EFFECTS OF DIETARY MACRONUTRIENT PROFILE AND FEEDING FREQUENCY ON CIRCULATING METABOLITES, POSTPRANDIAL ENDOCRINE RESPONSE, AND VOLUNTARY PHYSICAL ACTIVITY OF HEALTHY ADULT CATS

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DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Sciences in the Graduate College of the University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

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ABSTRACT

For many years, the incidence of obesity has been one of the most common health issues in domestic cats. Obesity is a risk factor for the development of insulin resistance and type II diabetes mellitus. The prevalence of highly palatable commercial pet foods, a sedentary indoor lifestyle, and inappropriate feeding strategies largely contribute to obesity in cats. This dissertation focused on diet composition and feeding strategies to understand how dietary factors may affect appetite regulation, metabolism, and energy expenditure of cats and in the development of weight-loss programs. Herein, six studies were designed to address two major research objectives: 1) to evaluate how dietary macronutrient composition influences circulating metabolites and appetite-regulating hormones in healthy adult cats; and 2) to investigate whether different feeding strategies promote voluntary physical activity in healthy adult cats. Because very little is known regarding appetite-regulating hormone concentrations in feline plasma and their relationship with feeding frequency, Study 1 was designed to monitor daily fluctuations of circulating glucose, insulin, total ghrelin, and leptin concentrations in cats fed 2 or 4 meals per day. Results from this study suggested that cats fed 4 meals daily had greater leptin incremental area under the curve (AUC) \(0-24\) and less variable concentrations of glucose and insulin compared to cats fed 2 meals. Study 2 was a macronutrient bolus experiment, similar to a glucose tolerance test, to measure the acute response of a single macronutrient dose on postprandial glucose, insulin, total ghrelin, and leptin concentrations in healthy adult cats. Circulating hormone data were highly variable and
indicated changes due to dietary macronutrients. We observed that a carbohydrate bolus increased glucose and insulin incremental AUC\textsubscript{0-6h}, and tended to increase leptin net AUC\textsubscript{0-6h}. Study 3 evaluated the effects of three isoenergetic diets rich in protein, fat, or carbohydrate (vs. a control diet with balanced macronutrients) on fasting and postprandial glucose, insulin, total ghrelin, and leptin concentrations in healthy adult cats. We reported that cats fed control and high-carbohydrate (HC) diets had greater glucose incremental AUC compared to those fed the high-fat (HF) and high-protein (HP) diets. Study 4 used a high-throughput metabolomics approach to characterize fasting blood metabolite profiles of cats fed various dietary macronutrient concentrations. Cats fed HP, HF, and HC diets formed distinct metabolite profiles and three potential biomarkers, gamma-glutamylleucine, 3-hydroxyisobutyrate, and 3-indoxyl sulfate were identified to distinguish cats fed the three macronutrient-rich diets. This study revealed that cats fed the HP diet had decreased metabolites associated with nucleotide catabolism, but increased metabolites associated with amino acid and fat metabolism for energy, as well as increased metabolites associated with gut microbial metabolism. Cats fed the HF diet had increased metabolites associated with lipid metabolism and oxidative status. Besides the diet, appropriate feeding strategies to promote physical activity have been suggested as a key contributing factor to manage body weight (BW), but research is lacking. Study 5 evaluated the effects of feeding frequency on voluntary physical activity in cats fed to maintain BW. Our data suggested that increased meal frequency promoted overall physical activity by increasing the food anticipatory activity (FAA). Study 6 evaluated the effect of increasing dietary water content without changing energy intake on
voluntary physical activity in cats. Our results suggested that increased dietary water content also increased daily physical activity, but appeared to occur by increasing the physical activity level after meals. Manipulating dietary macronutrient composition and designing appropriate feeding strategies appear to impact physiological and physical outcome variables in the cat.
This dissertation is dedicated to my grandfather, Jianbing Zhang, my husband, Ye Yuan, and my family in China
ACKNOWLEDGEMENTS

I thank my advisor and mentor, Dr. Kelly Swanson, for giving me the opportunity to pursue a doctoral degree in companion animal science, for his patience, encouragement, and for his constant guidance and support. He gave me confidence and inspired me to pursue a career in companion animal science. He helped me solve research problems, revised my numerous writing drafts, and always kept me on track. I will never have enough words to thank him.

I also thank my committee members, Drs. George C. Fahey, Jr., Kelly A. Tappenden, and Thomas K. Graves, for taking their time to review my dissertation and assist me with my research. I especially thank Dr. Thomas K. Graves and Tonya K. Ridge for their support on the catheter surgery and sample collections.

I sincerely thank all the members of the Swanson-Fahey laboratory group for their help throughout the animal trials and laboratory analyses for my research, along with their friendship. It would not have been possible to complete those studies without their help.

Finally, I thank my family for their endless support and love. I miss my grandfather who inspired me in my childhood and seeded my dream to be a scientist. Special thanks to my Aunt Shu and Uncle Huiyi, who inspired me to study in the area of animal nutrition. I thank my parents for always encouraging me when I fail and cheering for me when I succeeded. I especially thank my husband, Ye, for always standing by my side and giving me his understanding, support, and love.
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CHAPTER 1: LITERATURE REVIEW

Introduction

Obesity is one of the most common diseases and is a key risk factor for many comorbidities in humans and companion animals, including insulin resistance and type II diabetes mellitus. The prevalence of obesity in the United States has dramatically increased over the past few decades. Data from the National Health and Nutrition Examination Survey (2009–2010) showed that more than one third of U.S. adults were considered obese and two thirds were classified as overweight or obese (Flegal et al., 2012). Not surprisingly, the incidence of obesity in the dog and cat population is also increasing. An investigation in 31 private veterinary hospitals in the Northeastern United States in 1994 revealed that 29% of 2000 cats studied were overweight or obese (Scarlett et al., 1994). Nine years later, another investigation in private veterinary hospitals in the United States reported 35% of more than 8000 adult cats studied were observed to be overweight or obese (Lund et al., 2005). The latest nationwide survey conducted by the Association for Pet Obesity Prevention in 2011 reported that 55% of cats were classified as overweight or obese by their veterinarians. Moreover, the prevalence of feline diabetes mellitus reported by veterinary hospitals increased from eight cases per 10,000 in 1970 to 124 cases per 10,000 in 1999 (Prahl et al., 2007). Besides genetic factors, diet, age, neutering, and inactivity are highly associated with obesity and diabetes (Rand et al., 2004; Prahl et al., 2007).
In humans, large research initiatives pertaining to all aspects of obesity development have been supported by federal funding agencies. This research has provided an enormous amount of information on the metabolic and hormonal changes that occur with obesity, which may help in developing successful management, treatment, and prevention strategies against obesity. In companion animals, however, especially in cats, the fundamental knowledge in this area is very limited. Cats, as true carnivores, relied on high-protein animal tissue to meet their specific nutritional requirements in the wild and are metabolically adapted to a lower glucose utilization and higher protein metabolism (MacDonald et al., 1984). The unique metabolic needs of cats underscores the importance of conducting metabolic and endocrine studies in this species to increase our understanding and develop more specialized strategies for weight management in cats.

**Weight management in cats**

Obesity often is considered to be a result of energy imbalance, either excessive food intake or insufficient physical activity, which results in a state of positive energy balance. A successful weight maintenance or weight loss program should include dietary management with proper feeding strategies, increased exercise, and behavioral enrichment. To be successful, veterinarians and pet food professionals need to educate pet owners to increase their awareness of companion animal obesity as a serious health concern and the risk factors for developing obesity.
Determination of obesity

Obesity is considered as an accumulation of excessive body fat. In humans, diagnosis of overweight or obesity is usually based on an indirect measurement of body fat mass. The most common method is by calculating the body mass index (BMI), which only requires the measurements of height and weight \[ \text{weight (kg)/height (m)}^2 \]. A BMI greater than 25 is considered overweight and above 30 is considered obese as defined by the World Health Organization (WHO).

In companion animals, one report classified dogs and cats as overweight when their body weight (BW) was 15% above their ‘optimal BW’ and as obese when their BW exceeded 30% of optimal (Burkholder and Toll, 2000). However, limited data or epidemiologic studies exist to confirm or standardize the concept of an optimal BW. Therefore, various techniques are available to measure body composition to evaluate obesity in dogs and cats, including body condition scoring (BCS), morphometric measurements, ultrasound, electrical conductance, dual energy X-ray absorptiometry (DEXA), and magnetic resonance imaging (MRI). Among these, BCS is a simple and economical way for pet owners and clinicians to monitor weight status. A 9-point BCS system developed by Laflamme (1997) is commonly used in dogs and cats. This approach has been demonstrated to be highly correlated with DEXA analysis of body fat percentage (German et al., 2006). Body condition score is a good starting point for pet owners’ involvement in BW maintenance of their pet.
Morphometric measurement is another method to analyse body composition which refers to various measured criteria including BMI, skin fold thickness, and ultrasound technique. These measurements are more objective to determine body fat mass of cats than BCS. Taking the BMI as an example, it is determined by measuring the rib cage circumference at the level of the 9th cranial rib and the leg index measurement (LIM, the distance from the patella to the calcaneus). Then the percent body fat can be calculated as the equation: [(ribcage/0.7067-LIM)/0.9156]-LIM (Butterwick, 2000).

