.15 ± 0.63 [b]	0.027 [4]	ns	0.026 [5]	ns
	WK 8	5.32 ± 0.65	4.51 ± 0.59	6.99 ± 1.13	5.74 ± 0.43		ns	ns	ns
Lipoprotein-Cholesterol									
Preβ + β (LDL)									
	WK 1	1.00 ± 0.12	1.01 ± 0.15	1.01 ± 0.18	1.40 ± 0.24	ns	ns	ns	ns
	WK 4	0.90 ± 0.11	0.91 ± 0.09	1.27 ± 0.23	1.09 ± 0.17		ns	ns	ns
	WK 8	1.33 ± 0.21	1.11 ± 0.10	1.44 ± 0.27	1.15 ± 0.13		ns	ns	ns
α-2 (HDL-1)									
	WK 1	1.71 ± 0.26 [a]	1.23 ± 0.18 [a]	1.59 ± 0.33 [a]	2.38 ± 0.29 [b]	0.025 [3]	ns	ns	0.026
	WK 4	1.10 ± 0.06 [a]	1.35 ± 0.27 [a]	1.64 ± 0.37 [b]	2.39 ± 0.33 [b]	0.009 [4]	ns	0.012 [5]	ns
	WK 8	1.50 ± 0.23 [a]	1.26 ± 0.20 [a]	2.36 ± 0.50 [b]	1.84 ± 0.26 [b]		ns	0.04 [5]	ns
α-1 (HDL-2,3)									
	WK 1	2.49 ± 0.22	2.35 ± 0.34	2.27 ± 0.25	3.05 ± 0.35	0.010 [3]	ns	ns	ns
	WK 4	2.23 ± 0.25	2.77 ± 0.56	2.84 ± 0.50	3.66 ± 0.19		ns	ns	ns
	WK 8	2.49 ± 0.28	2.14 ± 0.32	3.20 ± 0.44	2.75 ± 0.21		ns	ns	ns

[1] Mean ±SEM, n=6; ns represents no statistical significance. Values in a row without a common letter differ, P<0.05.
[2] The *P*-value indicates main time effect. [3] The *P*-value indicates time-by-oil interaction. [4] The *P*-value indicates main starch effect.[5] Significance was eliminated after adjusting the data by % body weight.

(A)



(B)

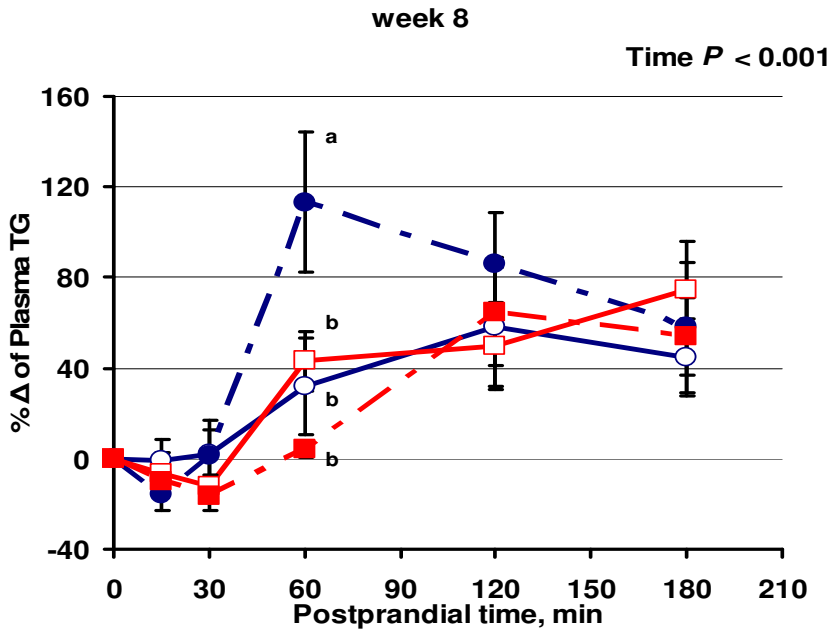


Figure 3. Postprandial plasma TAG concetrations. (A) week 1 and (B) week 8. Values are mean ± SEM. *P*-Values are for GLM repeated measures ANOVA. An asterisk denotes a significant difference between oil types by two-way ANOVA within time, $P = 0.009$. Letters not in common for a time point denote significant differences among diets by two-way ANOVA within time, $P = 0.034$.

postprandial TAG concentrations in the LT diet peaked at 60 min and was significantly greater than other three diets (Figure 3, $P = 0.034$). This greater TAG concentrations in the LT diet group remained statistically significant even after TAG concentration was adjusted by % body weight (ANCOVA, % body weight; $P = 0.003$).

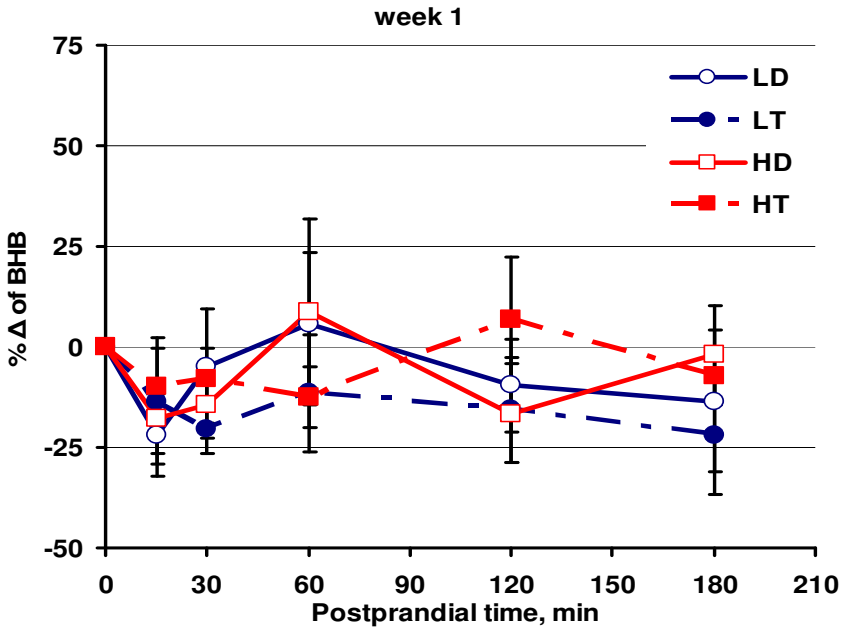
Plasma BHB

At week 1, neither starch nor oil type affected the postprandial plasma BHB concentrations (Figure 4). However, at week 8, the DAG containing diets were found to modestly increase BHB concentrations at 60 min (Figure 4, $P = 0.089$). Furthermore, this trend became significant at 120min (P=0.038). The elevation of BHB concentrations by the DAG diets was significant at 60 and120 min after BHB concentrations were adjusted by % body weight ($P \leq 0.05$).

Plasma TC

A significant time × oil interaction and main starch effect was observed in fasting plasma TC among weeks 1, 4, and 8. Further analyses using two-way ANOVA at each week found statistically significantly higher TC concentrations with the HGI diets at week 4 (Table 11, $P = 0.026$). This starch effect was blunted somewhat at week 8 (Table 11, $P = 0.069$). When % body weight was included as covariate, these starch effects were eliminated at both weeks 4 and 8.

(A)



(B)

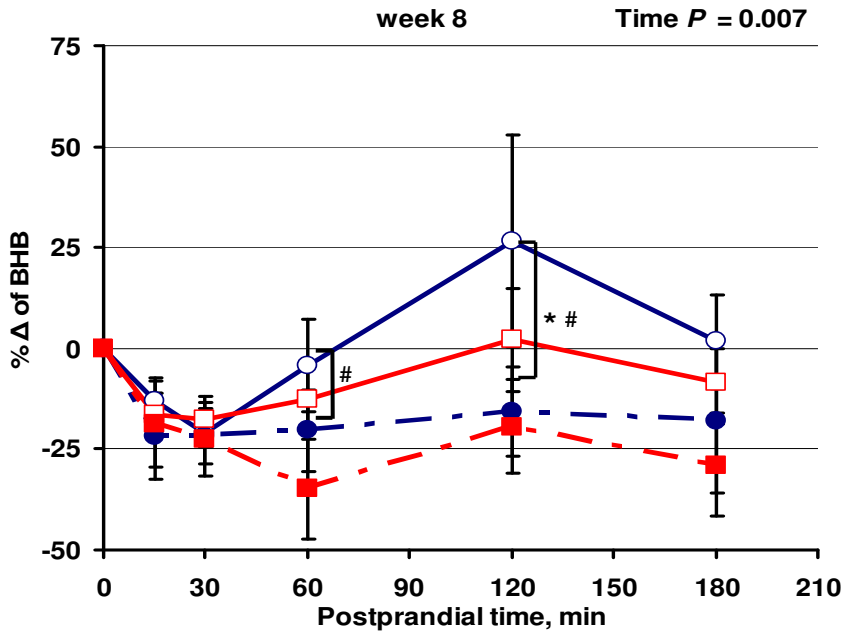


Figure 4. Postprandial plasma BHB concentrations. (A) week1 and (B) week 8. Values are mean ± SEM. P-Values are for GLM repeated measures ANOVA. An asterisk denotes significant differences between oil types by two-way ANOVA within time, $P = 0.038$. Pounds indicate significant differences between oil types by ANCOVA using % body weight as covariates, $P \leq 0.05$.

For postprandial TC concentrations, at week 1, although a significant oil × starch interaction was observed, further tests did not show any significance (Figure 5). By contrast, at week 8, repeated measures ANOVA found a week × oil × starch interaction with significantly reduced TC concentrations in the LGI containing diets at 15 min (Figure 5, $P = 0.013$). Adjusting the TC concentrations with % body weight as covariates revealed significantly lower TC concentrations in the LGI diets at 15 min (ANCOVA, $P = 0.036$). Moreover, this analysis revealed a significant oil × starch interaction in which the LD diet was significantly lower than the other diets at 120 min (ANCOVA, $P = 0.019$).

Plasma LPs

Plasma LPs including preβ, β, α-2, and α-1 fractions were measured at weeks 1, 4, and 8. Because pre β lipoprotein fraction was not clearly separated in some of the samples, it was combined with the β lipoprotein fraction and data is presented as pre β + β. Relative percentages of the LP fractions were multiplied by plasma TC concentrations and the results presented as LP cholesterol (LP-C) concentrations. Preβ + β LP-Cs were not different among weeks 1, 4, and 8. Moreover, these concentrations were the same independent of diets (Table 11). Furthermore, no differences were found after the concentrations were corrected by ANCOVA model using % body weigh as covariate.

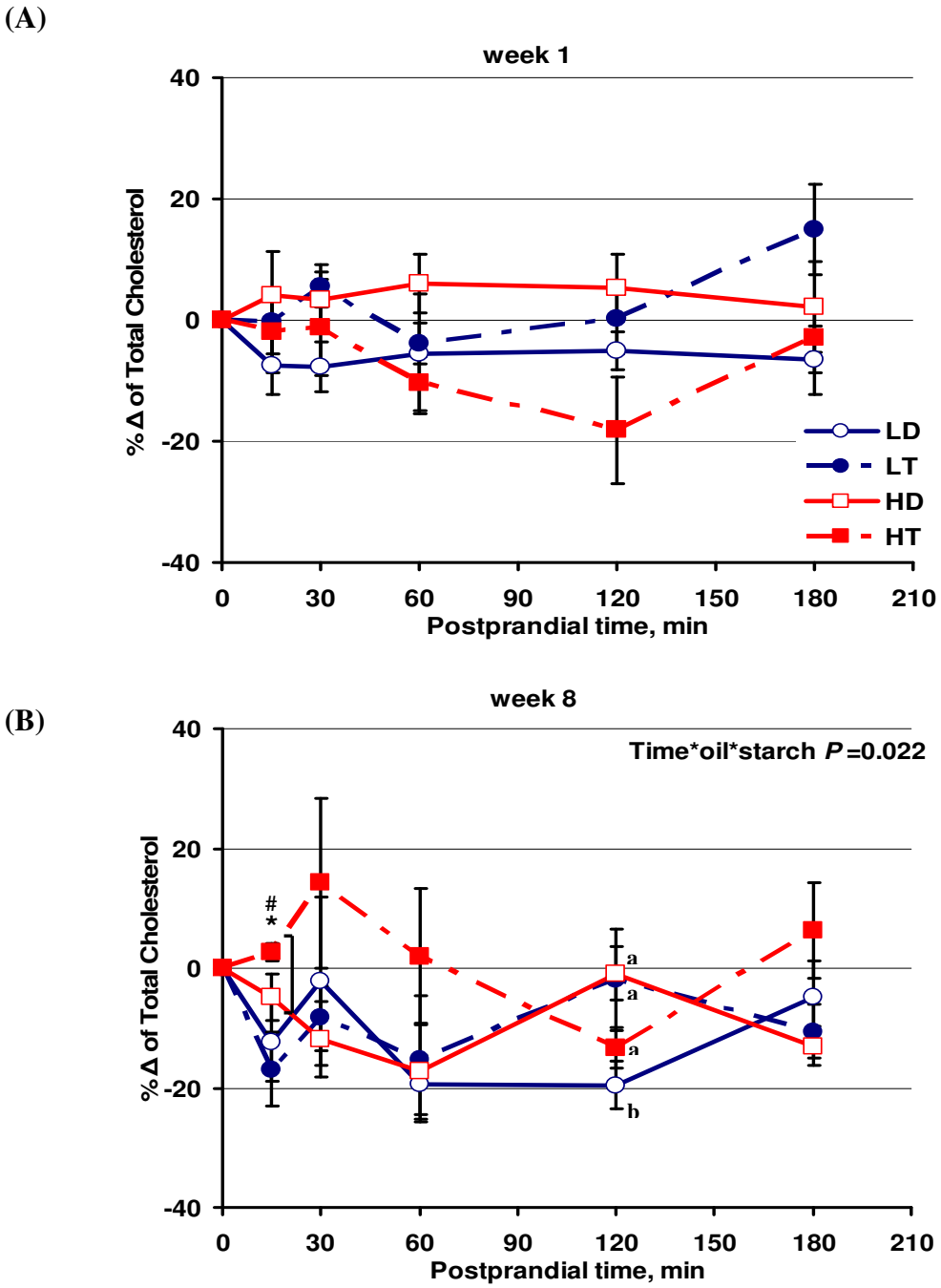


Figure 5. Postprandial plasma TC concentrations. (A) week1 and (B) week 8. Values are mean ± SEM. *P*-Values are for GLM repeated measures ANOVA. An asterisk denotes significant differences between starch types by two-way ANOVA within time, $P = 0.013$. A pound indicates significant differences between starch types by ANCOVA using % body weight as covariates, $P = 0.036$. Numbers without common letter significantly differ by ANCOVA using % body weight as covariates, $P = 0.019$.

By contrast, several differences were observed in α-2 and-1 LP-Cs. First, α-2 LP-C concentrations were greater at week 8 compared with week 1 and week 4 (time × oil interaction, $P = 0.025$). Moreover, a main starch effect was also observed. When diet effects at each week were analyzed by two-way ANOVA, the HT diet showed a significantly higher α-2 LP cholesterol than the other three diets at week 1 (Table 11). At weeks 4 and 8, the HD diet numerically increased its concentrations. Thus, when considered together with the HT diet, the HGI containing diets resulted in significantly higher values than those dogs fed the LGI containing diets (Table 11, $P < 0.05$). However, these increase in α-1 and -2 LP-Cs were eliminated when % body weight was included in ANCOVA model as a covariate. Second, a week × oil interaction was observed by repeated measures ANOVA with decreased α-1 LP-C concentrations in the TAG containing diet group at week 8 compared with weeks 1 and 4 ($P = 0.01$). Further ANOVA analyses within each week found that α-1 LP-C showed no significant difference among the four diets.

The results of α-2 and -1 LP-Cs appeared closely related to TC. Consequently, linear regression analysis was conducted to evaluate possible correlations with plasma TC. Strong correlations between TC and α -2 LP cholesterol ($\gamma = 0.922$, $\gamma^2 = 0.855$, $P<0.001$) as well as TC and α -1 LP cholesterol ($\gamma = 0.916$, $\gamma^2 = 0.833$, $P<0.001$) were found (Appendix F).

Alterations of LP-Cs were measured at weeks 1 and 8 at 120 min during the postprandial feeding study. Chylomicrons were not observed at 0 min as expected. Because of food ingestion, chylomicrons were observed at 120 min ($P < 0.05$), but these concentrations were not different among four diets (Table 12). By contrast, pre β + β LPs significantly decreased at 120 min at both weeks 1 and 8, but this was not related to the starch or oil types (Table 12). For α -2 LP cholesterol, a significant time × oil × starch interaction was observed at week 1 in which the HT diet group was significantly decreased at 120 min compared with 0 min ($P = 0.009$). At week 8, on the other hand, significantly higher α -2 LP with both of the HGI diets was observed at 120 min (Table 12, $P = 0.025$). This diet effect was eliminated when % body weight was included in the ANCOVA model (Table 12).

