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FISH OIL AND BARLEY SUPPLEMENTATION IN DIETS FOR ADULT DOGS: EFFECTS ON LIPID AND PROTEIN METABOLISM, NUTRIENT DIGESTIBILITY, FECAL QUALITY, AND POSTPRANDIAL GLYCEMIA

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DISSERTATION

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A dissertation submitted in partial fulfillment
of the requirements for the degree of Doctor of Philosophy
in the College of Agriculture
at the University of Kentucky

By

Maria Regina Cattai de Godoy

Lexington, Kentucky

Director: Dr. David L. Harmon, Professor of Animal Science

Lexington, Kentucky

2011

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ABSTRACT OF DISSERTATION

FISH OIL AND BARLEY SUPPLEMENTATION IN DIETS FOR ADULT DOGS:
EFFECTS ON LIPID AND PROTEIN METABOLISM, NUTRIENT DIGESTIBILITY,
FECAL QUALITY, AND POSTPRANDIAL GLYCEMIA

Obesity is the most prevalent nutritional disorder encountered in small animal medicine. Problems related with obesity are the higher incidence of morbidity and mortality. Nutritional and physical activity interventions have been common strategies employed; however, they have shown low compliance rates. Because of it more attention has been given to the nutrient composition of diets. Using the canine model, three experiments were conducted to examine the effect of fish oil or barley on protein and lipid metabolism, as well as postprandial glycemia, and nutrient digestibility in mature and in young adult dogs.

In Exp. 1, seven female dogs were randomly assigned to one of two isonitrogenous and isocaloric diets, control (CO) or fish oil (FO), in a crossover design. Animals fed the FO diet tended to be more sensitive to glucose, showing a lower glucose half life. Cholesterol and HDL decreased ($p<0.05$) on the FO treatment. Overall, the supplementation of fish oil may improve glucose clearance rate and is effective in decreasing cholesterol in mature overweight dogs.

In Exp. 2, eight female Beagles were randomly assigned to one of two isonitrogenous and isocaloric diets, control (CO) or fish oil (FO), in a crossover design. Overall, feeding a FO containing diet showed a protective effect against the rise of plasma CHOL and it increased plasma ghrelin levels. However, it did not appear to improve protein metabolism or postprandial glycemia in adult lean dogs.

In Exp. 3, sixteen female dogs were randomly assigned to four experimental diets; control (40% corn) or three levels of barley (10, 20, 40%). The data suggest that inclusion of barley up to 40% in diets for adult dogs is well tolerated and does not negatively impact nutrient digestibility of the diets. However, inclusion of barley did not improve aspects related to fecal odor, postprandial glycemia, or plasma cholesterol.

Overall, the research presented herein suggests that different nutritional strategies - dietary lipid or carbohydrate manipulation - may be beneficial in ameliorating health issues (e.g., hyperlipidemia) or in improving the health status of dogs (e.g., gut health by increased SCFA production).

KEYWORDS: dog, fish oil, barley, postprandial glycemia, lipid metabolites

Maria Regina Cattai de Godoy
Student's Signature

November 30, 2011
Date

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This dissertation is dedicated to my dear friend Caroline Morais, who has an incredible spirit. Carol has shown to all of her friends that life is a gift and we should never give up. She is a whole example of courage, perseverance and faith. I am very privileged to be her friend. I also dedicate this thesis in the memory of my friend Jeronymo Peixoto Athayde Pereira.

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LIST OF SYMBOLS AND ABBREVIATIONS

Item	Meaning
AA	Amino acids
AACC	American Association of Cereal Chemists
AHF	Acid hydrolyzed fat
Akt	Protein kinase
AOAC	Association of Official Analytical Chemists
AUC	Area under the curve
BA	Barley diet
BCFA	Branched-chain fatty acids
BHB	Beta-hydroxybutyrate
BMI	Body mass index
BW	Body weight
°C	Degrees Celsius
CETP	Cholesterol ester transfer protein
CHOL	Cholesterol
CH_4	Methane
CO	Control
CO_2	Carbon dioxide
CP	Crude protein
CPT - 1	Carnitine palmitoyltransferase - 1
d	Day (s)
DAUC	Decremental area under the curve

DHA	Docosahexaenoic acid
DM	Dry matter
EPA	Eicosapentaenoic acid
FOS	Fructooligosaccharide
g	Gram (s)
GE	Gross energy
GLUT	Glucose transporter
h	Hour (s)
HCl	Hydrochloric acid
HDL	High density lipoprotein
HMG-CoA	Hydroxy-3- methyl-glutaryl coenzyme A
HPLC	High performance liquid chromatography
H3PO4	Phosphoric acid
H2S	Hydrogen sulfide
IAUC	Incremental area under the curve
IRS	Insulin receptor substrate
IVGTT	Intravenous glucose tolerance test
JNK	c-Jun N-terminal kinase (s)
kcal	Kilocalorie (s)
kg	Kilogram (s)
LCAT	Lecithin-cholesterol acyl transferase
LDL	Low density lipoprotein
LPL	Lipoprotein lipase

LXR	Liver X receptor
m	Meter
MCT	Monocarboxylate transporter
ME	Metabolizable energy
μU	Microunits
mg	Milligram (s)
min	Minute (s)
ml	Milliliter
MOS	Mannanoligosaccharide
MW	Molecular weight
N	Nitrogen
NA	Nitrogen absorption
NADPH	Nicotinamide adenine dinucleotide phosphate
NEFA	Non esterified fatty acid (s)
NFE	Nitrogen free extract
NR	Nitrogen retention
OM	Organic matter
%	Percent
PI3K	Phosphatidyllinositol 3-kinases
PPAR	Peroxisome proliferator activated receptor (s)
PUFA	Polyunsaturated fatty acid (s)
RXR	Retinol X receptor
SCFA	Short-chain fatty acid (s)

SGLT-1	Sodium glucose co-transporter
SREBP-1c	Sterol regulatory element-binding protein - 1c
$T_{1/2}$	Half-life
TRIG	Triglyceride
TDF	Total dietary fiber
VLDL	Very low density lipoprotein
wk	Week (s)
yr	Year (s)
Zglu	Zeroed plasma glucose

CHAPTER 1: INTRODUCTION

In U. S., approximately, 68% of adult humans are obese (Flegal et al., 2010). Similarly, it is estimated that 55% of dogs and 53% of cats are overweight or obese (Calabash, 2011). Annually, human obesity costs 150 billion dollars for the U. S. government (CDC, 2011), and Veterinary Pet Insurance claims are over 14 million dollars for weight-related health problems (Ward, 2010). Obesity has become the leading health threat in pets and the major cause of death in humans in the 21th century (Ward, 2010 and CDC, 2010), as well as the most common form of malnutrition in dogs (Case et al., 2000) and the most prevalent nutritional disorder encountered in small animal medicine (Jeusette et al., 2005).

The obesity epidemics have been closely related to the contemporary lifestyle of Western populations, which has been characterized by lower physical activity and increased consumption of a highly processed and caloric-dense diet. It has been suggested that before the development of modern agriculture and animal husbandry, dietary choices were limited to seasonal availability of plant and animal food sources that were minimally (if at all) processed; a scenario that has changed with the introduction of modern agriculture and animal husbandry, and more recently, with the advent of industrialization. All of which have resulted in profound changes in social and environmental conditions that set up the stage for a drastic nutritional shift including: glycemic load, fatty acid composition, and fiber content (Cordain et al., 2005).

According to the similarities between human and pet obesity, it sounds fair to state that dogs and cats have also been "Westernized" by their owners. Companion animals have been exposed to highly palatable, energy-dense, human-like foods in

conjunction with a decreased energy requirement mainly due to sedentary life, and neutering and spaying practices. Similar to humans, overweight or obese animals are more predisposed to develop other chronic illnesses, which contributes to the estimated deaths associated with obesity.

In order to control and to prevent obesity, nutritional and physical activity interventions have been common strategies employed. However, they have proved to be difficult for the general population (Rossmeisl e al., 2009). Besides food restriction, nutritional approaches that show a potential in the prevention of weight-related diseases is the dietary consumption of ω3 PUFA, DHA and EPA, commonly found in fatty fish oils and β-glucans primarily present in cereal grains such as barley and oats.

Therefore, the main goals of the research discussed in this dissertation were to 1) investigate the effects feeding a fish oil-containing diet on nutrient digestibility, lipid and protein metabolism, postprandial glycemia, and body weight in mature overweight and young lean adult dogs and 2) determine the effects of incremental levels of barley; rich in β-glucans a highly fermentable type of dietary fiber, on apparent nutrient digestibility, fecal quality and odor, postprandial glycemic and insulinemic responses, and plasma cholesterol levels in adult dogs.

CHAPTER 2: LITERATURE REVIEW

Obesity is a pandemic problem, most prevalent in developed nations. In the U. S. approximately two thirds of the human population is either overweight or obese (WHO, 2011; Hedley et al., 2004). Obesity is defined as excess body fat accumulation that may impair health. Subjects can be considered obese when their body mass index (BMI) exceeds or equals 30 kg/m^2; BMI is a relationship between weight (in kilograms) and the square of height (in meters). Overweight is considered a pre-obese stage where BMI is equal or superior to 25 kg/m^2 (WHO, 2011). Energy imbalance between calorie intake and expenditure is the main cause in the development of obesity. The contemporary life-style and the advancement in food processing technology may be the major contributors for the increase in the incidence of obesity. The contemporary human population has a decreased physical activity due to more sedentary forms of work, combined with increased availability of highly palatable and energy-dense foods; high in fat and in processed and refined carbohydrates and sugars (WHO, 2011). Medical co-morbidities associated with obesity or overweight are: hypertension, diabetes or insulin resistance, cardiovascular diseases, metabolic syndrome, musculoskeletal disorders (e.g., osteoarthritis and fractures), hyperlipidemia, and some cancers (American Cancer Society, 2011; American Heart Association, 2011; WHO, 2011; Allison et al., 1999). Along with the increased incidence of chronic diseases, a recent epidemiological study reported that life expectancy might be reduced by 6 to 7 y in obese subjects (Peeters et al., 2003).

Similarly to humans, the incidence of obesity in the companion animal population has also skyrocketed and it has been considered the most common nutritional disorder in

pets (German, 2006). Differently than in humans, dogs and cats are considered overweight when their body weight exceeds 15% of their ideal body weight and obese when their body weight surpasses 30% of optimal (Burkholder and Toll, 2000). In parallel with increased obesity, higher manifestation of chronic diseases, similar to humans, have also being observed in veterinary clinics.

The domestication of dogs and cats and more recently, their anthropomorphism, have drastically changed the environmental and social behavior of these animals. During this process, dogs moved away from hunting, living in groups, and fighting for survival and reproduction for the maintenance of their species to live, mostly, indoors, individually housed or maybe with a companion dog; 62% of the household have only one dog (APPA, 2011) and the average of dogs owned per household is 1.7 (AVMA, 2007), and with minimal reproduction purpose; 75% of the pet population is neutered (ASPCA, 2011). All of these changes have contributed to the increased incidence of obesity in the pet population, mainly when combined with lack of exercise, overfeeding, or unbalanced diet (Jeusette et al., 2005).

Common therapeutic options in the management or in the control of obesity have been: dietary management, mainly by energy restriction, and increased physical activity. Although conceptually well based, in practice they are not easily achieved by humans and consequently by pets, since they rely on the will of the owners. Therefore, alternative strategies in the dietary management of obesity have included considerations about the nutrient composition of diets or use of supplements as a mean to aid in weight loss or avoid weight gain, as well as to ameliorate some of the chronic health problems related to obesity. Among them, ω3 PUFA, namely docosahexanoic acid (DHA) and

eicosapentanoic acid (EPA), mainly found in fatty cold-water fish, and β-glucans primarily present in cereal grains such as barley and oats have shown positive results. They have been effective in promoting weight loss or in preventing weight gain (Sato et al., 2007; Kunesová et al., 2006; Ruzickova et al., 2004; Baillie et al., 1999; Hainault et al. 1993), in lowering plasma cholesterol and triglycerides (Queenan et al., 2007; Kim and Choi., 2005; Mori et al., 1999; Anderson et al., 1990), in improving glycemic response (Mello and Laaksonen, 2009; Ajiro et al., 2000; Feskens et al., 1991), or in enhancing protein metabolism (Hayashi et al., 1999; Selleck et al., 1994; Mendez et al., 1992; Teo et al., 1989).

This review will focus on the general processes related to the digestion, absorption and metabolism of dietary lipids and carbohydrates, as well as on specific nutraceutical effects related to the consumption of fish oil and barley; rich in ω3 PUFA and β-glucans, respectively, in the management or prevention of canine obesity and other chronic diseases related to weight bearing.

LIPIDS

Digestion, absorption, and metabolism

Lipids are organic compounds that are relatively insoluble in water but relatively soluble in organic solvents and serve important biochemical and physiological functions in plant and animal tissues. In companion animal nutrition, lipid is an important dietary component since it serves many functions: concentrated energy supply, source of essential fatty acids, carrier of the fat-soluble vitamins, integral constituents of cell membranes, and enhancer of palatability and food texture (NRC, 2006; Pond et al.,

1995). The most important lipid constituents in animal nutrition include: fatty acids, mono-, di-, and triglycerides, and phospholipids. Glycolipids, lipoproteins, and sterols are important in metabolism but are present in lower amounts in the body than triglycerides, the main form of energy storage in animal tissue (Pond et al., 1995).

Dietary lipids are derived, primarily, from animal fat or seed oils. Animal fats are predominantly comprised of saturated fatty acids ($\geq$ 50%), which reflect *de novo* synthesis of fatty acids from dietary carbohydrates (Aldrich, 2005; NRC, 2006). When animals are fed diets containing sources of unsaturated fatty acids, they can be incorporated into the animals' fat tissue (Leskanich et al., 1997; Scollan et al., 2001), resulting in changes to the fatty acid profile of these tissues. This is true for monogastric and ruminant animals, even though in the latter this process is less efficient due to the ruminal biohydrogenation of dietary fibers (Scollan et al., 2001). Lipids from vegetable sources will have a more variable composition of fatty acids. Seed oils can be rich in ω3 or ω6 18-C fatty acids, and some may have varying amounts of both (NRC, 2006). Marine fat sources, e.g., fish and algae oils, are rich in ω3 long-chain PUFA. Because of their nutritional benefits, their use in companion animal diets has increased. However, the high unsaturation of marine oils predisposes them to fat oxidation, challenging their use in pet food. Therefore, fish oil is used in small amounts, approximately 1% (Aldrich, 2006), while poultry, mammal fat or oil seeds comprise the predominant lipid sources in dietary formulation for dog and cat food.

The majority of the dietary lipids are in the form of triglycerides. In order to be absorbed, triglycerides need to be broken down into monoglycerides and free fatty acids. In some animal species, lipid digestion starts with the action of lingual lipase. Dogs and

cats lack the activity of this enzyme (Iverson et al., 1991; Knospe and Plendl, 1997). Therefore, the first step in the hydrolysis of lipid in dogs and cats takes place in the stomach by the action of gastric lipase (Carriere et al., 1992; Knospe and Plendl, 1997). The lumen of the small intestine is the major site of lipid digestion. Bile salts and pancreatic secretions are required for proper lipid digestion. In dogs and cats, bile acids are conjugated with taurine and form the following bile salts: taurocholic acid, taurodeoxycholic acid, and taurochenodeoxycholic acid (NRC, 2006). These bile salts emulsify the lipids into smaller fat droplets facilitating the activity of pancreatic lipase-colipase complex (Borgstrom, 1975). Pancreatic lipase hydrolyzes the ester bonds between the glycerol backbone and each fatty acid of triglycerides, yielding β-monoglycerides and free fatty acids (from positions 1 and 3 of the triglyceride molecules). Final solubilization of products of lipid digestion occurs through the action of bile salts (Pond et al., 1995). The later will be responsible for the micelle formation. Micelles have an amphipathic property, once in contact with the brush border membrane of the enterocytes, micelles diffuse through the phospholipid bilayer membrane into the cells (Swenson and Reece, 1996).

Fatty acid appropriate transport and absorption processes are compromised by their poor solubility in an aqueous environment (Vorum et al., 1992). The capacity of lateral diffusion of fatty acids through the phospholipid bilayer of endothelial plasma membrane is too low to account for all the fatty acids taken up and metabolized by the body cells (Glatz and Van der Vusse, 1996). This fatty acid demand relates to the intestinal uptake of dietary fatty acids, their storage in, and mobilization from, triacylglycerols in adipose cells, energy production from fatty acid oxidation, the

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incorporation of fatty acids into complex lipids such as phospholipids and cholesterolesters, fatty acylation of specific proteins, and the function of fatty acids and metabolites as signaling compounds (Glatz and Van der Vusse, 1996). Thus, a number of specific proteins have been identified which can reversibly and non-covalently bind fatty acids, greatly enhancing their aqueous solubility and thus facilitating their transport. Such proteins are generally considered to take part in both extracellular and intracellular fatty acid transport. In blood plasma and interstitial fluid, albumin is the main carrier of fatty acids (Glatz and Van der Vusse, 1996).

Short chain fatty acids and some medium chain fatty acids (up through C14) are absorbed directly into the blood via the intestinal capillaries and travel through the portal vein similarly to other nutrient absorptive processes. Long chain fatty acids and some medium chain fatty acids, however, are too large to be directly released into the small intestinal capillaries. Instead, in the enterocyte these fatty acids bind to fatty acid binding proteins that will carry them to endoplasmatic reticulum where they will be re-esterified back to triglycerides. The triglycerides and cholesterol are coated by enterocyte-produced apolipoprotein B_{48} into a compound called chylomicron (Bauer, 2004). Within the villi, the chylomicra leave the enterocytes by reverse pinocytosis into lacteals (Pond et al., 1995); which merges into larger lymphatic vessels. Chylomicra are transported via the lymphatic system and the thoracic duct, and are emptied by the thoracic duct into the bloodstream. At this point the chylomicra can transport the triglycerides to where they are needed (Swenson and Reece, 1996).

Because lipids are not soluble in water, their metabolism is mediated by plasma lipoproteins. These molecules differ in size, density, and electrophoretic motility (Jones

and Manella, 1990). Chylomicra and very low-density proteins (VLDL) are the largest and lighest classes of lipoproteins, whereas low-density lipoproteins (LDL) and high-density lipoproteins are smaller and denser (Jones and Manella, 1990). Chylomicra are responsible for the transport of dietary lipids from the small intestine. Once these molecules enter the circulation, they acquire apo C and E peptides from HDLs, which determines their metabolic fate (Bauer, 2004). Apo C peptides facilitate the delivery of triglycerides to adipose and muscle tissues working as a cofactor for lipoprotein lipase. This enzyme is present at the capillary endothelium wall and anchored by proteoglycan chains of heparin sulfate, not being found freely in the blood (Bauer, 2004). In contrast, apo E peptide will remove chylomicron remnants from systemic circulation by binding with hepatic receptors (Hui et al., 1986).

Differently than chylomicra, VLDL, HDL and LDL are, predominatly, responsible for the metabolism of endogenous lipids. The VLDL are mainly synthesized in the liver and responsible for the transport of triglycerides during post-absorptive state. In the circulation, they also attain apo C and E peptides and therefore have similar metabolic fate as the chylomicra (Bauer, 2004). Remnant particles of VLDL can be removed by the liver or be transformed to LDL (Jones and Manella, 1990). The LDL are involved in the supply of cholesterol to extrahepatic tissues to be used in the synthesis of steroid hormones and cell membranes (Jones and Manella, 1990 and Brown and Goldstein, 1984); whereas HDL do the reverse cholesterol transport. Dogs and cats have copious amounts of HDL. They function as donors and acceptors of apo C, apo E and lipids from other lipoproteins. Triglycerides brought back to the liver by HDL are hydrolyzed by hepatic lipase, whereas cholesterol ester will be secreted in the bile or

redistributed among lipoproteins (Bauer, 2004). In humans, lecithin-cholesterol acyl transferase (LCAT) and cholesterol ester transfer protein (CETP) are involved in the reverse cholesterol transport. However, in dogs and cats the activity of CETP has not been determined (Tsutsumi et al., 2001; Watson et al., 1995). This difference in the HDL metabolism of dogs and cats might be responsible for lower susceptibility of these animals in developing atherosclerosis. The enzyme CETP is responsible for the exchange between triglycerides from LDL and VLDL with cholesterol from HDL, and also the endocytosis of apo B-containing LDL and VLDL remnants. Normally, the cholesterol present in these remnant molecules would be catabolized in the liver, however if the receptors are overwhelmed they may accumulate increasing the risk for atherosclerosis (Bauer, 2004).

Similar to humans, obese dogs have higher predisposition to insulin resistance and hyperlipidemia, defined as supraphysiological levels of triglycerides, cholesterol, or both (Jeusette et al., 2005). Omega 3 PUFA, DHA and EPA may alter lipid metabolism. Some of their beneficial effects on health have been: anti-atherogenic ability, decrease in plasma triglyceride and cholesterol concentrations, protective effect against weight again or promotion of weight loss, and reduction of chronic inflammatory processes. All of which, are promising in the management and prevention of obesity and chronic diseases associated with it, as well as being a means to improve the life quality and longevity of the companion animals.