DEXA is a sensitive method of determining body fat mass, bone mineral content, and lean body mass which is more often used in research studies. Speakman et al. (2001) validated DEXA to measure body composition by comparison to chemical analysis in cats. They reported that the DEXA estimates were strongly correlated with estimates derived from chemical analysis: total body mass (r = 1.00), lean tissue mass (r = 0.999), body water content (r = 0.992), and fat mass (r = 0.982). Although, on average, DEXA compared very well to chemical analysis, variables may exist including the fat content of skeletal muscle, the lean content of mesenteric fat, and skeletal muscle hydration.

Risk factors for obesity

Increasing awareness of risk factors for feline obesity by pet owners is as important as being alert to obesity itself. Feline obesity prevalence is influenced by numerous factors including genetics, neuter status, age, breed, indoor environment with inactivity, and diet (excessive food intake, incorrect food selection, or inappropriate feeding strategies). For example, many studies have confirmed that neutering is an
important risk factor for developing obesity, with neutered cats being 3 times more likely to be overweight or obese than intact cats, which may be due to a decrease in metabolic rate after neutering (Root et al., 1996; Harper et al., 2001; Vester et al., 2009). Middle-age cats (5-11 yr) are also considered as a higher risk population of developing obesity (Lund et al., 2005). Several breeds, including domestic shorthairs and Manx, were reported to have a higher incidence of obesity (Lund et al., 2005). Slingerland et al. (2009) reported that indoor confinement and low physical activity were significantly correlated with the development of obesity-induced type II diabetes. Dietary factors can be one of the most critical factors to lead to the development of feline obesity. Moreover, Kienzle et al. (2000) reported that obese cats more commonly had a free choice of food.

Dietary management

Successful dietary management may not be as simple as feeding a cat a lower amount of its current diet. Not only will that likely increase hunger, but the intake of all nutrients will be decreased, possibly below their required concentrations. Therefore, it is important to establish a weight-loss diet that has been formulated to have a reduced caloric density, and increased protein content and other essential nutrients. According to Association of American Feed Control Officials (AAFCO), “low calorie” or “light” dry kibble diets is defined to contain no more than 3250 kcal ME/kg, however, there is no legal definition of a “high protein” diet. It also may be wise to include low-glycemic-index ingredients to prevent insulin resistance, low dietary fat (no more than 10% crude fat for dry kibble diets containing less than 20% moisture) to decrease caloric density, as
well as including some functional components (e.g., L-carnitine and conjugated linoleic acid).

Dietary protein has several effects that benefit weight loss. Increased protein percentage is important to prevent or minimize the loss of lean mass during weight loss (Vasconcellos et al., 2009). Laflamme and Hannah (2005) demonstrated conservation of lean mass during weight loss in cats fed a diet containing 45% metabolizable energy from protein. High-protein diets also may stimulate satiety or have thermogenic effects, although this effect has been studied very little in cats (Halton and Hu, 2004). Burkholder and Toll (2000) recommended that cat foods for weight loss should contain at least 35% crude protein (dry matter (DM) basis). Not only is the amount of protein important in protecting against loss of lean body mass during weight loss, so is the protein quality. Dietary protein stimulates postprandial thermogenesis and protein turnover. Hoenig et al. (2007) reported that feeding a high-protein diet (27.5% of protein) led to greater heat production in lean cats. Less net energy was reported available when animals consumed high-protein (39.1% of protein) compared to control (30% of protein) diets (Laflamme and Hannah, 2005). The heat generated during the postprandial period was approximately 68% greater than that generated from carbohydrate sources (Robinson et al., 1990). Therefore, when an animal consumes protein, it burns more energy (more heat), which appears to be associated with increased protein turnover (increased protein synthesis).

Fat contains more than twice the calories per gram of protein or carbohydrate. Increasing dietary fat intake is a risk factor for inducing obesity and generally are not
considered optimum for a weight loss diet. Water provides another approach for diluting calories of the food. Canned foods generally contain between 70% and 82% water and have a lower energy density (as is basis) as compared with dry foods. However, canned foods typically have a higher fat and calorie content on a DM basis and are more palatable. Controlling the provided portion is the key to select a canned food to manage overweight cats.

Several human studies have highlighted the benefits of dietary fiber, especially soluble fiber, which resulted in decreased postprandial hyperglycemia (Chandalia et al., 2000), greater insulin sensitivity (Pereira et al., 2002), and altered gastrointestinal peptide release (Karhunen et al., 2010), while insoluble fiber can reduce energy density and may be associated with reduced food palatability (Nelson et al., 1998). Therefore, dietary fiber helps produce weight loss by diluting calories, increasing satiety, and limiting food consumption as a result of more bulk being present during its transit through the GI tract (Levine and Billington, 1994). Oats, barley, legumes, and by-products of fruit or vegetables are good sources of soluble fibers, providing β-glucans, pectin, gums, and other nondigestible polysaccharides. Insoluble fiber, including cellulose, hemicelluloses, and lignin, are found particularly in wheat, cereal fibers, and most plants. However, it must be noted that increased levels of dietary fiber will have noticeable effects on normal defecation habits. For example, Fahey et al. (1990) reported that dogs fed a meat-based diet supplemented with beet pulp (Total dietary fiber, TDF: 11.8%) or peanut hulls (TDF: 13.1%) had increases in the amount of fecal material and frequency of defecation compared to dogs fed the control diet (TDF: 6.1%). Burkholder and Toll (2000)
recommended that the range for dietary fiber content of cat diets used for weight loss is between 15 and 20% DM and after the weight loss, the range for weight maintenance is 6 and 15% DM.

Dietary supplementation of L-carnitine stimulates fat oxidation (Ibrahim et al., 2003; Wutzke and Lorenz, 2004) and increases weight loss without altering the fat to lean body mass ratio in cats (Ibrahim et al., 2003). Conjugated linoleic acid (CLA), derived from linoleic acid, is mainly found in the tissues and milk fat of ruminants. A study in pigs reported that CLA (5.0 g/kg) lowered body fat and increased lean body mass (Ostrowska et al., 1999). However, CLA, used as an antiobesity agent, did not show its effect in cats (Leray et al., 2006).

The future direction is to identify the relationship between dietary components and appetite regulation so that a weight maintenance or loss diet for cats can be developed to help control appetite and achieve energy balance.

*Feeding strategy*

In addition to diet, feeding strategies and exercise are critically important to successful weight management as well. Feeding strategies may include restricted feeding (rather than ad libitum), feeding multiple small meals (rather than a single large meal), and avoiding unnecessary snacks. However, research with appropriate study designs in this area has been lacking and very little research has been conducted to elucidate the mechanisms involved. It must be noted that even when BW is lost using more conventional caloric restriction, a considerable percentage of the loss comes from lean
tissues. This loss of lean body mass ultimately decreases an animal’s resting energy requirements. Therefore, the high incidences of unsuccessful weight loss or subsequent weight re-gain usually result from a mass-adjusted decrease in energy expenditure in cats during energy restriction (Villaverde et al., 2008). A recent study investigated the potential impact of feeding frequency on physical activity (Deng et al., 2011). However, strategies to increase physical activity have been lacking. Besides feeding strategies, pet owners should be encouraged to develop the routine of interactive play to enhance their cats energy expenditure.

Dietary macronutrients and appetite regulation

Appetite is regulated by a complex interaction of central and peripheral pathways. Circulating hormones are important signals communicating between the central and peripheral pathways, and interacting with neuronal signals to control energy intake. Some of these hormones such as ghrelin and leptin have been discovered only in the last 20 yr. The biological functions of these and other hormones such as insulin, cholecystokinin (CCK), incretin hormones, and peptide YY (PYY) are actively being studied in human and rodents. There is some evidence that dietary macronutrient composition can influence the release and circulating concentrations of these hormones, which could affect sensations of hunger, satiety and, ultimately, energy intake.