LPL and HL activities

LPL and HL activity are shown in Figures 6 and 7. No statistically significant starch, oil or interactions were observed.

Table 12. Postprandial lipoprotein cholesterol concentrations.[1]

	Postprandial time, min	Diet: LD	LT	HD	HT	*P*-value: Repeated measures ANOVA	ANOVA: Oil	Starch	O × S
Chylomicrons				*mmol/L*					
WK1	**0**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	ns	ns	ns	ns
	120	0.05 ± 0.01	0.05 ± 0.01	0.07 ± 0.03	0.06 ± 0.02		ns	ns	ns
WK8	**0**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	ns	ns	ns	ns
	120	0.06 ± 0.03	0.04 ± 0.02	0.05 ± 0.03	0.07 ± 0.03		ns	ns	ns
Preβ + β (LDL)									
WK1	**0**	1.00 ± 0.12	1.01 ± 0.15	1.01 ± 0.18	1.40 ± 0.24	0.046 [2]	ns	ns	ns
	120	0.73 ± 0.09	0.83 ± 0.11	1.03 ± 0.13	0.77 ± 0.14		ns	ns	ns
WK8	**0**	1.33 ± 0.21	1.11 ± 0.10	1.44 ± 0.27	1.15 ± 0.13	0.001 [2]	ns	ns	ns
	120	0.58 ± 0.10	0.67 ± 0.06	1.11 ± 0.14	1.03 ± 0.19		ns	ns	ns
α-2 (HDL-1)									
WK1	**0**	1.71 ± 0.26	1.23 ± 0.18	1.59 ± 0.33	2.38 ± 0.29	0.021 [3]	ns	ns	ns
	120	1.54 ± 0.24	1.51 ± 0.19	1.65 ± 0.20	1.52 ± 0.19 *	0.009 [4]	ns	ns	ns
WK8	**0**	1.50 ± 0.23	1.26 ± 0.20	2.36 ± 0.50	1.84 ± 0.26	0.018 [5]	ns	ns	ns
	120	1.22 ± 0.13 [a]	1.28 ± 0.22 [a]	1.95 ± 0.24 [b]	1.60 ± 0.23 [b]		ns	0.025 [6]	ns
α-1 (HDL-2,3)									
WK1	**0**	2.49 ± 0.22	2.35 ± 0.34	2.27 ± 0.25	3.05 ± 0.35	ns	ns	ns	ns
	120	2.29 ± 0.23	2.10 ± 0.32	2.29 ± 0.27	2.25 ± 0.20		ns	ns	ns
WK8	**0**	2.49 ± 0.28	2.14 ± 0.32	3.20 ± 0.44	2.75 ± 0.21	ns	ns	ns	ns
	120	2.17 ± 0.28	2.29 ± 0.45	2.74 ± 0.43	2.39 ± 0.22		ns	ns	ns

[1] Mean ±SEM, n=6. ns represents statically no significance. Values in a row without a common letter differ, $P < 0.05$. An asterisk denotes significant difference from 0 min within diets.
[2] The *P*-value indicates main time effect. [3] the *P*-value indicates time-by-starch interaction.
[4] The *P*-value indicates time-by-oil-by-starch interaction.
[5] The *P*-value indicates main starch effect.
[6] Significance was eliminated after adjusting its data by % body weight.

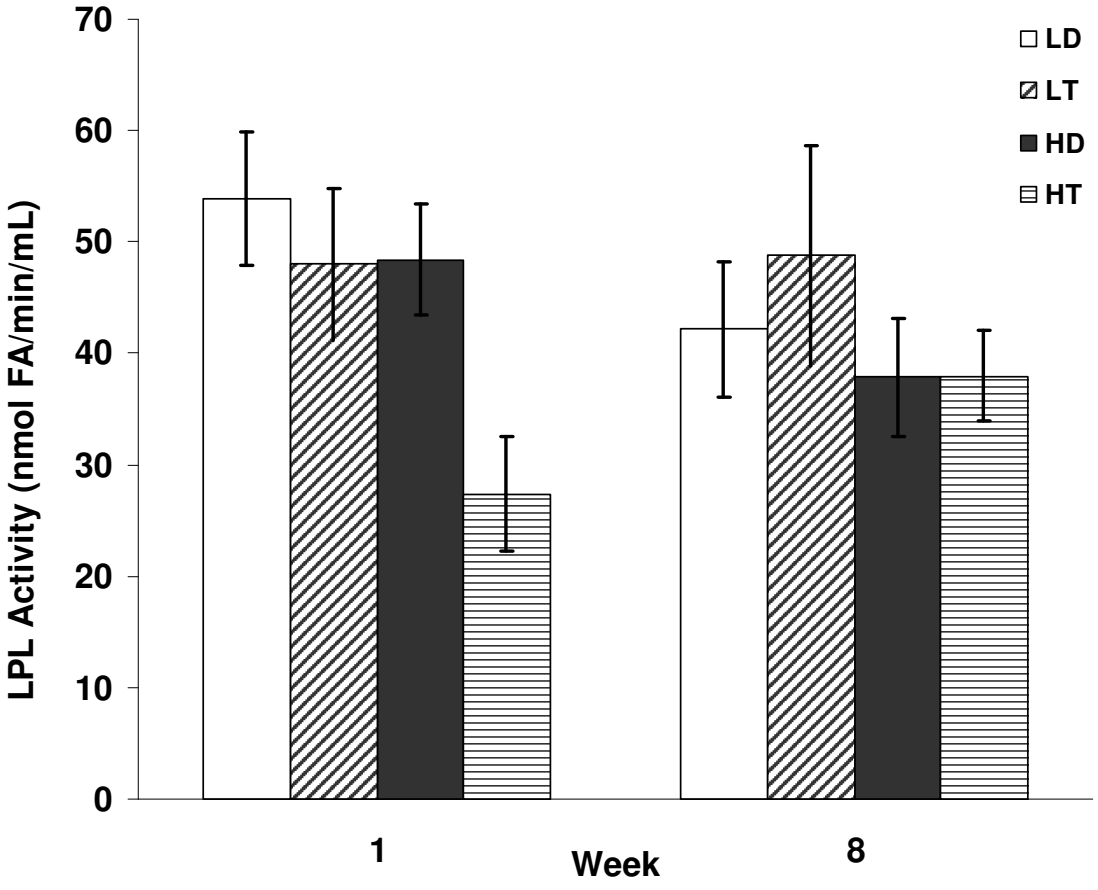


Figure 6. Fasting LPL activity at week 1 and week 8. Values are mean ± SEM.

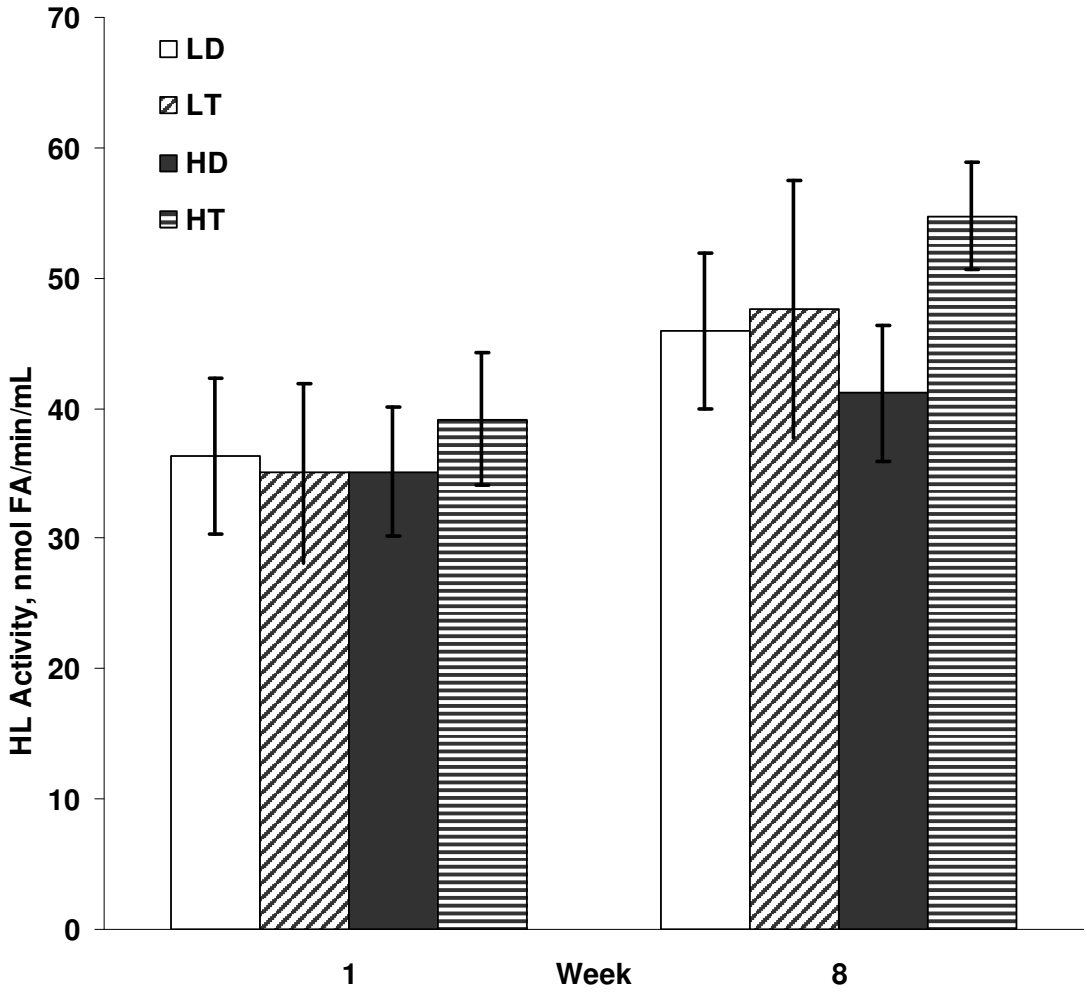


Figure 7. Fasting HL activity at week 1 and week 8. Values are mean ± SEM.

Discussion

The aim of this chapter of study was to determine the effects of DAG, LGI, and combination of DAG and LGI on plasma lipid and lipoprotein metabolism in adult Beagles during an 8 week weight loss period. During this period, dogs were fed an amount to maintain their obese body weight (ca. 3980 kJ/d). However, all dogs voluntarily consumed only 68 % of energy offered. Therefore, dogs lost body weight and body fat overall. Although the reductions of body weight and body fat were not controlled by the experimental design, it should be noted that dogs lost their weight ranging from 1.0 to 1.9 % per week. The safe rate of body weight loss is recommended to be between 1.0 and 2.0 % per week (21). Moreover, 35 to 40 % food restriction is commonly used in veterinary practice as one of the strategies for providing healthy weight loss. Therefore, the weight loss in this study was within a safe and acceptable weight loss rate.

The results of the digestibility study revealed lowered carbohydrate and protein digestibilities in the LGI diets compared with the HGI diets. The source of the LGI starch used in this study contained high amount of amylose (ca. 70 %) while the HGI starch contained 100 % amylopectin. High amylose corn starch is considered as a resistant starch which is poorly absorbed in the small intestine (152). It is widely recognized that both resistant starch and dietary fiber decrease protein digestibility due to several mechanisms including increased passage rate, increased endogenous nitrogen secretion, increased microbial production of organic acids, reduced ammonia production and absorption, increased bacterial nitrogen fixation, and adhesion of nutrients on fiber

(153, 154). Thus, some or all of these physiological properties may possibly result in the lowered carbohydrate, protein, and total digestibilities observed in this study. The ME was calculated using the data on total energy loss via feces and energy loss by urinary excretion, the latter of which is based on fecal protein loss. Thus, the lower ME of the LGI diets were likely due to decreased protein and total digestibilities compared with the HGI diet group. Consequently, dogs fed the LGI diets consumed less energy overall resulting in a higher percentage of their starting obese body weight loss than those fed the HGI diets. It is noteworthy that dogs voluntarily consumed the same dry matter weight of food independent of diets. Therefore, although the LGI diet group lost more weight, associated with less energy intake, the LGI diets would be expected to provide the same degree of satiety as the HGI diets.

In addition to the LGI beneficial effect on body weight, a DAG beneficial effect was observed associated with elevated postprandial BHB concentrations at week 8 but not at week 1. This result suggests that a DAG effect become pronounced later on in the study. DAG effects involving lipid catabolism (i.e. β-oxidation enhancement, TAG lowering, body fat accumulation reduction) seems to be dependent on the dose of 1,3-DAG inclusion in experimental diets (155). In this study, 1,3-DAG comprised approximately 7.56% in DAG containing diets. Therefore, this relatively smaller inclusion of 1,3-DAG in the diets may have prolonged the occurrence of DAG effect until 8 weeks.

There are some concerns whether % body weight change should be taken into account as a covariate in the ANOVA model in order to examine the BHB alteration as a

possible consequence of body weight change. Intuitively, it would appear that the BHB concentrations and body weight change during weight loss are metabolically linked. However, after BHB concentrations were adjusted by % body weight change, the oil effect remained significant. This result may be because body weight change was strongly affected by starch, but not oil, types. In addition, it is likely that some amount of fatty acids were lipolyzed from dietary DAG directly via β-oxidation in the small intestine or liver (84). The latter interpretation is supported by our finding that body fat loss was not directly affected by increased postprandial BHB concentrations. Finally, body fat was also less strongly correlated with body weight change. Therefore, some other factors may influence the body fat loss in this study.

A beneficial DAG effect was consistently observed on postprandial TAG concentrations. At both weeks 1 and 8, DAG containing diets lowered postprandial TAG concentrations. This observation has been consistently reported as a primary effect of DAG compared with TAG among various species including dogs (73, 88, 89, 156, 157). The unique finding in this study was that the TAG elevation was observed only in the LT combination diet but not the HT diet at week 8. In this study, postprandial insulin significantly increased in the HT diet (data not shown). Insulin is a lipogenic hormone which stimulates adipose LPL, fatty acid synthase, and DAG acyl transferase while directly inhibiting hormone sensitive lipase, and indirectly inhibiting carnitine palmitoyl transferase I (158). Thus, more insulin may stimulate LPL activity *in vivo*, resulting in decreased plasma TAG in the HT diet masking any effect of a plasma TAG elevation with TAG ingestion compared with DAG ingestion. On the other hand, the LT diet

appeared to have a synergistic effect on increased TAG concentrations because both diet components act on TAG elevation.

It was speculated that LPL activity may be enhanced by the HGI diets due to an increase of insulin concentration. Thus, we examined plasma LPL and HL activity *in vitro.* While the DAG effect appears to be dynamically altered more likely during postprandial periods, one study that determined the *in vitro* postprandial lipoprotein lipase mass revealed that the LPL activity was not altered between fasting and DAG feeding conditions (159). Therefore, we decided to collect fasting post-heparin plasma for the source of LPL and HL in order to avoid blood sampling complications. The result revealed that the mixture of oil and starch types did not alter fasting LPL and HL activity *in vitro* which is consistent with data obtained in our preliminary study (112). Our finding demonstrated that LPL and HL activities exist to the same extent when artificial emulsions are used as substrate *in vitro*. However, physicochemical alteration of chylomicrons by DAG ingestion was recently observed by Yasunaga et al. They found more monoacylglycerol, NEFA, and 1,3-DAG and less TAG in the chylomicrons obtained from DAG ingested mice. If this same result occurs in dogs, more 1,3-DAG may appear at the oil-water interface of chylomicrons (78). Consequently, because lipases such as LPL and HL hydrolyze the *sn* 1, 3-position of TAG, 1,3-DAG may actually have higher affinity for LPL and HL. Results of the present study are unable to address this possibility due to the usage of an artificial substrate for LPL determination. Therefore, further study will be needed to determine whether LPL and HL mediated

hydrolysis is altered by dietary DAG and TAG oil types in dogs and is the topic of Chapter IV this dissertation.

Regarding fasting TC, the HGI containing diets increased fasting TC concentrations at week 4. However, this effect was the result of body weight loss and not diet because ANCOVA with % body weight as covariate eliminated the increased TC concentrations seen at this time. α-1 and 2 LP-C concentrations were also altered due to body weight loss which was expected because these LP-Cs have a strong correlation with TC. No diet effect was observed on preβ + β, so called VLDL + LDL cholesterols both during fasting and the postprandial period.

By contrast, postprandial plasma TC concentrations were lower at week 8 when the LD diet was fed. This finding remained significant after eliminating the effect of weight loss. High amylose corn starch, the source of LGI starch in this study, has been reported to have a TC lowering effect involving an increase in the bile acid pool in the intestine and associated increase of bile acid secretion into feces due to its physiological property as resistant starch (153, 154). DAG has also been found to have a cholesterol lowering effect. However, this effect is somewhat controversial (160-163). Our finding that the LD diet lowered postprandial TC suggests that a synergistic response occurred with the combination of LGI and DAG.