ESSENTIALITY AND NUTRACEUTICAL EFFECTS OF ω3 PUFA

In nutrition, the term "essential" refers to the inability or inefficiency of the body to synthesize a nutrient in adequate amount. The fatty acids that belong to the ω6 and ω3 families contain the first double bond between carbons 6 and 7, and 3 and 4 counting from their methyl end, respectively. Because *de novo* desaturation of fatty acids occur from the carboxyl end by the activity of Δ9 desaturase, vertebrates, through *de novo* synthesis, can synthesize ω9 fatty acids that are non-essential fatty acids. Linoleic acid and α-linolenic acid are members of the ω6 and ω3 fatty acid families, respectively, and they cannot be *de novo* synthesized. Therefore, theybare considered essential for dogs and cats (NRC, 2006). They also serve as the "parent" fatty acids of the ω6 and ω3 series fatty acids, which are competitively synthesized through several elongation and desaturation reactions. The final products are arachidonic acid and docosahexaenoic acid (DHA). The process of elongation and desaturation of ω6 and ω3 fatty acids from linoleic acid and α-linolenic acid is not very efficient in dogs and cats due to limited activity of Δ6 desaturase (NRC, 2006). Nonetheless, the products from this process have important physiological functions, and because of it, their longer-chain derivatives are considered "conditionally essential" in the nutrition of dogs and cats. Good examples of conditionally essential nutrients are DHA and eicosapentanoic acid (EPA), both ω3 fatty acids. Currently, the recommendation for dietary supplementation for ω6 and ω3 fatty acids in diets for adult dogs is as follow: 9.5 g/ kg DM of linoleic acid, 0.36 g/ kg DM of α-linolenic acid, and 0.44 g/kg DM of DHA plus EPA in diets with an energy density of 4,000 kcal ME/ kg (NRC, 2006).

Mechanisms of ω3 PUFA in the Treatment of Obesity and Metabolic Syndrome

The anti-obesity property of ω3 fatty acids has been attributed to their role in adipocyte hyperplasia (cell proliferation) and hypertrophy (cell enlargement; Azain, 2004). Fatty acids and their metabolites are ligands of peroxisome proliferator activated receptors (PPAR). The stimulation of PPAR result in increased fatty acid oxidation (Kliewer et al., 1997). Additionally, unsaturated fatty acids are potent inhibitors of hepatic lipogenesis by down regulation of fatty acid synthase enzyme (Azain, 2004). Prevention of adipocyte hypertrophy and hyperplasia has also been observed by increased activity of CPT-1 in several tissues (adipose, and cardiac and skeletal muscles), which increases fat oxidation, by increased activity of malonyl-CoA decarboxylase, which inhibits lipogenesis (Power et al., 1997), and by increased gene expression of PPAR-γ co-activator 1α and nuclear respiratory factor-1; both are involved in mitochondrial biogenesis and oxidative metabolism (Flachs et al., 2005).

Kunesová et al. (2006) reported that severely obese subjects supplemented with 2.8g/d of ω3 PUFA lost more weight than the control group. This outcome was observed in conjunction with higher serum levels of β-hydroxybutyrate (BHB), which was related to an up-regulation of β-oxidation caused by the activation of hepatic PPARα (Kunesová et al., 2006).

A well-documented effect of ω3 PUFA is their hypolipidemic capability. However, the mechanism behind it has not been completely elucidated (Bays et al., 2008). Several mechanisms have been proposed, one of them suggests that EPA and DHA reduce triglyceride synthesis and enhance triglyceride plasma clearance (Ikeda et al., 2001; Agren et al., 1996). The latter being potentially mediated by apolipoprotein E.

Hepatic lipogenesis is suppressed by down regulation of sterol regulatory element-binding protein-1c (SREBP-1c) activity and by hepatic and muscular PPAR activation (Davidson, 2006). Dietary supplementation of fish oil has been shown to decrease hepatic mRNA expression of SREBP-1c (Le Jossic-Corcos et al., 2005). Omega 3 PUFA may inhibit the formation of the nuclear liver X receptor (LXR) α and retinol X receptor (RXR) α heterodimers and consequently their binding with the SREBP-1c promoter, which in turn leads to suppression of mRNA expression of SREBP-1c (Yoshikawa et al., 2002).

Another possible mechanism for the triglyceride-lowering effects of $\omega 3$ PUFA relates to the up regulation of β-oxidation; increasing fatty acid degradation, results in less fatty acids available for triglyceride and VLDL synthesis (Bays et al., 2008). Rats fed an $\omega 3$ PUFA - containing diet increased mitochondrial and peroxisomal β-oxidation (Harris and Bulchandani, 2006). Increased fat oxidation in the peroxisome may also explain some of the anti-obesity effect of $\omega 3$ PUFA, because more energy will be lost as heat with lower ATP synthesis (Wanders, 2004). In the study by Harris and Bulchandani (2006), rats fed the $\omega 3$ PUFA diet also had lower activity of hepatic enzymes involved in the synthesis of triglycerides: phosphatidic acid phosphatase and diacylglycerol acyltransferase.

Omega 3 PUFA also have hypocholesterolemic properties. This hypocholesterolemic effect is achieved by inhibition of cholesterol synthesis. Polyunsaturated fatty acids are potent inhibitors of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, which is important in the regulation of the rate limiting step in cholesterol synthesis: conversion of HMG-CoA to mevalonate

(Notarnicola et al., 2010). Fish oil supplementation has been shown to decrease cholesterol levels in dogs (Pasquini et al., 2008; Wright-Rodgers et al., 2005) and in murine models (de Silva et al., 2004; le Morvan et al., 2002; Dallongeville et al., 2001).

A less consistent nutraceutical effect of ω3 PUFA is related to improvements in glycemic and insulinemic responses. While evidence exists for the beneficial effects of their supplementation on the prevention and management of insulin resistance and glucose intolerance (Samane et al., 2009; Lombardo et al., 2006; Storlien et al., 1987), a few studies reported no health benefits (Gillam et al., 2009; Holness et al., 2004; Sasagawa et al., 2001). Omega 3 PUFA may improve insulin sensitivity and glucose uptake by a PPARα-dependent mechanism (Poudyal et al., 2011). Fish oil reduced phosphorylation of c-Jun N-terminal kinases (JNK), tau and insulin receptor substrate-1 (IRS-1), which are normally high in diabetes or insulin resistance (Ma et al., 2009). In addition, in liver and adipose tissues of mice fed fish oil, genes for GLUT2 and GLUT4, as well as IRS-1 and IRS-2 were up regulated (Gonzalez-Periz et al., 2009). To our knowledge only one study has examined the effects of ω3 PUFA, DHA and EPA, on insulin sensitivity and glucose tolerance in healthy non-obese dogs (Irvine et al., 2002). Dietary ω3 PUFA supplementation was not effective. The authors suggested the animals were less responsive to the ω3 PUFA treatment because they were insulin sensitive.

Omega 3 PUFA are important mediators of inflammatory processes; they suppress inflammation by production of anti-inflammatory metabolites or by competitive inhibition of the ω6 fatty acid pathway and pro-inflammatory metabolites such as cytokines, series-2 prostaglandins and series-4 leukotrienes (Poudyal et al., 2011). The anti-inflammatory effects of ω3 PUFA seem to be mediated by lipoxygenase and

cyclooxygenase, which generate series-3 prostaglandins and thromboxanes, and series-5 leukotrienes (Larsson et al., 2004). Recently, it was demonstrated that DHA and EPA are also involved in the formation of macrophage and inflammatory mediators such as: protectins, maresins, resolvins, and pro-resolvins (Serhan et al., 2009). This effect comprises a novel mechanism by which DHA and EPA contribute to anti-inflammatory processes.

In canine nutrition, supplementation of ω3 PUFA has shown positive effects in the control of osteo, renal and cardiac diseases (Fritsch et al., 2010; Brown et al., 1998; Freeman, 1998; Kang and Leaf, 1996), which can be partially attributed to the anti-inflammatory properties of these fatty acids. Supplementation of fish oil (27 mg/ kg BW of EPA and 18 mg/ kg BW DHA) for dogs suffering from congestive heart failure decreases the anti-inflammatory marker Il-1 and improves cardiac cachexia, which is characterized by a depletion of muscle mass (Freeman, 1998). In another study, dietary supplementation of DHA and EPA demonstrated an antiarrhythmic effect in dogs with heart problems (Kang and Leaf, 1996). While Brown et al. (1998) reported that nephrectomized dogs supplemented with fish oil (15% of dietary fat intake) had improved renal function, decreased proteinuria, serum cholesterol and triglyceride levels.

Two recent studies evaluating the effects of ω3 PUFA in dogs suffering from osteoarthritis have reported that supplementation of fish oil (0.8% DHA and EPA) improved animals' ability to rise from resting, positioning and their ability to walk (Roush et al., 2010[a,b]). A third study, analyzing the effects of increasing dosages of fish oil in osteoarthritic dogs found supplementation of fish oil ω3 PUFA (2.94% DHA+EPA) to be effective in the improvement of lameness (Fritsch et al., 2010). The previous

authors speculated that the beneficial effects observed by supplementation of fish oil ω3 PUFA was due to their ability to suppress inflammation and to promote the synthesis of anti-inflammatory eicosanoids.

CARBOHYDRATES

Digestion, absorption, and metabolism

Carbohydrates comprise up to 70% of the dry matter of plants, whereas in animal tissues, carbohydrates made up less than 1% of the weight of the animal, consisting mainly of glucose and glycogen (Pond et al., 1995). In nutrition, carbohydrates are used as an energy source, however, like other animal species dogs and cats do not have a nutritional requirement for carbohydrates (Kettlehut et al., 1980 and Romsos et al., 1976). Under specific physiological conditions, for example gestation and lactation, scientific data exist showing that carbohydrate intake improves the animals' performance by sparing other nutrients like protein and lipids from being used as energy sources (Romsos et al, 1981 and Kienzle et al., 1985). In companion animal nutrition carbohydrates comprise 30-60% of the pet food formulations (Carciofi et al., 2008) but also are fundamental in the plasticity and gel-forming ability of extruded and canned products.

Carbohydrates are very heterogeneous compounds; including low and high molecular weight sugars, starches, and structural polysaccharides (Bach-Knudsen, 1997). Their unique molecular arrangement: type of monomers and linkages among them, dictates their function in plants and in animals. Dietary carbohydrates can be classified into four functional groups: absorbable, digestible, fermentable and non-fermentable (NRC, 2006). Monosaccharides (glucose, fructose, and galactose) are considered

absorbable carbohydrates because they do not require hydrolytic digestion to be absorbed by the enterocytes. Digestible carbohydrates are represented by non-structural saccharides that are hydrolytically digested by the animal. Starch is probably the most common digestible carbohydrate present in human and animal diets along with low molecular weight sugars, such as lactose, maltose, and sucrose. Carbohydrate fractions that escape hydrolytic/enzymatic digestion in the small intestine, also named dietary fibers, will enter the large intestine where they have the potential to be fermented by the hindgut microbiota. Common fermentable carbohydrates are: resistant starches, oligosaccharides (e.g., stachyose, raffinose, fructooligosaccharides, mannanoligosaccharide, galactooligosaccharides, and lactulose), and certain polysaccharides (e.g., β-glucans, pectins, gums, and hemicellulose). Cellulose and lignin (a phenolic compound), are classic examples of non-fermentable dietary fibers (NRC, 2006).

In some monogastric species (e.g., humans and pigs), the digestion of carbohydrates starts in the mouth in the presence of salivary α-amylase (ptyalin). In the case of the dogs and cats, carbohydrate digestion takes place in the small intestine by the action of pancreatic α-amylase (lumen) and disaccharidases (e.g. maltase, lactase, and sucrase) present in the brush border membrane. In contrast to proteins and lipids, which can be absorbed as single amino acids and small peptides, or as free fatty acids and monoglycerides, respectively, carbohydrates have to be digested down to monomers (glucose, fructose, and galactose) in order to be absorbed in the small intestine. In the small intestine brush border membrane the absorptive process of monosaccharides is achieved mainly by apical membrane transporters such as: sodium glucose co-transporter

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(SGLT-1) and a family of glucose transporters (GLUT2, GLUT5). Through SGLT-1 glucose and galactose can be actively absorbed while fructose is absorbed by the facilitated transporter GLUT5. During the post-prandial period the concentration of monosaccharides may saturate SGLT-1 and GLUT5. The transporter GLUT2 can be translocated to the apical membrane of the enterocyte to aid in the facilitated absorption of glucose and fructose (Kellet et al., 2008; Helliwell and Kellet, 2002; Kellet and Helliwell, 2000). Once in the enterocytes, glucose, galactose and fructose are transported out of the enterocyte through GLUT2 in the basolateral membrane. These monosaccharides then diffuse in a concentration gradient into capillary blood and into the portal circulation towards the liver.

Glucose plays a major rule in the mammalian metabolism. Once absorbed, glucose enters the blood pool being distributed among the body tissues. In order to be metabolized, glucose will be phosphorylated to glucose-6-phosphate, which is a key intermediate for many metabolic pathways. In a post-prandial state glucose may have several metabolic fates: it can be used in glycogenesis mainly in the liver and at some extent in skeletal and cardiac muscle to form glycogen; it can also enter glycolysis and it can be converted to pyruvate that in turn enters the citric acid cycle or lipogenesis. Glucose-6-phosphate can be oxidized in the pentose phosphate cycle, generating reducing equivalents (NADPH) and ribose-5-phosphate important for synthesis of nucleotides and nucleic acids.

Fructose and galactose are metabolized predominantly in the liver. They are first phosphorylated by fructokinase and galactokinase, respectively, and through a series of metabolic steps will be converted to pyruvate that then can enter the citric acid cycle, be

used in the synthesis of fatty acids (as previously discussed for glucose), or as substrates for glycogen synthesis.

Most carbohydrate sources are not completely digested by hydrolytic and enzymatic process. Therefore, di, oligo and polysaccharides that resisted the digestion at the small intestine will enter in the large intestine where they may be fermented by enteric bacteria. Degradation of these compounds will result in the formation of short chain fatty acids (SCFA), mainly, acetate, propionate and butyrate, whereas non-fermentable fibers will be excreted in feces. The absorption of SCFA across the intestinal mucosa has been shown to occur through the nonionic diffusion of protonated SCFA and through a carrier-mediated anion exchange (Charney et al., 1998; Cook and Sellin, 1998). More recently, Gill et al. (2005) have proposed a new mechanism in the absorption of SCFA by a family of monocarboxylate transporters (MCT). In this model, MCT1 is responsible for mediating the SCFA from the intestinal lumen into the colonocytes, while MCT4 and MCT5 are present in the basolateral membrane and facilitate the exit of the SCFA from the colonocytes into the portal circulation (Gill et al., 2005).

A primary role of SCFA is to be used as an energy source by the colonic mucosa, meeting approximately 60-70% of the energy requirement of these cells. In this matter, butyrate is the preferred substrate being followed by propionate and acetate (Wong et al., 2006). Acetate is the predominant SCFA being produced by fermentative processes in the hindgut, succeeded by propionate and butyrate (Englyst et al., 1987). Acetate is also involved in cholesterol and fatty acid synthesis and serves as energy fuel for many tissues, particularly the cardiac muscle during fasting periods (Wong et al., 2006).

Proprionate is a precursor of glucose through gluconeogenesis, but also inhibits cholesterol synthesis in rats and pigs (Hamer et al., 2008; Illman et al., 1988; Boila et al., 1981). Butyrate is extensively metabolized by colonic epithelia and therefore little of it reaches the liver (Cummings, 1991). Similarly to acetate, butyrate can be used as a substrate for fatty acid synthesis or as a precursor for the citric acid cycle. Along with SCFA other gases: methane (CH_4), hydrogen sulfide (H_2S), and carbon dioxide (CO_2) are formed during fermentation.

In addition to the production of SCFA, dietary fibers play an important role in gut health and in the management of some pathological conditions. Fermentable and soluble fibers increase digesta viscosity, decrease gastric emptying, increase satiety, reduce the rate of glucose uptake, lower blood cholesterol levels, and promote growth of gut commensal bacteria (Jenkins et al., 2008; Brennan and Cleary, 2005; Tungland, 2003; German et al., 1996). Conversely, non-fermentable and insoluble fibers decrease gastric transit time, increases fecal bulk and moisture, and aid in laxation (Wenk, 2001).

BARLEY AND THE ROLE OF BETA-GLUCANS IN NUTRITION

Traditionally in companion animal nutrition, corn and rice have been probably the most popular ingredients used as sources of carbohydrates (mainly starch), while beet pulp has been considered the gold standard as a dietary fiber source. Because of the increased awareness of the beneficial effects of dietary fibers in health, as well as the popularity of holistic and natural diets, novel carbohydrates have become widespread in human and pet nutrition. Consequently, cereal grains with low-glycemic index (defined as a lower glycemic response as a percent of the response of a standard carbohydrate

source, e.g., glucose or white bread) and rich in fermentable and soluble (viscous) fibers such as; barley (*Hordeum vulgare*) and oats (*Avena sativa*) have received increasing interest.

Cereal β-glucans are classified as soluble dietary fibers and have rheological properties similar to guar gum and other random coil polysaccharides (Wood, 2007). On average, the β-glucan content in oats and barley ranges between 3-7% and 5-11%, respectively (Skendi et al., 2003) and is a prevalent component of the cell wall of these cereal grains (Wood, 2007). The molecular arrangement of β-glucans consists of D-glucose molecules connected by a series of β- (1→3) and β- (1→4) linkages (Queenan et al., 2007). The viscous behavior of these non-starch polysaccharides is related to their physical entanglements; the presence of β- (1→3) linkages leads to bends in the straight chain of the polymer allowing water to permeate and conferring the high water solubility and viscosity of β-glucans (Vasanthan and Temelli, 2008; Guillon and Champ, 2003).

The beneficial physiological effects related to the consumption of β-glucans have been associated with their capacity to form high viscous solutions (Wood, 2007). A previous study (Wood et al., 1994) demonstrated that the viscosity of β-glucans accounts for 79-96% of the changes in glycemic and insulinemic responses. Additional factors that influence the bioactivity of β-glucans are: the molecular weight (MW) and concentration, the nature of the extract, the form of delivery, and the dose ingested. Viscosity increases exponentially with concentration and MW. Biorklund et al. (2005) reported that oat β-glucan with a MW of 70,000 lowered serum cholesterol and postprandial glucose and insulin levels, whereas barley β-glucan with a lower MW of 40,000 did not. In addition, it has been suggested that the use of an intact food as a

source of β-glucans as oppose to the use of a more purified form can interfere in their behavior (Wood, 2007). It has also been proposed that bioactivity of β-glucans may be increased when they are consumed with a meal, instead of between meals (Wolever et al., 1994), and that their ingestion as a drink is more effective than when incorporated into food matrixes (Kerckhoffs et al., 2003). According to FDA (2005), a daily intake of 3g of barley or oat β-glucans may have positive effects on health. Dose response studies have reported a direct relationship between amount of β-glucans consumed and health improvements (Cavallero et al., 2002; Davidson et al., 1991). Furthermore, rheological properties of β-glucans can be modified by thermal treatment; cooking and freezing processes may decrease their solubility (Anderson et al., 2004; Beer et al., 1997). This effect on solubility cannot be overlooked by the pet food manufacturers because of the harsh thermal treatments (e.g., extrusion and canning) applied in their products that may decrease the bioactivity of β-glucans. In addition to intrinsic characteristics of cereal β-glucans, the physiological responses observed after their ingestion can also be driven by other factors. They may include: age, gender, and physiological status of the subjects consuming them (Ripsin et al., 1992).

β-glucans and Nutrient Digestibility

Cereal β-glucans affect several aspects of the digestive process: site and extent of digestion (Wilfart et al., 2007 and Bach Knudsen and Hansen, 1991), enzymatic activity (Hedemann et al., 2006), nutrient absorption (Bach Knudsen et al., 2000), and systemic and gastrointestinal hormonal responses (Ellis et al., 1995; Serena et al., 2009). Results from animal studies, have shown controversial effects of dietary β-glucan in nutrient

digestibility and absorption, as well as in animal performance. In studies using swine, dietary β-glucan decreased nutrient digestibility and absorption, as well as growth performance (Bach Knudsen et al., 2000; Back Knudsen et al., 1993), while others have found positive or no detrimental effects of supplementation of β-glucan on nutrient digestibility and performance (Renteria-Flores et al., 2008; Hahn et al., 2006; Fortin et al., 2003; Jensen et al., 1998).

To our knowledge, literature available about the effect of cereal β-glucans on nutrient digestibility in dogs is practically nonexistent. The only study found on this topic reported that inclusion of 40% of extruded barley to a basal diet, predominantly made of corn, wheat, and animal fat, resulted in decreased fecal dry matter and consequently looser stools. Calculated barley nutrient digestibility was high for organic matter (92%, OM) and for nitrogen free extract (98%, NFE), whereas crude protein (CP) digestibility was lower being reported as 71% (Groner and Pfeffer, 1997). Lower apparent CP digestibility in dogs fed diets containing soluble dietary fibers has been attributed to an increased fecal excretion of microbial mass, due to increased fermentative process in the large intestine (Middelbos et al., 2007).