The central nervous system

In order to discuss how hormones regulate appetite, the central pathways are reviewed briefly. Specific areas in the hypothalamus and brainstem are important in
coordinating the signals in the pathway of the central nervous system (CNS). The arcuate nucleus (ARC) in the hypothalamus contains two main neuronal subsets for conveying information from circulating hormones to various regions of the brain. One group contains neurons expressing the orexigenic (stimulating appetite) peptides, neuropeptide Y (NPY), and agouti-related peptide (AgRP). The other group expresses anorexigenic (suppressing appetite) peptides derived from pro-opiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART). Generally, these two groups of neurons act in such a way that when one group is activated, the other is inhibited, thus promoting a net increase or decrease in energy intake (Schwartz et al., 2000). Other regions in the hypothalamus, including the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA), are also targets of these neurons and are involved in appetite regulation (Schwartz et al., 2000). Together with regions in the hypothalamus, regions in the brainstem, such as the nucleus of tractus solitarius (NTS) and area postrema (AP) (Schwartz et al., 2000), are all linked in a complex neuronal network, integrating and responding to circulating hormonal signals.

**Appetite-regulating hormones**

The discovery of ghrelin was reported by Kojima et al. in 1999. Ghrelin is predominantly secreted by endocrine cells in the stomach and is known to stimulate growth hormone release. It has been recognized as the only orexigenic hormone in the gastrointestinal tract, with concentrations peaking before a meal and decreasing postprandially (Tschop et al., 2001). In the central neutral pathway, ghrelin directly
stimulates the NPY/AgRP neurons and inhibits the POMC/CART neurons of the ARC to stimulate food intake (Gil-Campos et al., 2006). In rodents and normal weight humans, ghrelin release is macronutrient-specific. Notably, carbohydrates appear to be effective at suppressing ghrelin concentration (Monteleone et al., 2003). Fat appears to have a relatively weak ghrelin-suppressing capacity compared to carbohydrate and protein (Monteleone et al., 2003; Sanchez et al., 2004; Overduin et al., 2005). There is conflicting evidence as to the effect of dietary protein on ghrelin suppression. Protein has been reported to have prolonged suppression of ghrelin, suggesting that it is the most satiating macronutrient (Blom et al., 2006; Browen et al., 2006; Tannous et al., 2006). The prolonged suppression of ghrelin after protein intake might relate to the delayed gastric emptying of protein or the larger thermic effect coming from protein metabolism (Blom et al., 2006). Moreover, increased circulating concentrations of amino acids after protein intake stimulated hepatic gluconeogenesis to prevent hypoglycemia and, thus, increased satiety (Blom et al., 2006). However, there have been a few studies in humans suggesting that protein stimulated (Erdmann et al., 2004) or had no effect on (Moran et al., 2005) ghrelin concentration.

Leptin was discovered in 1994 and is a hormone mainly produced by adipose tissue and has effects on regulating energy balance (Zhang et al., 1994). In general, leptin functions to decrease food intake, increase energy expenditure, and modulate glucose and fat metabolism through central and peripheral pathways (Houseknecht et al., 1998). In the central pathway, leptin regulates food intake and energy expenditure primarily through inhibition of NPY/AgRP neurons and direct stimulation of POMC/CART neurons.
(Ahima and Flier, 2000). Leptin also acts peripherally. Leptin has diverse roles in the gastrointestinal tract (Yarandi et al., 2011) and affects insulin secretion (Houseknecht et al., 1998). Leptin is usually thought as a long-term regulator of BW. However, it was reported that the change in leptin concentrations observed under conditions of fasting and overfeeding were greater than that expected from the minor changes in adipose tissue stores (Weigle et al., 1997), indicating leptin action also occurs in the short term. Postprandial leptin concentrations have been reported to be dependent on dietary macronutrient composition, and high-carbohydrate, low-fat meals (81% of energy from carbohydrate, 1% of energy from fat) resulted in higher postprandial leptin concentrations compared to high-fat, low-carbohydrate meals (79% of energy from carbohydrate, 3% of energy from fat) in healthy humans (Romon et al., 1999). In addition, high-fat feeding (56% of energy from fat) resulted in leptin resistance and increased hypothalamic NPY concentrations when rats were fed the high-fat diet for five days (Lin et al., 2001). However, dietary protein has not been shown to impact postprandial leptin concentration (Groschl et al., 2003).

Insulin is produced by pancreatic β cells and is known as a major regulator of blood glucose concentrations. Many studies have demonstrated the actions of insulin in the hypothalamic feedback loop of energy balance regulation (Schwartz et al., 1992). Insulin resistance and hyperinsulinemia are well known characteristics of human (Smith, 1996) and canine or feline obesity (Hoenig, 2002). High fat-induced obesity (54% of energy from fat) in dogs decreased insulin accessibility to skeletal muscle, resulting in decreased insulin sensitivity (Ellmerer et al., 2006). In cats, it has been reported that
obesity decreases insulin sensitivity (Appleton et al., 2001) and weight loss corrects impaired insulin sensitivity and hyperinsulinemia in overweight cats (Hoenig et al., 2007). Even though a high-carbohydrate diet (protein 25%, fat 26%, carbohydrate 47%) resulted in a greater glucose response when compared to high-protein (protein 46%, fat 26%, carbohydrate 27%) or high-fat (protein 26%, fat 47%, carbohydrate 26%) diets in healthy cats, insulin secretion only tended to be increased (Farrow et al., 2002). In addition, it was reported that a high-protein diet (46% of protein) fed to lean and obese cats led to higher postprandial concentrations of amylin (co-secreted with insulin) than the other diets, indicating a possible effect of amino acids on pancreatic β-cell secretion (Martin et al., 2010).

Cholecystokinin (CCK) is secreted by I cells in the proximal small intestine and is known to promote nutrient digestion and to induce a negative feedback inhibition on appetite through the hypothalamus both directly and indirectly (Moran and Kinzig, 2004). Both pathways are mediated via CCKA receptors expressed on vagus nerves, the NTS, and circular muscle cells of the pyloric sphincter (Moran and Kinzig, 2004). Gastrointestinal release of CCK is macronutrient-specific, with fat and protein leading to greater postprandial concentrations than carbohydrates (Moran and Kinzig, 2004). Although fat is effective in increasing CCK release, it has been reported that high-fat, low-carbohydrate feeding reduced CCK-induced satiety in rats (Covasa and Ritter, 1998; Covasa et al., 2000). Moreover, there is evidence that high-fat, low-carbohydrate (58% energy from fat) diets may impair CCK-induced satiety through the down-regulation of CCK receptors in humans (French et al., 1995).
The ‘incretin effect’, whereby oral glucose elicits a much greater insulin response than an isoglycemic intravenous glucose infusion, has been attributed to two hormones: glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (Wu et al., 2010). Glucagon-like peptide-1 contributes to the regulation of glucose homeostasis in a number of ways, including increased insulin secretion, improvement of insulin sensitivity and peripheral glucose disposal, inhibition of gastric emptying, and suppression of glucagon secretion and inhibition of food intake. The actions of GIP on the pancreatic β-cell are similar to those of GLP-1 in healthy subjects, but diminished in diabetics. In contrast, GIP in a diabetic state promotes energy storage by direct action on adipose tissue and is likely to exacerbate insulin resistance. In healthy humans, GIP stimulates glucagon secretion under euglycemic conditions and has no effect on gastric emptying (Wu et al., 2010). Wu et al. (2010) reviewed a number of recent studies that resulted in fundamental insights into the influence of individual nutrients on incretin release and the mechanisms involved in the detection of carbohydrates, fats, and proteins by enteroendocrine cells, including the KATP channel, sodium-glucose cotransporter1 (SGLT1), G-protein-coupled receptors (GPRs), and oligopeptide transporter1 (PepT1). Dietary modification, namely, varying macronutrient composition, or the consumption of ‘preloads’ prior to a meal, represents a novel approach to manipulate the incretin response and thereby regulates glucose homeostasis in patients with type 2 diabetes (Wu et al., 2010).

Peptide YY (PYY) is co-produced with GLP-1 by endocrine L cells in the distal gastrointestinal tract. Peptide YY and GLP-1 have very similar biological functions,
suggesting that these two hormones complement each other (Huda et al., 2006). Peptide YY primarily inhibits food intake through reducing NPY secretion in the ARC (Huda et al., 2006). Its release is stimulated by macronutrient intake in proportion to energy content. There is evidence suggesting that PYY is particularly stimulated by fat intake compared to carbohydrate and protein meals with similar calorie content (Huda et al., 2006).

*Current research in cats*

Compared to the studies in humans and rodents, less research in cats was conducted to determine the effect of dietary macronutrients on appetite-regulating hormonal responses. These studies in cats are listed in Table 1-1.

*Meal frequency and energy balance*

In addition to diet composition, increased meal frequency has become a popular dietary approach in human weight management programs. Several epidemiological studies reviewed by Bellisle et al. (1997) reported a strong inverse relationship between people’s habitual frequency of eating and BW, leading to the suggestion that a ‘more frequency’ meal pattern may help manage weight. However, Palmer et al. (2009) reviewed 176 trials with eating frequency ranging from 1 to 9 eating occasions per day within a short period of time (2-8 wk). There was no relationship between the changes in body weight and varying eating frequency. Of the 176 studies identified, 25 relevant studies matched the criteria and only 10 of these were weight-loss interventions. Many of these trials were short-term studies with small sample sizes (7-19 subjects) and a wide
array of definitions of eating occasion. Therefore, it is difficult to discern whether eating frequency affects BW or energy regulation.