It is notable that at week 1 of feeding the HT diet that decreased postprandial HDL concentrations were found. Decreased HDL cholesterol is one risk factor for atherosclerosis (164). However, the LGI and DAG containing diets did not decrease HDL cholesterol postprandially. Therefore, these two ingredients may be further

beneficial in this regard. Although the incidence of atherosclerosis in dogs has been rarely seen because of their unique lipoprotein metabolism (165), this finding is nonetheless important for understanding the effect of the DAG and LGI containing diets on lipoprotein metabolism in dogs and may apply to other species such as humans.

In summary, LGI, DAG and combined LGI/DAG effects were demonstrated in adult dogs fed a combination of starch (LGI vs. HGI) and oil (DAG vs. TAG) types for 9 weeks. First, although all dogs consumed the same amount of food on a dry matter weight basis, the LGI containing diets resulted in increased body weight loss due to the lowered ME (LGI effect). Second, the DAG containing diets lowered postprandial TAG and increased fat oxidation later on the study (DAG effect). Postprandial TC was lowered with the combination of LGI and DAG (LGI and DAG combination effect). Finally, postprandial HDL cholesterol was not decreased by the LGI and DAG containing diets. These findings indicate the combination of LGI and DAG favored more lipid catabolism while maintaining lipoprotein metabolism. Therefore, this combination diet has potential to be used as a weight management diet in dogs. LPL and HL activities were not affected by LGI and DAG in this study. However, further research will better define lipase mediated effects on chylomicron metabolism.

CHAPTER IV

THE EFFECT OF DAG ADMINISTRATION ON CANINE TRIGLYCERIDE RICH LIPOPROTEIN METABOLISM

Introduction

In this study our aim is to determine the effect of DAG on canine chylomicron and chylomicron remnant metabolism. The previous studies have shown that amount of LPL and HL enzymes were not altered by DAG or LGI ingestion. However, these diet components may alter substrate suitability for LPL and HL. Yasunaga et al. recently found that murine nascent chylomicrons isolated from 2 h postprandial oral DAG containing emulsion administration contained a small but significantly increased amount of 1,3-DAG, MAG, and non esterified fatty acids (NEFAs), in addition to a significantly decreased amount of TAG compared to nascent chylomicrons isolated from TAG emulsion administration (78). Because Yasunaga et al. found that the lipid compositions of chylomicrons obtained from DAG and TAG emulsion administrations were different, the possibility exists that DAG may alter the suitability for LPL and HL for their substrate chylomicron and chylomicron remnants. If so, corresponding chylomicron metabolism such as the clearance rate of chylomicron remnants from the blood circulation and their hepatic uptake may be altered. In order to address this series of research questions, the present study was conducted to evaluate the effect of DAG on canine triglyceride rich lipoprotein (TRL) metabolism, which include chylomicron and chylomicron remnants. In order to better understand TRL metabolism, the lipid profiles

of canine postprandial TRLs were first evaluated. Second, enzyme kinetics, including Km, Vmax, and initial velocity of LPL and hepatic lipase (HL) using the DAG and TAG enriched TRLs as substrates were investigated. Finally, the *in vivo* clearance rate and hepatic uptake of these substrates was evaluated after their intravenous injection into young adult mice. We hypothesized that enzyme kinetics of LPL and HL would change as a function of TRLs obtained from DAG ingestion compared to TRLs obtained from TAG ingestion and therefore, TRLs obtained from DAG ingestion would be cleared from blood circulation more rapidly and more efficiently taken up by hepatic tissue.

Materials and methods

Animals

This study was approved by the Texas A&M University Animal Care and Use Committee under animal use protocol # 2008-267 and #2008-293 entitled "Metabolism of diacylglycerol enriched lipoproteins in the circulation". Both dogs and mice were used in the study. First, for dogs, eight normal intact female adult Beagles were used. The age and body weights of dogs were 8.0 ± 1.0 (SEM) years old and 12.7 ± 0.9 kg, respectively. They were given water *ad libitum* and allowed exercise once a day outside and freely inside their kennels during the study. Second, wild-type female C57BL/6J mice (7-8 weeks old) were purchased from Jackson Laboratory. The mice were allowed to recover from transportation for 1-3 weeks before the experiment. All Beagle dogs were housed individually in an 8ft long × 9ft high × 4ft wide kennel and mice were caged ≤ 5 per a cage at the Laboratory Animal Research and Resources (LARR) facility,

Texas A&M University according to the American Physiological Society Guidelines for Animal Research and guidelines set forth by Texas A&M University Care and Use Committee. Twelve-hour light and dark cycles were maintained during the study.

Prior to the study, complete blood counts and serum biochemistry profile tests were performed on all dogs to assure their normal clinical status.

Study designs

A randomized cross-over design was performed. Dogs were fed Hills Science Diet® Adult Original dry food prior to the study at each dog's maintenance energy requirement (MER) amount. Two experimental diets were freshly prepared at the time of feeding which contained 49.6 wt % of either DAG or TAG enriched oil (kindly provided by Kao Corporation, Tokyo, Japan), 19.2 wt % of an instant mashed potato mix, and 30.2 wt % of hot water to fully cook the potato. In order to increase palatability, 1.0 wt % chicken bouillon (Wyler's instant bouillon, Chicken flavor, H. J. Heinz Co. LP. Pittsburg, PA) was also added to the diets. DAG enriched oil contained 31.3 % of 1,2-DAG, 46.3 % of 1,3-DAG, and 22.4 % of TAG while acylglycerol compositions of TAG oil was predominantly TAG (94.9 %) while 1,2-DAG and 1,3-DAG were less than 3.5 % (Table 13). These compositions reflected the overall diet acylglycerol distributions (Table 14). In addition, the fatty acid compositions of oils and diets were similar (Tables 1 and 2). It should be noted that Idahoan® original mashed potatoes (Idahoan foods, Lewisville, ID) was selected in the present study because this brand contained the lowest

Table 13. Acylglycerol and fatty acid compositions of oils (relative %).

	DAG	TAG
Acylglycerol	*Relative %*	
MAG	0.00	0.00
1,2-DAG	31.30	2.10
1,3-DAG	46.30	3.06
TAG	22.41	94.85
Fatty acid	*g/100 g fat*	
16:0	2.60	5.33
16:1	0.12	0.14
18:0	1.01	1.96
18:1 n9	35.29	31.49
18:1 n7	2.07	1.91
18:2 n6	48.80	48.69
18:3 n3	8.19	7.87
20:0	0.30	0.50
20:1	0.62	0.65
22:0	0.22	0.27
22:1	0.00	0.00
Unidentified	0.77	1.17

Values are the average of two representative samples with duplicate analyses.

Table 14. Acylglycerol and fatty acid compositions of diets (relative %).

Fatty Acid	DAG	TAG
Acylglycerol	*Relative %*	
MAG	0.00	0.00
1,2-DAG	32.73	2.72
1,3-DAG	45.36	3.30
TAG	21.91	93.98
Fatty acid	*g/100 g fat*	
16:0	2.66	5.24
16:1	0.13	0.13
18:0	1.09	2.10
18:1 n9	35.17	31.62
18:1 n7	2.18	1.95
18:2 n6	48.69	48.67
18:3 n3	8.23	7.82
20:0	0.30	0.56
20:1	0.63	0.72
22:0	0.23	0.33
22:1	0.00	0.00
Unidentified	0.70	0.86

Values are the average of two representative samples with duplicate analyses.

1, 3-DAG concentration (< 5 % in the total diet lipid) among other commercially available potato brands.

Before the study, dogs were assigned into two diet groups carefully controlling the age difference among them. Dogs were fasted overnight and one of the two diets was offered. This study employed a single meal feeding, therefore, after one of the diets was fed, a 21 day washout period was provided, after which the opposite diet type was given to each dog.

Chylomicron collection

A total of 70 mL blood at the approximate postprandial peak of chylomicron content was collected. Although the timing of chylomicron secretion varies in each dog, a preliminary study evaluating the postprandial effect of DAG revealed peak chylomicron presence in blood circulation at 3-4 h after meal ingestion in our colony dogs. Thus, 10 mL of postprandial blood was drawn every 10 minutes between 3 h and 4 h. Plasma was separated by low speed centrifugation and then placed into 13.2 mL ultra clear ultracentrifuge tubes. Two mL of saline (ρ <1.006 g/mL) was added to fill each tube and samples were ultracentrifuged at 118,500 ×g for 60 mim at 20 °C using a Sorvall TH-641 rotor to obtain TRLs (166). The TRLs isolated at the postprandial period were pooled for each dog and stored at 4 ºC until analyses. Because TRL is not highly stable, all analyses and procedures were performed within 3 days.

Post heparin plasma sample collection

Fasting post heparin plasma samples were collected prior to the study. Three dogs were selected randomly and 100 I.U. Na heparin/kg-body weight was injected intravenously into each animal via a cephalic vein. Ten minutes after heparin injection, 7 mL of blood was taken from a jugular vein into a clean tube. Plasma was separated by low speed centrifugation, pooled, aliquoted, and stored at -80 ºC until use.

TRL TG measurement

TRL TG concentrations were measured with an enzymatic colorimetric assay previously described.

TRL protein measurement

The relative size of TRL was compared in each dog by measuring TRL protein using bicinchoninic acid (BCA) assay. The principle of BCA assay utilizes two reactions: 1) the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium known as the biuret reaction and 2) the highly sensitive and selective colorimetric detection of the Cu^{1+} using a reagent containing BCA. Two BCAs bind with one Cu^{1+} which produces a purple color, providing a strong absorbance at 562 nm. The procedures of the BCA assay were as follows: First, 2 mg/mL of stock bovine serum albumin (BSA) was prepared. Several concentrations of BSA diluted with distilled water were prepared which covered the linear range of desired protein concentrations. Twenty five µL of prepared standards and samples were placed into wells in a 96 microplate in triplicate. A working reagent (WR) was then prepared by the mixing the BCA™ protein assay reagent A (Thermo Scientific, Rockford, IL) and BCA™ protein assay reagent B (Thermo Scientific,

Rockford, IL) in a 50:1 ratio. BCA™ protein assay reagent A contains sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartate in 0.1 mol/L sodium hydroxide while BCA™ protein assay reagent B consists of 4 % cupric sulfate. Two hundred μL of WR were added into each well. The microplate was mixed thoroughly in a plate shaker for 30 seconds and incubated at 37 ºC for 30 min. The plate was cooled to room temperature and absorbance measured at 550 nm in a plate reader (Molecular Devices Corporation, Menlo Park, CA) and its software (SoftMax® Pro, Molecular Devices Corporation, Sunnyvale, CA). Distilled water served as a blank.

TRL lipid composition determination

TRL lipids (Phospholipids, cholesterol, cholesterol ester, NEFA, MAG, 1,2-DAG, 1,3-DAG, and TAG) were determined by high performance thin layer chromatography (HPTLC). Lipids in TRLs were extracted using the methodology previously mentioned. After drying under nitrogen gas, the extracted lipids were reconstituted with 100 μL of chloroform.

HPTLC

A two dimensional development system was used to fractionate the lipids. Two glass chambers (Analtech, Inc., Newark, DE) were prepared and filled with hexane:ethyl ether:acetic acid (30:70:1,v/v/v) (1st development chamber) and hexane:ethyl ether:acetic acid (70:30:1, v/v/v) (2nd development chamber) before use in order to equilibrate the chambers. Four μL of the extracted lipids were placed on 10 × 10 cm silica gel plates (TLC Silica gel 60, Merck KGaA, Darmstadt, Germany) and the spot was dried at room temperature. The plate was placed into the 1st development chamber, dried, and again

placed into the 2nd development chamber at 90° to the direction of the first development. After completely drying, the plate was immersed into a charring solution which contained 10 % $CuSO_4$ in 8 % phosphoric acid. The plate was blotted carefully and charred at approximately 180 ºC, allowing visualization of the fractionated lipids. Relative amounts of each lipid were determined via a densitometer (Model GS-700 Imaging Densitometer, Bio-Rad, Hercules, CA), and its software (Quantity One, Bio-Rad, Hercules, CA).

TRL particle size measurement

The TRL (d < 1.006 g/mL fraction of plasma) diameters were determined by dynamic laser light-scattering analysis using a ultrafine particle analyzer with a laser probe tip (UPA-250, Microtrac, Clearwater, FL) and appropriate software (Microtrac, Honeywell, Washinton, PA) (167). The TRL were suspended as a 1:5 dilution in a 0.9 % NaCl (d =1.0063 g/mL) and placed into sample tubes. The laser probe was placed gently into the tubes in order to prevent air bubbles at the probe-liquid interface. Light scattering from TRL particles were recorded for 1 min in triplicate and deconvoluted by system software. The results of particle diameter obtained from the system software were expressed as a distribution of particle number, particle volume, or particle area (167). Particle number distribution describes the frequency distribution of TRL particle diameter. In contrast, particle volume distributions provide the distribution of total TRL volume among particles of different diameters. This distribution is sensitive to the presence of small numbers of larger diameter particles because the volume increases in a cubic manner to particle radius (volume of sphere shape of particle = $4/3\pi\gamma^3$). Finally,

particle area distribution considers both particle number and volume distributions and therefore is less sensitive to the presence of a few large particles (168).

Diet and TRL fatty acid compositions determination

Lipid extraction

Total lipid in diets and TRLs were extracted with the methodology described by Folch (169). For lipid extraction from diets, a 500 mg of homogenized diet were placed into a teflon-lined screw-capped clean tube. For TRL samples, 150 µL of isolated TRLs from plasma were pipetted into a test tube. Twenty mL and 9 mL chloroform-methanol (2:1, v/v) with 0.2 % glacial acetic acid (HAc) were added to the diet and TRL containing tubes, respectively, and mixed for 20 min at room temperature with a shaker (Model S-500, Kraft apparatus, Mineola, NY). Ten and 2 mL of distilled water were then added to the diet and TRL containing tubes, respectively, and again mixed for 10 min. After that, tubes were centrifuged at 1825 ×g for 10 min which separated two layers: chloroform as a bottom layer, and methanol/water as a top layer. The bottom layer, containing the extracted lipid was then transferred to a clean test tube. Five mL chloroform-methanol-distilled water (3:48:47, v/v/v) was added into each tube, the solution was mixed for 10 min with the shaker, and again centrifuged at 1825 ×g for 10 min. The bottom layer was again transferred to a clean test tube and evaporated under nitrogen at 37 °C. One hundred µl of chloroform were added and a stream of nitrogen gas was placed into the tube to avoid fatty acid degradation. If the samples were stored at -20 ºC, 1 mL chloroform was added to each tube under nitrogen gas.

Lipid methylation

Fifty µL of extracted lipids and 2 mL of 4% surferic acid in methanol were placed into a clean Teflon-lined screw-capped test tube. After mixing, the tube was heated at 90 ºC for 1 hour. Because the caps were often loosened during heating, the tightness of the caps was checked during the first 10 min of the heating process. The tubes were removed, and cooled at room temperature for 5 min. Three mL of hexane was then added. After mixing, tubes were centrifuged at 1825 ×g for 15 min, which separated a top layer containing hexane and fatty acid methyl esters (FAMEs) and a bottom layer containing methanol. The top layer was then transferred to a clean test tube, and dried under nitrogen gas. The samples were reconstituted using 1 mL of hexane and stored at -20 ºC until analyses.

Gas chromatography

Twenty µL of FAMEs in hexane were loaded into a gas chromatography (GC) vial insert and placed on an autosampler of Hewlett Packard 5890 Series II Gas Chromatograph (Hewlett Packard Co., Palo Alto, CA). Three µL of the sample were injected onto a FAMEWAX^{TM} fused silica capillary column (30 m long, 0.25 µm thickness, and 0.32 mmID) (Restek, Bellefonte, PA) which was attached to a flame ionization detector. Helium was used as a carrier gas at an initial velocity of 28.3 cm/sec and flow of 1.25 mL/min. Initial oven temperature was held at 160 ºC for 8 min, then increased at a rate of 1.8 ºC/min until 225 ºC and maintained for 10 min. After that time, the temperature was increased at a rate of 8 ºC/min until 250 ºC and maintained for another 10 min to eliminate any debris on the column. Chromatograms were generated

via the instrument's software (ChemStation B.03.01, Agilent Technologies, Waldronn, Germany). FAME standards (#68-B, plus 17:0, 18:1 n7, 18:3 n6, 20:5 n3, 22:4 06, 22:5 n3, Nu-Check Prep, Inc., Elysian, MN) were used to identify individual fatty acid peaks based on retention times.