Few other mechanisms have been proposed in order to explain how β-glucans may positively or negatively impact nutrient digestibility and absorption. Improvements in nutrient digestibility could result from delayed gastric emptying rate and longer mean retention times induced by increased viscosity of the digesta. This long retention time would extend the contact of the nutrients in the gastrointestinal tract with digestive enzymes (Owusu-Asiedu et al., 2006; Bray 2002; Schneeman, 1998) or could be a reflection of a greater digestibility of dietary fiber due to the fermentation of β-glucans

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(Le Goff and Noblet, 2001). Conversely, decreased nutrient digestibility and absorption could indicate reduced digestive enzyme activity by non-specific binding of the enzymes or presence of specific enzyme inhibitors (Dunaif and Schneeman, 1981).

β-glucans in the Management of Obesity and Chronic Diseases

In human trials, consumption of cereal β-glucan improved glycemic response (Jenkins et al., 2008; Panahi et al., 2007). Likewise, dietary β-glucan decreased insulin responses in healthy humans (Juvonen et al., 2009; Nazare et al., 2009), and in obese and type II diabetic patients (Kim et al., 2009; Liatis et al., 2009). In contrast, other studies have failed to detect improvements in insulin response by the dietary supplementation of β-glucan using human (Frank et al., 2004) and animal models (Bach Knudsen et al., 2000; Bach Knudsen et al., 2005). Insulin is quickly cleared in the liver and this may impact variability and accuracy of its measurements. C-peptide is produced by the pancreas in an equimolar ratio with proinsulin and because it has a lower hepatic clearance rate, and renal excretion, when compared to insulin (Polonsky and Rubenstein, 1984), it may be an alternative for use in the evaluation of insulinemic responses.

The effect of β-glucan in blunting glycemic response is possibly mediated by the delay in gastric emptying, resulting in slower and gradual absorption of glucose (Lo et al., 2006). This mechanism may also postpone the feeling of hunger caused by a rapid decrease in blood glucose (Ludwig, 2003). Beta-glucans have also been implicated in increasing phosphatidylinositol 3-kinases and protein kinase (PI3K/ Akt) activity (Chen and Seviour, 2007). In insulin sensitive subjects, insulin binds with its receptor and activates the intracellular subunit tyrosine kinase domain, which initiates a cascade

response, activating IRS- proteins that will activate PI3K/Akt pathway. However, insulin resistant or diabetic subjects have compromised ability in insulin signaling due to diminished insulin-induced tyrosine phosphorylation of IRS-proteins, which impairs the PI 3-kinase/Akt activation and thereby the translocation of GLUT4 to the surface of muscle and fat cells (Asano et al., 2007).

Because insulin also mediates fatty acid uptake in muscle cells, subjects with insulin resistance or diabetes will have higher levels of circulating free fatty acids, which will result in increased triglyceride synthesis and assembly of VLDL (Hobbs, 2006). The proposed mechanism by which β-glucan aids in the control of hyperlipidemia is by adhering with bile acids present in the gastrointestinal tract, increasing their elimination in feces, and as a consequence LDL will be utilized in the hepatocytes as substrate for bile acid synthesis (Nilsson et al., 2007). An alternative mechanism that may also contribute in the hypocholesterolemic effect of β-glucans is the increased production of SCFA by fermentative process, mainly propionate that suppresses hepatic cholesterol synthesis (Wright et al., 1990).

OVERALL OBJECTIVES

The hypothesis of the research project was that dietary supplementation of fish oil or barley would be well tolerated by adult dogs, resulting in no detrimental effects on nutrient digestibility. In addition, these ingredients would attenuate postprandial glycemic responses, as well as blood lipid metabolites (e.g., triglycerides, cholesterol, HDL and LDL). Therefore, the experiments presented in this dissertation were designed with the following objectives:

1. To determine the effect of feeding a fish oil-containing diet on nutrient digestibility, lipid and protein metabolism, postprandial glycemia, and body weight in mature overweight dogs.

2. To verify the effect of feeding a fish oil-containing diet on nutrient digestibility, lipid and protein metabolism, postprandial glycemia, and body weight in adult lean dogs.

3. To examine the effects of incremental levels of barley; rich in β-glucans a highly fermentable type of dietary fiber, on apparent nutrient digestibility, fecal quality and odor, postprandial glycemic and insulinemic responses, and plasma cholesterol levels in adult dogs.

CHAPTER 3: INFLUENCE OF FEEDING A FISH OIL CONTAINING DIET TO MATURE OVERWEIGHT DOGS: EFFECTS ON LIPID METABOLITES, POSTPRANDIAL GLYCEMIA, AND BODY WEIGHT

INTRODUCTION

Currently, there are more than 77.5 million pet dogs in the United States and 93.6 million pet cats, and nearly 62% of the households have at least one pet (APPA, 2011). In 1995, the prevalence of obesity in US dogs was around 34% (Lund et al., 2006). A recent study by the Association for Pet Obesity Prevention (APOP) found that approximately 55% of dogs and 53% of cats were overweight or obese (Calabash, 2011), which demonstrates that the population of obese and overweight dogs continues to increase. Obesity has become the most common form of malnutrition in dogs in the United States (Case et al., 2000) and the most prevalent nutritional disorder encountered in small animal medicine (Jeusette et al., 2005[a]).

The high incidence of obesity in the pet population can be attributed to several factors including; genetic predisposition, sedentarism, neutering and spaying practices, ingestion of highly palatable and energy-dense foods. Similar to humans, obese pets are more prone to be affected by weight-related diseases such as diabetes mellitus, hyperlipidemia, arthritis, and kidney disease. In order to aid in the control of pet obesity, common management strategies have been adopted: energy restriction, increased exercise activity, or a combination of the two. Problems observed with these strategies have included exacerbated behavioral stress and lower compliance of pet owners (Mitsuhashi et al., 2010). Because of the low success rate of these management strategies, more attention has been given to the nutrient composition of diets. Scientific evidence suggests

that different carbohydrate and lipid sources can aid in weight loss. Among them ω3-PUFA, docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) mainly found in fatty fish oil, have shown positive results on weight loss in obese women and in rats (Kunesova et al., 2006; Sato et al., 2007). Additionally, interactions between dietary lipids, proteins and carbohydrates on attenuation of plasma glucose and insulin concentrations, as well as body weight loss have been observed (Collene et al., 2005; Gentilcore et al., 2006; Gunnarsson et al., 2006; Sato et al., 2007). Mechanisms involved in the positive outcomes with fish oil consumption and the interactions between fat and protein sources with carbohydrates are not completely understood. Additionally, most of the research conducted in this area has been performed using rodents as the experimental model and these findings may not always reflect canine metabolism. Thus, the objective of this study was to determine the effect of feeding a fish oil-containing diet on lipid and protein metabolism, postprandial glycemia, and body weight in mature overweight dogs.

MATERIALS & METHODS

Animals and Model

All animal care procedures were conducted under a research protocol approved by the Institutional Animal Care and Use Committee, University of Kentucky, Lexington. Seven purpose-bred mature female dogs (Marshall Bioresources, North Rose, NY), with an average age of 9 years, and initial BW of approximately 27 ± 1.3 kg were used in the experiment. Dogs were randomly divided into two groups, and each group was randomly assigned to one of two diets, control (CO) or fish oil (FO), in a cross-over design. There were two experimental periods and each was 69 days in length. Experimental periods

were separated by a wash out period of 30 days to minimize carry-over effects. The dogs were individually housed in kennels (1 x 1.5 m) with a slotted floor in a temperature-controlled room with a light: dark cycle of 12 hours at the Division of Laboratory Animal Research Facility at the University of Kentucky. Each kennel was connected to a concrete outside run (1 x 2 m) where animals had free diurnal access. Dogs were also allowed to exercise and to have social interaction in outside and/ or inside play areas for an average of 3 hours daily, except during urine and fecal collection periods. During nitrogen balance collections, dogs were confined to the indoor portion of their kennels for a period of 6 days, and human interaction was provided for a minimum of 20 minutes per dog daily. For urine collection following glucose tolerance tests animals were confined to the indoor portion of their kennels until the next morning.

Treatments and Feeding

Two dry, extruded kibbled diets were used. All diets were formulated to be isonitrogenous and isocaloric. Diets had similar ingredient compositions and nutrient profiles (Tables 3.1 and 3.2, respectively), except that for source of oil. This replacement led to alterations in the fatty acid profile of these diets (Table 3.2). Diets were formulated in accordance with the Association of American Feed Control Officials (2002) nutrient guide for dogs and balanced to meet maintenance requirements.

Prior to the start of the experiment the dogs were fed ad libitum (Canine Active Diet Hills Science Diet, Topeka, KS), a high fat (28%), high protein (30%) diet to ensure that the animals would increase their body weight to 20% over ideal body weight (based on each dog's records for the previous year). The 20% increase body weight aimed a

body condition score (BCS) of approximately 7, which is described as: ribs palpable with difficulty; heavy fat cover, with noticeable fat deposits over lumbar area and base of tail, and with waist absent or barely visible, whereas the ideal body weight was considered a BCS=5 (ribs palpable without excess fat covering, with waist observed behind ribs when viewed from above and abdomen tucked up). Larson et al. (2003) suggested that approximately 20% body fat was the critical point at which dogs became insulin resistant. At the beginning of each experimental period dogs were gradually transitioned onto experimental diets over a five day period. During the experiment the dogs were fed to maintain their increased BW, which was established at the beginning of each experimental period. Energy requirements were calculated using the formula, 100kcal ME $*$ kgBW $^{0.75}$ (Gross et al., 2000). Dogs were fed twice a day, at 0630 and 1830 in stainless steel bowls. All dogs were allowed 20 minutes to consume the food. Any remaining orts were collected, weighed, and recorded. Water was available ad libitum throughout the experiment. On the days when a glucose tolerance test was performed, animals were not fed in the morning, and their daily ration was offered at the afternoon feeding time.

Experimental Procedures

Blood Sampling

Venous blood samples (8 mL) were collected on d 0, 30, 60 and 69 via jugular puncture into a sodium heparin containing vacutainer (Becton Dickson, Franklin Lakes, NJ), and placed on ice for plasma glucose, triglycerides, total cholesterol, HDL, LDL, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), insulin, glucagon, ghrelin,

and lipoprotein lipase (LPL) determination. Blood samples were kept on ice and centrifuged at 4,000 x g for 15 min at 4°C to collect plasma. Plasma samples were stored at -20°C until analysis.

Glucose Tolerance Test

Glucose tolerance tests were performed at the beginning of the experiment to establish baseline values (prior to treatment), and at 30 and 60 days of feeding the experimental diets. Prior to the test, animals were fasted overnight and the next morning the area over the cephalic vein was prepared by clipping hair and disinfecting with alcohol and an i.v. catheter (20Gx 1 ¼) was placed in the cephalic vein and secured with tape and an injection cap and filled with heparinized saline (20 units/mL) to ensure patency. The dogs were infused with a 500 g/L solution (Hospira Inc., Lake Forest, IL) of D-glucose (2g/kg body weight) through the intravenous catheter (Larson et al., 2003). After D-glucose infusion, blood samples (4mL) were collected via intravenous catheter at 5, 10, 15, 30, 45, 60, 90, 120, 150 and 180 min counted from the starting time of glucose infusion. Between sampling times, a heparin solution (20 units of heparin per mL) was infused to prevent blood from clotting in the catheters. Once blood sampling was completed, catheters were removed and animals received their daily amount of food. Blood and plasma samples were processed and stored in a similar manner as described for baseline samples.

Concomitantly with the glucose tolerance test, urine was collected for approximately 20 h after D-glucose infusion (1230 and 0630 on the next morning) to monitor glucose loss via urine. Urine was collected via catch pans that were

approximately 1 x 1.5 m. These pans were made of stainless steel and had one inch raised edges on three sides, with a flat edge on one of the 1m sides. The pans were placed under the slotted floor of the kennels and suspended by wire at the back of the kennels so that they were sloping towards the front of the kennel. Fiberglass screens with stainless steel frames were placed on the top of each pan to keep hair and feces out of the urine. A watertight, plastic trough was placed under the front edge of the pan to collect the excreted urine. Eight milliliters of 6 N H_3PO_4 was added into the plastic troughs at the time of placement in order to acidify and preserve the urine.

Nitrogen Balance and Protein Turnover

Sampling periods began at 0630 on day 63 of each experimental period, when dogs entered confinement, and ended at 0630 on day 69. Dogs were weighed prior and at end of collection periods.

Total urine was collected at 0630, 1230 and, 1830 daily during the collection period. Urine was collected using the system described above. Eight mL of 6 N H_3PO_4 was put into the plastic troughs at placement. The urine catch pan was rinsed down with water three times daily (0630, 1230 and, 1830) during the sampling period to ensure complete collection. Each rinse was approximately 80 mL spread evenly over the entire pan. Total urine collected for each individual dog was placed into a tared plastic container. Fifty percent of the total urine collected from a 24 hour period was composited for each dog within experimental period and frozen.

Just before the 0630 feeding on day 66 of each period a single dose (7.5 mg/kg) of ^{15}N-glycine (Andover, MA, Cambridge Isotope Laboratories, Inc.) was administered

orally to each dog via a gelatin capsule (Size 000, Park City, UT, Nutraceutical Corp.). From day 66 at 0630 through day 69 at 0630 25% of acidified urine from each dog was separated, composited and frozen for later analysis for ^{15}N-glycine enrichment. Total feces were collected at the same intervals, by removing fecal matter from the slotted floor of the kennels as well as from the fiberglass screen on top of the urine catch pan. After collection, feces were weighed, placed in labeled plastic bags and frozen until analysis. At the end of each collection period fecal samples were composited by animal.

Blood Metabolite Analyses

Plasma glucose, triglycerides, total cholesterol, and HDL concentrations were analyzed by Konelab 20 XTi, using available commercial kits (Infinity™, Thermo Electron Corporation, Louisville, CO). Low density lipoprotein (LDL) concentration were determined similarly to the methodology above using an enzymatic method; LDL Cholesterol Reagent (Thermo Electron Corporation, Pittsburg, PA). Plasma NEFA was assayed using an enzymatic method (Wako NEFA C kit, Wako Diagnostics, Richmond, VA, USA). Plasma BHB was analyzed by enzymatic method (LiquiColor Reagent Kit, Stanbio Laboratory, Boerne, TX, USA).

Aliquots of plasma were collected, and analyzed for plasma insulin, glucagon, ghrelin, and lipoprotein lipase (LPL) concentrations as described previously (Yamka et al., 2006).

Feed, Fecal and Urine Sample Analyses

Samples from the two test diets were collected throughout the experimental periods and stored at -20˚C until chemical analyses were conducted. Diet samples were ground in a Wiley mill (model 4, Thomas Swedesboro, NJ) through a 2-mm screen. Each diet was analyzed for dry matter (AOAC, 2000), crude protein; calculated from total N values (Vario Max CN, Elementar, Hanau, Germany), total dietary fiber (Prosky et al., 1992), gross energy using an oxygen bomb calorimeter (model 1756, Parr Instruments, Moline, IL) and fat content; measured by acid hydrolysis (AACC, 1983) followed by ether extraction (Budde, 1952). Fatty acid profile of experimental diets was analyzed as described previously (Fritsch et al., 2010).

Fecal samples were composited for each dog, within period and homogenized. Similarly to the diets, feces samples were analyzed for DM, CP and fat content. Approximately, 1 g samples of wet feces were dried in a 105°C circulating air oven overnight, and difference in weight was used to determine absolute dry matter. Approximately 500 mg of wet feces were weighed into crucibles and analyzed as is for nitrogen content. Dried (55°C) ground fecal samples were used to determine fecal fat content as described above. Urine composites were thawed, homogenized and 1 g aliquots were weighed into crucibles containing 100 mg of steel wool and 200 mg of sucrose to determine nitrogen content. Urea and ^{15}N nitrogen enrichment contents in urine composites prior and post ^{15}N-glycine dosing were analyzed as previously described by Reeder et al. (2011). All samples were analyzed in duplicate with a 5% error allowed between duplicates; otherwise, analyses were repeated.

Calculations

Glucose Tolerance Test

Incremental area under the curve (IAUC) for plasma glucose was calculated according to method of Wolever et al. (1991) using GraphPad Prism 5 Software (GraphPad Software, Inc., San Diego, CA).   Peak area for plasma glucose was also calculated using GraphPad Prism 5 Software (GraphPad Software, Inc., San Diego, CA). This software calculates the area under the curve using the trapezoid rule. It permits the separation of the data into different regions as a fraction of the total area and to calculate the highest point of each region (peak).   Glucose fractional clearance rate, k, was calculated as the slope of the line of natural log glucose concentration against time between 5 and 90 min following glucose i.v. administration. Half life, which is the time required for glucose concentration to fall by half, was estimated between 5 and 90 min postinfusion, using the formula (Kaneko et al., 2008):

$$T_{1/2} = \frac{0.693}{k} \times 100 = \text{minutes,}$$

Zeroed plasma glucose concentration at peak (5min) was calculated as:

Zglu= [glucose] at 5 min postinfusion – [glucose] at baseline (preinfusion)

The zero correction is important to remove variation from differing plasma glucose baseline concentrations and to facilitate comparisons of glucose concentration post infusion on a similar scale and the incremental response based on glucose concentration at baseline.

Fraction of glucose in urine was calculated using the formula:

$$\text{GLUfrac (\%)} = 100 - \left(\frac{\text{(dextrose dose,mg)} - \text{(glucose excreted in urine,mg)}}{\text{dextrose dose,mg}} \right) \times 100$$

Nitrogen Balance

All nitrogen balance calculations were done on a dry matter (DM) basis. Nitrogen from feed was considered nitrogen intake, while nitrogen from urine composites and feces were nitrogen outputs. Apparent nitrogen absorption (NA), nitrogen retention (NR), %DM digestibility, and apparent %nitrogen (N) digestibility were calculated, with all values on a g/d basis, as:

NA (g/d) = (N from intake – N from feces)

NR (g/d) = N absorbed – N from urine

$$\% \text{ DM digestibility} = \frac{(\text{DM intake} - \text{DM output})}{(\text{DM intake})} \times 100$$

$$\% \text{ N digestibility} = \frac{(\text{N intake} - \text{N from feces})}{(\text{N intake})} \times 100$$

$$\% \text{ N balance} = \frac{(\text{N intake} - (\text{N from feces} + \text{N urine})}{(\text{N intake})} \times 100$$

Whole body protein turnover was measured using the ^{15}N-glycine single-dose urea end-product method (Waterlow et al., 1978). This procedure assumes that ^{15}N-glycine will be partitioned mainly between protein synthesis and oxidation in the same proportion as total amino acid turnover, and that oxidized end products are excreted quantitatively in the urine. Therefore, by measuring the amount of ^{15}N-glycine not incorporated into body tissues, protein turnover (g N/d) can be calculated (Wessels et al., 1997). Whole body protein turnover was calculated as:

$$Q = d/G$$

where Q represents protein turnover (g N/d), and d is the rate of urea N excretion (g/d) from day 66 1230 through day 69 at 0630. The samples from day 66 0630 were not included since they represented urine that was excreted before the ^{15}N-glycine dose was administered. In the equation, G represents the fractional recovery of ^{15}N from ^{15}N-glycine in urine urea. The ^{15}N enrichment of all urine samples was corrected for background ^{15}N enrichment (Wessels et al., 1997). From whole body protein turnover, protein synthesis (PS) and protein degradation (PD) were calculated using the following equations:

$$PS \text{ (g N/d)} = [Q - (N \text{ excreted in urine})]$$

$$PD \text{ (g N/d)} = [Q - (N \text{ intake} - N \text{ in feces})]$$

where N excreted in urine, N intake, and N in feces were all represented on a (g N/day) basis.

Statistical Analysis

Data from glucose tolerance test, lipid and blood metabolites were analyzed as repeated measurements in a cross-over design using the Mixed procedure of SAS (SAS Inst., Inc., Cary, NC). All other variables were analyzed by the Mixed procedure of SAS (SAS Inst., Inc., Cary, NC). The statistical model included the fixed effect of treatment, and the random effects of period and animal. All treatment least squares means were compared to each other and Tukey adjustment was used to control for experiment wise error. Plasma β-hydroxybutyrate, ghrelin, and glucagon data were not normally

distributed, so a log transformation was applied for analysis. Differences between means with $P<0.05$ were considered significant and means with $P \leq 0.10$ were considered trends.

RESULTS

Glucose Tolerance Test

Incremental area under the curve (IAUC), peak area and zeroed glucose concentration at peak (Table 3.3) did not differ between treatments or among sampling days within treatment. A tendency for a day x treatment interaction was noted for glucose half life ($p<0.10$), which was decreased in the FO treatment on d30 when compared to its baseline value (d0). In contrast, half life was not affected by the control diet. Only a day effect was observed for glucose fractional clearance rate ($p=0.0073$); d 30 and 60 differed from baseline. The fraction of dextrose in urine did not differ between dietary treatments or among sampling days within treatment. Similarities of baseline values between CO and FO treatments suggest that the wash out period (30d) was adequate to remove any potential carry over effect of dietary treatments. No body weight change was observed during the experiment.