Previous research has focused on the effect of meal frequency on energy expenditure. Meal frequency appears to have no effect on daily energy expenditure in humans (Bellisle et al., 1997; Taylor and Garrow, 2001; Smeets and Westerterp-Plantenga, 2008). Therefore, recent studies have been more focused on the other side of the energy balance equation, namely appetite control and food intake, and how it may be affected by eating frequency. Appetite control is typically measured in 3 ways, including assessment of the perceived appetite or satiety sensations, measurement of calorie intake, and investigation of appetite-regulating hormones. These data suggested that increasing meal frequency leads to improvements in appetite control, including attenuated responses of perceived appetite or less daily fluctuations in glucose, insulin, and ghrelin (Speechly et al., 1999; Solomon et al., 2008; Leidy et al., 2010). Moreover, the reduction in meal frequency appears to negatively influence appetite control (Stote et al., 2007). However, conflicting data exist in the literature. Smeets and Westerterp-Plantenga (2008) reported that eating three meals compared with two meals per day increased feelings of satiety over a 24 h period in healthy women. They also observed an increased 24 h fat oxidation with increased meals.

Limited data exist regarding the impact of feeding frequency on circulating hormones and food intake by dogs and cats. Lubbs et al. (2010) evaluated the effect of feeding frequency on the daily patterns of glucose, insulin, ghrelin, and GLP-1 over a 24-
h period in healthy dogs. Eight dogs were randomly allotted to 1 of 2 treatments (fed once or twice daily) in a crossover design. After a 14-d adaptation period, blood samples were collected every 2 h for 24 h. Even though overall area under curves did not differ, the overall incremental concentrations of insulin and GLP-1 differed because of feeding frequency. To our knowledge, there are no data available for cats regarding how feeding frequency may impact appetite.

**Nutritional metabolomics**

Metabolomics can be defined as the comprehensive analysis of the whole metabolome (Goodacre et al., 2004). It provides an analytical platform to understand the global metabolic regulation of organisms. Compared with global gene expression and protein analysis that might only indicate the potential for physiological changes, metabolomics, including the global metabolite concentrations and their kinetic changes in cells, tissues, and organs, represents the end-point of physiological regulatory processes (Nicholson and Wilson, 2003). Nutritional metabolomics is applying this analytical platform to nutritional sciences to allow investigation of the complexity of interactions of all of the nutrients within complex biosystems. These developments are critical to facilitate transition of nutritional sciences from population-based to individual-based criteria for nutritional research, assessment, and management (Jones et al., 2012).

Small-molecular-weight metabolite profiling is achieved by several analytical methods along with multivariate statistical methods, bioinformatics, pathway mapping, and computational modeling. Nuclear magnetic resonance spectroscopy (NMR) and
liquid chromatography/gas chromatography mass spectroscopy (LC/GC MS) are the primary analytical platforms. Both NMR and MS data are coupled with multivariate data analysis and pattern recognition methods for biomarker discoveries.

Principal components analysis (PCA) and random forest analysis (RF) are the most common multivariate methods for clustering, classifying, and modeling metabolomics data sets. PCA is usually used as a starting point for clustering the metabolomics data by visualizing the biological datasets based on the inherent similarity/dissimilarity of samples regarding their biochemical compositions with relation to physiological variations. It provides an overview of the information hidden behind the data. RF analysis enables estimation of how well individuals can be classified in the dataset into each dietary group (Breiman, 2001). To determine which variables (metabolites) make the largest contribution to the classification, a “Mean Decrease Accuracy (MDA)” measure is computed. The MDA is determined by randomly permuting a variable, running the observed values through the trees, and then reassessing the prediction accuracy. If a variable is important to the classification, the prediction accuracy will drop after such a permutation. Thus, the RF analysis provides an importance rank ordering of metabolites. In addition to these two multivariate methods, partial least-square discriminant analysis (PLS-DA) and its variant, the orthogonal partial least-square discriminant analysis (O-PLS-DA) are also commonly used, but a priori knowledge to the class of the samples is required. They provide a means to filter out metabolic information that is not correlated to the predefined class relationships (Trygg and Wold, 2002). PLS-DA loading is similar to PCA that yield information on which
spectral signals are associated with the observed clustering, giving a basis for metabolic interpretation.

**Metabolomics application in humans**

Metabolomics provides a wide range of applications in biomedical research. One of the applications is to identify biomarkers to predict or detect the stages of disease. Gall et al. (2010) conducted a metabolite profiling study in a cohort of 399 nondiabetic subjects to search for a novel early biomarker of insulin resistance and impaired glucose regulation. Fasting plasma samples from 399 subjects were analyzed using a mass spectrometry, non-target method, with 485 biochemicals measured. They performed a random forest analysis to assess the ability to classify subjects as insulin-sensitive or insulin-resistant. They found the organic acid, $\alpha$-hydroxybutyrate ($\alpha$–HB), was the top-ranked metabolite contributing to the separation of insulin-sensitive or insulin-resistant subjects, which can be used as an early marker for insulin resistance and type II diabetes. Suhre et al. (2010) used a multiplatform analytical approach to investigate the metabolic footprint of diabetes. Overnight fasted plasma samples were collected from 40 subjects with type II diabetes and 60 randomly age healthy subjects. They incorporated two analytical platforms: ultra-high performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS2) and GC/MS. Their study depicted the promising potential of metabolomics in diabetes research by identification of a series of dysregulated metabolites that are associated with diabetes. Key observations included perturbations of metabolic pathways linked to kidney dysfunction (3-indoxyl sulfate),
lipid metabolism (glycerophospholipids, free fatty acids), and interaction with the gut microflora (bile acids). These studies suggested that early metabolic markers hold the potential to detect diabetes-related complications under sub-clinical conditions in the general population.

Focusing on nutrition, metabolomics is a useful tool for assessing nutritional status, food consumption, and the biological consequences of a nutritional intervention of an individual. It is also useful in studying metabolic mechanisms in response to dietary intervention depending on a particular metabolic phenotype. Population studies have suggested that food plays an important role in influencing urinary metabolic profiles (Lenz et al., 2004; Dumas et al., 2006). Some initial investigations have applied a metabolomic approach to assess metabolic changes to a particular food item (e.g., vitamin E, isoflavones, cocoa) in humans and rodent models (Griffin et al., 2002; Solanky et al., 2005; Walsh et al., 2006; Llorach et al., 2009). Later, there have been studies designed to investigate the effect of complex dietary modulation on human metabolic phenotype. Stella et al. (2006) applied NMR-based metabolomics to characterize the effects of vegetarian, low-meat (60 g/d meat), and high-meat (420 g/d meat) diets on the urinary metabolic profile in twelve healthy men (age range of 25-74 yr; BW range of 60-91 kg) using a crossover design. Each dietary period lasted 15 d and three 24-h urine collections were performed after the subjects had consumed each diet for 10 d. All food was provided and carefully controlled throughout the study. After consuming the high-meat diet, subjects had higher urine concentrations of creatine, carnitine, acetyl carnitine, and trimethylamine-N-oxide (TMAO), all being proposed by
the authors to be the potential biomarkers of meat intake. Subjects consuming a vegetarian diet had higher concentrations of p-hydroxyphenylacetate (a microbial mammalian co-metabolite) and a lower concentration of N, N, N-trimethyllysine, suggesting an alteration in the bacterial composition or metabolism in response to diet.

Applying metabolomics technology to nutritional science may be difficult due to high variations within and among individuals over time. Therefore, studies must be designed with enough power to differentiate metabolic profile changes resulting from dietary changes vs. those from normal physiologic variation. Walsh et al. (2006) investigated the acute effect of standardizing diet on reducing metabolic variation in healthy humans. Urine, plasma, and saliva samples were collected from 30 healthy participants (23 females and 7 males; age range of 19-31 yr; BMI range of 18.5-30 kg/m²) after standardizing all food consumed on the day before collection. The inter-individual metabolic variability was analyzed by NMR-based metabolomics and PCA. They reported that the dietary standardization reduced the inter-individual variation in urine (from 30% to 18%), but not in plasma or saliva. When the diet was not standardized, the metabolites that contributed most to variation in the urinary profiles were hippurate and creatinine. This study suggested that future metabolomics studies should consider dietary and sampling factors (time of sample collection) as a means of reducing normal physiologic variation. In addition to dietary interventions, nutritional metabolomics can be used to explore the roles of dietary functional components in health maintenance and disease prevention and development.
Metabolomics applications in pets

The application of metabolomics to feline and canine nutrition has been very limited. In dogs, metabolomics techniques have been applied to identify the impact of breed on the urinary metabolic profile (Viant et al., 2007), breed-specific dietary or metabolism differences (Beckmann et al., 2010), and the effect of life-long energy restriction on urinary metabolic profile (Wang et al., 2007).