LPL activity determination

LPL activity was determined *in vitro* using the isolated TRLs as substrates. To determine the suitability of chylomicrons as a substrate for the lipolytic enzymes present in the post-heparin plasma (PHP), enzyme kinetic assays were conducted using variable substrate concentrations to obtain apparent Km and Vmax for each dog. Moreover, an initial velocity based upon enzyme concentrations was investigated using an amount of substrate similar to the physiological postprandial plasma TAG concentration.

Labeled TRL preparation

In a clean vial, an amount of TRL containing 7 mg TG was placed and stirred under a nitrogen gas stream. A cryovial was prepared and 1.0×10^7 cpm/μmol ^{3}H-triolein was pipetted into the vial and evaporated under nitrogen. The ^{3}H-triolein was reconstituted with 100 μL acetone. In order to incorporate ^{3}H-triolein into the TRL without disrupting the TRL, the reconstituted ^{3}H-triolein was immediately placed into a solution containing the TRLs and incubated with stirring for more than 1.5 h under a stream of nitrogen gas. The final volume was adjusted to provide 13.2 mmol/L TRLs TAG concentration in substrate with 0.2 mol/L Tris-HCl buffer (pH 8.0).

Determination of labeled TRL integrity

Labeled TRL integrity was measured as described by Qi et al. with modifications (170). Small aliquots of radiolabeled TRLs were placed into 7.5 cm long glass capillary tubes. After low speed centrifugation (3000 ×g) for 20 min, the tubes were cut into five fractions (1cm each), and TAG concentrations and radioactivity of each fraction were measured. If ^{3}H-triolein is uniformly incorporated into the TRLs, the distribution of TAG and ^{3}H-triolein concentrations in the corresponding fractions should be the same.

Reagent preparations

Four % (w/v) BSA in 0.2 M Tris-HCl buffer (pH 8.0) was prepared and aliquot of fasted dog plasma obtained as described earlier was heated at 56 °C for 15 min.

Determination of incubation time

Ten tubes were prepared, each of which contained 20 µL labeled TRLs (1.32 mmol/L), 10 µL of 4 % BSA, 10 µL heat treated plasma, and 60 µL Tris-HCl buffer (pH 8.0). One Hundred µL of diluted PHP (4 µL sample, 96 µL 0.15 mol/L NaCl) were pipetted into each tube. The tubes were incubated in duplicate for 5 various incubation times (10, 20, 30, 40, and 60 min) at 37 °C with constant shaking. One hundred µL of 0.15 mol/L NaCl instead of diluted PHP served as a blank. In order to stop the reaction, 3.25 mL methanol: chloroform: heptane (1.41:1.25:1 (v/v/v)) were added to the tubes. 1.05 mL of 0.1 mol/L potassium carbonate buffer (pH 10.5) were then added to each tube and then mixed for 15 sec followed by centrifugation for 15min at 4000 ×g (Allegra® X-15R Centrifuge, Beckman Coulter, Fullerton, CA). One mL of the upper layer containing methanol: water was placed into a Pony Vial™ (PerkinElmer, Inc,

Waltham, MA). Ten µL of substrate mixture were also prepared in a separate Pony Vial™. Finally, 5 mL of liquid scintillation counting cocktail (Ultima Gold™ , PerkinElmer, Inc, Waltham, MA) were added and the labeled fatty acid released measured by scintillation counting (1900TR Liquid Scintillation Analyzer, Packard Instrument Company, Downers Grove, IL).

Apparent Km and Vmax determination

In order to evaluate the kinetics of each substrate, 12 various concentrations of labeled TRLs were placed into different tubes ranging from 0 to 5.28 mmol/L (0, 0.132, 0.264, 0.528, 0.66, 0.99, 1.32, 1.65, 1.98, 2.64, 3.96, and 5.28 mmol/L). In each tube, 10 µL of 4% BSA, 10 µL of heat treated plasma, and an appropriate amount of buffer to provide 100 µL total volume were added to each tube. One hundred (100) µL of diluted PHP (3 µL sample, 97 µL 0.15 M NaCl) were then added to each tube and the tubes were incubated for 30 min at 37 °C with constant shaking. One hundred (100) µL of 0.15M NaCl instead of diluted PHP served as a blank which was prepared for each substrate concentration. All samples were prepared in duplicate. Lineweaver–Burk plots were drawn from the obtained data using Microsoft Excel® graphic software. The reciprocal number of the y-intercept in the plot indicated Vmax while the negative reciprocal number of the x-intercept showed Km.

Initial velocity determination

Five varying different enzyme concentrations were prepared: 1, 2, 4, 10, and 20 µL PHP. In each tube, 10 µL of 4% BSA, 10 µL of heat treated plasma, 40 µL labeled TRLs (2.64 mmol/L) , and an appropriate amount of Tris-HCl buffer (pH 8.0) to provide total 200 µL volume were added to each tube. The tubes were incubated for 30 min at 37 °C with constant shaking. One hundred µL of 0.15 mol/L NaCl instead of diluted PHP served as a blank and all samples were prepared in duplicate. Michaelis-Menten plots were drawn from the obtained data using Microsoft Excel® graphic software. The initial linear velocity was calculated from the slope of a linear equation of the data.

HL activity determination

HL activity was also determined *in vitro* using TRL as a substrate. To determine the suitability of TRLs as a substrate for this lipolytic enzyme present in the PHP, similar techniques and methodologies were used as in the LPL activity determination with minor technical differences as follows: The tris-HCl buffer was prepared at pH 9.0. In addition, apo CII is not required to enhance HL activity, thus heat treated plasma was not added. Moreover, diluted PHP was prepared using a 2-fold dilution (50 µL sample: 50 µL 2 mol/L NaCl). Furthermore, for initial velocity determination, the following PHP amounts were used: 0, 10, 30, 50, 60, and 80 µL.

Determination of TRL clearance

Isolated TRLs from the dogs were injected into mice intravenously to measure chylomicron clearance *in vivo*. Cholesteryl hexadecyl ether (^{3}H-CEt, cholesteryl-1,2-^{3}H(N)-ether, PerkinElmer, Inc., Boston, MA) was used as a tracer of TRL catabolism.

^{3}H-CEt is incorporated into the core of the chylomicron particle and is therefore not hydrolyzed in blood circulation. Although cholesterol ester transfer protein (CETP) transports core lipids from triacylglycerol rich LPs such as chylomicrons to high density LP, CETP activity is absent in both rodents and dogs. Thus, the selection of cholesteryl ether as a labeled material allowed us to trace canine TRL clearance in a mouse model.

Radiolabeled TRLs were prepared using the same methodology as for labeled TRLs in the HL and LPL assays. 1.3×10^{5} cpm/μmol amount of ^{3}H-CEt was dried and reconstituted using 70 μL of acetone and then immediately pipetted into an amount of TRLs containing 0.028 mmol TG and stirred under nitrogen gas for more than 1.5 hours.

Wild-type C57BL/6J mice (7-8 weeks old, female) were purchased from Jackson Laboratories. The mice were allowed to recover from transportation for 1-3 weeks before the experiment. They were randomly assigned into two oil (TAG vs. DAG) groups. General anesthesia was obtained via intraperitoneal injection of 3.3μl/g body weight of ketamine (15 mg/mL) and xylazine (3 mg/mL). Mice received 50 μL of labeled TRLs diluted with 0.9% NaCl containing 4 mg of TG by a bolus injection via a femoral vein. Twenty μL of blood were drawn before and after labeled TRL injection via retro orbital plexus at the following intervals: 0, 2, 4, 6, 10, 15, and 30 min into heparinized capillary tubes. Plasma was separated via low speed centrifugation, pipetted into Pony Vials^{TM} with 3.5 mL of scintillation cocktail, and ^{3}H radioactivity was measured by the scintillation spectrometry.

Mice were euthanized immediately by injection of an overdose of pentobarbital after the final blood collection at 30 min. The mice tissues were flushed with 20 mL of 4

mol/L phosphate-buffered saline (pH 7.4) through the left ventricle to clear whole blood. The liver was dissected, weighed, and homogenized using a tissue homogenizer (Tri-R, Model S63C, Jamaica, NY) with 5 mL 4 M phosphate buffer (pH 7.4). One mL of homogenized liver was transferred to a Poly Vial^{TM} along with 3.5 mL scintillation cocktail. ^{3}H radioactivity was measured by scintillation spectrometry to determine TRL uptake in the liver. A quench curve was constructed for the yellow color of liver samples and was used for the correction of radioactivity obtained for those samples.

Statistical analyses

Data were expressed as means ± SEM and analyzed by SPSS 15.0 for Windows. Distributions were checked by Shapiro-Wilks test and skewness value and considered to be normal if the range was within +1.0 to -1.0. If data was normally distributed, a paired t test was used to evaluate a diet effect of LPL, HL, and TRL lipid compositions. Otherwise, the Wilcoxon signed-rank test was performed. The TRL fatty acid composition data were analyzed by Student's t-test. The particle diameter from number, volume, and area distributions were not normally distributed. Therefore, population percentiles for TRLs were converted to an accumulation percentile plot. The paired-t test was used to evaluate diet difference at every 10^{th} percentile from the 10^{th} to the 90^{th}, and 95^{th}. Repeated Measures ANOVA was performed for the data obtained for plasma TRL recovery with diet as the between subject variable and post labeled TRL injection time as the within subject variable. At each post injection time, Student's t-test was conducted to evaluate the diet effect. The data for liver uptake of TRLs was evaluated using Mann-Whitney U test because the data showed skewed distributions. Homogeneity of variance

in all data was not violated. Differences were considered significant at $P \leq 0.05$ and α level was maintained at 0.05.

Results

Plasma and TRL TAG concentrations and TRL protein concentrations

Postprandial plasma was collected between 3 and 4 h after meal ingestion at 10 min intervals. The plasma was pooled and its TAG concentrations were measured. It was found that plasma TAG concentrations were significantly decreased in the DAG group compared to the TAG group ($P = 0.050$). Similarly, the TAG concentration of the DAG derived TRLs was significantly lower than that of the TAG derived TRLs ($P = 0.036$, Table 4). TRL protein concentrations were not different between diet types (Table 15).

Table 15. Plasma and TRL TAG concentrations and TRL protein concentrations.

		Diet		
		DAG	TAG	P-value
Plasma		*mmol/L*		
	TG	2.1 ± 0.6	3.3 ± 1.0	0.05
TRL		*mmol/L plasma*		
	TG	2.0 ± 0.6	3.1 ± 1.0	0.036
	Protein	0.15 ± 0.04	0.25 ± 0.06	NS

Values are means ± SEM. *P*-values are for paird-t test or Wilcoxon signed rank test between DAG (n=8) and TAG (n=8). $P \leq 0.05$ is considered significant. NS represents non significant. Molecular weight of protein used was 70,000 g/mol

Lipid compositions of TRLs

The TRLs collected from dogs fed the DAG diet (DAG derived TRLs) had a significantly greater amount of 1,3-DAG ($P = 0.003$) and less TAG ($P = 0.013$) than the TRLs collected from dogs fed the TAG diet (TAG derived TRLs). In addition, the DAG derived TRLs contained a greater amount of MAG ($P = 0.005$) and NEFAs ($P = 0.028$) than the TAG derived TRLs (Figure 8 (A)). Because MAG and DAG are more hydrophilic than TAG due to the existence of hydroxyl groups in their glycerol backbone, the possibility exists that these MAG and DAG orient to the surface of TRLs instead of being located in their core along with TAG and cholesteryl esters. If so, the characteristics of DAG derived TRLs may be functionally and structurally different. In order to better define this possibility, the relative amount of hydrophilic and hydrophobic contributions to the TRLs were calculated. In addition, hydrophobic-hydrophilic ratio was also estimated as an index of the characteristic difference between DAG and TAG derived TRLs. Phospholipids, MAG, cholesterol,1,2-DAG, and 1,3-DAG were categorized as hydrophilic while TAG and cholesteryl esters were considered as hydrophobic components. Because it was not possible to determine whether NEFAs were ionized, NEFAs were not included in this calculation. The results showed that the DAG derived TRLs contained significantly greater hydrophilic substrates ($P = 0.005$), lower hydrophobic substrates ($P = 0.001$), and therefore a lower hydrophobic/hydrophilic substrate ratio ($P=0.007$) than the TAG derived TRLs (Figure 8 (B)). These results suggest that the DAG derived TRLs have distinct TRL lipid

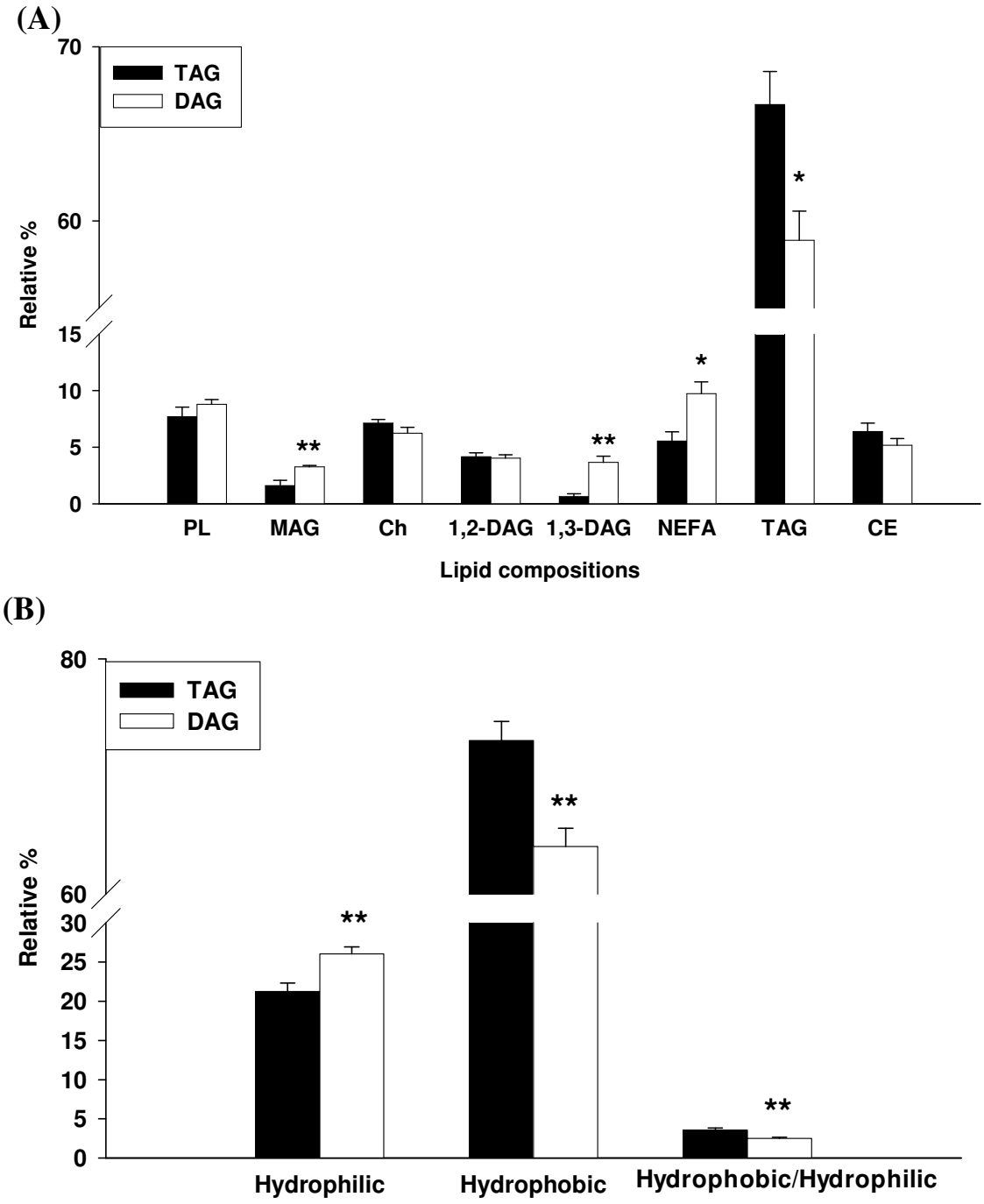


Figure 8. Lipid composition of TRLs (relative %). (A) Lipid compositions. PL=phospholipids, MAG=monoacylglycerol, Ch=cholesterol, 1,2-DAG=1,2-diacylglycerol, 1,3-DAG=1,3-diacylglycerol, NEFA=nonesterified fatty acid, CE=cholesteryl ester. (B) Lipid distributions. All data are shown as mean ± SE. The asterisks denote statistically significant differences between DAG and TAG groups (* $P<0.05$, ** $P<0.01$).

distributions and may be structurally and functionally different compared to the TAG derived TRLs.