Blood metabolites

β-hydroxybutyrate, NEFA and triglycerides (Table 3.4) did not differ between treatments or among sampling days within treatment. There was a treatment x day interaction for fasting plasma cholesterol concentration ($p=0.0002$). Cholesterol did not differ in the CO treatment, however, it was decreased ($p<0.05$) in the FO treatment on

d30, d60 and d69 when compared to baseline (d0). Similarly, a treatment x day interaction (p=0.0051) was observed for high density lipoprotein (HDL). While it did not differ in the CO treatment, HDL was decreased in the FO treatment on d69 when compared to baseline (d0). For the low density lipoprotein (LDL) there was a treatment and day effect. The FO treatment LDL was lower than the CO treatment. A treatment effect was also observed for plasma ghrelin; the FO group was higher than the CO group (p= 0.0036). There were no treatment effect seen for fasting plasma glucose, insulin, glucagon, or LPL concentrations (P>0.05).

Nutrient Digestibility, Nitrogen Balance and Protein Turnover

Feed refusals were minimal and nutrient intakes were similar between treatments (Table 3.5). Fecal excretion, dry matter and fat digestibilities were not affected by treatment. Similarly, nitrogen intake, fecal and urine N excretion, N absorbed, N digestibility, N retained, and N balance did not differ between the treatments (Table 3.6). In addition, there was no change in body weight during the nitrogen balance periods in both treatments. Protein turnover, synthesis and degradation were similar in dogs fed the CO and the FO diets.

DISCUSSION

Previous research has demonstrated health benefits associated with the consumption of fish oils, particularly those rich in DHA and EPA. These ω3 fatty acids have been related to lower plasma triglyceride (Kim and Choi., 2005), to promote weight loss (Kunesova et al., 2006; Sato et al., 2007), and to improve glycemic response (Ajiro

et al., 2000 and Feskens et al., 1991). However, most of the research conducted in this area has been performed using murine and human models and these findings may not always translate to the canine. Additionally, obesity is an increasing concern in the pet population and fish oil may be an effective strategy to attenuate problems related to this metabolic syndrome. Therefore, the objective of this study was to determine the effect of feeding a fish oil containing diet on lipid and protein metabolism, postprandial glycemia, and body weight in mature overweight dogs.

Glucose Tolerance Test

In this study, the intravenous glucose tolerance test was used to assess the dogs' ability to utilize glucose and as an indirect means to assess insulin sensitivity in these animals. In dogs, it has been demonstrated that basal insulin level increases with age and that fat mass is positively correlated with glucose peak and incremental increase after IVGTT (Larson et al., 2003). In the latter study, senior dogs fed at maintenance had increased glucose half life of approximately 43% in comparison to energy-restricted leaner dogs; suggesting that a threshold of 20% fat mass existed at which the dogs became less insulin sensitive (Larson et al., 2003). Therefore, the mature overweight model utilized in this experiment was expected to show signs of insulin resistance, which could have been depicted by increased glucose IAUC as well as longer glucose half-life and lower glucose clearance rate. The dogs had fasting plasma insulin levels above the normal physiological range suggesting that they were insulin resistant and therefore, a valid model to test our hypothesis. In support of our results and use of the experimental

model, similar glucose half life values, 26.2 and 27 ± 14 min, have been reported for overweight or obese senior dogs (Verkest et al., 2011; Larson et al., 2003).

In this study, it was also anticipated that dietary supplementation of FO would alleviate or resolve some of the problems related to glucose utilization and metabolism. However, after a maximal challenge with infusion of 2 g/ kg BW glucose (Larson et al., 2003) the CO and FO treatments had similar glucose IAUC, peak area, zeroed glucose at peak and fractional clearance rate. A similar peak plasma glucose concentration (3 min), 51 ± 4 mmol/L, has been reported for obese dogs during i.v. administration of glucose at a dose of 1g/ kg of body weight (Verkest et al., 2011). In the current study, a decrease of 11.5% of glucose half-life on d 30 in the FO treatment was observed and it tended to differ from its baseline value, indicating that the dogs fed the FO diet improved their ability to clear glucose following a glucose tolerance test, probably by an increased sensitivity to insulin signaling.

In support of our results, similar glucose clearance rate of 2.5 %/min has been noted in non-obese dogs supplemented with ω3 PUFA (Irvine et al., 2002). Evidence exists for the beneficial effects of ω3 PUFA supplementation on the prevention and management of insulin resistance and glucose intolerance (Samane et al., 2009; Lombardo et al., 2006; Storlien et al., 1987). However, this topic is still controversial because a few studies reported no health benefits (Gillam et al., 2009; Holness et al., 2004; Sasagawa et al., 2001). To our knowledge only one study has examined the effects of ω3 PUFA, DHA and EPA, on insulin sensitivity and glucose tolerance in healthy non-obese dogs (Irvine et al., 2002). The authors found the supplementation to not be effective. They suggested the animals were less responsive to the ω3 PUFA treatment

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because they were insulin sensitive. In the current study, the reason for the lack of response to dietary FO supplementation is unclear. Factors that might have contributed are the large animal variation in response to IVGTT, the low power (n=7), the need for a longer treatment period, and the FO dose.

Two recent studies evaluating the effects of ω3 PUFA in dogs suffering from osteoarthritis have reported that similar doses to the one utilized in the current study (around 0.8% DHA and EPA) improved weight bearing (Roush et al., 2010[a,b]). A third study, analyzing the effects of increasing dosages of FO in osteoarthritic dogs found that a higher dose of FO ω3 PUFA (2.94% DHA+EPA) to be more effective in the improvement of weight bearing and lameness (Fritsch et al., 2010). In contrast to our study, the aforesaid studies had a larger number of dogs per treatment, ranging from 22 up to 71, and longer experimental periods, varying from 90 to 168 d.

Blood metabolites

Total CHOL values in the present study were similar to literature values reported for dogs over 6y of age (Pasquini et al., 2008). In addition, a previous study (Jeusette et al., 2005[a]) demonstrated that morbid obese dogs have increased total plasma CHOL concentration compared to lean controls or obese dogs maintained at a low energy intakes. Both studies support our results and appear to be good comparisons to our findings because of the similarities with the animals' physiological status (age or body condition) and species used as the experimental models.

Physiological serum CHOL concentration for healthy dogs ranges from 3.6-5.4 mmol/L (Jones and Manella, 1990), therefore the dogs utilized in this study presented

signs of hypercholesterolemia. In contrast to humans, hypercholesterolemia in dogs does not increase the incidence of atherosclerosis mainly due to differences in the lipoprotein metabolism such as; predominant HDL fraction and absence of the enzyme cholesteryl ester transfer protein (Xenoulis and Steiner, 2010; McAlister et al., 1996; Jones and Manella, 1990). Canine hyperlipidemia, however, has been related to pancreatitis, liver and ocular diseases, seizures, and increased morbidity (Xenoulis and Steiner, 2010; Jones and Manella, 1990). Low fat diets have been employed to control hyperlipidemia in dogs; and fish oil supplementation has been suggested as an alternative dietary approach. In the current study, fasting plasma CHOL concentration in the FO treatment was decreased throughout the experiment when compared to its baseline value. Plasma CHOL concentration in the FO group was closer to normal physiological levels, indicating that FO supplementation ameliorated hypercholesterolemia in these dogs. Likewise, lower cholesterol levels were observed in response to fish oil supplementation during maintenance in healthy lean dogs (Pasquini et al., 2008), as well as during gestation, lactation and perinatal period in dogs (Wright-Rodgers et al., 2005). In contrast to FO, the CO group maintained a steady cholesterol concentration during the entire experiment.

The lipoprotein fractions, HDL and LDL, in healthy dogs have been reported to range from 2.5-5.2 mmol/L and 0.49-2.25 mmol/L, respectively (Pasquini et al., 2008; Jeusette et al., 2005[a]; and Downs et al., 1997). The mean HDL and LDL values for the current study were 5.31 and 0.32 mmol/L respectively for the CO and 5.04 and 0.28 mmol/L respectively for the FO treatments. Previously it has been shown that obese female Beagles have higher plasma HDL and LDL (5.77 and 0.78 mmol/L)

concentrations compared to lean controls (3.88 and 0.49 mmol/L) or obese energy-restricted dogs (4.63 and 0.36 mmol/L) (Jeusette et al., 2005[a]). In contrast, Bailhache et al. (2003) reported lower levels of plasma HDL cholesterol in obese male Beagles and no change in the LDL fraction. The reason for the discrepancy between these two studies is not clear, but it could be related to differences in the experimental design, for example, the nutrient composition of the diet, the daily energy intake and the gender of the animals.

Pasquini et al. (2008) and Barrie et al. (1993) reported that female dogs had higher plasma HDL cholesterol concentration than male dogs. From our results, HDL plasma concentration falls within the range reported in the literature, whereas LDL plasma concentration is below the lowest values reported. Differences in methodology assaying LDL concentration could be a possible explanation for the lower levels of LDL observed in the current study; other studies have used fast-protein liquid chromatography (Bailhache et al., 2003) and ultracentrifugation and precipitation methods to determine LDL concentration, in contrast to the enzymatic method used in this study. The latter could be not as efficient in detecting LDL or in separating the lipoprotein fractions, leading to an underestimation of LDL plasma concentration. Additionally, the LDL fraction is normally low in dogs in relation to the HDL_1 fraction, which is the predominant HDL fraction. The LDL and HDL_1 lipoproteins also have similar molecular size and hydrated density in dogs (Bauer, 2004), which could also played a role in the accuracy of the assay method.

Supplementation with FO resulted in a numerically lower HDL concentration, but on d 69 it was decreased by 20% in relation to its baseline value. Similar lipid lowering

effects were observed in response to FO supplementation in dogs (Pasquini et al., 2008; Wright-Rodgers et al., 2005) and in murine models (de Silva et al., 2004; le Morvan et al., 2002; Dallongeville et al., 2001). In these studies, the period of exposure to the test diets varied substantially, anywhere from 1 wk (Dallongeville et al., 2001) up to 16 wks (le Morvan et al., 2002). In the study by Wright-Rodgers et al. (2005), the lipid lowering effects of supplementation of FO were observed during gestation and lactation periods in female dogs, which corresponded to supplementation periods of 56 and 28 d, respectively. Controversial results have been found on the effects of FO supplementation on plasma LDL concentration. Dietary supplementation of FO has shown to increase LDL concentration in hamsters (de Silva et al., 2004). In contrast, in this study LDL was decreased in the FO treated dogs, which agrees with other studies using the canine model (Pasquini et al., 2008; Wright-Rodgers et al., 2005).

It is important to acknowledge that other studies have found no beneficial effect with supplementation of FO or ω3 fatty acids, DHA and EPA, in lowering total CHOL levels in several experimental models (Fakhrzadeh et al., 2010; Mori et al., 1999; Dunstan et al., 1997; Goh et al., 1997; McAllister et al., 1996). The most remarkable and consistent effect of FO supplementation on lipid metabolism has been in decreasing triglycerides (Mori et al., 1999; Lombardo et al, 1996; Williams et al., 1992), mainly in hypertriglycerimic models (Skulas Ray et al., 2011; Harris et al., 1997; Singer et al., 1985). In this experiment, fasting plasma TRIG concentration was greater (1.0 and 0.93 mmol/L for the CO and FO treatment, respectively) than literature values (0.6 ± 0.3 mmol/L) for healthy dogs (Pasquini et al., 2008 and Jones and Manella, 1990). However, higher levels of TRIG have been reported in chronically and grossly obese dogs, 1.04 ±

0.15 mmol/L (Jeusette et al., 2005[a]). The latter finding supports our results and seems a more appropriate data to be compared with, since the animal model used in current study was the mature overweight dog. Even though, the plasma TRIG concentration was increased, it was still within the normal physiological range and no signs of hyperlipidemia were observed in these animals. McAlister et al. (1996) did not find a TRIG-lowering effect after FO supplementation in dogs; the authors attributed the lack of response to the treatment to be associated with the normally low plasma levels of TRIG in dogs, which in their opinion made it harder to detect significant treatment differences. In addition, the TRIG-lowering capacity of fish oil and ω3 fatty acids seems to be dose dependent. Reduced serum TRIG concentration has been reported in studies with daily ω3 fatty acid supplementation of 3-4 g (Harris, 1999; Mori et al., 1999). Utilization of such high doses of ω3 fatty acids in dog food may pose a challenge for the pet food industry due to the high susceptibility of fish oil to fat oxidation and the need of extended shelf-life for its products. It is also important to emphasize that TRIG response to supplementation of ω3 fatty acids varies among species. Studies using Rhesus monkey and swine models found no effect of fish oil in serum TRIG concentration (Davis et al., 1987; Weiner et al, 1986). Therefore, caution must be taken in doing extrapolations among experimental models, as they may not be appropriate. Future studies looking at the ideal dose of fish oil to lower triglyceride levels in the canine model are needed.

It has been suggested that obesity leads to insulin resistance, which results in increased plasma concentration of BHB and NEFA by stimulation of adipose tissue lipolysis (Rand et al., 2003; Leong and Wilding, 1999; Hall et al., 1984). In the current study, mean plasma BHB and NEFA concentrations were similar to NEFA values

reported in adult lean dogs; 0.57±0.07 mmol/L (Jeusette et al. 2005[a]) and BHB reference ranges for dogs; 0.02-0.15 mmol/L (Duarte et al., 2002). These findings suggest that insulin resistance did not occur in the canine mature overweight model utilized herein. Supplementation of FO did not lead to differences in plasma BHB or NEFA concentrations. It differs from studies by Kunesová et al. (2006) and Murata et al. (2002) that found significantly higher serum BHB concentration in severely obese women supplemented with $\omega 3$ PUFA and in Male Sprague-Dawley rats fed a FO-containing diet. However, Kunesová et al. (2006) observed no differences in serum NEFA levels between the control and $\omega 3$ PUFA groups. Fish oil has been implicated to increase ketogenesis and hepatic β-oxidation by activation of peroxisome proliferator activate receptor alpha (PPARα). It has also been suggested a potential synergistic effect of fish oil (or $\omega 3$ PUFA) supplementation in conjunction with dietary caloric restriction in obese subjects (Kunesová et al., 2006). It is unclear the reasons for the lack of response to FO supplementation in the present study, since its dietary supplementation has been beneficial in obese and lean models. Supplementation of fish oil or $\omega 3$ PUFA, DHA and EPA, is expected to stimulate LPL activity in adipose and muscle tissues due to increased sensitivity to insulin. Because of the non-invasive nature of this study, tissue biopsies (muscle and adipose) were not taken; therefore fasting plasma LPL concentration was measured as an attempt to detect dietary treatment effects on lipid metabolism. In the present study, there were no differences in plasma LPL concentration between dietary treatments or among sampling days within treatment. It's important to recognize, however, that plasma LPL concentration may not reflect local regulation and activity of this enzyme in muscle and adipose tissues. In agreement with our findings, few studies

demonstrated that FO supplementation did not influence plasma LPL activity in humans (Nozaki et al., 1991; Harris et al., 1988), whereas others found FO supplementation to be effective in increasing plasma LPL activity (Rudkowska et al., 2010; Harris et al., 1997; Kasim-Karakas et al., 1995).

In obese humans, rodents and dogs suppressed levels of fasting plasma ghrelin have been indicated (Jeusette et al., 2005[a,b]; Ariyasu et al., 2002, Shiiya et a., 2002; Tschop et al., 2001). To our knowledge, few studies have been published looking at plasma ghrelin levels in dogs (Bosch et a., 2009; Jeusette et al., 2005[a,b]) and none has investigated the effects of the supplementation of FO or its ω3 PUFA in this species. In this experiment, fasting plasma ghrelin concentration is in agreement with the canine literature that shows that obese dogs have lower plasma ghrelin than lean ones (Jeusette et al., 2005[a,b]). From our results, dogs fed the FO diet had a 30% increase in plasma ghrelin concentration when compared to the CO group. In support of our results, energy-restricted young overweight female subjects supplemented with DHA and EPA had increased plasma ghrelin concentration compared to the control group (Ramel et al., 2009). In the latter, the increase in plasma ghrelin was partially explained by weight loss and gender, and was associated with a decrease in fasting plasma insulin, which contrasts to our results where no difference in fasting plasma insulin or body weight was observed. The authors speculate that, in this study, the higher levels of plasma ghrelin in dogs fed the FO-containing diet may reflect a change in body composition; decreased fat mass and increased lean mass, potentially mediated by an improvement in insulin sensitivity. Dogs on FO diet tended to have lower glucose half-life (d 30) after a glucose tolerance test, which matched with the highest level of plasma ghrelin. However, fasting insulin

concentration was numerically higher in the FO treatment and body composition was not determined in this experiment.

Excessive body fat is considered a risk factor for the development of diabetes type 2, which is characterized by insulin resistance and beta cell dysfunction, and by hyperglucagonemia during fasting and lack of glucagon suppression following oral glucose and exaggerated glucagon responses to mixed meal ingestion (Unger and Orci, 1981) due to a suggested reduced glucose sensitivity and/or insulin resistance in diabetic alpha pancreatic cells (Bagger et al., 2011). In this experiment, a mature overweight canine model was used and it was hypothesized that these animals would have higher fasting glucagon concentrations. It was also expected that the animals on the FO treatment would have lower fasting glucagon concentrations when compared to the CO treatment. Fasting plasma glucagon concentrations of, approximately, 90 and 56 pg/ml have been reported for alloxan-diabetic and healthy dogs, respectively (Sakurai et al., 1974). Even though dietary treatment did not affect mean glucagon values in this study, numerically higher mean glucagon levels were noticed for the CO treatment when compared to the FO treatment. The fasting plasma glucagon concentrations observed herein are above the values reported for healthy dogs, which may be suggestive of a decreased sensitivity to insulin. Fasting plasma insulin concentrations were also increased in these dogs. Serum insulin concentration for normal and obese type 2 diabetic dogs range between; 5-20 μU/ ml (or 6-24 pg/ml) and 20-130 μU/ ml (or 24-155 pg/ml), respectively (Kaneko, 2008). The fasting insulin values in the current study were at least 3, up to almost 5 times, greater than the maximum value mentioned above. No effect of FO supplementation was observed in fasting plasma glucagon and insulin levels.

Despite their elevated concentrations, fasting plasma glucose levels were within normal physiological range for healthy adult dogs, 3.6-6.5 mmol/L (Kaneko, 2008) and there was no dietary treatment effect. In this study, the remarkably increased levels of fasting plasma insulin and glucagon suggest the presence of insulin resistance in these animals, and the euglycemic values observed could be a consequence of a compensatory secretion of pancreatic insulin, as it has been suggested by Verkest et al. (2011).

Nutrient Digestibility, Nitrogen Balance and Protein Turnover

Food intake did not differ between dietary treatments as the inclusion of 2% of fish oil in the diet matrix was not expected to result in food refusals. Fecal excretion, N intake, DM and N digestibilities were also similar between the dietary treatments. Similarly, fat digestibility was not different between dietary treatments and as expected was high; 95.8 and 95.9% for the CO and FO diets, respectively. Dogs are well designed to digest and assimilate high amounts of dietary fat (Case et al., 2000). Omega-3 fatty acids from fish oil, mainly DHA and EPA, have been shown to improve protein metabolism and to have a protein sparing effect in catabolic illness (Hayashi et al., 1999; Selleck et al., 1994; Mendez et al., 1992; Teo et al., 1989; DeMichele et al., 1988; Trocki et al 1987; Alexander et al., 1986). Aging animals have increased bodily catabolic and inflammatory processes, thus it was expected that the FO diet would ameliorate such processes in mature overweight dogs. However, in the current study, N metabolism, protein turnover, protein synthesis and degradation were not affected by the FO containing diet. Discrepancies between the current study and the literature cited above could be related to differences in experimental design. In this study, the mature

overweight model represented chronic inflammatory and catabolic processes, in contrast to the murine models that were young lean animals that were exposed to acute inflammatory and catabolic processes created by burn injuries (Selleck et al., 1994; Hayashi et al., 1999; DeMichele et al., 1988) or by injections of tumor cells (Mendez et al., 1992). In addition, previous studies differed in the form of FO administration; parenteral (Hayashi et al., 1999; Mendez et al., 1992) or enteral nutrition (Selleck et al., 1994; Teo et al., 1989; DeMichele et al., 1988) and in the dosage of FO provided. Body weight did not differ between treatments. This finding contradicts some of the literature that has found fish oil to be effective in weight loss treatment in obese humans (Kunesova et al., 2006; Thorsdottir et al., 2007), and in mice (Flachs et al., 2006; Ruzickova et al., 2004). Although fish oil has been shown to be beneficial in weight loss, the data still not conclusive and few studies have found no significant effect using mouse and rat models (Gaiva et al., 2001; Takahashi and Ide, 2000; Hun et al., 1999;). Overall, it seems that FO supplementation may aid in the glucose clearance rate in mature overweight dogs. In addition, the FO lipid-lowering effects observed can be of help in the management of hyperlipidemia in dogs. It also may pose an important tool for the pet food industry in developing diets for breeds predisposed to this disease or to obese dogs that are more prone to suffer from lipid metabolism abnormalities. Pet owners have become increasingly aware of nutritional strategies to improve the health of their companion animals; nutraceutical effects of dietary supplementation of fish oil may become a determinant factor in the selection of food by pet owners. Further studies in this area, however, are needed to advance and to verify our findings.