Wang et al. (2007) conducted a study to monitor urinary metabolic profiles throughout the lifetimes of control-fed (CF) and diet-restricted (DR) dogs using $^1$H NMR-based metabolomics method. A total of 48 Labrador dogs were allotted to a paired feeding design by sex and weaning weight with litter. Dogs were randomly assigned to either the CF or DR group in each pair. The DR group was designed to have 75% of the food consumption of the CF group. Besides that, dogs were all housed and fed under the same environmental conditions for life. Urinary metabolic trajectories were constructed for each dog, and metabolic variation was found to be predominantly influenced by age. Urinary excretion of creatinine increased with age, reaching a maximum between ages 5 and 9 yr, and declined thereafter. Excretion of mixed glycoproteins was noted at earlier ages, which may be a reflection of growth patterns. In addition, consistent metabolic variation related to diet also was characterized, and energy-associated metabolites, such as creatine, 1-methylnicotinamide, lactate, acetate, and succinate, were depleted in urine from diet-restricted dogs. Both aging and diet restriction altered activities of the gut microbiota, manifested by variation of aromatic metabolites and aliphatic amine
compounds. This study allowed the metabolic response to two different physiological processes to be monitored throughout the lifetime of the canine population and may form part of a strategy to monitor and reduce the impact of age-related diseases in dogs, as well as providing more general insights into extension of longevity in mammals.

Compared to the studies in dogs, even fewer applications of metabolomics to cats have been made. In cats, there is one data set (Colyer et al., 2011) that was generated from a study that was designed to identify the variation in plasma metabolite profiles in an environmentally-controlled study. Fourteen neutered female cats (age range of 1-9 yr) were randomly assigned to two feeding groups. Cats were fed a similar diet to maintain starting BW for 3 wk. Plasma samples were collected from each cat on d 14, 16, and 18 in each group. PCA was used to investigate the variation associated with individual, sampling day, feeding group, age, or sibling group. They observed that the individual is a major driver of plasma metabolite profile variance. In another study published only as an abstract, a simple nutritional intervention (3.85% additional glucose) was tested to evaluate whether a change in the fasted plasma metabolome was observed in cats after a 3-wk feeding period. Fourteen neutered female cats were randomly assigned to one of two diets (basal diet vs. basal diet + 3.85% glucose) in a two-way, crossover design. Plasma samples were collected on d 14, 16, and 18. They observed that the plasma metabolite profiles were dominated by inter-individual differences rather than the effect of dietary glucose. They reported that 44% of the plasma metabolites related to amino acid, carbohydrate, and energy metabolism had a significant (P < 0.05) change in the fasted pool size when glucose was added to the basal diet (Allaway et al., 2010).
Determining the influence of dietary patterns on the metabolic profiles may further enhance our understanding of feline metabolism and may aid in studying obesity and diabetes in the future.

**LITERATURE CITED**


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TABLES

Table 1-1. Effects of dietary macronutrient intake on glucose homeostasis, insulin resistance, and appetite-regulating hormone response

<table>
<thead>
<tr>
<th>Study design</th>
<th>Diets</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Backus et al., 1995</td>
<td>Six healthy cats were given aqueous nutrient suspensions by oral-gastric tube to determine the supplementation effect of fat, protein, or starch on plasma CCK.</td>
<td>(1) Intact protein: 5 g casein (2) Amino acids: 5 g mixture (3) Fat: 15 g corn oil (4) Protein: 15 g whey protein (5) Starch: 15 g cooked corn starch</td>
</tr>
<tr>
<td>Backus et al., 2007</td>
<td>Twenty-four healthy cats were assigned to 4 dietary groups (6 cats per group) until the end of the study. The dietary effect was studied 13 wk before and after 17 wk after gonadectomy.</td>
<td>(1) Diet 1 (HFLC): protein, 46.8%; fat, 40%; NFE, 4% (2) Diet 2 (LFHC): protein, 32.7%; fat, 4%; NFE, 56.4% (3) Diet 3: protein, 35.8%; fat, 12%; NFE, 44.8%</td>
</tr>
<tr>
<td>Study</td>
<td>Description</td>
<td>Data</td>
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<td>-----------------------</td>
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<td>----------------------------------------------------------------------</td>
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<tr>
<td>Hoenig et al., 2007</td>
<td>Twelve lean and 16 obese cats were fed two diets for 4 mo each in a cross-over design. After 8 mo, food intake of obese cats was decreased to obtain 1.5% decrease in BW per wk. Insulin sensitivity was tested using the EHC.</td>
<td>(1) HP: protein, 45.2%; fat, 15.8%; NFE, 24.7%</td>
</tr>
<tr>
<td>Martin et al., 2010</td>
<td>Five lean and 5 obese cats were fed three different diets for 6 wk each in a randomized cross-over design. Food amount (70 g/d) provided was greater than the consumption.</td>
<td>(1) HP: protein, 46%; fat, 12%; NFE, 22.9%</td>
</tr>
</tbody>
</table>
Table 1-1. (cont.)

Verbrugghe et al., 2010
Nine healthy lean cats were fed 3 isoenergetic, homemade diets for a period of 3 wk each in a $3 \times 3$ Latin square design. Intravenous glucose tolerance tests were performed at the end of each period.

(a) The LP diet decreased ($P = 0.019$) insulin AUC.
(b) The LC diet increased ($P = 0.019$) insulin AUC and delayed second insulin peak.

Wei et al., 2010
Twenty overweight adult cats were randomly assigned to be fed either HP or MP diet ad libitum for 4 mo.

(a) There were no differences between the diets for blood glucose and leptin concentration.
Gilor et al., 2011

Ten healthy cats were used in a repeated measurements design. Intravenous glucose infusion (IGC) with no prior treatment served as control. Glucose (OGTT), lipid (LIGC), and amino acids (AAIGC) were administrated through nasoesophageal tube.

(a) Total GIP AUC was larger ($P < 0.001$) after lipid compared to amino acids. But total GLP-1 AUC was similar after all three oral stimulations.

(b) Insulin and GIP concentrations were positively correlated after lipid and amino acids challenges, but not after oral glucose stimulation.

(c) Insulin and GLP-1 concentrations were positively correlated after glucose, amino acids, or lipids stimulations.

(b) There was a trend towards an increase ($P = 0.054$) of serum insulin concentration in cats fed the HP diet.
Table 1-1. (cont.)

<table>
<thead>
<tr>
<th>Study</th>
<th>Description</th>
<th>LCHP: protein, fat, NFE</th>
<th>HCLP: protein, fat, NFE</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coradini et al., 2011</td>
<td>Thirty-two lean cats were paired to be fed either LCHP or HCLP diet for 4 wk maintenance feeding and 8 wk ad libitum feeding.</td>
<td>(1) 46%; 12%; 22.8%</td>
<td>(2) 21.5%; 11.7%; 51%</td>
<td>(a) The HCLP diet fed at maintenance requirements resulted in higher ($P &lt; 0.01$) postprandial glucose and insulin concentrations. (b) The LCHP diet fed ad libitum resulted in greater ($P &lt; 0.01$) weight gain and was associated with higher ($P &lt; 0.01$) energetic efficiency.</td>
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<tr>
<td>Hoenig et al., 2011</td>
<td>Thirty-seven cats (13 young lean, 12 old lean, and 12 old obese) were fed 3 different diets for 5 mo each in a cross-over design.</td>
<td>(1) 48%; 11.4%; 24.2%</td>
<td>(2) 46.6%; 12.9%; 23.4%</td>
<td>(3) 32.6%; 10.5%; 37.7%</td>
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<td>Table 1-1. (cont.)</td>
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<tr>
<td>Vester et al., 2012</td>
<td>This study evaluated the effects of in utero and postnatal exposure of a HP (n = 9) or MP (n = 16) diet on ghrelin and leptin concentrations during the first 4 mo of life in kittens.</td>
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<tr>
<td>(1) MP: protein, 34.3%; fat, 19.2%; NFE, 25.1%</td>
<td>(a) Kittens fed MP tended to have greater serum ghrelin concentrations.</td>
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<tr>
<td>(2) HP: protein, 52.9%; fat, 23.5%; NFE, 5.6%</td>
<td>(b) Leptin concentrations were not affected by diet, but changed over time.</td>
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</tbody>
</table>