TRL particle size

The raw density function plots for TRL particle number, volume, and area distributions are shown in Fig 9. Particle diameter in each distribution was right skewed. Median diameter in the number distribution data was 143.0 nm for DAG derived TRLs and 137.1 nm for TAG derived TRLs (Fig 9 (A)). In contrast, median diameters in the volume distribution from the DAG derived TRLs and the TAG derived TRLs were 414.2 nm and 309.6 nm (Fig 9 (B)). Because particle area distribution takes into account both number and volume of particles, a larger median diameter than the diameter based on number distribution was observed in both oil derived TRLs and was 216 nm for the DAG derived TRLs and 194.4 nm for the TAG derived TRLs (Fig 9 (C)). In order to better understand these differences, percentile distributions of TRL number, volume, and area distributions were calculated from the appropriate accumulation percentile plots (Fig 10). The TRL number distribution showed no significant differences at each percentile level (Fig 10 (A)). However, the diet difference in each percentile level of TRL volume distribution showed that the DAG derived TRL had a significantly larger particle diameter between 40^{th} to 70th population percentiles ($P < 0.05$, Fig 10 (B)). The TRL area distribution, when converted to the percentile population plot, found that DAG derived TRLs had significantly larger particle diameter at 90^{th} and 95^{th} percentile ($P < 0.05$, Fig 10 (C)).

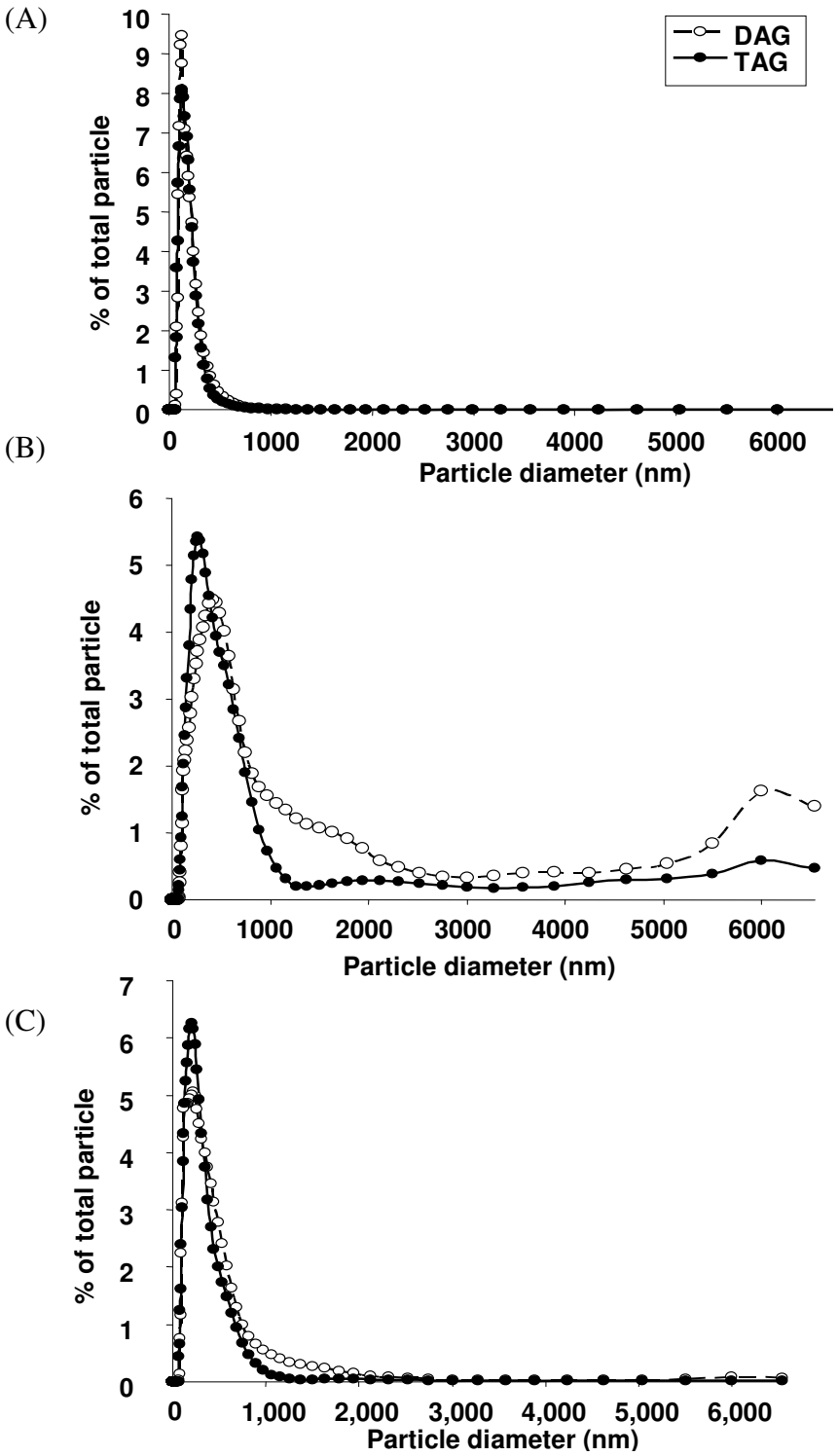


Figure 9. Diameter distributions of DAG and TAG derived TRLs. TRL diameters are expressed as a distribution of (A) particle number, (B) particle volume, and (C) particle area.

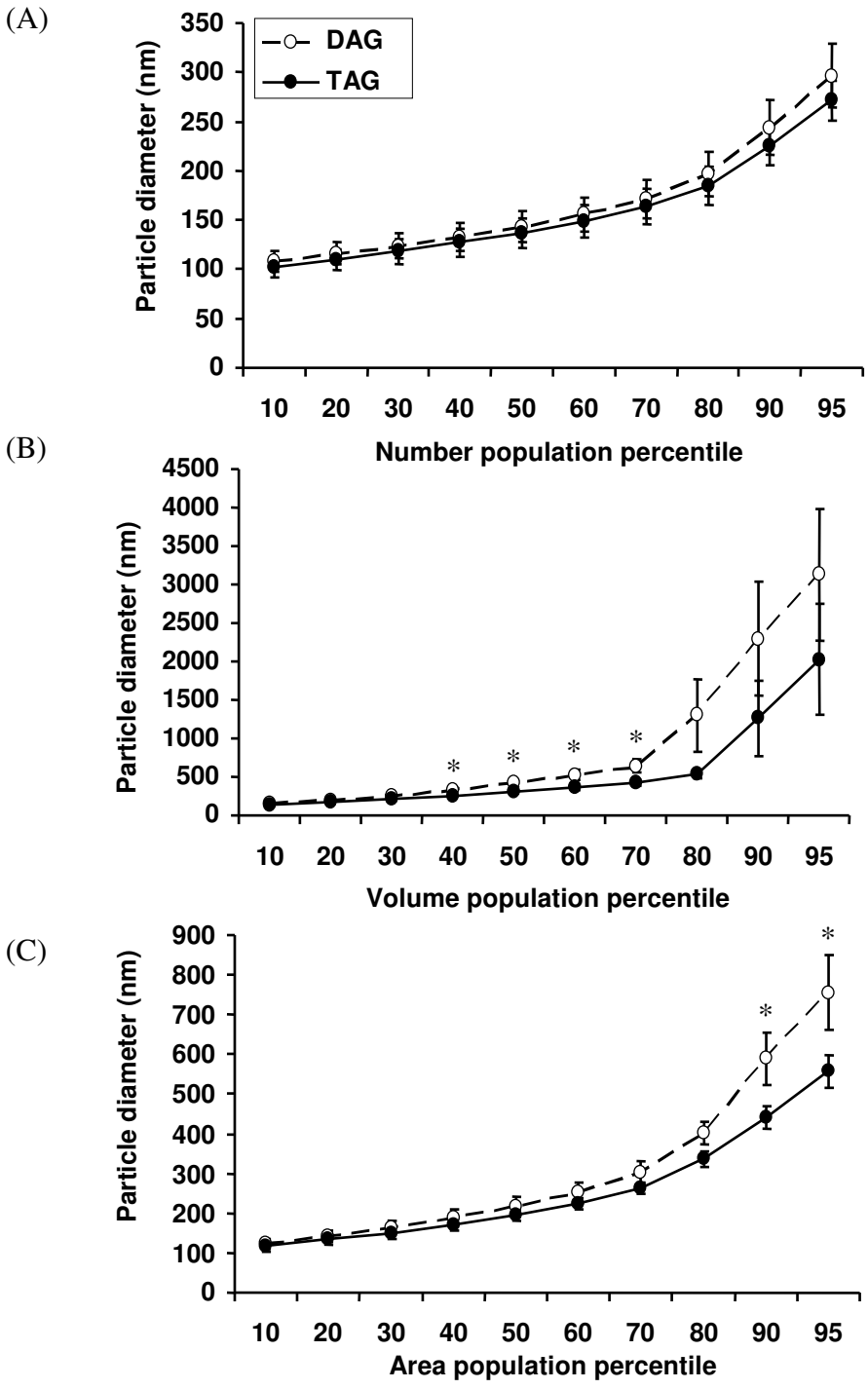


Figure 10. Distribution percentile of DAG and TAG derived TRLs. TRL diameters were drawn against the percentile of (A) number population, (B) volume population, and (C) area population. Asterisks denote significant differences between DAG and TAG derived TRLs using the paired t-test ($P < 0.05$).

TRL fatty acid composition

The DAG derived TRLs contained statistically significantly lower 16:0, 20:0, and 20:1 concentrations than the TAG derived TRLs ($P<0.05$, Table 16). Moreover, the DAG derived TRLs had significantly higher 18:3 n3 concentrations than the TAG derived TRLs ($P = 0.048$). It should be noted, however, that these differences were of small magnitude (≤ 2 relative %). In addition, similar fatty acid patterns were observed in both the oils and diets, therefore, the few differences seen in fatty acid pattern likely originated from these same differences in fatty acid composition between the DAG and TAG oils.

Labeled TRL integrity

Labeled TRLs were prepared in order to determine their suitability as substrates for LPL and HL *in vitro*. Because it is critical to introduce ^{3}H-triolein into core lipids to accurately measure the lipase activities, the integrity of TRLs was measured. As a check on the method, an artifact of TRL integrity was induced by sonication at higher voltage (150 watt for 20 sec with 3 intervals) than generally used for labeling such particles and the resultant label and lipid distributions were used as a marker of TRL disintegration (21). Pre-sonication (i.e. pre-incubation) was prepared as a baseline comparison. It was found that most of TAG was distributed in the 1st capillary tube fraction. In this fraction, the ratio of relative % TAG and radioactivity at pre-sonication, 1 h-, 1.5 h-, and 2 h-incubations were 0.51, 0.65, 0.83, and 0.84, respectively (Figure 11). Although 1h-incubation resulted in more ^{3}H-triolein incorporation into the core than pre-sonication,

Table 16. TRL fatty acid compositions (relative %).

Fatty Acid	DAG	TAG	*P* -value
	g/100 g fat		
14:0	0.10 ± 0.03	0.13 ± 0.01	NS
16:0	5.19 ± 0.26	7.20 ± 0.07	0.002
16:1	0.20 ± 0.05	0.18 ± 0.02	NS
18:0	4.12 ± 0.52	5.15 ± 0.39	NS
18:1 n9	31.02 ± 0.79	28.96 ± 0.51	NS
18:1 n7	2.48 ± 0.08	2.23 ± 0.08	NS
18:2 n6	44.42 ± 0.74	43.12 ± 0.44	NS
18:3 n3	6.92 ± 0.19	6.27 ± 0.14	0.048
20:0	0.27 ± 0.06	0.47 ± 0.04	0.002
20:1	0.44 ± 0.02	0.52 ± 0.02	0.014
20:3	0.00 ± 0.00	0.03 ± 0.03	NS
20:4n6	3.20 ± 0.59	3.57 ± 0.51	NS
22:0	0.00 ± 0.00	0.03 ± 0.03	NS
22:4n6	0.25 ± 0.06	0.29 ± 0.03	NS
24:0	0.53 ± 0.20	0.74 ± 0.17	NS
Inidentified	0.86 ± 0.18	1.12 ± 0.08	NS

Values are means ± SEM. *P*-values are for Student's t test between DAG (n=6) and TAG (n=8). $P < 0.05$ is considered significant. NS represents non significant.

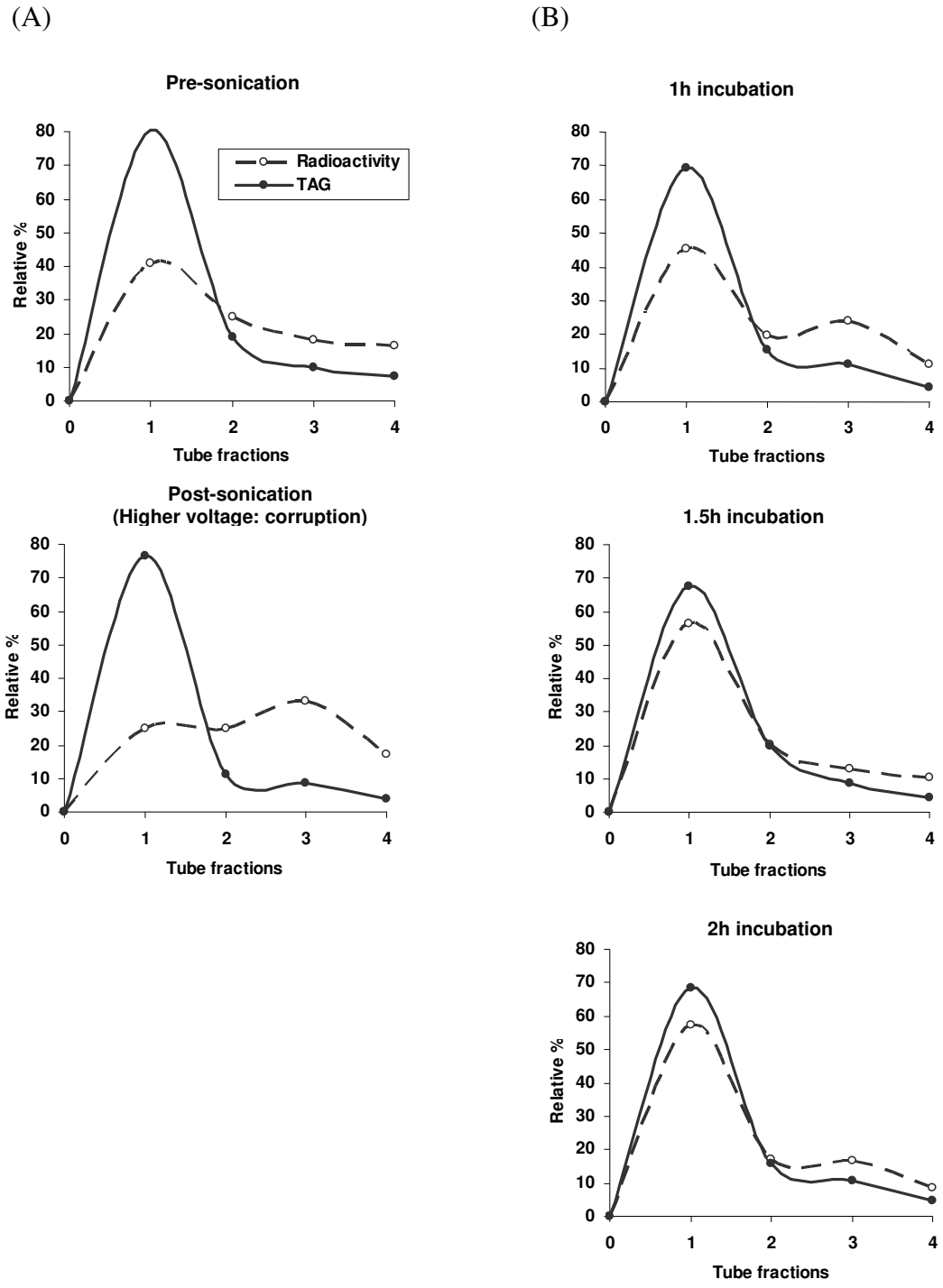


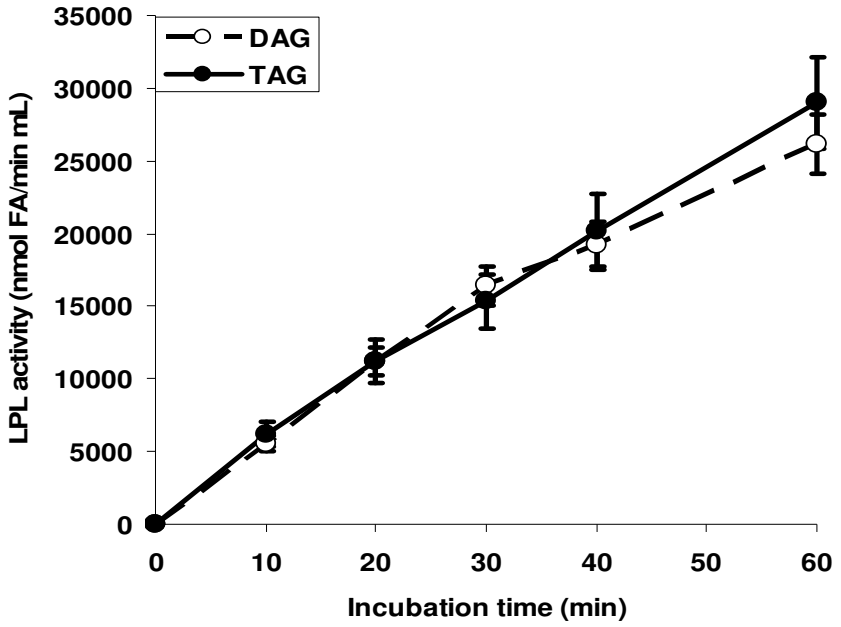
Figure 11. Determination of labeled TRL integrity from two separate methodologies. (A) sonication. (B) incubation with stir under nitrogen.