Table 3. 1. Ingredient composition of control (CO) and fish oil (FO) diets fed to mature overweight dogs.

	Treatment	
	CO	FO
Ingredients, % as fed basis		
Rice, Brewers	43.70	43.70
Corn-Starch	22.60	22.60
Poultry Meal	13.80	13.80
Poultry Fat	7.60	7.60
Corn Gluten Meal	4.50	4.50
Soybean oil	2.00	--
Fish Oil	--	2.00
Cellulose	2.00	2.00
Potassium Chloride	1.00	1.00
Palatability Enhancer	1.00	1.00
Dicalcium Phosphate	0.71	0.71
L-Lysine	0.40	0.40
Salt, Iodized	0.25	0.25
Choline Chloride	0.17	0.17
Calcium Carbonate	0.10	0.10
Vitamin mix	0.10	0.10
Mineral Mix [a]	0.05	0.05
Magnesium Oxide	0.03	0.03

[a] Formulated to supply at least (g/kg of food): 0.6 Mg, 1.8 Na, 7.0 K, 7.6 Cl, (mg/kg of food) 211 Fe, 163 Zn, 13 Cu, 13 Mn, 0.4 Se, 1.5 I (IU/g of food) 18.2 Vitamin A, 1.0 Vitamin D, 0.18 Vitamin E (mg/kg of food) 0.3 Biotin, 1484 Choline, 1.9 Folic acid, 62 Niacin, 18 Pantothenic acid, 8.6 Pyridoxine, 8.0 Riboflavin, 41 Thiamin, and 0.13 Vitamin B12

Table 3. 2. Chemical composition of control (CO) and fish oil (FO) diets fed to mature overweight dogs.

	Treatment	
	CO	FO
Item		
DM, %	92.40	92.80
	%, DM basis	
Acid hydrolyzed fat	14.70	14.33
Total dietary fiber	5.1	5.3
Crude protein	19.3	18.7
Ash	4.06	3.95
Gross energy, kcal/kg	4,608	4,584
C18:0 Octadecanoic (Stearic)	1.40	1.32
C18:1 Octadecenoic (Oleic)	5.23	4.81
C18:2 Octadecadienoic Omega 6	3.29	2.00
C18:3 Octadecatrienoic Omega 3	0.19	0.10
C18:4 Octadecatetraenoic Omega 3	< 0.01	0.06
C20:0 Eicosanoic (Arachidic)	0.03	0.03
C20:2 Eicosadienoic Omega 6	0.06	0.06
C20:3 Eicosatrienoic Omega 3	0.01	0.01
C20:4 Eicosatetraenoic Omega 6	0.06	0.09
C20:5 Eicosapentaenoic Omega 3	0.02	0.39
C21:5 Heneicosapentaenoic Omega 3	< 0.01	0.01
C22:2 Docosadienoic Omega 6	< 0.01	< 0.01
C22:3 Docosatrienoic Omega 3	< 0.01	< 0.01
C22:4 Docosatetraenoic Omega 6	0.02	0.02
C22:5 Docosapentaenoic Omega 3	0.01	0.04
C22:6 Docosahexaenoic Omega 3	0.01	0.25
Total Omega 3	0.24	0.86
Total Omega 6	3.47	2.21

Table 3. 3. Glucose response to intravenous glucose tolerance test in mature overweight dogs consuming soy (CO) or fish oil (FO) containing diet.

| | Treatment | | | | | | |
| | CO | | | FO | | | |
	d0	d30	d60	d0	d30	d60	SEM
Item							
Incremental area under the curve (0-180 min), (min•mmol)/L	1422	1354	1317	1353	1338	1351	141.5
Peak area	1388	1331	1278	1288	1292	1324	157.2
Fractional clearance rate, %/min	2.7	2.7	2.9	2.6	2.9	2.8	0.12
Half life, min	26.1	25.4	23.6	26.6	23.6[†]	24.5	1.09
Zeroed glucose concentration at peak, mM	51.2	50.5	52.1	49.3	60.0	55.1	4.90
Urine glucose excretion, mM	514.3	383.6	415.2	413.6	492.5	402.3	62.17
Fraction of dextrose dose in urine, %	16.7	14.7	14.0	15.0	14.4	13.2	1.23
Body weight, kg	27.2	26.6	26.3	27.8	27.3	27.0	1.39

[†] Tendency (P<0.10) to differ from its baseline value (d 0). SEM=pooled SEM, n=7

Table 3. 4. Fasting plasma lipid metabolites, lipoprotein lipase (LPL), ghrelin, glucagon, insulin, and glucose in mature overweight dogs consuming soy (CO) or fish oil (FO) containing diet.

	Treatment								
	CO				FO				
	d0	d30	d60	d69	d0	d30	d60	d69	SEM
Item, mM									
Cholesterol*	5.95^{abc}	5.89^{abcd}	5.96^{abc}	6.05^{ab}	6.28^a	5.41^{cd}	5.33^d	5.49^{bcd}	0.231
HDL*	5.12^{ab}	5.38^{ab}	5.33^{ab}	5.39^{ab}	5.63^a	4.91^{ab}	4.98^{ab}	4.61^b	0.282
LDL**	0.33	0.31	0.30	0.30	0.32	0.26	0.24	0.25	0.029
Triglycerides	0.98	0.95	1.03	0.84	0.82	0.80	0.81	0.70	0.150
Non esterified fatty acids	0.73	0.44	0.54	0.42	0.60	0.46	0.55	0.46	0.092
β-Hydroxybutyrate[1]	0.048	0.054	0.047	0.043	0.053	0.048	0.045	0.044	0.0076
LPL, ng/ml	74.7	79.7	67.2	74.5	76.0	74.3	74.7	74.9	5.31
Ghrelin, ng/ml[1]**	1.7	1.4	1.4	1.5	1.8	2.3	1.6	2.0	0.14
Glucagon, pg/ml[1]	72.1	77.3	80.3	79.2	56.2	55.4	69.0	85.6	25.49
Insulin, pg/ml	436.1	482.4	392.0	557.5	511.8	621.3	504.8	631.6	87.94
Glucose, mM	5.5	5.4	5.4	5.2	5.7	5.4	5.2	5.1	0.15

[a-d] Means in the same row not sharing common superscript letters are different (P<0.05). SEM=pooled SEM, n=7

* Day x treatment interaction (p<0.05).

** Treatment effect (p<0.05).

[1] Mean BHB, ghrelin and glucagon values reported are from not transformed data.

Table 3. 5. Food intake (DM), fecal excretion, dry matter and fat digestibility in dogs consuming soy (CO) or fish oil (FO) containing diet.

	Treatments			
Item	CO	FO	SEM	P- value[1]
Food Intake (g DM/d)	291.9	287.7	13.54	0.7712
Fecal Excretion (g DM/d)	28.42	28.32	1.749	0.9476
DM Digestibility (%)	90.22	90.12	0.446	0.8372
Fat Digestibility (%)	96.80	96.53	0.447	0.5470

[1] Main treatment effect. SEM=pooled SEM, n=7.

Table 3. 6. Nitrogen metabolism in dogs administered ^{15}N – glycine and body weight change of dogs fed control (CO) and fish oil (FO) diets.

	Treatments			
Item	CO	FO	SEM	P-value[1]
Food N Intake, g/d	9.00	8.60	0.406	0.3532
Fecal N Excretion , g/d	1.59	1.62	0.072	0.6791
Urine N Excretion, g/d	4.84	4.78	0.490	0.9404
N Absorbed, g/d	7.41	6.97	0.426	0.2914
N Digestibility, %	82.26	81.22	0.864	0.2753
N Retained, g/d	2.58	2.20	0.523	0.6141
N balance, %	28.63	24.74	5.709	0.6386
Protein turnover (g N/d)	18.53	17.02	1.545	0.3656
Protein synthesis (g N/d)	13.56	12.73	2.037	0.7010
Protein degradation (g N/d)	11.11	10.03	1.274	0.5001
Body weight change, kg	-0.03	-0.17	0.082	0.2431

[1] Main treatment effect. SEM=pooled SEM, n=7.

CHAPTER 4: INFLUENCE OF FEEDING A FISH OIL CONTAINING DIET TO ADULT YOUNG LEAN DOGS: EFFECTS ON LIPID METABOLITES, POSTPRANDIAL GLYCEMIA, AND BODY WEIGHT

INTRODUCTION

Obesity is a serious health concern, as it continues to increase worldwide in human and companion animal populations. According to the National Center for Disease Control and Prevention (CDC), 33.8% of the adult population in United States is obese (CDC, 2011). Similarly, a recent study by the Association for Pet Obesity Prevention (APOP) found that approximately 21% of dogs and 32% of cats were obese (Calabash, 2011). Annually, human obesity costs 150 billion dollars for the US government (CDC, 2011), and Veterinary Pet Insurance claims are over 14 million dollars for weight-related health problems (Ward, 2010).

Besides the high medical cost the biggest problem faced with obesity is the higher incidence of morbidity and mortality due to increased predisposition of overweight and obese subjects to develop chronic diseases (e.g. insulin resistance, diabetes mellitus, heart disease, osteoarthritis, cancer, etc.). A life-long study in dogs has shown that lean animals had a lifespan of about 1.8y longer than dogs with increased body fat mass (Kealy et al., 2002).

Because obesity is the leading health threat in pets and the major cause of death in humans (Ward, 2010; CDC, 2010), many programs have been developed to increase awareness and prevention. Nutritional and physical activity interventions have been common strategies employed, however they have proved to be difficult for the general population (Rossmeisl e al., 2009). Besides food restriction, a nutritional approach that

shows a potential in the prevention of weight-related diseases is the dietary consumption of ω3 PUFA, DHA and EPA, commonly found in fatty fish oils (Rossmeisl et al., 2009; Storlien et al., 1987).

Besides the antiobesity properties of ω3 PUFA (DHA and EPA), they also exhibit hypolipidemic and antidiabetic effects in murine and human models (Kim and Choi., 2005; Mori et al., 1999; Ruzickova et al., 2004; Baillie et al., 1999; Hainault et al. 1993). In addition, enteral supplementation of fish oil in crossbred steers has been shown to increase amino acid and glucose disposal by improvement in muscle insulin sensitivity (Gingras et al., 2007). Maintenance of muscle mass can be a valuable strategy in aiding in the control of obesity, since muscle mass partially defines the body's basal metabolic rate (Gingras et al., 2007).

Despite the potential health benefits observed with fish oil intake, the mode of action of the ω3 PUFA found in fish oil (DHA and EPA), as well as their interaction with protein and carbohydrate metabolism is not completely comprehended. Moreover, the majority of the literature available in this topic was performed using murine models and these findings may not always relate to the canine metabolism. Therefore, the objective of this study was to investigate the effect of feeding a fish oil containing diet on lipid and protein metabolism, postprandial glycemia, and body weight in adult lean dogs.

MATERIALS & METHODS

Animals and Model

All animal care procedures were conducted under a research protocol approved by the Institutional Animal Care and Use Committee, University of Kentucky, Lexington.

Eight female Beagles (Marshall Bioresources, North Rose, NY), with an average age of 2.5 years, and initial BW of approximately 8.0 ± 0.7 kg were used in the experiment. Dogs were randomly divided into two groups, and each group was randomly assigned to one of two diets, control (CO) or fish oil (FO), in a cross-over design. There were two experimental periods and each was 69 days in length. Experimental periods were separated by a wash out period of 30 days to minimize carry-over effects. The dogs were individually housed in kennels (1 x 1.5 m) with a slotted floor in a temperature-controlled room with a light:dark cycle of 12 hours at the Division of Laboratory Animal Research Facility at the University of Kentucky. Each kennel was connected to a concrete outside run (1 x 2 m) where animals had free diurnal access. Dogs were also allowed to exercise and to have social interaction in outside and/ or inside play area for an average of 3 hours daily, except during urine and fecal collection periods. During nitrogen balance collections, dogs were confined to the indoor portion of their kennels for a period of 6 days, and human interaction was provided for a minimum of 20 minutes per dog daily.

Treatments and Feeding

Two dry, extruded kibbled diets were used. All diets were formulated to be isonitrogenous and isocaloric. Diets had similar ingredient compositions and nutrient profiles (Tables 4.1 and 4.2, respectively), except that for source of oil. This replacement led to alterations in the fatty acid profile of these diets (Table 4.2). Diets were formulated in accordance with the Association of American Feed Control Officials (2002) nutrient guide for dogs and balanced to meet maintenance requirements.

Prior to the start of the experiment the dogs were fed Canine Adult Maintenance Diet (Hills Science Diet, Topeka, KS), to maintain a constant lean body weight. At the beginning of each experimental period dogs were gradually transitioned onto experimental diets over a five day period. During the experiment the dogs were fed to maintain their BW, which was established at the beginning of each experimental period. All dogs were with a body condition score (BCS) between 4 and 5 (using a scale from 1 to 9), which is considered ideal and relates to an animal with ribs easily palpable without excess fat covering, waist observed behind ribs and abdomen tucked up. Dogs were fed once a day at 0700 in stainless steel bowls. All dogs were allowed 20 min to consume the food. Any remaining orts were collected, weighed, and recorded. Water was available ad libitum throughout the experiment.

Experimental Procedures

Blood Sampling

Venous blood samples (8 mL) were collected on d 0, 30, 60 and 69 via jugular puncture into a sodium heparin containing vacutainer (Becton Dickson, Franklin Lakes, NJ), and placed on ice for plasma glucose, triglycerides, total cholesterol, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), insulin, glucagon, ghrelin, and lipoprotein lipase (LPL) determination. Blood samples were kept on ice centrifuged at 4,000 g for 15 min at 4°C. Plasma samples were stored at -20°C until analyses.

Postprandial Glycemia

Postprandial glycemia tests were performed at the beginning of the experiment to establish baseline values (prior to treatment), and at 30 and 60 days of feeding the

experimental diets. In the morning of the test, an i.v. catheter (22G x 1) was placed in the cephalic vein of the dogs and secured with tape and an injection cap and filled with heparinized saline (20 units/mL) to ensure patency. After blood baseline collection and subsequent placement of catheters, the dogs were fed their daily ration and allowed 15 min to consume it. Postprandial blood samples (3mL) were collected via intravenous catheter at 15, 30, 45, 60, 90, 120, 150 and 180 min counted from the starting time of food ingestion. Between sampling times, a heparin solution (20 units of heparin per mL) was infused to avoid blood clotting in the catheters. Once blood sampling was completed, catheters were removed. Blood and plasma samples were processed and stored in a similar manner as described for baseline samples.

Nitrogen Balance and Protein Turnover

Sampling periods began at 0700 on day 63 of each experimental period, when dogs entered confinement, and ended at 0700 on day 69. Dogs were weighed prior and at the end of collection periods.

Total urine was collected at 0700, 1300 and, 1900 daily during the collection period. Urine was collected via catch pans that were approximately 1 x 1.5 m. These pans were made of stainless steel and had one inch raised edges on three sides, with a flat edge on one of the 1m sides. The pans were placed under the slotted floor of the kennels and suspended by wire at the back of the kennels so that they were sloping towards the front of the kennel. Fiberglass screens with stainless steel frames were placed on the top of each pan to keep hair and feces out of the urine. A watertight, plastic trough was placed under the front edge of the pan to collect the excreted urine. Eight milliliters of 6

N H_3PO_4 was added into the plastic troughs at the time of placement in order to acidify and preserve the urine. The urine catch pan was rinsed down with water three times daily (0700, 1300, and 1900) during the sampling period to ensure complete collection. Each rinse was approximately 80 mL spread evenly over the entire pan. Total urine collected for each individual dog was placed into a tared plastic container. Fifty percent of the total urine collected from a 24 hour period was composited for each dog within experimental period and frozen.

Just before the 0700 feeding on day 66 of each period a single dose (7.5 mg/kg) of ^{15}N-glycine (Andover, MA, Cambridge Isotope Laboratories, Inc.) was administered orally to each dog via a gelatin capsule (size 000, Park City, UT, Nutraceutical Corp.). From day 66 at 0700 through day 69 at 0700 25% of acidified urine from each dog was separated, composited and frozen for later analysis for ^{15}N-glycine enrichment. Total feces were collected at the same intervals, by removing fecal matter from the slotted floor of the kennels as well as from the fiberglass screen on top of the urine catch pan. After collection, feces were weighed, identified, and placed in plastic bags and frozen until analysis. At the end of each collection period fecal samples were composited by animal.

Blood Metabolite Analyses

Plasma glucose, triglycerides, and total cholesterol concentrations were analyzed by Konelab 20 XTi, using available commercial kits (Infinity™, Thermo Electron Corporation, Louisville, CO). Plasma NEFA was assayed using an enzymatic method (Wako NEFA C kit, Wako Diagnostics, Richmond, VA, USA). Plasma BHB was analyzed by enzymatic method (LiquiColor Reagent Kit, Stanbio Laboratory, Boerne,

TX, USA). Aliquots of plasma were analyzed for insulin, glucagon, ghrelin, and lipoprotein lipase (LPL) concentrations as previously described by Yamka et al. (2006).

Feed, Fecal and Urine Sample Analyses

Samples from the two test diets were collected throughout the experimental periods and stored at -20˚C until chemical analyses were conducted. Diet samples were ground in a Wiley mill (model 4, Thomas Swedesboro, NJ) through a 2-mm screen. Each diet was analyzed for dry matter (AOAC, 2000), crude protein; calculated from total N values (Vario Max CN, Elementar, Hanau, Germany), total dietary fiber (Prosky et al., 1992), gross energy using an oxygen bomb calorimeter (model 1756, Parr Instruments, Moline, IL) and fat content; measured by acid hydrolysis (AACC, 1983) followed by ether extraction (Budde, 1952). Fatty acid profile of experimental diets was analyzed as described previously by Fritsch et al. (2010).

Fecal samples were composited for each dog, within a period, and homogenized. Similarly to the diets, feces samples were analyzed for DM, CP and fat content. Approximately, 1 g samples of wet feces were dried in a 105°C circulating air oven overnight, and difference in weight was used to determine absolute dry matter. Approximately 500 mg of wet feces were weighed into crucibles and analyzed as is for nitrogen content. Dried (55°C) ground fecal samples were used to determine fecal fat content as described above. Urine composites were thawed, homogenized and 1 g aliquots were weighed into crucibles containing 100 mg of steel wool and 200 mg of sucrose to determine nitrogen content. Urea and ^{15}N nitrogen enrichment contents in urine composites prior and post ^{15}N-glycine dosing were analyzed as previously

described by Reeder et al. (2011). All samples were analyzed in duplicate with a 5% error allowed between duplicates; otherwise, analyses were repeated.

Calculations

Postprandial Glycemia

Incremental area under the curve (IAUC) for plasma glucose values was calculated according to method of Wolever et al. (1991) using GraphPad Prism 5 Software (GraphPad Software, Inc., San Diego, CA). Peak area for plasma glucose was also calculated using GraphPad Prism 5 Software (GraphPad Software, Inc., San Diego, CA). This software calculates the area under the curve using the trapezoid rule. It permits to separation of the data into different regions as a fractional of the total area and to calculate the highest point of each region (peak).

Nitrogen Balance

All nitrogen balance calculations were done on a dry matter (DM) basis. Nitrogen from feed was considered nitrogen intake, while nitrogen from urine composites and feces were nitrogen outputs. Apparent nitrogen absorption (NA), nitrogen retention (NR), %DM digestibility, and apparent %nitrogen (N) digestibility were calculated, with all values on a g/d basis, as:

$$NA \ (g/d) = (N \ from \ intake - N \ from \ feces)$$

$$NR \ (g/d) = N \ absorbed - N \ from \ urine$$

$$\% \ DM \ digestibility = \frac{(DM \ intake - DM \ output)}{(DM \ intake)} * 100$$

$$\% \text{ N digestibility} = \frac{(\text{N intake} - \text{N from feces})}{(\text{N intake})} *100$$

$$\% \text{ N balance} = \frac{(\text{N intake} - (\text{N from feces} + \text{N urine})}{(\text{N intake})} *100$$

Whole body protein turnover was measured using the ^{15}N-glycine single-dose urea end-product method (Waterlow et al., 1978). This procedure assumes that ^{15}N-glycine will be partitioned mainly between protein synthesis and oxidation in the same proportion as total amino acid turnover, and that oxidized end products are excreted quantitatively in the urine. Therefore, by measuring the amount of ^{15}N-glycine not incorporated into body tissues, protein turnover (g N/d) can be calculated (Wessels et al., 1997). Whole body protein turnover was calculated as:

$$Q = d/G$$

where Q represents protein turnover (g N/d), and d is the rate of urea N excretion (g/d) from day 66 1300 through day 69 at 0700. The samples from day 66 0700 were not included since they represented urine that was excreted before the ^{15}N-glycine dose was administered. In the equation, G represents the fractional recovery of ^{15}N from ^{15}N-glycine in urine urea. The ^{15}N enrichment of all urine samples was corrected for background ^{15}N enrichment (Wessels et al., 1997). From whole body protein turnover, protein synthesis (PS) and protein degradation (PD) were calculated using the following equations:

$$\text{PS (g N/d)} = [Q - (\text{N excreted in urine})]$$

$$\text{PD (g N/d)} = [Q - (\text{N intake} - \text{N in feces})]$$

where N excreted in urine, N intake, and N in feces were all represented on a (g N/day) basis.