BW, body weight; EHC, Euglycemic hyperinsulinemic clamp; HP, high-protein diet; HF, high-fat diet; HC, high-carbohydrate diet; LP, low-protein diet; LF, low-fat diet; LC, low-carbohydrate diet; MP, moderate-protein diet; HPPUFA: high-protein with polysaturated fatty acids; AUC, area under the curve; NFE, nitrogen-free extract; NEFA, nonesterified fatty acids; OGTT, oral glucose tolerance test; IGC, isoglycemic clamp; LIGC, oral lipid challenge with an isoglycemic clamp; AAIGC, Amino acids challenge with an isoglycemic clamp; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide 1; LCHP, low-carbohydrate, high-protein diet; HCLP, high-carbohydrate, low-protein diet.
CHAPTER 2: EFFECTS OF DIETARY MACRONUTRIENT COMPOSITION AND FEEDING FREQUENCY ON FASTING AND POSTPRANDIAL HORMONE RESPONSE IN DOMESTIC CATS

ABSTRACT: The objective was to evaluate the effects of dietary macronutrients and feeding frequency on blood glucose, insulin, total ghrelin, and leptin. Twelve adult lean neutered male cats were used in three tests, all crossover studies composed of a 15-d adaptation and blood sampling on d 16. In Study 1, differences between two- and four-meal feeding was tested. On d 16, blood samples were collected every 2 h for 24 h. In Study 2, macronutrient boluses were tested. Instead of the control diet, the morning meal on d 16 was replaced with an isoenergetic bolus of carbohydrate (maltodextrin), protein (chicken meat), fat, or water. Fasted and 10 post-prandial blood samples were collected. In Study 3, diets high in fat (HF), protein (HP), carbohydrate (HC), or control were tested. On d 16, fasted and 10 post-prandial blood samples were collected. Data were analyzed to identify baseline and area under the curve (AUC) changes. Cats fed 4 meals daily had greater \( P = 0.03 \) leptin incremental \( \text{AUC}_{0-24h} \) compared to cats fed twice daily. Carbohydrate bolus increased glucose \( P < 0.001 \) and insulin \( P < 0.001 \) incremental \( \text{AUC}_{0-6h} \), and tended to increase \( P = 0.09 \) leptin net \( \text{AUC}_{0-6h} \). Cats fed control and HC diets had greater \( P = 0.03 \) glucose incremental AUC compared to HF and HP. Circulating hormone data were highly variable in all three studies and indicated changes due to dietary macronutrients and feeding frequency, but the complete diets with different
macronutrient compositions did not show differences. Further study is needed to identify impacts on appetite and contributing mechanisms.

**INTRODUCTION**

In humans and companion animals, obesity is one of the most common diseases and is a key risk factor for many other diseases. As in humans, the incidence of obesity and type II diabetes mellitus in domestic cats has rapidly increased in recent decades (Prahl et al., 2007). In addition to the sedentary indoor lifestyle, the prevalence of highly palatable commercial pet foods (e.g., high-fat dry diets) and/or inappropriate feeding strategies (e.g., the amount and frequency of food provision) contribute to obesity (Scarlett et al., 1994), insulin resistance (Rand et al., 2004), and diabetes (Freskens et al., 1995) in domestic cats.

Diets containing different macronutrient concentrations may influence the release and circulating concentrations of appetite-regulating hormones, which could affect sensations of hunger, satiety, and ultimately energy intake (Stubbs et al., 1999; Potier et al., 2010; Brennan et al., 2012). Ghrelin and leptin play competing roles in appetite regulation (Cummings and Foster, 2003) and the release of both have been reported to be impacted by dietary nutrient composition (Foster-Schubert et al., 2008; Blom et al., 2006; Monteleone et al., 2003; Poppitt et al., 2006; Sanchez et al., 2010). Ghrelin, an orexigenic gastric hormone, stimulates food intake and supports lipogenesis (Tschop et al., 2000; Choi et al., 2003). In rodents and normal weight humans consuming isoenergetic meals, ghrelin release is suppressed following a meal, but is macronutrient-specific (Monteleone
et al., 2003; Sanchez et al., 2004; Overduin et al., 2005). Notably, fat appears to have a relatively weak ghrelin-suppressing capacity compared to carbohydrate and protein (Monteleone et al., 2003; Sanchez et al., 2004; Overduin et al., 2005). In contrast, leptin, mainly produced from adipose tissue, is an indicator of body energy status. It contributes to the long- and short-term regulation of food intake, acting on the hypothalamus to reduce appetite (Ahima and Flier, 2000; Yarandi et al., 2011) in rodents and humans. In humans, postprandial leptin concentrations have been reported to be dependent on dietary macronutrient composition and that high-carbohydrate, low-fat meals resulted in higher postprandial leptin concentrations compared to high-fat, low-carbohydrate meals (Romon et al., 1999; Romon et al., 2003). Very little is known regarding the effects of dietary macronutrients on the ghrelin and leptin response in cats. The cat, as a true carnivore, has relied on high-protein animal tissue to meet its specific nutritional requirements in the wild and are metabolically adapted to a lower glucose utilization and higher protein metabolism when compared to most omnivores (MacDonald et al., 1984). The unique metabolic need of cats underscores the importance of conducting this fundamental study in this species to increase our understanding and develop more specialized dietary strategies for weight management in cats.

In addition to diet composition, increased feeding frequency has been suggested to manage BW. To manage weight loss in cats, owners often are instructed to offer the daily food ration in several meals (2-4) throughout the day rather than in a single meal (German and Martin, 2008). Although feeding frequency has been studied for its potential impact on physical activity, recent studies have been more focused on the other side of
the energy balance equation, namely, appetite control and food intake, and how it may be affected by meal frequency (Leidy and Campbell, 2011). Feeding frequency may have an impact on appetite control by influencing the release of appetite-regulating hormones, including insulin, ghrelin, and leptin (Leidy and Campbell, 2011; Steelman et al., 2006; Lubbs et al., 2010; Solomon et al., 2008).

Three tests were conducted in healthy adult cats to investigate how appetite-regulating hormone concentrations fluctuated over a 24-h period and responded to dietary manipulation. Our objectives were 1) to monitor patterns of glucose, insulin, ghrelin, and leptin concentrations over a 24-h period in cats fed a dry diet twice or four times daily; 2) to measure the acute response of a single protein, fat, or carbohydrate dose on postprandial glucose, insulin, ghrelin, and leptin concentrations; and 3) to measure the effects of a protein-, fat-, or carbohydrate-rich dry diet on fasting and postprandial glucose, insulin, ghrelin, and leptin concentrations.

MATERIALS AND METHODS

Animals and diets

A total of twelve healthy adult, neutered, male domestic shorthair cats (initially 3 years old; 4.74 (SEM 0.16) kg BW; ~5 BCS on a 9-point scale) were used in these three tests. Cats were group-housed in the same room (26.67 m² × 3 m) most of the day but were individually housed (0.61 m x 0.61 m x 0.61 m) to access diets in the animal facility of the Edward R. Madigan Laboratory at the University of Illinois (Urbana, IL, USA). The room was environmentally controlled (20 °C) with a 16 h light: 8 h dark cycle. Cats
were provided access to various toys and scratching poles and socialized with each other and humans for behavioral enrichment.

Dietary ingredient and chemical composition of the 4 test diets are presented in Table 2-1. Test diets were extruded dry kibble diets based on milled brewer’s rice, poultry by-product meal, corn gluten meal, whole yellow corn, whole wheat, soy protein isolate, and fish meal. Test diets included: 1) control diet (33% kcal ME from each macronutrient), 2) high-fat diet (HF diet: ~50% kcal ME from fat), 3) high-protein diet (HP diet: ~50% kcal ME from protein), and 4) high-carbohydrate diet (HC diet: ~50% kcal ME from carbohydrate). All diets were formulated to contain similar concentrations and types of dietary fiber so that any changes were due to macronutrient differences. Diets were formulated to meet all nutrient recommendations provided by the Association of American Feed Control Officials (AAFCO, 2009) and were manufactured at Nestlé Purina PetCare Product Technology Center (St. Louis, MO, USA). Prior to the nutritional studies, food intake was determined by calculating the daily maintenance energy requirement of lean domestic cats (ME requirement (kcal) = 100 × BW kg$^{0.67}$) (National Research Council, 2006) and by using previous feeding records. Cats were weighed weekly and food intake was adjusted to maintain BW and BCS throughout the study. If not consumed, food was removed, weighed, and recorded. Water was available ad libitum throughout all studies.

Prior to Study 1, body composition was determined using dual energy x-ray absorptiometry (DEXA). Cats were sedated and anesthetized and placed in ventral
recumbency on the bed of a scanner (Hologic model QDR-4500 Fan Beam x-ray Bone Densitometer; Hologic Inc., Waltham, MA, USA), and with the use of computer software (Hologic Inc.) for cats, DEXA data were used to determine body fat, lean and bone mineral content. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee before the studies.