1.5 h and 2 h-incubations provided better incorporations into the core substrate overall. In addition, more than 1.5 h-incubation showed the strongest correlations between TRL TAG concentrations and radioactivities in each tube fraction ($\gamma^2 > 0.99$, $P \leq 0.003$). Because the ratio and correlations of incorporation of ^{3}H-triolein into the core TRLs showed no differences between 1.5 h and 2 h-incubation, ≥ 1.5 h incubation time was considered to most efficiently label the TRLs with the least artifact.

LPL activities using DAG and TAG TRL substrates

First, various incubation times at constant substrate concentration (1.32 mmol/L TG in the labeled TRLs) were measured to evaluate a possible DAG effect and determine an appropriate incubation time for further studies to estimate the apparent Km, Vmax, and initial velocities (V_0). No significant differences in LPL activities vs. time between oil types were observed (Figure 12 (A)). LPL-mediated lipolysis and incubation time were linearly related for at least 60 min. Consequently, 30 min incubation times were used in studies for apparent Km, Vmax, and V_0 determinations. Michaelis-Menten plots (Figure 13 (A)) were transferred to Lineweaver-Burk plots which were used for calculating Vmax and apparent Km (Figure 14 (A)). V_0 was calculated using the first order kinetics of Figure 15 (A). The results showed that the DAG derived TRLs as substrate for LPL had a moderately decreased Vmax and V_0 compared with the TAG derived TRLs (P=0.058, and P=0.069), but these were not significant. In contrast, the apparent Km of LPL for the DAG derived TRLs was statistically significantly lower than that of the TAG derived TRLs demonstrating that the DAG derived TRLs showed more affinity for the LPL enzyme (Table 17).

(A)



(B)

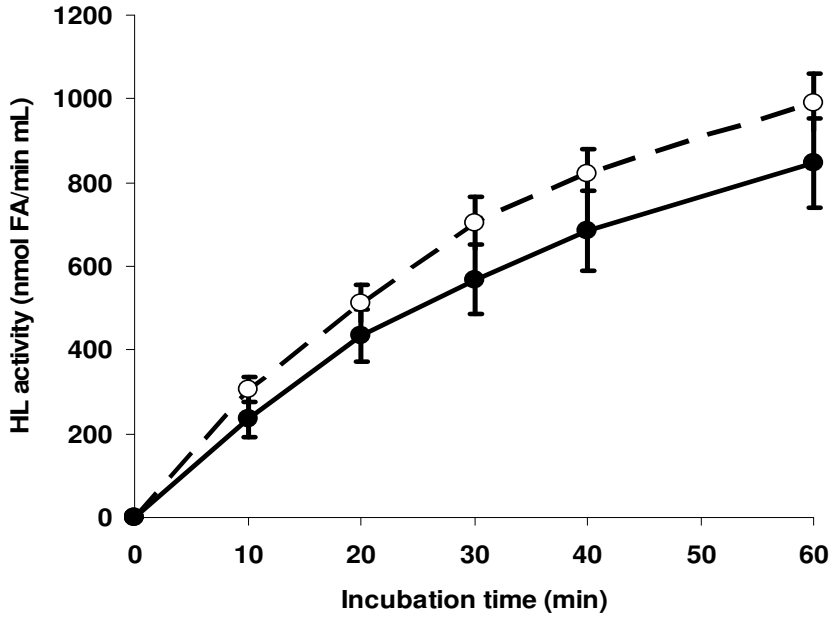
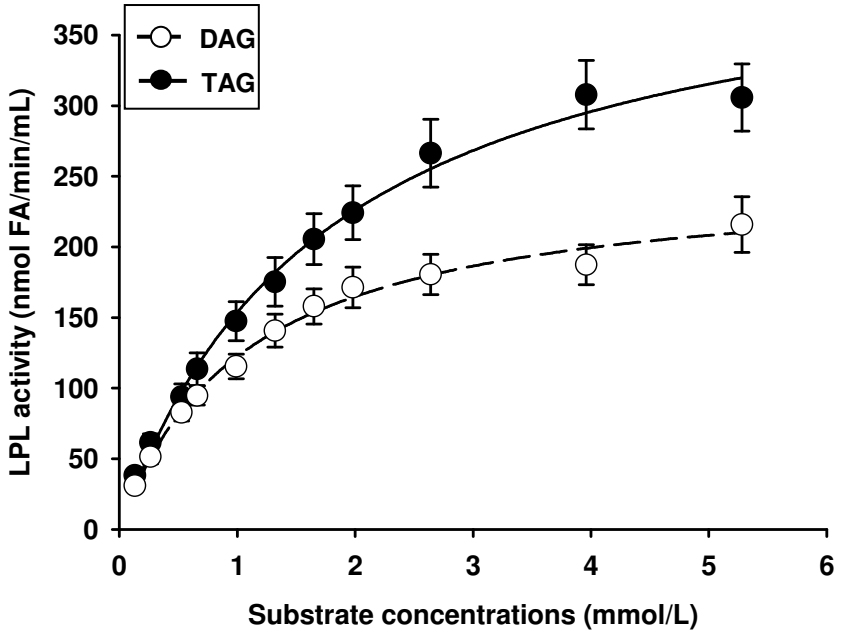


Figure 12. Effects of incubation time on lipase activities. (A) LPL activity, (B) HL activity. Data are mean ± SEM. No significant differences were observed between DAG and TAG.

(A)



(B)

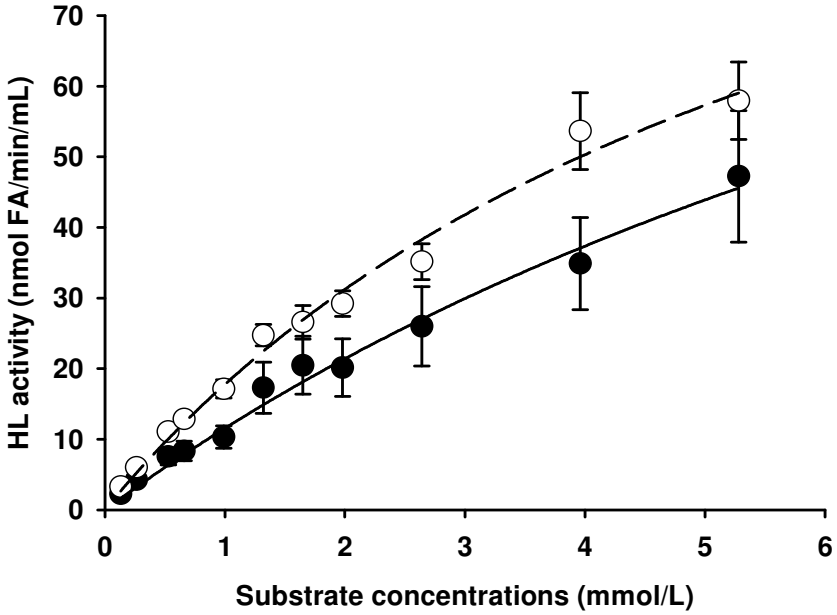
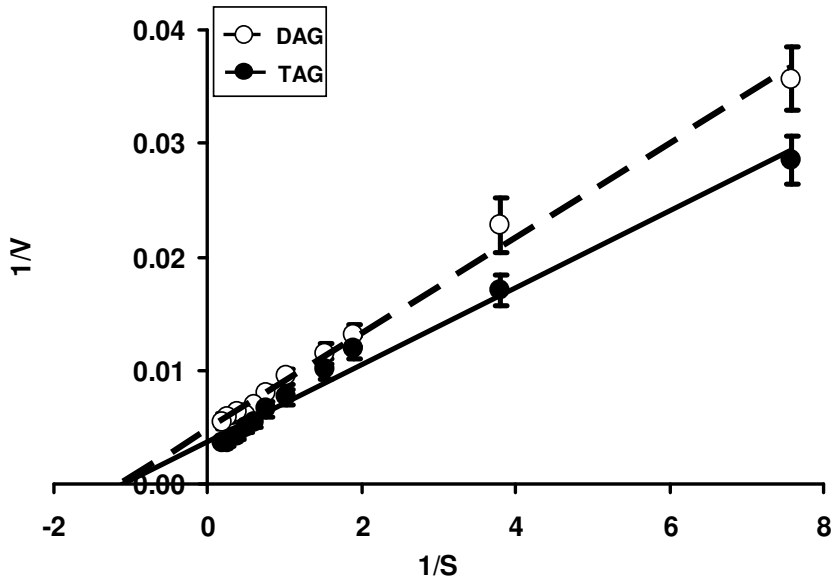


Figure 13. Effects of substrate concentrations on lipase activities (Michaelis-Menten's plots). (A) LPL activity, (B) HL activity. Data are mean ± SEM.

(A)



(B)

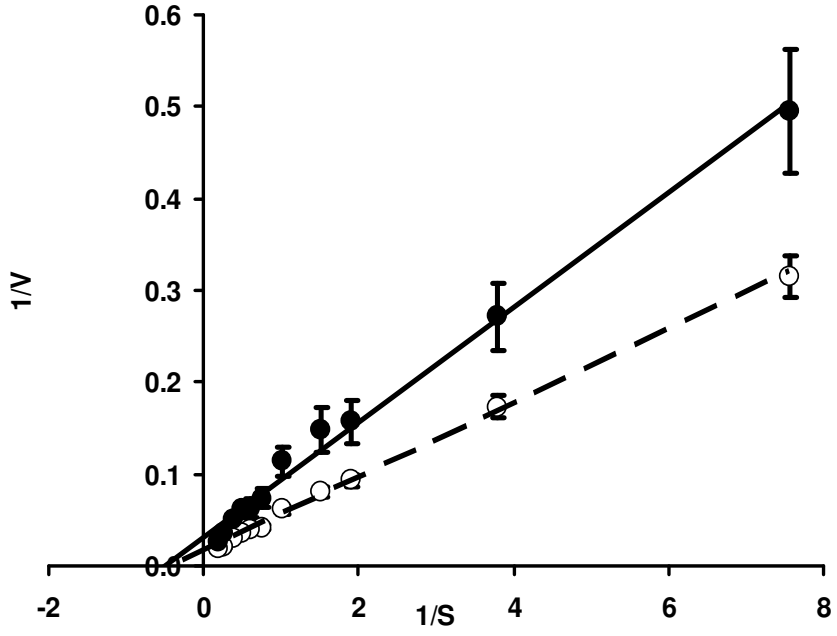
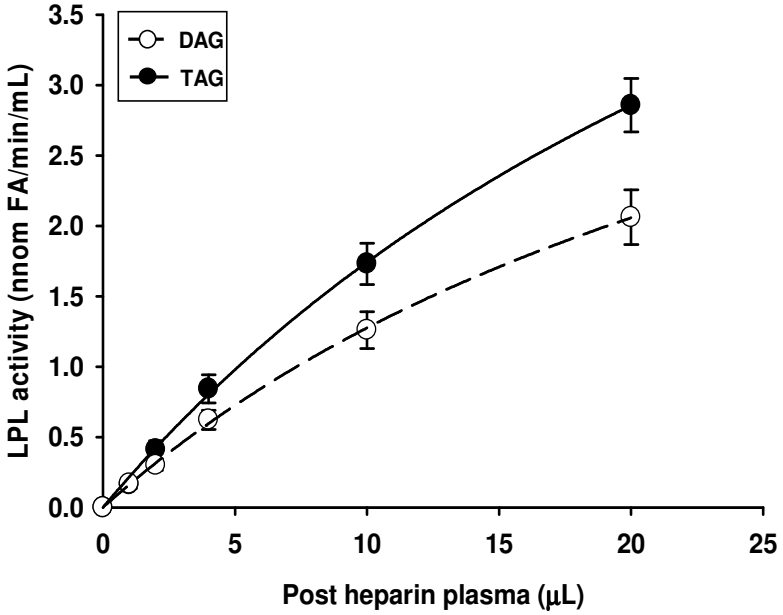


Figure 14. Effects of substrate concentrations on lipase activities (Lineweaver–Burk plots). (A) LPL activity, (B) HL activity. Data are mean ± SEM.

(A)



(B)

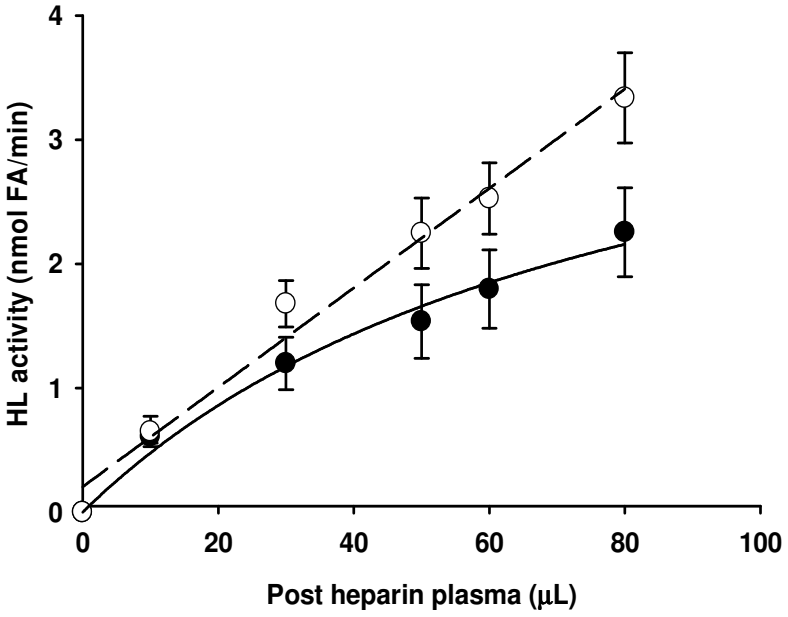


Figure 15. Effects of enzyme concentrations on lipase activities. (A) LPL activity, (B) HL activity. Data are mean ± SEM.

Table 17. Vmax, apparent Km, and V_0 of LPL and HL.

Lipase		DAG	TAG	*P* -value
LPL	Vmax, *nmolFA/min/mL*	215.80 ± 25.96	340.95 ± 44.47	NS
	Recalculated Vmax, *nmolFA/min/mL*	217.02 ± 25.56	340.95 ± 44.47	NS
	Km, *mmol/L*	0.82 ± 0.09	1.24 ± 0.12	0.047
	Recalculated Km, *mmol/L*	0.70 ± 0.08	1.13 ± 0.11	0.026
	Vo	0.15 ± 0.02	0.21 ± 0.03	NS
HL	Vmax, *nmolFA/min/mL*	76.65 ± 12.95	88.69 ± 27.94	NS
	Recalculated Vmax, *nmolFA/min/mL*	76.09 13.09	88.69 27.94	NS
	Km, *mmol/L*	3.07 ± 0.63	6.23 ± 1.46	0.05
	Recalculated Km, *mmol/L*	2.59 0.55	5.65 1.33	0.039
	Vo	0.04 ± 0.00	0.03 ± 0.01	NS

Values are means ± SEM, *n*=8 (DAG) and *n*=8 (TAG). *P*-values are for paired t test between DAG and TAG. $P \leq 0.05$ is considered significant.

The methodology used for TRL TAG determination measured acylglycerols, all of which were considered to be TAG. It should be noted, however, that significantly different TAG and 1,3-DAG concentrations were observed between oil types. Therefore, it may be more appropriate to recalculate TRL TAG concentrations based on the ratio of TAG in the total acylglycerols of TRLs in order to specify the degree of TAG lipolysis due to LPL. Thus, the apparent Km and Vmax were recalculated using re-drawn Michaelis-Menten and Lineweaver-Burk plots based on these revised TAG concentration. Although Vmax values were not affected by this re-calculation, statistical significance became more prominent for the recalculated Km values compared to the initial method of pre-calculation ($P = 0.026$, Table 17).