Statistical Analysis

Data from post-prandial glycemia test and plasma hormones and lipid metabolites were analyzed as repeated measurements in a cross-over design using the Mixed procedure of SAS (SAS Inst., Inc., Cary, NC). All other variables were analyzed by the Mixed procedure of SAS (SAS Inst., Inc., Cary, NC). The statistical model included the fixed effect of treatment, and the random effects of period and animal. All treatment least squares means were compared to each other and Tukey adjustment was used to control for experiment wise error. Differences between means with P < 0.05 were considered significant and means with P<0.10 were considered trends.

RESULTS

Postprandial Glycemia

Incremental AUC and peak area for glucose (Table 4.3) were not affected (p>0.05) by dietary treatment or among sampling days within treatment. Likewise, no difference (p>0.05) in body weight was observed between the CO and FO treatments throughout the experiment.

Blood Metabolites

A treatment x day interaction was observed for cholesterol (p=0.0271). While it did not differ in the FO treatment, fasting plasma CHOL was increased throughout the experiment when compared to baseline value for the CO diet (Table 4.4). Plasma CHOL

concentration also tended to be lower in the FO when compared to CO diet on d 30. On d 60, CHOL concentration was decreased in the FO versus the CO treatment. There was no treatment x day interaction (p=0.1103) for fasting plasma triglyceride concentration; however, there was an effect of treatment (p=0.0289). The FO treatment had a higher TRIG than the CO treatment. A significant treatment x day interaction existed for fasting plasma NEFA concentration (p=0.0053). In the FO treatment, NEFA was decreased on d 30 in comparison to d 69 (p=0.0010) and d 69 tended to be increased when compared to baseline (p=0.09), whereas no difference was observed in the CO treatment. Likewise, a treatment x day interaction was observed for fasting BHB (p=0.0392). In the CO treatment, BHB was only increased on d69 when compared to baseline, whereas on the FO diet, BHB decreased at d 30 when compared to d0 and increased at d 69 when compared to d 0, 30 and 60.

For fasting plasma LPL concentration (Table 4.4) a day effect was observed (p=0.0083) and a tendency (p=0.1051) for the FO treatment to have a higher LPL value versus the CO treatment. For fasting plasma ghrelin concentration there was a main effect of treatment and day. The FO treatment had increased levels of ghrelin (p=0.0051) and all days differed from each other except between d 30 and 60 when no effect of day was depicted. There was no treatment x day interaction or main treatment effect for fasting plasma glucagon, insulin, or glucose.

Nutrient Digestibility, Nitrogen Balance and Protein Turnover

Food intake was similar between the CO and FO treatments (Table 4.5) and fecal excretion was increased (p=0.0361) in the FO treatment. This resulted in decreased dry

matter digestibility (p=0.0247) and fat digestibility tended to be lower (p=0.1084) for the FO diet. Nitrogen intake, fecal and urine N excretion, N absorbed, N digestibility, N retained, and N balance did not differ between the treatments (Table 4.6). In addition, there was no change in body weight during the nitrogen balance periods in both treatments. Protein turnover, synthesis and degradation were similar in dogs fed the CO and the FO diets.

DISCUSSION

Omega 3 fatty acids, mainly DHA and EPA, from fatty cold water fish have been alluded to have anti-obesity properties (Ruzickova et al., 2004; Baillie et al., 1999; Hainault et al. 1993), to enhance protein metabolism (Hayashi et al., 1999; Selleck et al., 1994; Mendez et al., 1992; Teo et al., 1989), to decrease plasma cholesterol and triglycerides (Kim and Choi., 2005; Mori et al., 1999), and to improve glycemic response (Ajiro et al., 2000; Feskens et al., 1991). Most of the research conducted in this area, however, has been performed using murine and human models and these findings may not always mirror the canine metabolism. Additionally, not much attention has been paid to the supplementation of fish oil to healthy models. Fish oil may have a preventive effect on the onset of metabolic syndrome, as well as on metabolic processes relate to aging; decreased insulin sensitivity and increased protein catabolism. Thus, the objective of this study was to determine the effect of feeding a fish oil containing diet on lipid and protein metabolism, postprandial glycemia, and body weight in adult lean dogs.

Postprandial Glycemia

In this study, the postprandial glycemic test was used to determine the effect of FO treatment on the dogs' ability to clear blood glucose after ingestion of a mixed meal. The animals in this experiment were young adult lean female dogs, therefore it was expected that these dogs would not have disorders related to the metabolism of carbohydrates, such as insulin resistance, which has been suggested in animals with increased fat mass (Larson et al., 2003). It was hypothesized in this study, that supplementation of FO would further improve insulin sensitivity in these dogs, which could result in smaller glucose IAUC and peak area. Dietary intake of FO has been shown to have an antiobesity effect, thus it was also anticipated that there would be a protective effect of FO supplementation against body weight gain in these animals. Pet foods are energy-dense and formulated to exceed the dogs' minimum nutrient requirements, which may predispose dogs to become obese.

In contrast to our hypothesis, the results of the current experiment showed no effect of FO treatment on glucose IAUC or peak area, as well as body weight. In this study, glucose IAUC fell within ranges reported for healthy dogs (Lubbs et al., 2010). In support of our data, dietary supplementation with ω3 PUFA did not improve insulin sensitivity or glucose disposal in healthy non-obese dogs (Irvine et al., 2002). In the latter study, the authors suggested that the lack of response to the FO supplementation was due to the fact that the dogs were insulin sensitive. Similar factors could have played a role in the current study. Additionally, it is possible that a longer treatment period, higher dosage of FO, and increased number of animals would be necessary in order to improve the effectiveness of the FO treatment. Fritsch et al. (2010) reported that a

supplementation of 2.94% of EPA and DHA during 90 days was effective in improving weight bearing and lameness in 60 osteoarthritic dogs. In murine models a preventive effect of FO supplementation on the development of insulin resistance has been indicated (Clarke, 2000; Podolin et al., 1998). Therefore, further research in this area may be warranted before the preventive role of the supplementation of FO in disorders related to carbohydrate metabolism in the canine model is precluded.

Blood Metabolites

Physiological serum CHOL concentration for healthy dogs ranges from 3.6-5.4 mmol/L (Jones and Manella, 1990). In this study, plasma CHOL concentrations were within the normal physiological range and in agreement with literature values reported for adult young lean dogs, 4.47 to 5.39 mmol/L (Pasquini et al., 2008; Jeusette et al., 2005[a]). Fasting plasma CHOL concentration in the FO treatment did not differ throughout the experiment when compared to its baseline value. In contrast, CHOL levels were consistently increased during the experimental period in the CO group. This suggests that supplementation of FO had a protective effect against a plasma CHOL rise in adult lean dogs, specially at d 60 of supplementation when dogs on the FO diet had a 10% decrease in plasma cholesterol compared to the dogs on the CO diet. In support of our data, Wright-Rodgers et al. (2005) reported that gestating and lactating dogs fed a diet rich in marine ω3 PUFA had lower CHOL concentrations (4.9 mmol/L) in contrast to dogs fed diets with low levels of FO or high levels of α-linolenic acid from linseed oil. In this experiment, fasting plasma TRIG concentration was within the limits reported for healthy adult dogs, 0.6 ± 0.3 mmol/L (Jones and Manella, 1990) and supplementation

with FO led to an increase in plasma TRIG. These findings differ from those seen in human, rodent and canine studies in which dietary FO either decreased or had no effect on plasma TRIG levels (Ulven et al. 2011; Fakhrzadeh et al., 2010; Harris, 1999; Moris et al., 1999; Sanchez-Muniz et al., 1999; McAlister et al., 1996). The increased plasma TRIG (3%) observed in the FO treatment seems to be an effect of higher baseline values observed on d0 rather than a treatment effect. In addition, the dogs on this diet were not hyperlipidemic, which is characterized by plasma TRIG concentrations above 1.7 mmol/L (Jones and Manella, 1990). Mean plasma NEFA and BHB concentrations were similar to the values reported for adult dogs 0.97 ± 0.09 mmol/L and 0.02-0.15 mmol/L, respectively (Bailhache et al. 2003; Duarte et al., 2002). Changes in plasma NEFA followed similar patterns observed for plasma BHB concentrations.

Lipoprotein lipase is an important enzyme in the regulation of glucose disposal by the adipose and muscle tissues. Because of the non-invasive nature of this study, tissue biopsies (muscle and adipose) could not be performed. Instead, fasting plasma LPL concentration was analyzed as indirect method to verify dietary treatment effects on lipid metabolism. A recent study has demonstrated a numeric increase in plasma LPL activity after $\omega 3$ PUFA supplementation (Rudkowska et al., 2010). In the present study, no differences in plasma LPL concentration were detected by supplementation of FO, although the FO treatment showed a tendency toward higher plasma LPL concentration. It's important to acknowledge, however, that plasma LPL concentration may not reveal local regulation and activity of this enzyme in muscle and adipose tissues. In agreement studies demonstrated that FO supplementation did not influence plasma LPL activity in humans (Nozaki et al., 1991; Harris et al., 1988), whereas others found FO

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supplementation to be effective in increasing plasma LPL activity (Harris et al., 1997; Kasim-Karakas et al., 1995).

A consistent indirect relationship between fasting plasma ghrelin and fat mass has been reported in several experimental models (Jeusette et al., 2005[a,b]; Ariyasu et al., 2002; Shiiya et a., 2002; Tschop et al., 2001). In this study, plasma ghrelin concentrations for the CO and FO treatments are slightly above values reported for lean adult dogs; 4.1 ± 0.46 ng/ml (Jeusette et al., 2005[a,b]). However, our data are agreement with the literature that shows that lean animals have higher ghrelin levels when compared to obese animals and that FO treated subjects have increased ghrelin concentration versus the control (Ramel et al., 2009). In this study, an increase in plasma ghrelin of approximately 12% was observed in dogs supplemented with FO. A previous study conducted in our laboratory (presented in chapter 3) also supports that FO augments plasma ghrelin concentration and that obese dogs have lower levels of this hormone.

No change in fasting glucagon concentration or treatment effect was observed. A range for fasting plasma glucagon of 48-57 pg/ml has been reported for adult dogs (Ionut et al., 2008; Sakurai et al., 1974) whereas ours ranged from 60-100 pg.mL. Serum insulin concentrations for dogs range between; 6-24 pg/ml (Kaneko, 2008) and fasting insulin values in the current study were at least ten times greater. While it is not known the reason for the higher plasma levels of these hormones, differences in experimental design and laboratory analyses should not be eliminated. Fasting glucose levels were within normal physiological range for healthy adult dogs (Kaneko, 2008) and unaffected by treatment.

Nutrient Digestibility, Nitrogen Balance and Protein Turnover

Food intake was not affected by dietary treatments. This result was expected because fish products are known to be palatable to dogs (Folador et al., 2006). Despite the similarities in the ingredient composition between the diets, fecal excretion was increased in the FO treatment resulting in decreased dry matter and fat digestibilities. Although, DM and fat digestibility values were lower than the CO diet, they are in agreement with the literature which has shown DM digestibility of dog food with equivalent ingredient composition (e.g. majority of nutrients coming from poultry meal and brewers rice) to be around 80-90% (Faber et al., 2010; Barry et al., 2009; Zinn et al., 2009; Middelbos et al., 2007) and fat digestibility to be greater than 90% (Faber et al., 2010; Zinn et al., 2009; Middelbos et al., 2007). The reason for the lower DM and fat digestibilities for the FO treatment for the current study is not known. These results also differ from the data obtained from the previous study using the mature overweight canine model, where there was no effect of dietary treatment on fecal excretion, DM and fat digestibilities. It has been suggested that older dogs have increased fat, protein and energy digestibilities (Harper, 1998; Sheffy et al., 1985; Lloyd and McCay, 1954), which could be a possible explanation for the findings observed in the current study. Our data also suggests that younger dogs would be less capable of digesting lipid sources rich in PUFA, since only the fat digestibility for the FO diet was decreased when compared to the fat digestibility observed in our previous study using the mature canine model, where fat digestibility for both diets (CO and FO) where around 96-97%. However, it's important to acknowledge that conflicting information exist on the increased nutrient

digestibility of older dogs, as some research indicated no significant age-related change (Taylor et al., 1995; Buffington et al., 1989).

Food nitrogen intake, N digestibility, N retention, and N balance were similar between the dietary treatments. Likewise, protein turnover, synthesis and degradation were not affected by the FO diet. Omega 3 PUFA from fish oil has been shown to improve protein metabolism and to have a protein sparing effect in catabolic illness (Hayashi et al., 1999; Selleck et al., 1994; Mendez et al., 1992; Teo et al., 1989; DeMichele et al., 1988; Trocki et al 1987; Alexander et al., 1986). No data could be found about protein kinetics and fish oil supplementation in lean and/ or healthy animal models. However, it is possible that young adult dogs have reduced catabolic and inflammatory processes, making them a more challenging model to detect health benefits associated with fish oil supplementation, mainly in a short-term experiment.

Body weight did not differ between treatments. Although fish oil has been shown to be beneficial in weight loss, the data still not conclusive and some studies have found no significant effect using mouse and rat models (Gaiva et al., 2001; Takahashi and Ide, 2000; Hun et al., 1999). In addition, the alleged effects of fish oil in weight loss treatments seem to be most effective in obese models (Kunesova et al., 2006; Thorsdottir et al., 2007), which differs from the young lean adult dog model used in this study. In support to our data, Brilla and Landerholm (1990) found no beneficial effect of fish oil supplementation and/ or exercise treatment in healthy adult men. The authors suggested the lack of effect of fish oil and exercise to be related to the limiting capacity of the subjects in further reduce their fat mass. In the current study, similar constrains could have blunted the effect of fish oil treatment, since the dogs were lean and very active. It

was expected that the FO diet, would improve protein metabolism and help maintaining the lean body mass of the animals, which was measured indirectly by N balance and protein turnover. Even though fish oil did not boost the protein metabolism and probably did not affect lean mass in this model (not measured in the experiment), it is important to emphasize that these animals were fed to maintain their ideal body weight. Animals fed ad libitum may benefit from fish oil supplementation. Research has shown supplementation of DHA and EPA to be favorable in weight loss of mice fed free-choice (Flachs et al., 2006). Additionally, no research has been done looking at the long term effects of feeding a fish oil containing diet to dogs. Early supplementation of fish oil may delay the onset of aging processes; protein catabolism and development of insulin insensitivity. Wray-Cahen et al. (1997) and Davis et al. (1998) stated that there is a decline in the sensitivity of muscle protein metabolism to insulin from neonatal period to maturity. Gringas et al. (2007) suggested that maintenance of muscle mass during adulthood could also be an important strategy in addressing the obesity epidemic because muscle mass partially defines basal metabolic rate.

In summary, feeding a FO containing diet showed a protective effect against the rise of CHOL and to be effective in increasing plasma ghrelin. However, it did not appear to improve protein metabolism and postprandial glycemia in adult lean dogs. Discoveries on the long term supplementation of FO in the canine model might assist the pet food industry as well as pet owners to improve the life quality and health of dogs.

Table 4. 1. Ingredient composition of control (CO) and fish oil (FO) diets fed to adult lean dogs.

	Treatment	
	CO	FO
Ingredients, % as fed basis		
Rice, Brewers	43.70	43.70
Corn-Starch	22.60	22.60
Poultry Meal	13.80	13.80
Poultry Fat	7.60	7.60
Corn Gluten Meal	4.50	4.50
Soybean oil	2.00	--
Fish Oil	--	2.00
Cellulose	2.00	2.00
Potassium Chloride	1.00	1.00
Palatability Enhancer	1.00	1.00
Dicalcium Phosphate	0.71	0.71
L-Lysine	0.40	0.40
Salt, Iodized	0.25	0.25
Choline Chloride	0.17	0.17
Calcium Carbonate	0.10	0.10
Vitamin mix	0.10	0.10
Mineral Mix[a]	0.05	0.05
Magnesium Oxide	0.03	0.03

[a] Formulated to supply at least (g/kg of food): 0.6 Mg, 1.8 Na, 7.0 K, 7.6 Cl, (mg/kg of food) 211 Fe, 163 Zn, 13 Cu, 13 Mn, 0.4 Se, 1.5 I (IU/g of food) 18.2 Vitamin A, 1.0 Vitamin D, 0.18 Vitamin E (mg/kg of food) 0.3 Biotin, 1484 Choline, 1.9 Folic acid, 62 Niacin, 18 Pantothenic acid, 8.6 Pyridoxine, 8.0 Riboflavin, 41 Thiamin, and 0.13 Vitamin B12

Table 4. 2. Chemical composition of control (CO) and fish oil (FO) diets fed to adult lean dogs.

	Treatment	
	CO	FO
Item		
DM, %	92.40	92.80
	%, DM basis	
Acid hydrolyzed fat	14.70	14.33
Total dietary fiber	5.1	5.3
Crude protein	19.3	18.7
Ash	4.06	3.95
Gross energy, kcal/kg	4,608	4,584
C18:0 Octadecanoic (Stearic)	1.40	1.32
C18:1 Octadecenoic (Oleic)	5.23	4.81
C18:2 Octadecadienoic Omega 6	3.29	2.00
C18:3 Octadecatrienoic Omega 3	0.19	0.10
C18:4 Octadecatetraenoic Omega 3	< 0.01	0.06
C20:0 Eicosanoic (Arachidic)	0.03	0.03
C20:2 Eicosadienoic Omega 6	0.06	0.06
C20:3 Eicosatrienoic Omega 3	0.01	0.01
C20:4 Eicosatetraenoic Omega 6	0.06	0.09
C20:5 Eicosapentaenoic Omega 3	0.02	0.39
C21:5 Heneicosapentaenoic Omega 3	< 0.01	0.01
C22:2 Docosadienoic Omega 6	< 0.01	< 0.01
C22:3 Docosatrienoic Omega 3	< 0.01	< 0.01
C22:4 Docosatetraenoic Omega 6	0.02	0.02
C22:5 Docosapentaenoic Omega 3	0.01	0.04
C22:6 Docosahexaenoic Omega 3	0.01	0.25
Total Omega 3	0.24	0.86
Total Omega 6	3.47	2.21

Table 4. 3. Postprandial glycemic response in adult lean dogs consuming soy oil (CO) or fish oil (FO) containing diet.

	Treatment						
	CO			FO			
	d0	d30	d60	d0	d30	d60	SEM
Item							
Incremental area under the curve (0-180 min), (min•mmol)/L	125.0	104.2	105.0	115.2	87.2	118.7	16.46
Peak area	123.5	106.7	106.1	111.0	64.0	126.9	17.97
Body weight, kg	8.2	8.3	8.4	8.2	8.3	8.3	0.37

SEM=pooled SEM, n=8.

Table 4. 4. Fasting plasma lipid metabolites, lipoprotein lipase (LPL), ghrelin, glucagon, insulin, and glucose in adult lean dogs consuming soy oil (CO) or fish oil (FO) containing diet.

	Treatment								
	CO				FO				
	d0	d30	d60	d69	d0	d30	d60	d69	SEM
Item, mmol/L									
Cholesterol*	4.76^a	5.40^d	5.37^d	5.35^{cd}	4.82^{ab}	$4.91^{abcd\S}$	4.88^{abc}	5.31^{bcd}	0.350
Triglycerides**	0.33	0.39	0.37	0.49	0.42	0.40	0.37	0.51	0.020
Non esterified fatty acids	0.97^{ab}	1.24^{bc}	1.16^{bc}	1.17^{bc}	1.00^{abc}	0.86^a	1.04^{abc}	$1.28^{c\dagger}$	0.082
β-hydroxybutyrate	0.06^{ab}	0.05^{ab}	0.06^{ab}	0.09^{cd}	0.07^{bc}	0.04^a	0.05^{ab}	0.12^d	0.013
LPL, ng/ml†	64.0	69.2	70.3	72.3	61.4	78.0	80.7	74.5	6.76
Ghrelin, ng/ml**	5.2	6.0	6.0	7.8	5.7	7.3	6.9	8.1	0.66
Glucagon, pg/ml	60.8	80.3	70.1	97.0	63.9	85.7	67.5	67.6	18.24
Insulin, pg/ml	251.2	232.0	270.0	325.2	266.5	311.2	230.5	347.5	75.12
Glucose, mmol/l	5.1	5.0	5.3	5.3	5.0	5.4	5.2	5.6	0.24

[a-d] Means in the same row not sharing common superscript letters are different ($P<0.05$). SEM=pooled SEM, n=8.

* Day x treatment interaction ($p<0.05$).

** Treatment effect ($p<0.05$).

§ Trend to differ from d30 of CO treatment ($p<0.10$).

$\dagger$ Trend to treatment effect ($p<0.10$).

Table 4. 5. Food intake (DM), fecal excretion, dry matter and fat digestibility in dogs consuming soy (CO) or fish oil (FO) containing diet.