**Experimental design**

Study 1: Because very little is known regarding appetite-regulating hormone concentrations in feline plasma, the initial test was designed to monitor daily fluctuation of glucose, insulin, total ghrelin, and leptin concentrations in cats fed 2 or 4 meals per day. Twelve healthy adult male cats were used in a crossover study design consisting of 32 d (two 16-d periods). In the first period, cats (six animals per treatment) were fed either two meals or four meals daily and vice versa for the second period. The control diet was fed in this study. Half of the daily intake was offered to two-meal-fed cats at 08.00 and 20.00 h. At 08.00, 12.00, 16.00 and 20.00 h, one-quarter of the daily intake was offered to four-meal-fed cats, respectively. Cats were individually housed for access to diet from 08.00 to 09.00, 12.00 to 13.00, 16.00 to 17.00, and 20.00 to 21.00 h each day. In each period, a 15-d adaptation phase was followed by a blood sampling phase on d16. A small blood sample (1.5 ml) was collected before the 08.00 h meal (baseline samples) and then every 2 h for 24 h via catheter.

Study 2: This study was designed to measure the acute response of a single protein, fat, or carbohydrate bolus on postprandial glucose, insulin, total ghrelin, and
leptin concentrations. Twelve healthy adult male cats were used in a replicated 4 × 4 Latin square design for a total of 64 d (four 16-d periods). Cats were fed the control diet twice daily at 08.00 and 20.00 h. A 15-d adaptation phase preceded a blood collection phase on d 16. On d 16, rather than consuming the control diet, cats were dosed at 08.00 h with one of four treatments. Treatments included a 20 g carbohydrate load (20 g maltodextrin in ~20 mL water; ~80 kcal), 9 g fat load (lard; ~80 kcal), 27 g protein load (canned chicken; ~80 kcal; Sweet Sue Premium Chicken Breast, Tyson Foods, Springdale, AR, USA), and 20 mL water. Water was used as a control for the effect of stomach filling, which has been shown not to influence postprandial ghrelin response in humans (Blom et al., 2005). Carbohydrate and water solutions (~20 mL) were given by slowly dripping the solution from the syringe into the mouth of the cats to accurately measure intake. Because of their high palatability, cats were able to consume the fat and protein loads without assistance. If time became an issue, cats were hand fed to increase consumption. Cats consumed all of the water, carbohydrate, fat and protein loads within 15 min. Prior to Study 2, cats were accustomed to these dosing strategies to minimize stress during testing, which may affect the outcomes measured herein. Blood samples were collected prior to dosing (0 min) and at 15, 30, 60, 90, 120, 150, 180, 240, 300, and 360 min after dosing.

Study 3: In this study, fasting and postprandial response to HF, HP, or HC diets was evaluated. Twelve healthy adult male cats were used in a replicated 4 × 4 Latin square design for a total of 64 d (four 16-d periods). Cats were randomly allotted to one of the four test diets listed in Table 2-1. Cats were fed twice daily at 08.00 and 20.00 h.
and consumed their food within 10 min. After a 15-d adaptation phase, blood samples were collected on d 16 via jugular or saphenous catheter before (0 min) and 10, 20, 30, 60, 90, 120, 150, 180, 240, 300, 360, 720 min after the morning meal was consumed.

Chemical analyses

Diet subsamples were collected and ground using a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen and dry ice in preparation for chemical analyses. Diet samples were analyzed for dry matter (DM) and organic matter (OM) according to Association of Official Analytical Chemists (AOAC, 2006). Crude protein was measured using a Leco Nitrogen/Protein Determinator (model FP-2000, Leco Corporation, St. Joseph, MI) according to Association of Official Analytical Chemists (AOAC, 2006). Fat concentrations were determined by acid hydrolysis according to the American Association of Cereal Chemists (AACC, 1983) followed by ether extraction (Budde, 1952). Total dietary fiber (TDF) was determined according to Prosky et al. (1992). Gross energy was measured using a bomb calorimeter (Model 1261, Parr Instrument Co., Moline, IL).

Blood collection and analysis

The same blood collection and handling procedures for measurement of blood glucose, plasma insulin, and plasma total ghrelin and plasma leptin were used in all three tests. For all studies, jugular or saphenous catheters were placed 1 or 2 d prior to the collections to minimize stress. Cats were sedated and anesthetized while performing the catheter placement by intramuscular injection of a cocktail of butorphanol (0.2 mg/kg),
medetomadine (0.02 mg/kg), and atropine (0.04 mg/kg) along with the reversal atipamezole (0.02 mg/kg). Patency was maintained by flushing with heparinized saline daily until sampling began and following every sample. A total of 1.5 mL of blood was collected at each time point, maintaining the total volume of blood collection below the maximum recommended levels for the wellbeing of the cats. Catheters were removed after the last time point on the collection days.

Blood glucose concentration was immediately measured using the handheld AlphaTRAK blood glucose meter (Abbott Laboratories, Chicago, IL, USA). Blood was then immediately transferred into a pre-cooled Vacutainer tube (#367835, Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) containing EDTA and centrifuged at 1000 x g at 4 °C for 10 min. After centrifugation, plasma was collected into its respective cryovial and stored at -80 °C until further analysis.

Prior to analysis, the kits were validated for use in our laboratory using parallel determination from increasing linear dilutions of pooled feline plasma (at least 5 cats) (data not reported). Plasma insulin was determined using the Feline Insulin ELISA kit (Mercodia, Uppsala, Sweden) previously validated in cats (Strage et al., 2012). Following a 10-fold dilution, plasma total ghrelin concentration was analyzed using the Total Ghrelin Canine ELISA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). Plasma leptin concentration was measured using the Multi-species Leptin Radioimmunoassay kit (Millipore, St Charles, MO, USA). The ghrelin and leptin kits were validated for use in cats in our laboratory previously (Belsito et al., 2009).
**Statistical analyses**

For the baseline data (fasting samples), data were analyzed using the Mixed procedure of SAS 9.2 (SAS Inst. Inc., Cary, NC) testing the main effect (feeding frequency or test diet) and including random effects of cat and period. For postprandial data, incremental change from baseline (baseline subtracted) data were analyzed to minimize any differences in baseline concentrations among cats, and then analyzed using the Mixed procedure of SAS 9.2 as repeated measures. The main effects of treatment and time were tested and the treatment × time interaction was evaluated if significant. Random effects of cat and period were included in the model. Means were separated for diets using the PDIFF statement in the Mixed procedure for individual time points after detecting a significant treatment effect using SLICE/time. Area under the curve [AUC; incremental AUC (IAUC), decremental AUC (DAUC), net AUC (NAUC)] data were calculated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Differences in the AUC of glucose, insulin, total ghrelin, and leptin among treatments were tested for significance using the Mixed procedure of SAS 9.2. A probability of \( P \leq 0.05 \) was considered significant and \( P \leq 0.10 \) was considered a trend.

**RESULTS**

**Study 1**

Average food intake for all cats in this trial was 65.6 g/d (256.7 kcal ME/d) and was not different between periods. In cats fed 2 or 4 meals daily, the baseline concentrations of blood glucose (83.5 vs. 80.3 mg/dL; SEM 2.4 mg/dL), insulin (81.5 vs.
67.1 pmol/L; SEM 18.4 pmol/L), total ghrelin (7.2 vs. 7.2 ng/mL; SEM 1.0 ng/mL), and leptin (5.7 vs. 5.4 ng/mL; SEM 0.2 ng/mL) were not different. Figure 2-1 presents incremental changes in blood glucose, insulin, total ghrelin, and leptin concentrations over a 24-h period. Blood glucose concentrations of cats fed 2 meals daily were more variable than cats fed 4 meals daily during the light period. Cats fed two meals daily had two peaks of glucose concentration in both the light and dark periods. Similar to glucose, insulin concentrations of cats fed 2 meals daily also were more variable and maintained a higher concentration throughout the 24-h period compared to those fed 4 meals daily. Total ghrelin remained below baseline throughout the 24-h period in cats fed 4 meals daily, but its concentrations remained above baseline during the light period from 8 am to 4 pm in cats fed 2 meals daily. Cats fed 4 meals daily maintained higher leptin concentrations over the 24-h period than cats fed 2 meals daily. Cats fed 4 meals daily had greater ($P = 0.03$) leptin IAUC$_{0-24h}$ compared to cats fed twice daily (10.8 vs. 5.5 ng/mL, SEM = 2.1 ng/mL). However, AUC$_{0-24h}$ of glucose, insulin, and total ghrelin were not affected by feeding frequency.