HL activity of DAG and TAG TRL substrates

A diet effect was not observed when TRLs generated from post meal ingested plasma were used for measuring HL mediated lipolysis at various incubation times (Figure 12 (B)). The relationship between incubation times and LPL mediated lipolysis was approximately linear until 30 min, therefore, 30 min incubation times were used for subsequent studies evaluating apparent Km, Vmax, and V_0. Michaelis-Menten plots (Figure 13 (B)) were transferred to Lineweaver-Burk plots which were used for the calculating Vmax and apparent Km (Figure 14 (B)). Moreover, V_0 was calculated using the first order kinetics of Figure 15 (B). The apparent Km of HL for the DAG derived TRLs was significantly lower than that of the TAG derived TRLs indicating that the DAG derived TRLs had more affinity for the HL enzyme ($P = 0.05$). Vmax and Vo were not significantly different (Table 17). The TAG substrate concentrations in the TRLs were recalculated as mentioned in the section of LPL activities using DAG and TAG TRL substrates. The results showed that while Vmax values were not affected by this re-calculation, the significantly lower Km for the DAG derived TRLs became more pronounced ($P = 0.039$, Table 17).

TRL plasma clearance

Mice were intravenously injected with labeled canine TRLs containing 4 mg TG (approximately 4.6 mmol/L plasma per 20 g mouse) and blood samples were collected at intervals up to 30 min post-injection to measure the plasma clearance of the canine TRLs. In this study, it was necessary to evaluate efficient radiolabeling of the TRLs, successful intravenous injection of labeled TRLs, stable anesthetic conditions during the blood collections, and complete tissue perfusion as criteria for data accuracy and interpretations. Consequently, although ≤ 10 mice were used in each diet group, only 7 mice in the TAG group and 5 mice in the DAG group satisfied the established criteria and therefore utilized for data analyses. No main time and diet effects were observed by repeated measures ANOVAs. However, when comparing the DAG vs. TAG groups at each post TRL injection time, the DAG group had significantly lower plasma ^{3}H-CEt recovery than the TAG group at both 15 and 30 min post injection ($P = 0.03$ and $P = 0.004$, Figure 16). This indicated that the DAG derived canine TRLs had a more rapid blood clearance rate than the TAG derived canine TRLs in this mouse model.

TRL liver uptake

A significantly greater TRL uptake in the liver was observed in the DAG group than the TAG group ($P = 0.045$, Figure 17). Possible outliers (≥ mean ± 2 SD) were found in each group, however, the statistical results remained significant whether those two data points were included or eliminated. Thus, these two data points were included in the analysis.

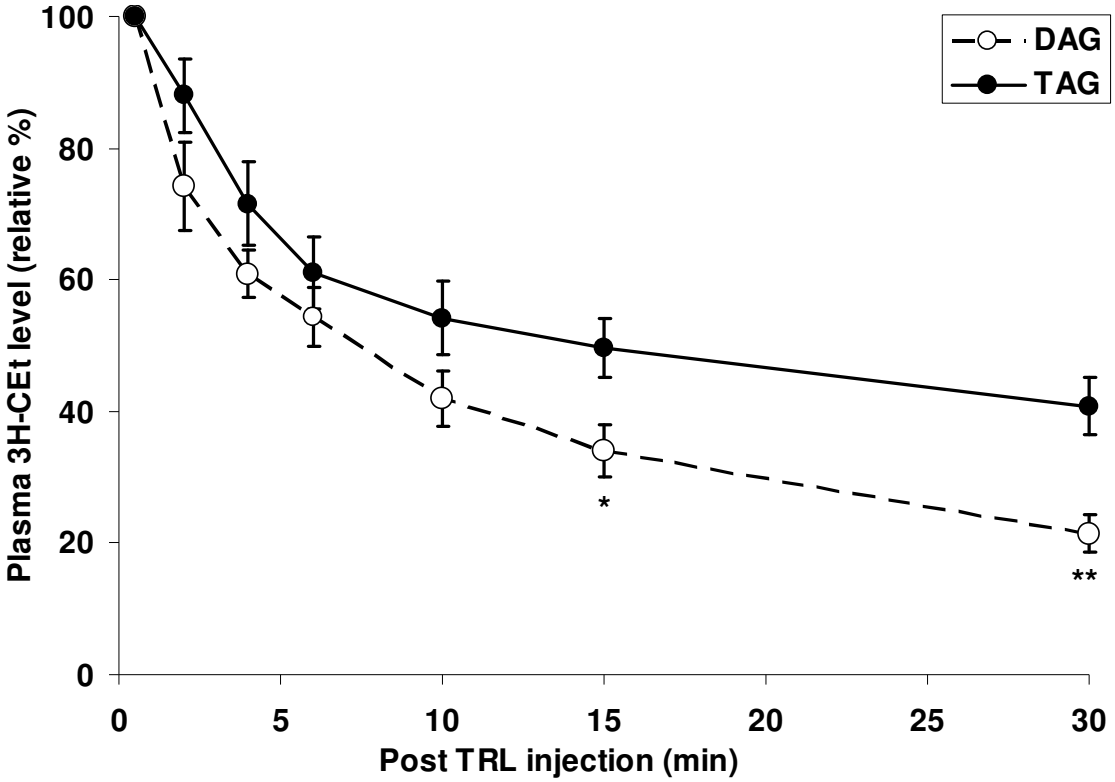


Figure 16. Blood clearance of TRLs. Data are shown as mean ± SE. The asterisks denote statistically significant differences between DAG (n=5) and TAG (n=7) (*P = 0.03, ** P = 0.004).

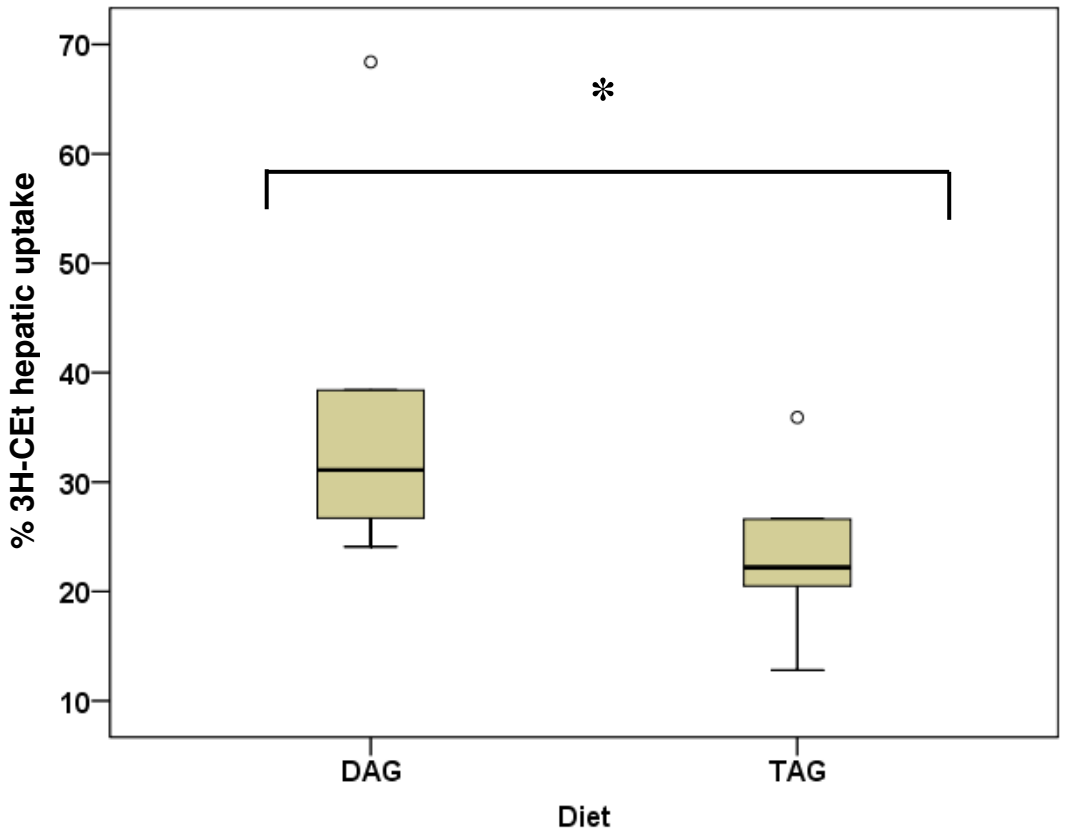


Figure 17. Hepatic uptake of TRLs. Boxes represent the middle 50 % of the data. Upper and lower lines in the box indicate the 75th and 25th percentile of the data set. The middle line in the box represents the median and whiskers indicate minimum and maximum values of data. Outliers (≤ mean ± 2 SD) are present as open circle. The asterisk denotes statistically significant differences between DAG (n=5) and TAG (n=6) (Mann-Whitney U test, P=0.045).

Discussion

The effect of DAG on chylomicron metabolism has not been widely studied to date. Recently, Yasunaga et al. used emulsions containing DAG as substrates and found their rapid blood clearance by the liver in a partially apo E dependent fashion (78). Based on their findings, the present study to evaluate the effect of DAG on TRL metabolism was designed and conducted. The metabolic effect of DAG on canine lipid metabolism has received little attention. However, it has potential as one of the functional dietary supplements for dogs for health management purposes. Thus, in the present study, dogs were fed a high fat meal enriched in 1,3-DAG using a cross-over design with dietary TAG. TRLs were isolated from 3-4 h postprandial plasma and they were used in the study. For comparison, TRLs that were isolated from plasma of dogs fed a high fat meal enriched in TAG were also prepared.

Blunting of the post-prandial increase of plasma TAG concentrations was observed in dogs fed the DAG vs. TAG enriched diet. This finding was expected because consistent findings have been observed in relevant DAG studies using rodent and human models (81, 83). Moreover, our preliminary study in dogs also showed less of an elevation of plasma postprandial TAG concentrations at 2 and 3 h after feeding a 19 wt % DAG enriched diet to dogs compared with a 19 wt % TAG diet (112). Furthermore, the plasma TAG lowering effect of DAG has been considered to be dose dependent (155). In the present study, the dose of DAG oil was 49.6 wt % and higher than those used in previous DAG studies. Similarly, TAG concentrations of the DAG derived TRLs were also lower compared to the TAG derived TAG. This result suggests

that the TAG lowering effect of DAG in TRLs can be attributed to plasma TAG concentrations postprandially.

Acylglycerol distributions of the DAG derived TRL lipid contents were distinctly different compared to the TAG derived TRLs. Similar lipid distributions have also been found in the rat intestinal mucosa. In that study, rats had been administered a 5 h continuous duodenal infusion of a DAG enriched emulsion followed by duodenal injection of ^{14}C-linoleic acid 10 min prior to mucosal sample collection and radiolabel measurement. Another study found that nascent chylomicrons obtained from mice fed ^{14}C-diolein using a DAG emulsion also showed similar acylglycerol distributions (78). Together these results indicate that the increase in NEFAs, MAG, and 1,3-DAG along with less TAG in the DAG derived TRLs likely occurred during the process of chylomicron assembly. It should be noted that the presence of TAG in both DAG and TAG derived TRLs was approximately 30 % lower than the finding from those studies investigating intestinal mucosa and nascent chylomicrons. The explanation for this decrease is that the TRLs isolated from dog plasma between 3 and 4 h after feeding very likely contained both chylomicron and chylomicron remnants because once nascent chylomicrons are secreted into the blood circulation, they are rapidly lipolyzed by LPL (173).

Because 1,3-DAG and MAG are more hydrophilic than TAG, their presence in the DAG derived TRLs vs. the TAG derived TRLs may result in them more specifically partitioning to the amphiphilic surface of TRLs. Yasunaga et al. found that DAG was more readily hydrolyzed by LPL when chylomicrons generated by ^{14}C-diolein labeled

DAG ingestion was used as substrate compared with those generated by ^{14}C-triolein labeled TAG ingestion. This finding suggests that the surface of DAG containing TRLs was more readily hydrolyzed by LPL than core TAG. This phenomenon is reasonable to expect because lipases such as LPL and HL specifically hydrolyze *sn*-1 and 3 positions of acylglycerols and thereby allowing surface 1,3-DAG to interact more freely than core TAG (174). However, under normal physiological conditions, the 1,3-DAG concentrations in DAG derived chylomicrons would be only a small molar component compared with TAG concentrations (77, 78). Consequently, hydrolysis of core TAG would still be expected to occur to produce chylomicron remnants from those chylomicrons. Thus, in the present study, ^{3}H-triolein was used to label both DAG and TAG derived TRLs. This approach allowed us to measure the effect of modified lipid distributions by DAG on LPL mediated hydrolysis targeting the core TAG. Our results demonstrated that the DAG derived TRLs showed greater *in vitro* affinity of LPL and HL for TRLs than the TAG derived TRLs. The explanation for this finding includes the possibility that more 1,3-DAG and MAG and less TAG in the DAG derived TRLs may alter TRL structure to the extent that hydrophilic components of lipids (i.e. DAG, and MAG) in the TRLs may orient at the oil-water interface of the TRL surface. This structural difference may consequently alter the particle diameter of the DAG derived TRLs given that their area distributions were larger than the TAG derived TRLs. Lipase affinity is largely reflected on larger lipoprotein particle diameter (175, 176). Therefore, the greater affinity of LPL and HL for the DAG derived TRLs would not be unexpected. Alternatively, there is the possibility that the larger particle diameter of the DAG derived

TRLs were the result of artifact. This may be possible because the presence of more hydrophilic components in the DAG derived TRLs may show a greater aggregability. This may be true especially in those few percentiles that were appoximately 6000 nm in diameter as that found in the volume distribution data. However, it should be noted that particle size based on particle number distribution did not reveal any particles at approximately 6000 nm, which suggests that only a few particle numbers were present having this very large particle diameter. Chylomicron diameter is generally considered to be maximally ≈1200 nm (178). If so, particle diameters more than 1200 nm may be an artifact due to aggregation. The area distribution data, which consider both particle number and volume, may be therefore more informative in this regard, especially because the population percentile of particles > 1200 nm was only 2 %. Therefore, even though if these larger particles were the artifact, they may not have been the primary contributor to the difference in the affinity for LPL and HL for the TRLs in this study.

It was found that the initial velocity under the physiological TAG substrate concentrations of both lipases using the DAG vs. TAG derived TRLs were either not different for HL or moderately lower for LPL. However, this finding was inconsistent with Yasunaga et al. who observed higher LPL mediated lipolysis using DAG radiolabeled chylomicrons compared to TAG radiolabeled chylomicrons. One possible explanation for this discrepancy involves methodological differences in which Yasunaga et al. traced LPL mediated DAG hydrolysis using DAG generated chylomicrons while our study targeted core TAG hydrolysis using the DAG derived TRLs. Unfortunately, substrate concentrations in Yasunaga et al. study were not described.

In order to evaluate the *in vivo* clearance of DAG derived canine TRLs, the isolated TRLs from canine plasma were injected into mice to model this phenomena. It is unknown previously whether canine TRLs are substantially different from murine TRLs. Nontheless, regarding lipoprotein metabolism, dog and mouse have been shown to be similar in several respects. For example, cholesteryl ester transfer protein, a protein that exchanges TAG and cholesteryl ester between HDL and apo B containing lipoproteins are low in both species. In addition, mice and dogs both have a predominant HDL particle relative to apo B lipoprotein particles compared to humans (165, 173, 174). In addition, our findings were comparable to Yasunaga et al. who used artificial emulsions injections in mice. Therefore, mice were considered to be suitable species to measure canine TRL metabolism *in vivo*.

In the i*n vivo* study, the blood clearance of the DAG derived TRLs was more rapid and was associated with greater hepatic uptake vs. TAG. This may be because the DAG derived TRLs had a larger particle diameter due to its structural difference compared to the TAG derived TRLs. This larger size of TRLs in the DAG group altered affinity to the LPL and HL, and thereby leading to possibly enhance lipolytic activity *in vivo*. Our findings indicate that the DAG derived TRLs show more affinity with HL but with similar Vmax as the TAG derived TRLs. Therefore, once entering the space of Disse, the DAG derived TRLs may more actively interact with HL for further hydrolysis or perhaps participate in some way as a ligand during hepatic remnant uptake. It is known that HL has a considerable phospholipase activity and, although controversial, generation of lysophospholipid may be an important factor for the transformation of a

chylomicron into a chylomicron remnant (178-181). If so, this greater affinity of the DAG derived TRL may contribute to greater TRL uptake in the liver overall. While there is considerable debate whether HL has a role in chylomicron remnant metabolism as a ligand (182-185), the present study was not designed to investigate this possibility.

Finally, fatty acid compositions of the TRLs were slightly different between diets. This difference occurred due to the slightly different fatty acid compositions of the oils used. It is unknown whether these modest differences would partially affect blood clearance and/or TRL hepatic uptake. However, the differences are considered to be too small ($\leq$ 2 relative %) to have any major effect on metabolic alterations seen in the experiment.