	Treatments			
Item	CO	FO	SEM	P- value[1]
Food Intake (g DM/d)	151.3	151.1	4.81	0.9819
Fecal Excretion (g DM/d)	14.3	16.7	0.76	0.0361
DM Digestibility (%)	90.6	88.9	0.47	0.0247
Fat Digestibility (%)	96.4	94.9	0.61	0.1084

[1] Main treatment effect. SEM = pooled SEM, n=8.

Table 4. 6. Nitrogen metabolism in dogs administered ^{15}N – glycine and body weight change of dogs fed control (CO) and fish oil (FO) diets.

	Treatments			
Item	CO	FO	SEM	P-value
Food N Intake, g/d	4.62	4.57	0.147	0.8177
Fecal N Excretion , g/d	0.28	0.32	0.019	0.1229
Urine N Excretion, g/d	3.43	3.37	0.143	0.7616
N Absorbed, g/d	4.34	4.24	0.146	0.6395
N Digestibility, %	93.95	92.82	0.470	0.1124
N Retained, g/d	0.91	0.87	0.140	0.8577
N balance, %	19.50	18.78	2.759	0.8553
Protein turnover (g N/d)	8.53	8.44	0.524	0.8449
Protein synthesis (g N/d)	5.93	5.83	0.488	0.8325
Protein degradation (g N/d)	4.19	4.20	0.516	0.9888
Body weight change ,kg	0.10	0.14	0.040	0.5430

[1] Main treatment effect. SEM = pooled SEM, n=8.

CHAPTER 5: INCREMENTAL LEVELS OF BARLEY IN CANINE DIETS: INFLUENCE ON APPARENT NUTRIENT DIGESTIBILITY, FECAL QUALITY AND ODOR, AND POSTPRANDIAL GLYCEMIA AND INSULINEMIA IN ADULT DOGS

INTRODUCTION

In recent years, the human population has become progressively aware of the importance of nutrition for the maintenance of optimal health. Despite it, the incidence of obesity and other chronic diseases is still increasing worldwide. Similarly to humans, companion animals also have a high incidence of chronic diseases. Dogs and cats have shared the same environment, food and life style of their owners; therefore it is not surprising these animals suffer from similar health problems. In the area of human nutrition, several approaches have been investigated to address this serious public health issue. The interest in functional ingredients has increased because they have shown to be beneficial in the control or prevention of obesity, diabetes mellitus, cancer, hypercholesteremia, as well as other chronic diseases. Among these functional ingredients, barley (*Hordeum vulgare L.*) has become a popular carbohydrate choice due to its high concentration of complex carbohydrates and β-glucans, two important constituents of dietary fiber (Asare et al., 2011).

Some of the health benefits observed with the ingestion of dietary fibers are related to their lower energy density as compared to other carbohydrate fractions such as, starch, but also to their potential to increase satiety and to slow down digestive and absorptive processes due to slower rate of gastric emptying and due to increased viscosity of the digesta in the gastrointestinal tract. Additional benefits associated with the

ingestion of barley or its fiber components are: the cholesterol-lowering property (Queenan et al., 2007; Anderson et al., 1990) and the decreased insulinemic and glycemic response observed in healthy and diabetic patients (Mello and Laaksonen, 2009).

Because humans have developed strong emotional bonds with their companion animals, pet owners have been particularly concerned with the health of their pets and have sought for similar nutritional approaches as to their own, to improve the life quality and longevity of their pets. However, limited information on the health benefits of dietary supplementation of barley is available for the canine. Therefore, the objective of this study was to examine the effects of incremental levels of barley on apparent nutrient digestibility, fecal short chain fatty acid concentrations, fecal quality and odor, and postprandial glycemic and insulinemic responses in adult dogs.

MATERIALS & METHODS

Animals and Model

All animal care procedures were conducted under a research protocol approved by the Institutional Animal Care and Use Committee, University of Kentucky, Lexington. Sixteen female dogs; eight purpose-bred mature female dogs with an average age of 10 years, and initial BW of approximately 22.6 ± 3.2 kg and eight young female Beagles with an average age of 3.5 years and initial BW of 8.2 ± 0.8 kg were used in the experiment. Dogs were blocked by age and 4 experimental diets; control (40% corn) or three levels of barley (10, 20, and 40%) were randomly assigned within each block giving 4 dogs per diet, 2 young and 2 senior. This process was repeated for a second

experimental period with one constraint, no dog received the same diet twice, giving a total of 8 dogs for each diet. In order to facilitate sample collection, dogs were divided in two groups within experimental period intercalated by a week. Each group was comprised of four dogs of each age block. Experimental periods were 14 days in length; a 9-d adaptation phase followed by a 4-d confinement for collection of urine and feces, followed by a 1-d post-prandial glycemia test, and were separated by a wash out period of 7 days to minimize carry-over effects. The dogs were individually housed in kennels (1 x 1.5 m) with a slotted floor in a temperature-controlled room with a light: dark cycle of 12 hours at the Division of Laboratory Animal Research Facility at the University of Kentucky. Each kennel was connected to a concrete outside run (1 x 2 m) where animals had diurnal access. Dogs were also allowed to exercise and to have social interaction in outside and/ or inside play area for an average of 3 hours daily, except during urine and fecal collection periods. During nitrogen balance collections, dogs were confined to the indoor portion of their kennels for a period of 4 days, and human interaction was provided for a minimum of 20 minutes per dog daily.

Treatments and Feeding

Four dry, extruded kibbled diets were used. All diets were formulated to be isonitrogenous and isocaloric. Diets had similar ingredient compositions and nutrient profiles (Tables 5.1). The dietary treatments were: control (40% corn, CO) and three diets with incremental levels of barley (10, 20 or 40%; BA10, BA20 and BA40) added at the expensive of whole yellow corn. This incremental substitution of barley led to alterations in the total dietary fiber content of the diets (Table 5.1). Diets were

formulated in accordance with the Association of American Feed Control Officials (2002) nutrient guide for dogs and balanced to meet maintenance requirements.

All dogs were adapted to the control diet prior to the start of the experiment. During the experiment the dogs were fed to maintain their BW, which was established at the beginning of each experimental period. Dogs were fed once a day at 0700 in stainless steel bowls. All dogs were allowed 15 minutes to consume the food in order to condition them to meal feeding. Any remaining orts were collected, weighed, and recorded. At the beginning of each experimental period, dogs were abruptly switched to their assigned experimental diets to evaluate potential adaptation concerns with newly introduced carbohydrate sources; measured by fecal score (48h). Water was available ad libitum throughout the experiment.

Experimental Procedures

Apparent Nutrient Digestibility and Nitrogen Balance

Sampling periods began at 0700 on day 10 of each experimental period, when dogs entered confinement, and ended at 0700 on day 14. Dogs were weighed prior and at the end of collection periods. Total urine was collected at 0700, 1300 and, 1900 daily during the collection period. Urine was collected via catch pans that were approximately 1 x 1.5 m. These pans were made of stainless steel and were placed under the slotted floor of the kennels and suspended by wire at the back of the kennels so that they were sloping towards the front of the kennel. Fiberglass screens with stainless steel frames were placed on the top of each pan to keep hair and feces out of the urine. A watertight,

plastic trough was placed under the front edge of the pan to collect the excreted urine. Eight milliliters of 6 N H_3PO_4 was added into the plastic troughs at the time of placement in order to acidify and preserve the urine. The urine catch pan was rinsed down with water three times daily (0700, 1300, and 1900) during the sampling period to ensure complete collection. Each rinse was approximately 80 mL spread evenly over the entire pan. Total urine collected for each individual dog was placed into a tared plastic container. Fifty percent of the total urine collected from a 24 hour period was composited for each dog within experimental period and frozen.

Total feces were collected at the same intervals, by removing fecal matter from the slotted floor of the kennels as well as from the fiberglass screen on top of the urine catch pan. After collection, feces was weighed, identified, and placed in plastic bags and frozen at -20°C. At the end of each experimental period 50% of the daily fecal excretion was composited by animal. All fecal samples were scored for consistency throughout each experimental period according to the following system: 1 = hard, dry pellets; small, hard mass; 2 = hard-formed, dry stool; remains firm and soft; 3 = soft, formed, and moist stool, retains shape; 4 = soft, unformed stool; assumes shape of container; and 5 = watery; liquid that can be poured. On day 4 of fecal collection, an aliquot of fresh feces was collected and frozen (-20°C) until analysis of phenol, indole, and biogenic amine concentrations. A second aliquot of fresh feces (2 mg) was collected and weighed into 5 mL of 5% metaphosphoric acid for short chain fatty acid (SCFA) analysis.

Postprandial glycemia

Postprandial glycemia tests were performed on day 14 of each experimental period. In the morning of the test, venous blood samples (5 mL) were collected via jugular puncture into a sodium heparin containing vacutainer (Becton Dickson, Franklin Lakes, NJ), and placed on ice for plasma glucose and insulin analyses. Subsequently, an i.v. catheter, 22gx1 or 20gx 1 ¼ (Terumo Medical Corporation, Somerset, NJ), was placed in the cephalic vein of the Beagles and purpose-bred dogs, respectively, and secured with tape and an injection cap and filled with heparinized saline (20 units/mL) to ensure patency. After blood baseline collection and placement of catheters, the dogs were fed their daily ration and allowed 15 min to consume it. Postprandial blood samples (3mL) were collected via intravenous catheter at 15, 30, 45, 60, 90, 120, 150, 180, 240, and 300 min counted from the starting time of food ingestion. Between sampling times, heparin solution was infused to avoid blood clotting in the catheters. Once blood sampling was completed, catheters were removed. Blood samples collected in sodium heparin containing vacutainer were kept on ice and centrifuged at 4000 x g for 20 minutes at 4°C (Sorvall RT6000B, Newton, CT). Plasma samples were stored at -20°C until analysis.

Feed, Fecal and Urine Sample Analyses

Samples from the four test diets were collected throughout the experimental periods, composited and stored at -20˚C until chemical analyses were conducted. Diet samples were ground in a Wiley mill (model 4, A.H. Thomas Swedesboro, NJ) through a

2-mm screen. Each diet was analyzed for dry matter, ash (AOAC, 2000), crude protein; calculated from total N values (Vario Max CN, Elementar, Hanau, Germany), total dietary fiber (Prosky et al., 1992), gross energy using an oxygen bomb calorimeter (model 1756, Parr Instruments, Moline, IL) and fat content; measured by acid hydrolysis (AACC, 1983) followed by ether extraction (Budde, 1952).

Similarly to the diets, fecal samples were analyzed for DM, CP and fat content. Approximately, 1 g samples of wet feces were dried in a 105°C circulating air oven overnight, and difference in weight was used to determine absolute dry matter. Approximately 500 mg of wet feces was weighed into crucibles and analyzed as is for nitrogen content. Dried (55°C) ground fecal samples were used to determine fecal fat and TDF contents as described above. Fecal SCFA concentrations were determined using gas chromatography according to methods described previously (Strickling et al., 2000). Phenol and indole were extracted from fecal samples according to methods described by Flickinger et al. (2003) and determined via HPLC according to methods described by Niwa (1993). Biogenic amines were quantified via HPLC according to methods described by Flickinger et al. (2003).

Urine composites were thawed, homogenized and 1 g aliquots were weighed into crucibles containing 100 mg of steel wool and 200 mg of sucrose to determine nitrogen content as described above.

Blood Metabolite Analyses

Plasma glucose and cholesterol concentrations were analyzed by Konelab 20 XTi, using available commercial kits (Infinity™, Thermo Electron Corporation, Louisville,

CO). Plasma insulin concentrations were determined by radioimmunoassay using available commercial kit (Canine Coat-A-Count –Siemens Healthcare Diagnostics, Los Angeles, CA).

Calculations

Apparent Nutrient Digestibility and Nitrogen Balance

All apparent nutrient digestibility and nitrogen balance calculations were done on a dry matter (DM) basis. Nutrient digestibility was calculated based on total fecal matter excreted during the collection phase of each period. For the nitrogen balance, nitrogen from feed was considered nitrogen intake, while nitrogen from urine composites and feces were nitrogen outputs. Apparent nitrogen absorption (NA), nitrogen retention (NR), %DM digestibility, and apparent % nitrogen (N) or nutrient digestibility were calculated, with all values on a g/d basis, as:

$$\% \text{ DM digestibility} = \frac{(\text{DM intake} - \text{DM output})}{(\text{DM intake})} *100$$

$$\% \text{ N digestibility} = \frac{(\text{N intake} - \text{N from feces})}{(\text{N intake})} *100$$

$$\text{NA (g/d)} = (\text{N from intake} - \text{N from feces})$$

$$\text{NR (g/d)} = \text{N absorbed} - \text{N from urine}$$

$$\text{N balance} = \frac{(\text{N intake} - (\text{N from feces} + \text{N urine})}{(\text{N intake})} *100$$

Postprandial glycemia and insulinemia

Glucose incremental and decremental area under the curve (IAUC and DAUC, respectively) and insulin AUC were calculated according to method of Wolever et al. (1991) using GraphPad Prism 5 Software (GraphPad Software, Inc., San Diego, CA). This software calculates the area under the curve using the trapezoid rule. It allows to separate the data in different regions as a fractional of the total area and to calculate the highest point of each region (peak).

Statistical Analysis

Data were analyzed as a generalized randomized complete block design, with period as a repeated measurement using the Mixed procedure of SAS (SAS Inst., Inc., Cary, NC). Data for discontinuous variables were analyzed by the GLIMMIX procedure (SAS Inst., Inc., Cary, NC). The statistical model included the fixed effects of treatment and period, and the random effects of animal and block (age). All treatment least squares means were compared using contrasts that tested for linear and quadratic effects of incremental dietary supplementation of barley. Fecal concentrations of agmatine, cadaverine, tryptamine, total biogenic amines, 4-ethylphenol, P-cresol, isobutyrate, isovalerate and total branched-chain fatty acids (BCFA) were not normally distributed, so a log transformation was applied for statistical analysis. Differences between means with $P<0.05$ were considered significant and means with $P \leq 0.10$ were considered tendencies.

RESULTS

Nutrient Intake and Digestibility, and Fecal Scores

Dry matter (DM) intake (Table 5.2) was based on their metabolizable energy requirements for maintenance thus, it was consistent across dietary treatments. Total dietary fiber (TDF) intake increased quadratically with incremental addition of barley (p=0.001), while acid hydrolized fat (AHF) intake did not differ among treatments. Despite the differences in TDF intake, fecal DM and TDF output were not different among dietary treatments. Fecal AHF output tended to increase quadratically as dietary barley increased (p=0.0602). Apparent DM digestibility was similar among treatments. Total dietary fiber digestibility increased quadratically (p=0.0307), being highest for BA20 and BA40 treatments, while AHF digestibility decreased in the same fashion (p=0.0344) with lowest values observed in the BA20 and BA40 treatments. No differences were observed for fecal scores after 48h or 15d of feeding the experimental diets.

Nitrogen Metabolism

Nitrogen (N) intake (Table 5.3) had a quadratic response (P<0.001), being lower in the CO and BA10 treatments than in the BA20 and BA40 treatments. Fecal and urine N excretion were not different among treatments. Nitrogen absorbed increased quadratically (p=0.0069), being lowest for CO and highest for the BA20 treatments. Nitrogen digestibility was unaffected by treatment whereas nitrogen retention tended to respond in a quadratic fashion (p=0.0524) being highest in the BA20 treatment and lowest in the BA10. However, N balance was unaffected by treatment.

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Fermentation Metabolites

Fecal concentrations of acetate (p=0.0364), propionate (p=0.0004), and total SCFA (p=0.0203) increased quadratically as the inclusion of barley increased. In contrast, no treatment effect was observed for fecal concentration of butyrate or any of the BCFAs (Table 5.4).

Fecal concentration of 4-ethylphenol was unchanged among dietary treatments (Table 5.5), whereas P-cresol tended (quadratic, p=0.0599) to decrease as barley increased. Biogenic amines were unaffected by dietary treatment.

Postprandial Glycemic and Insulinemic Responses and Fasting Plasma Cholesterol Concentration

Incremental and decremental glucose AUC were not affected by the treatments (Table 5.6). Likewise, insulin AUC did not differ among treatments and no difference in fasting plasma cholesterol were observed among different dietary treatments.

DISCUSSION

Carbohydrates are heterogeneous organic compounds that include low and high molecular weight sugars, starches, and various cell wall and storage non-starch polysaccharides or dietary fibers (Bach-Knudsen, 1997). Because of their heterogeneity, carbohydrates can have different functional properties: absorbable, digestible, fermentable, and non-fermentable, which are the major contributors for modifying post-prandial glucose and insulin curves in dogs and humans (Nguyen et al., 1994 and Wolever and Bolognesi, 1996). In addition, fermentable dietary fibers have also shown

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to exert hypolipidemic effects in human and rodent models (Queenan et al., 2007; Nishima et al., 1990) and to have the potential to improve the host health by increased SCFA production and decreased production of carcinogenic compounds in the hindgut (Swanson et al., 2002). Because pets have become an essential family member in the American household, pet owners search for ways to maintain the health and to increase the longevity of their companion animals, as well as to discover new strategies to prevent or ameliorate common health conditions that affect the today's pet population such as: diabetes mellitus, hyperlipidemia, insulin resistance, etc. Thus, the aim of this study was to examine the effects of incremental levels of barley; rich in β-glucans a highly fermentable type of dietary fiber, on apparent nutrient digestibility, fecal quality and odor, postprandial glycemic and insulinemic responses, and plasma cholesterol levels in adult dogs.

Nutrient Intake and Digestibility, and Fecal Scores

The higher intake of TDF for the BA20 and BA40 treatments was anticipated and reflected the higher levels of fiber present in barley, mainly β-glucans. The observed quadratic increase of AHF output could possibly be a consequence of increased digesta viscosity in the small intestine, decreasing the effectiveness of enzymes related to the hydrolytic digestion of lipids in this site. It could also be related to an increased excretion of cholesterol due to a decrease in its recycling from the lower small and large intestine into hepatic portal blood circulation. Another possibility would be an increased lipid excretion due to higher excretion of microbial mass in feces. Fermentable fibers augment fermentative processes, which may lead to a higher number of microbes in the

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hindgut and consequently, a greater elimination of microbial cells in fecal matter that can result in a false negative effect of fermentable fibers in nutrient digestibility. As discussed later in this chapter, N digestibility was not negatively affected by incremental levels of barley, suggesting that it is unlikely that the observed decrease in AHF digestibility would be related to increased excretion of microbial mass. In support of our reasoning, higher ileal fat flow and fecal cholesterol excretion have been observed in ileum-cannulated pigs and humans after consumption of diets rich in β-glucans (Knudsen et al., 1993 and Lia et al., 1995). Despite the lower AHF digestibility observed with increased barley, AHF digestibility for all treatments was high and within and the expected range. Only a numerical decrease in TDF output of approximately 16% was observed for the BA40 treatment. The higher TDF digestibility of the BA40 diet, relates to the increased TDF intake and numerically lower TDF output; a consequence of the high fermentability of this substrate in the large intestine.

To our knowledge, no studies have been conducted looking at the effects of barley on nutrient digestibility in the canine model. However, several studies looking at other sources of fermentable fibers (e.g. fructoologosacharides, inulin, and pectin) have been performed in dogs and cats (Faber et al., 2011; Barry et al., 2010; Barry et al., 2009; Flickinger et al., 2003; and Silvio et al., 2000). In general, the data from these studies showed similar DM and AHF digestibilities ranging between 78-89% and 92.5-96%, respectively. Silvio et al. (2000) reported a TDF digestibility of 69% in a diet with high levels of soluble fiber (10% pectin). This result is comparable to the apparent TDF digestibility value obtained in the current study for the BA40 diet. Because barley is rich in β-glucans, which is a highly fermentable fiber source, a higher TDF digestibility was

anticipated with incremental inclusion of barley in the diets. Based on these results it appears that inclusion of barley (up to 40%) is well tolerated by adult dogs and can be adopted by the pet food industry in the manufacturing of commercial diets. In addition, studies looking at fecal DM digestibility of diets containing barley for swine have reported similar values ranging between 85.5-88% (Wilfart et al., 2007; Veum et al., 2007; Woyengo et al., 2009). In contrast, Wilfart et al. (2007) found lower fat (ether extract) and TDF digestibilities, 77 and 46%, respectively, for pigs fed a diet containing 41.1% barley.

Fecal scores for dogs fed all dietary treatments within the desirable range 2-3. This result indicates that inclusion of barley at the levels utilized in this study did not compromise stool quality, which is an important factor in considering the suitability of an ingredient in pet food. Currently, most dogs are indoors; causing pet owners to seek diets that result in small and well-shaped stools.

Nitrogen Metabolism

The slightly increased concentrations of crude protein in the BA20 and BA40 treatments led to the slight increase in N intake for these treatments. Even though, the diets were formulated to be isonitrogenous, barley grain has higher crude protein value than corn, 12.3 and 8.4%, respectively; which led to higher dietary CP in the diets containing higher levels of barley. The increased N absorbed for the BA20 and BA40 diets would seem to be a consequence of the increased N intake as well. Despite differences observed in N intake and absorption, N digestibility was not affected by treatment. The N digestibilities of the current study are in agreement with literature values for N digestibility using the canine or feline models, which varied from 74 up to

90.5% (Faber et al., 2011; Barry et al., 2010; Barry et al., 2009; Flickinger et al., 2003; and Silvio et al., 2000). Also in support of our data, studies investigating the impact of barley on nutrient digestibility in pigs, have found similar apparent fecal CP or N digestibilities, ranging from 83 up to 90% (O'Shea et al., 2011; Wilfart et al., 2007; Veum et al., 2007; Woyengo et al., 2009). In this study, inclusion of barley up to 40% did not negatively impact nitrogen digestibility. Similarly, inclusion of barley or oats in diets for broilers and cockerels, and pigs either improved or did not result in deleterious effects on N retention (O'Shea et al., 2011; Perttila et al., 2001).