In conclusion, plasma TAG concentrations were increased less due to decreased TRL TAG concentrations 3-4 h after DAG ingestion in dogs. The DAG enriched oil altered the lipid distributions of TRLs, which appeared to alter both structure and function of the TRLs. For example, particle size of the DAG derived TRLs was larger than that of TAG derived TRLs, and this provided increased affinity of core TAG in the DAG derived TRLs to LPL and HL enzymes *in vitro*. *In vivo*, the intravenous injection of the DAG derived TRLs underwent more rapid blood clearance associated with the greater hepatic uptake compared with the TAG derived TRL injection, which may also be due to the above mentioned structural and characteristic differences. These findings suggest that the postprandial plasma TAG lowering effect of DAG results, at least in part, from the efficient clearance of TRLs from blood circulation in dogs and their ability to act as a more efficient substrate for plasma lipolytic enzymes.

CHAPTER V

SUMMARY AND CONCLUSIONS

Obesity is the most common clinical disorder in the companion animal nutrition field and is associated with various medical conditions. Therefore, decreasing the incidence of obesity is important to maintain health. Appropriate dietary management should potentially provide weight loss in a safe, healthy, and efficacious manner, in which lipid metabolic alteration is one important factor. Some possible dietary nutrients for these purposes include several dietary fiber types, carnitine, LGI starch, and DAG, however, their effects on weight loss in dogs are largely unsettled. Therefore, the overall objective of the study was to evaluate the effect of these four nutrients with respect to their potential to support healthy weight loss by alteration of lipid metabolism and/or satiety in dogs. Three separate studies were therefore conducted in order to evaluate: 1) dietary fiber/carnitine feeding effect on short term (3 and 7 h) satiety and long term (6 weeks) weight loss, 2) dietary LGI and/or DAG feeding effect during a 9 week weight loss period, and 3) the dietary DAG enriched meal effect on TRL metabolism which were isolated from plasma 3-4h postprandially.

The combination of dietary fiber/carnitine supplementation mimicked a practical approach in which diets contain a variety of nutrients. This combination supplement decreased both food (gram basis) and energy intake 3 h but not 7 h post-meal, suggesting a better 3 h post-meal satiety. Although PYY has reported to be increased due to the presence of fermentable fiber in rats, its concentrations were not altered by increased dietary fiber/carnitine combination in this dog study. Different PYY sensitivity to

fermentable fiber ingestion in species and delayed onset of post-meal satiety in obese subjects may be the explanation of this difference. In addition to the beneficial effect on short term satiety, the effect of the combination of dietary fiber/carnitine was found in conjunction with increased postprandial plasma BHB at the end of the weight loss period. The supplement thus resulted in increased body fat and body weight loss at the end of the study and demonstrated successful weight loss along with greater degree of body fat utilization. Of additional interest, the high amount of vitamin A, primarily as β-carotene, included in this combination supplement. It did not result in any alteration of either fasting or early postprandial plasma vitamin A concentrations or retinyl ester distributions after 6 weeks in spite of the increased content of β-carotene. Longer term effects of the ingestion of high amount of β-carotene on plasma vitamin A have not been previously reported in the canine species.

In the second study, the LGI containing diets decreased total, protein, and carbohydrate digestibilities compared to the HGI containing diets. Because diet digestibilites reflect ME, a decreased ME was found in the LGI diets, which further contributed to less energy consumption in the LGI diets compared to the HGI diets. Consequently, although all diet groups lost body weight, the LGI containing diets resulted in increased body weight loss compared to the HGI containing diets. In addition to the LGI beneficial effects, DAG beneficial effects were observed. For example, the DAG containing diets increased postprandial BHB later on in the study, which is a marker of increased fat oxidation. Moreover, the DAG containing diets lowered postprandial TAG early on during the study. However, later on, the DAG containing

diets as well as the combination of HGI/TAG diet also showed decreased postprandial TAG concentrations. It should be noted, however, that the HGI containing diets increased postprandial circulating insulin concentrations, possibly increasing lipolysis which may explain this unique finding. Finally as a synergistic effect of DAG and LGI, the combination of DAG/LGI starch lowered postprandial TC later on in the study. Fasting TC and lipoprotein fractions were affected by body weight loss, but not diets. In addition, fasting LPL and HL activities were also not affected by diets although the methodology for LPL and HL activity determinations used an artificial substrate, *in vitro*, did not target of the lipolysis of the physiologic chylomicron lipid core. Because this limitation, the final study was examined to further elucidate this question.

In the final study, postprandial plasma TAG concentrations were less increased by DAG ingestion, and appeared to be the result of decreased TRL TAG concentrations compared to TAG ingestion. The DAG enriched meal increased NEFAs, MAG, and 1,3-DAG and decreased TAG in TRLs compared with the TAG meal. This lipid composition modification of the DAG derived TRLs may consequently alter the particle diameter of the DAG derived TRLs given that there area distributions were larger than the TAG derived TRLs. Lipase affinity is largely reflected on larger lipoprotein particle diameter. Therefore, the greater affinity of LPL and HL for the DAG derived TRLs were observed. The greater affinity in the DAG derived TRLs for LPL and HL may further provided more rapid blood clearance of intravenous injection of canine DAG derived TRLs into mice and was associated with increased hepatic uptake compared to the TAG derived TRL injection.

In conclusion, the combinations of dietary fiber/carnitine and DAG/LGI preferably reduce body weight with greater fat utilization compared with low dietary fiber/carnitine supplementation, or other combination diets (i.e. TAG/HGI, TAG/LGI, and DAG/HGI). DAG ingestion affected lipid metabolisms including postprandial plasma TAG reduction. This effect is the result, at least in part, from the efficient clearance of DAG derived canine TRLs from the blood circulation and their ability to act as a more efficient substrate for the plasma lipolytic enzymes.

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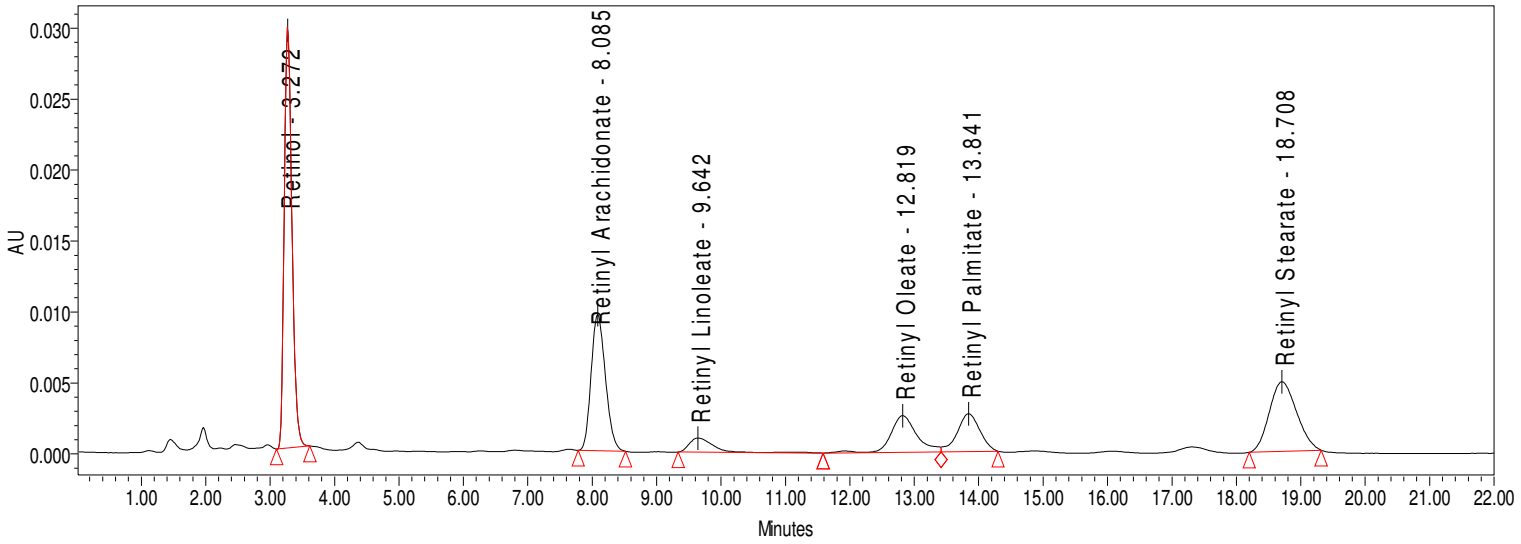
APPENDIX A

Typical separation of plasma retinol and retinyl esters with retinyl arachidonate as an internal standard.



APPENDIX B

Fatty acid composition of oils and boiled chickens (relative %)

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

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

APPENDIX C

Fatty acid composition of acclimation and experimental diets.

Fatty Acid	Acclimation diet	LD	LT	HD	HT
			Relative %		
14:0	0.3	0.23	0.19	0.25	0.38
16:0	13.77	11.5	12.54	10.3	16.4
16:1 n-7	2.37	2.15	1.89	2.26	3.52
17:0	ND	TR	0.1	TR	0.12
17:1	ND	0.11	ND	0.11	ND
18:0	5.11	4.11	5.36	3.89	5.66
18:1n-9	38.15	39.67	37.55	38.74	36.57
18:1 n-7	3.17	3.89	1.68	2.89	3.31
18:2 n-6	28.31	30.1	29.86	32.91	27.08
18:3 n-3	5.45	2.76	3.28	3.33	2.93
20:0	0.56	0.41	0.65	0.72	0.42
20:1 n-9	0.62	0.93	0.94	0.65	0.58
20:2 n-6	ND	ND	0.17	ND	ND
20:3 n-6	ND	ND	ND	ND	ND
20:4 n-6	0.41	0.37	0.28	0.43	0.37
22:0	ND	TR	0.6	0.39	0.41
22:1 n-9	ND	ND	0.19	TR	0.13
22:6 n-3	0.41	0.36	0.78	0.42	0.68
24:1 n-9	0.26	0.6	0.55	0.49	0.63
Unidentified	1.11	2.81	3.39	2.22	0.81

All diets contained 19.7% fat as fed. Values represent averages of two samples. ND and TR represent not detected and trace (< 0.1 relative %), respectively.

APPENDIX D

Calculations of concentration released fatty acid per 1 min per 1 mL basis by LPL and HL mediated lipolysis.

1. FA concentrations in 1μL substrate.

$$\frac{7\text{mg triolein (TO)}}{3\text{ mL substrate}} \times \frac{1\text{mmol TO}}{885.4\text{mg}} \times \frac{1\text{mL}}{1000\mu\text{L}} \times \frac{1\times10^{6}\text{ nmol}}{1\text{mmol}} \times \frac{3\text{nmol FA}}{1\text{nmol TO}} = \frac{7.89\text{ nmol FA}}{\mu\text{L substrate}}$$

2. Lipoprotein and hepatic Lipase activities calculations.

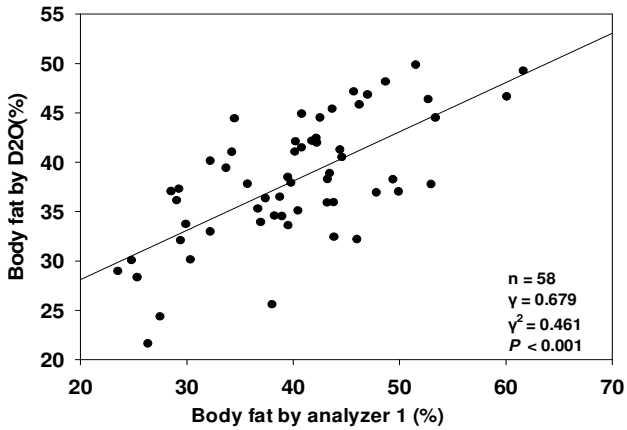
$$\frac{\text{nmol FA}}{\text{min mL}} = \frac{\text{Sample count (cpm)}}{\text{Incubation time (min)}} \times \frac{\text{substrate amt (}\mu\text{L)}}{\text{substrate count (cpm)}} \times \frac{7.89\text{ nmol FA}}{\mu\text{L substrate}}$$

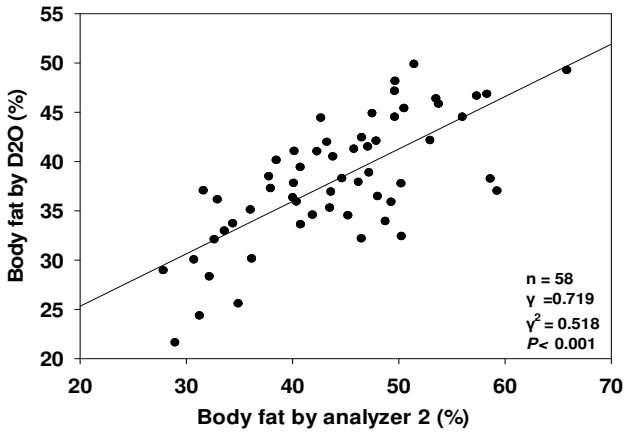
$$\times \frac{2.5\text{ mL total upper layer}}{1.0\text{ mL upper layer counted}} \times \frac{1}{0.76^{*} \times \text{post heparin plasma amt (mL)}}$$

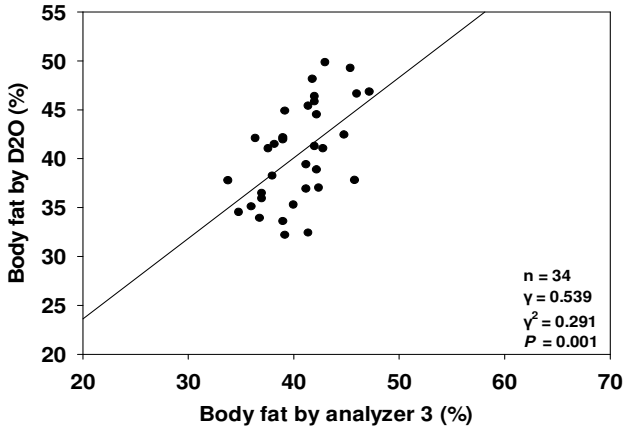
* This value represents the fraction extracted in this partition system

APPENDIX E

Relationship of % body fat based on the D2O technique and body fat analyzers.

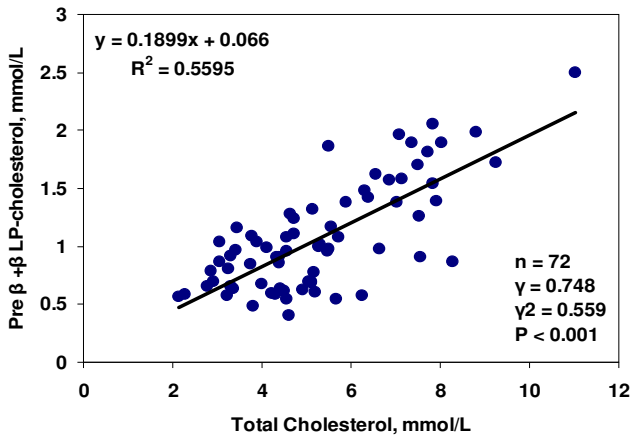


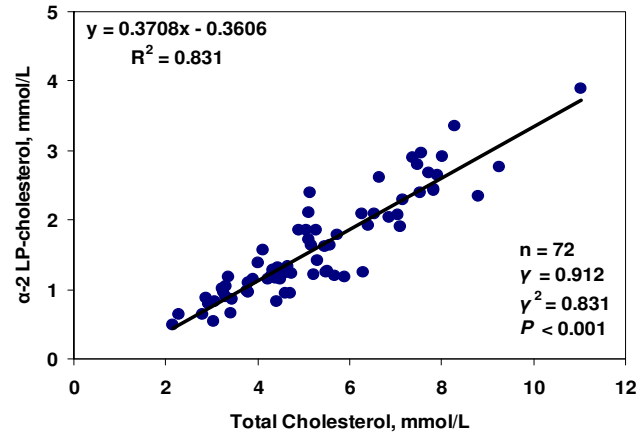


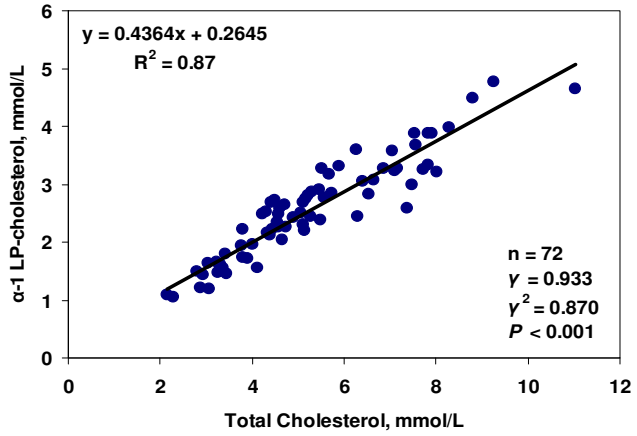


APPENDIX F

Correlation of lipoprotein fractions and total cholesterol.







VITA

Yuka Mitsuhashi received her Bachelor of Science degree in chemistry from Tokyo University of Agriculture and Technology in 2001. She entered the Nutrition program at Texas A&M University in September 2004 and received her Doctor of Philosophy degree in December 2009. Her research interests include companion animal nutrition and lipid metabolsim.

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