Fermentation Metabolites

Direct measurement of fermentation in the canine hindgut is difficult; therefore fecal concentration of SCFA is used as an indirect measurement. Even though changes in fecal SCFA profile or amount can be an indicative of increased or decreased colonic fermentative processes, fecal SCFA concentration may not always accurately reflect the amount produced. Barley is rich in β-glucans, which are rapidly fermented by the microbiota in the hindgut. Therefore, increased SCFA production was expected as a result of higher rate of fermentative process at this site. As dietary supplementation of barley increased, the fecal concentration of acetate, propionate, and total SCFA increased quadratically. In support of our data, pigs fed with a variety of commercial barley had increased distal colon concentrations of acetate, propionate and total SCFA, while butyrate remained unchanged (Bird et al., 2004). Other studies using canine or feline models have reported results similar to ours when animals were fed a source of fermentable fiber such as; beet pulp, fructooligosaccharides (FOS), galactoglucomannan,

mannanoligosaccharides (MOS), and pectin (Faber et al., 2011; Barry et al., 2010; Middelboss et al., 2007; Swanson et al., 2002;). Several studies have reported no change or decreased levels of butyrate with dietary supplementation of fermentable fibers (Faber et al., 2011; Bird et al., 2004; Flickinger et al., 2003; Swanson et al., 2002; Silvio et al., 2000), whereas others have found increased levels (Middelboss et al., 2007; Strickling et al., 2000; Sunvold et al., 1995). It has been suggested that the fecal concentration of butyrate may not reflect fermentative processes due to its rapid absorption and utilization as an energy source by colonocytes (Swanson et al., 2002). In agreement, in pigs fed different fiber sources (wheat bran, barley, and oat bran) higher numerical values were reported for SCFA at the cecum and proximal colon, when compared to mid and distal colon (Bird et al., 2004). In general, molar distribution of SCFA (%) in the current study is similar to studies that evaluated different fermentable fiber sources; with acetate being the predominant fraction, followed by propionate and butyrate (Branning and Nyman, 2011; Faber et al., 2011; Barry et al., 2010; Middelboss et al., 2007; Flickinger et al., 2003; Swanson et al., 2002; Silvio et al., 2000).

Branched chain fatty acids (BCFA), phenol and indole components, as well as biogenic amines are derivatives of the fermentation of amino acids and peptides, mainly when energy is limiting in the large intestine (Middelbos et al., 2007; Macfarlane et al., 1992). These nitrogenous compounds are responsible for fecal odor and at certain extent may be deleterious to gut health (Swanson et al., 2002). In the current study, inclusion of barley was expected to decrease the formation of these compounds, since it has been proposed that fermentable carbohydrates provide additional energy supply for the gut microbiota (Swanson et al., 2002). However, differently than hypothesized, barley

supplementation did not influence fecal concentration of BCFAs. In support of our findings, dietary supplementation of MOS and FOS did not affect fecal concentration of BCFAs in adult dogs (Middelbos et al., 2007; Flickinger et al., 2003; Swanson et al., 2002). Increased levels of isobutyrate, isovalerate, valerate, and total BCFA were seen after FOS and pectin supplementation for adult cats (Barry et al., 2010). These data may be due to the fact that cats are strict carnivores, have a higher consumption of dietary protein, and generally a higher population of proteolytic bacteria (clostridial species) in their hindgut.

In the current study indole was detected in very few samples. Fecal concentrations of 4-ethylphenol were not affected by treatment and p-cresol tended to be lower for the diets containing higher levels of barley. Lower numerical P-cresol concentration was reported for dogs fed beet pulp and 1.5% FOS plus 1% cellulose, however, the absolute values reported were, approximately, 10 fold lower than ours (Middelboss et al., 2007). No differences in biogenic amines were observed among dietary treatments, which agree with most of the canine literature that looked at the effect of fermentable fibers on these compounds (Middelbos et al., 2007; Flickinger et al., 2003; and Swanson et al., 2002). Recently, Faber et al. (2011) suggested that increased levels or lack of change in amine concentration, may be viewed as beneficial to colonic health, since some of the biogenic amines (e.g., putrescine, spermine, and spermidine) can modulate apoptosis and cell turnover processes.

Postprandial Glycemic and Insulinemic Responses and Fasting Plasma

Cholesterol Concentration

The health benefits associated with the consumption of β-glucans have been well documented and accepted by FDA, allowing products containing 0.75g of β-glucans per serving to claim health benefits against heart disease (FDA, 1997). The mechanism for the beneficial effects of barley consumption have been related to increased digesta viscosity in the gastrointestinal tract and decreased gastric emptying rate, which leads to a lower absorption rate of glucose and to a increased excretion of cholesterol and dietary fat (Lia et al., 1995). However, in this study no beneficial effects in postprandial glycemic and insulinemic responses were observed with incremental inclusion of barley. Factors that might have prevented our ability to detect differences among treatments could be related to the high variation in treatment response among dogs and the amount of β-glucan supplemented in the test diets. In barley (*Hordeum vulgare L.*), the amount of β-glucans can be affected by varietal and environmental conditions (Stuart et al., 1988). Average β-glucan content in barley has been reported to be approximately 4.2% (NRC, 2006). Based on this value, the average daily intake of β-glucan of dogs consuming the BA10, BA20, and BA40 diets would be 0.9, 1.9, 3.8 g/d, respectively. Thondre et al. (2009) suggested 4 g/d of dietary β-glucan as an ideal dose to reduce glycemic response. In contrast, a 12% inclusion of β-glucan was needed before improvements in postprandial glycemia and insulinemia were observed (Ostman et al., 2006). Although, the estimated β-glucan concentration in the BA40 diet was close to the optimal dose proposed by Thondre et al. (2009). The same authors also acknowledge that the amount of β-glucan to lower glycemic response may differ among various food

products. Therefore, additional research is required to determine the optimal level of barley β-glucan to lower glycemic and insulinemic responses in dogs, as well as to verify the effects of food processing (e.g., extrusion, canning, and baking) and diet matrix on the chemical behavior of barley β-glucans. It has been reported differences in postprandial glycemia from subjects receiving liquid and solid supplementation of barley β-glucans (Thondre et al., 2009; Panahi et al., 2007; Poppitt et al., 2007). Different responses in postprandial glycemia and in product viscosity also have been observed after β-glucans in barley have been processed using enzymatically or aqueous methods, with the former being the most efficacious to increase viscosity (Panahi et al., 2007). Other researchers have also mentioned blunted effects of β-glucans on glucose and insulin responses in diets that already have a lower glycemic response, e.g., pasta vs. bread (Bourdon et al., 1999; Jarvi et al., 1995). It is also possible that other nutrients in the diet may influence postprandial glycemic response, for example, it has been suggested a positive synergistic effect of the dietary amylose and β-glucan contents (Bourdon et al., 1999). In the current study, the CO diet used corn as the primary carbohydrate source. Corn is rich in amylose, which could have made more difficult to detect difference in postprandial responses among our treatments.

In the current study, fasting plasma cholesterol concentrations were within normal physiological range reported for adult healthy dogs, 3.6-5.4 mmol/L (Jones and Manella, 1990). The lack of response in fasting plasma cholesterol concentration by incremental levels of dietary barley could possibly be explained by the need of a higher consumption of β-glucan, or the requirement of a longer experimental period, or the use of a hypercholesterolemic model. Dietary supplementation of 6 g of oat β-glucans decreased

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total cholesterol and low density lipoprotein (LDL) levels in humans suffering from hypercholesterolemia (Queenan et al., 2007).

Overall, the inclusion of barley up to 40% in diets for adult dogs seems to cause no adverse effect on food intake or nutrient digestibility, which indicates that barley, is an adequate carbohydrate source to be used by the pet food industry. In addition, inclusion of barley led to an increased fecal concentration of acetate, proprionate and total SCFA, which may be beneficial in promoting or maintaining gut health in these animals. In the current study, however, no change was observed with dietary supplementation of β-glucans from barley on most of fecal metabolites from fermentative processes, on the attenuation of postprandial response of glucose and insulin, and on fasting plasma cholesterol concentration as it has been reported in other models (Behall et al., 2006; Wood et al., 1994; Braaten et al., 1991; Queenan et al., 2007; Anderson et al., 1990). Nonetheless, further research in this field is warranted before these nutraceutical effects of barley β-glucans are ruled out in the canine model.

Table 5. 1. Ingredient and analyzed nutrient composition of experimental diets.

	Treatments			
	Control	Barley 10	Barley 20	Barley 40
Ingredients, % as fed basis				
Corn yellow whole	40.00	30.00	20.00	0.00
Corn starch	27.82	27.82	27.82	27.82
Poultry meal low ash	19.94	19.94	19.94	19.94
Barley cracked	0.00	10.00	20.00	40.00
Pork fat	8.53	8.53	8.53	8.53
Cellulose	1.00	1.00	1.00	1.00
Chicken liver digest	1.00	1.00	1.00	1.00
Sodium chloride, iodized.	0.75	0.75	0.75	0.75
Potassium chloride	0.62	0.62	0.62	0.62
Choline chloride dry60	0.15	0.15	0.15	0.15
Vitamin premix 2352	0.06	0.06	0.06	0.06
Calcium carbonate	0.06	0.06	0.06	0.06
Mineral premix 2305 [a]	0.04	0.04	0.04	0.04
Antioxidant naturox plus	0.04	0.04	0.04	0.04
Nutrients, DM basis				
Crude protein, %	19.0	19.3	19.6	20.2
Acid hydrolized fat, %	15.0	14.8	14.7	14.3
Ash, %	4.5	4.6	4.6	4.7
Gross energy, Kcal/ kg	4,731.2	4,741.4	4,666.2	4,748.2
Total dietary fiber, %	9.7	10.2	10.8	11.7
Ca, %	0.6	0.6	0.6	0.6
P, %	0.5	0.5	0.6	0.6
K, %	0.7	0.7	0.7	0.7
Na, %	0.4	0.4	0.4	0.4
Cl, %	1.0	1.0	1.0	1.0
Mg, ppm	0.1	0.1	0.1	0.1
Zn, ppm	168.1	168.8	169.6	171.1
I, ppm	1.8	1.8	1.8	1.7
Se, ppm	0.5	0.5	0.5	0.5

[a] Formulated to supply at least (g/kg of food): 0.6 Mg, 1.8 Na, 7.0 K, 7.6 Cl, (mg/kg of food) 211 Fe, 163 Zn, 13 Cu, 13 Mn, 0.4 Se, 1.5 I (IU/g of food) 18.2 Vitamin A, 1.0 Vitamin D, 0.18 Vitamin E (mg/kg of food) 0.3 Biotin, 1484 Choline, 1.9 Folic acid, 62 Niacin, 18 Pantothenic acid, 8.6 Pyridoxine, 8.0 Riboflavin, 41 Thiamin, and 0.13 Vitamin B12

Table 5. 2. Nutrient intake and apparent digestibility of total dietary fiber (TDF) and acid hydrolyzed fat (AHF) and fecal score for dogs fed control or barley-containing diets.

	Treatment						
Item	CO	BA10	BA20	BA40	SEM	*Linear*	*Quadratic*
Intake, g/d							
Dry matter	214.8	217.6	219.3	227.8	6.30		
Total dietary fiber	20.8	22.2	23.7	26.7	0.79	0.0190	0.0001
Acid hydrolyzed fat	32.3	32.0	32.2	32.5	0.93	0.3004	0.2425
Excretion, g/d							
Dry matter	26.0	26.5	30.3	26.8	2.86	0.6144	0.5302
Total dietary fiber	10.0	10.0	10.7	8.5	1.24	0.7427	0.4809
Acid hydrolyzed fat	1.4	1.4	1.9	2.0	0.32	0.7428	0.0602
Apparent digestibility, %							
Dry matter	87.2	87.4	86.3	87.9	1.59	0.8765	0.9405
Total dietary fiber	49.9	54.3	56.0	67.7	7.64	0.5868	0.0303
Acid hydrolyzed fat	95.5	95.5	94.0	94.0	0.93	0.6977	0.0344
Fecal score – 48h	2.9	2.8	2.9	3.0	0.11	0.2847	0.3775
Fecal score – 15d	2.8	2.9	2.8	2.9	0.05	0.4232	0.2756

SEM=pooled SEM, n =16.

Table 5. 3. Nitrogen metabolism of dogs fed control (CO) or barley-containing diets.

Item	Treatments				SEM	*Linear*	*Quadratic*
	CO	BA10	BA20	BA40			
Food N Intake, g/d	6.8	6.9	7.6	7.4	0.22	0.0116	0.0001
Fecal N, g/d	1.3	1.2	1.4	1.4	0.15	0.8865	0.2559
Urine N, g/d	4.3	4.6	4.2	4.8	0.31	0.7049	0.6860
N Absorbed, g/d	5.5	5.6	6.3	6.1	0.24	0.2739	0.0069
N Digestibility, %	80.7	81.5	81.2	80.8	2.37	0.7527	0.9013
N Retained, g/d	1.3	0.9	1.8	1.2	0.23	0.6610	0.0524
N balance, %	18.8	11.8	25.3	15.5	2.97	0.2317	0.2668

SEM=pooled SEM, n =16.

Table 5. 4. Concentrations (mmol/g, DM basis) of fecal short-chain (SCFA) and branched-chain fatty acids (BCFA) for dogs fed a control (CO), or barley-containing diets.

	Treatment						
Item	CO	BA10	BA20	BA40	SEM	*Linear*	*Quadratic*
SCFA							
Acetate	0.23	0.22	0.25	0.29	0.023	0.7431	0.0364
Propionate	0.12	0.12	0.14	0.18	0.013	0.7604	0.0004
Butyrate	0.06	0.05	0.05	0.06	0.008	0.1996	0.9830
Total SCFA	0.40	0.39	0.43	0.53	0.042	0.7690	0.0203
BCFA							
Isobutyrate[1]	0.01	0.01	0.01	0.01	0.001	0.3048	0.7780
Isovalerate[1]	0.01	0.02	0.01	0.01	0.002	0.3140	0.1425
Valerate	0.002	0.002	0.001	0.002	0.0005	0.1624	0.8342
Total BCFA[1]	0.022	0.028	0.033	0.026	0.0641	0.2004	0.7924

[1] Mean values reported are from not transformed data. SEM=pooled SEM, n =16.

Table 5. 5. Fecal score, concentrations (DM basis) of fecal 4-ethylphenol, p-cresol and biogenic amines for dogs fed control (CO) or barley-containing diets.

Item	Treatment				SEM	*Linear*	*Quadratic*
	CO	BA10	BA20	BA40			
4-ethylphenol, ug/g[1]	50.0	148.9	78.2	42.4	48.09	0.4206	0.5105
P-cresol, ug/g[1]	336.6	398.1	256.3	270.1	44.25	0.6154	0.0599
Biogenic amines, mg/g							
Agmatine [1]	0.5	0.2	1.1	0.4	0.21	0.4866	0.1983
Cadaverine[1]	2.6	1.7	6.7	3.5	1.51	0.9059	0.3655
Putrescine	2.3	2.0	3.1	2.3	0.44	0.9919	0.3584
Spermidine	1.0	1.1	0.9	1.3	0.23	0.9592	0.5208
Spermine	1.0	1.2	0.7	0.9	0.23	0.8433	0.2075
Tryptamine[1]	1.0	0.7	1.5	0.4	0.49	0.8073	0.6633
Total[1]	8.4	6.7	14.6	8.6	2.75	0.8974	0.3452

[1] Mean values reported are from not transformed data. SEM=pooled SEM, n =16.

Table 5. 6. Postprandial glycemic and insulinemic responses, and fasting plasma cholesterol levels of dogs fed control (CO) or barley-containing diets.

Item	Treatment				SEM	*Linear*	*Quadratic*
	CO	BA10	BA20	BA40			
Incremental Glucose AUC	192.1	177.6	218.2	181.9	49.24	0.9658	0.7492
Decremental Glucose AUC	97.5	34.3	183.9	45.15	96.42	0.6745	0.9996
Insulin AUC	363.8	594.6	548.0	546.5	115.44	0.1576	0.6878
Cholesterol, mmol/L	4.3	4.4	4.3	4.3	0.15	0.3800	0.5338

SEM=pooled SEM, n =16.

CHAPTER 6: SUMMARY AND CONCLUSIONS

Nowadays, the role played by pets in the American household is very different from when dogs were first domesticated. In the course of these 14,000y of domestication, the dog moved from being a working animal (e.g., hunting, herding, protection, etc.) to become Men's best friend. In this process, a strong emotional human-animal bond was developed and the dog assumed a pivotal role in the families' systems. Pets became a source of emotional and psychological support, were brought inside of the homes and started sharing the same environment, food, and life style as of their owners. Ironically, in this process many human health maladies have also been manifested in pets. Among them, obesity and other chronic diseases such as: diabetes mellitus, hyperlipidemia, arthritis, etc.

In order to address or to prevent these health issues, human and pet populations have been encouraged to modify their eating habits and increase their physical activity. All of which, have shown low success rates as many of these ailments still increasing in both populations. An alternative strategy has been the modification of dietary nutrient composition. Different lipid and carbohydrate sources have shown to be beneficial in ameliorating some of these chronic health conditions. Among them fish oil and β-glucans have received considerable attention in human nutrition.

The overall objectives of this research were to evaluate how different nutritional approaches, lipid or carbohydrate dietary modification by the use of fish oil or barley (rich in β-glucans), would affect several parameters related to lipid, protein, and carbohydrate metabolism, which could be an useful tool during the management or in the prevention of chronic diseases such as, hyperlipidemia and insulin insensitivity, that

affect a large portion of the canine pet population. In the first experiment, the effect of feeding a fish oil containing diet on lipid and protein metabolism, postprandial glycemia, and body weight in mature overweight dogs was examined. Seven female dogs were randomly assigned to one of two isonitrogenous and isocaloric diets, control (CO) or fish oil (FO), in a crossover design. Overall, the FO diet seemed an effective strategy to decrease cholesterol and might improve glucose clearance rate in mature overweight dogs.

Similarly to the previous experiment, the second experiment aimed to assess how fish oil supplementation may affect lipid and protein metabolism, postprandial glycemia, and body weight in young lean adult dogs. In this study, eight female Beagles were randomly assigned to one of two isonitrogenous and isocaloric diets, control (CO) or fish oil (FO), in a crossover design. Feeding FO containing to young adult dogs showed a protective effect against the rise of plasma CHOL and it increased plasma ghrelin levels. However, it did not appear to improve protein metabolism or postprandial glycemia in adult lean dogs.

A different nutritional approach was used in the third experiment, which had the objective to examine the effects of incremental levels of barley on apparent nutrient digestibility, fecal short chain fatty acid concentrations, fecal quality and odor, postprandial glycemic and insulinemic responses, and plasma cholesterol levels in adult dogs. Sixteen female dogs; eight purpose-bred mature female dogs and eight female Beagles were randomly assigned to four experimental diets; control (40% corn) or three levels of barley (10, 20, 40%). The data gathered in this experiment suggested that inclusion of barley up to 40% in diets for adult dogs was well tolerated and did not

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negatively impact nutrient digestibility of the diets. In addition, dietary supplementation of barley might be beneficial in promoting gut health due to increased production of SCFA at this site; as suggested by the increased fecal concentration of these compounds. However, inclusion of barley (at the levels tested in this study) did not influence aspects related to fecal odor, postprandial glycemia, and plasma cholesterol.

In conclusion, the body of research presented herein suggests that different nutritional strategies - dietary lipid or carbohydrate manipulation - may be beneficial in ameliorating health issues (e.g., hyperlipidemia) or in improving the health status of dogs (e.g., gut health by increased SCFA production). In addition, no deleterious effects in nutrient digestibility were observed due to the incorporation of barley (up to 40%) or fish oil (2%) in the diets fed to adult dogs. The data gathered in this dissertation offer valuable information for the pet food industry, as well as for pet owners seeking for novel nutraceutical ingredients as a mean to enhance the health and longevity of their companion animals or to aid in the management of common maladies noted in today's pet population: hyperlipidemia, insulin resistance, diabetes mellitus, among others. Further research is necessary to determine optimal levels of supplementation of fish oil and barley to improve glycemic response and insulin sensitivity in the canine models and to advance our understanding on the metabolic processes involved in the onset of these chronic diseases and to determine how and when they can be best addressed by nutritional intervention. Future discovery of a broader array of nutraceutical ingredients in the prevention or control of these chronic health issues would help not only to increase the longevity and life quality of our beloved pets, but also of their owners as dogs have been a well accepted and utilized experimental model for humans.

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EXTRACURRICULAR ACTIVITIES

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