**Study 2**

Average food intake for all cats in this study was 56.9 g/d (222.7 kcal ME/d) and was not different among periods. Baseline glucose, insulin, total ghrelin, and leptin concentration of cats dosed with water, fat, protein, or carbohydrate were not different (Table 2-2). Figure 2-2 presents incremental blood glucose, insulin, total ghrelin, and leptin concentrations over a 6-h postprandial period. Carbohydrate load produced a
marked increase in incremental glucose concentration after 30 min and reached a plateau at 1 h. It remained elevated over 6 h and was greater ($P = 0.0002$) than water, fat, and protein in glucose concentration overall, resulting in greater ($P < 0.001$) IAUC$_{0-6h}$ than cats dosed with water, fat, or protein. Similar to glucose, incremental insulin concentrations of cats dosed with carbohydrate rapidly increased and reached its peak at 1 h and remained higher than the other treatments for 4 h postprandially (Figure 2-2B). Cats dosed with carbohydrate had greater ($P < 0.001$) insulin IAUC$_{0-6h}$ compared to cats fed the other 3 treatments (Table 2-2).

Incremental total ghrelin concentrations varied greatly in response to carbohydrate, fat, and protein and tended to stay below baseline values (Figure 2-2C). Although a treatment × time interaction ($P < 0.0001$) was significant, postprandial IAUC$_{0-6h}$, DAUC$_{0-6h}$, or NAUC$_{0-6h}$ of total ghrelin were not affected by treatments (Table 2-2). Total ghrelin concentrations remained close to baseline in cats dosed with water.

Incremental leptin concentrations decreased below baseline for all treatments. Although leptin continued to decrease in cats dosed with water, protein, and fat, incremental leptin concentrations in cats dosed with carbohydrate started increasing at 2.5 h and reached baseline at 5 h (Figure 2-2D)). When compared to cats dosed with carbohydrate, those dosed with protein tended to have a decreased ($P = 0.09$) leptin NAUC$_{0-6h}$ than carbohydrate load.

**Study 3**

Average food intake for cats fed control, HF, HP, and HC diets in this study was
51.5 g/d (201.5 kcal ME/d), 46.2 g/d (206.1 kcal ME/d), 54.4 g/d (202.0 kcal ME/d), and 54.3 g/d (208.3 kcal ME/d), respectively, which was not different (P>0.10) among diets. Diets were highly palatable by all cats (no food refusals) and no gastrointestinal symptoms or diarrhea issues were observed throughout the entire study.

Baseline glucose, insulin, total ghrelin, and leptin concentrations did not differ among dietary treatments (Table 2-3). Incremental blood glucose concentrations in cats fed the control diet was greater than for the other diets between 1.5 to 3 h postprandially, while cats fed the HC diet maintained a greater incremental glucose concentration after 5 h postprandially (data not shown). The glucose IAUC₀₋₆₉ was higher (P = 0.03) in cats fed the control diet compared with those fed HF and HP, and the glucose IAUC₀₋₁₂₉ was higher (P = 0.03) in cats fed the HC diet compared with HF and HP diets. The IAUC, DAUC, or NAUC of insulin, total ghrelin, and leptin were not affected by diets over 6 or 12 h postprandially (Table 2-3).

**DISCUSSION**

The aim of the present study was to investigate the potential role of dietary macronutrient composition on circulating appetite-regulating hormone concentrations in healthy cats. Three studies were conducted to: 1) observe the daily hormonal fluctuations in response to different meal patterns; 2) evaluate the postprandial response to oral ingestion of a single macronutrient; and 3) evaluate the fasting and postprandial response to macronutrient-rich diets.
Because very little is known regarding appetite-regulating hormone concentrations in cats and their relationship with feeding frequency, the initial study was designed to monitor daily fluctuation of circulating glucose, insulin, total ghrelin, and leptin concentrations in cats fed 2 or 4 meals per day. We hypothesized that increasing feeding frequency without changing daily food intake would prevent large metabolic and hormonal fluctuations. In that study, circulating glucose and insulin concentrations were less variable in cats fed 4 compared to 2 meals daily which is consistent with the data in healthy and overweight humans (Leidy et al., 2010; Leidy and Campbell, 2011) and with feeding strategies recommended for diabetic cats. We also observed that cats fed 4 meals daily had lower incremental ghrelin (Figure 2-2C) and greater incremental leptin (Figure 2-2D) concentrations and leptin IAUC0-24h compared to cats fed 2 meals daily. These data indicate that increasing feeding frequency may inhibit ghrelin secretion and stimulate leptin secretion that may aid in appetite control. Smeets and Westerterp-Plantenga (2008) reported that eating three compared to two meals increased fat oxidation and feelings of satiety over a 24 h period in healthy women. Leptin can activate the enzyme AMP kinase in peripheral tissues, and is important in regulating lipid oxidation (Minokoshi et al., 2002). While greater leptin secretion with increased feeding frequency may contribute to decreased appetite and/or to increased fat oxidation, these outcomes were not tested in this study. Limitations exist in our current test, with the primary one being the sampling times. Because the blood volume allowances over 24 h limited the number of samples we could collect, the changes occurring between every 2 h collection were unknown. Therefore, our perception of the hormonal responses could have been influenced by the
selection of blood sampling times. More accurate hormonal responses could be accomplished by smaller sample volume requirements in future studies. Another potential limitation of the protocol used is that we used meal feeding, which may differ from ad libitum feeding. Although this may result in physiologic and metabolic differences from some household cats who have free access to food, this method allowed concise and accurate food consumption and postprandial blood measurements.

Before evaluating complex diets, we designed a study to measure the acute response of a single macronutrient dose. To our knowledge, no one has tested the effect of a single macronutrient or a macronutrient-rich diet on postprandial ghrelin and leptin concentrations in cats. We hypothesized that a carbohydrate load would have the most rapid and effective influence on postprandial glucose and insulin concentrations. We hypothesized that the fat load would have a relatively weak effect on ghrelin suppression and leptin secretion, whereas protein, which is considered as the most satiating macronutrient in humans, would have a prolonged effect on ghrelin suppression and leptin secretion. Macronutrient sources that were deemed to be highly digestible and contained relatively large amounts of one macronutrient were selected. The amount of each macronutrient dose fed to cats was based on the amount of calories provided by each macronutrient (~ 80 kcal) and how it compared to daily intake (approximately 25% of daily ME). Based on a similar canine study performed in our laboratory (Lubbs et al., 2010) and human studies (Blom et al., 2006; Poppitt et al., 2006; Blom et al., 2005), a 6 h length of blood sampling was selected because the postprandial ghrelin response to diet was expected to return to baseline by then.
Similar to the previous studies in dogs (Lubbs et al., 2010) and cats (Hoenig et al., 2010), we observed that oral carbohydrate load elicited a rise in blood glucose and insulin, and their IAUC$_{0-6h}$ were higher than that for water, fat, and protein. Due to these robust increases in postprandial glucose and insulin in the present study, significant ghrelin suppression after carbohydrate load was expected to be observed simultaneously. Although we did not observe the influence of macronutrient loads (fat, protein, and carbohydrate) on ghrelin DAUC$_{0-6h}$, all macronutrient loads suppressed ghrelin secretion 6 h postprandially. Our observations that ghrelin was mostly responsive to carbohydrate and fat loads is consistent with previous findings in humans (Blom et al., 2005; Erdmann et al., 2003). Blom et al. (2005) reported that postprandial ghrelin responded rapidly and dose-dependently to carbohydrate intake and might be regulated through insulin. Erdmann et al. (Erdmann et al., 2003) reported that the fat-rich diet decreased plasma ghrelin levels, but reached a nadir later than that of carbohydrates. Protein load showed the weakest ghrelin response in cats compared to fat and carbohydrate loads. This finding is inconsistent with previous human studies in which protein induced a prolonged postprandial ghrelin suppression (Blom et al., 2006; Bowen et al., 2006; Al Awar et al., 2005; Tannous et al., 2006). There are a few other studies in humans, however, that suggested protein ingestion stimulated (Erdmann et al., 2003) or had no effect on (Moran et al., 2005) postprandial ghrelin concentration.

Factors other than macronutrient composition may affect ghrelin response. Arosio et al. (2004) reported that circulating ghrelin concentrations were decreased in humans as much by sham feeding as they were by meal consumption, suggesting the importance of
the cephalic response to nutrient intake and the role of vagal activity in the control of ghrelin secretion. However, the role for cephalic-vagal stimulation on ghrelin suppression is unclear in cats. It might be argued that the volume difference that existed among our macronutrient loads influenced postprandial ghrelin secretion. This was likely not the case, however, because previous studies demonstrated that the gastric factor alone (such as stomach expansion) does not play a role in the regulation of ghrelin secretion (Blom et al., 2006; Shiiya et al., 2002).

In contrast to our hypothesis that postprandial leptin secretion would increase, leptin actually decreased in our study. It is unknown whether the decreased leptin secretion in the initial 2.5 h after dosing carbohydrate was due to the large amount of the highly digestible macronutrient selected or the dosing method used in this study. A potential drawback of carbohydrate loads was incidence of diarrhea in the present study, which has also been reported in cats given an oral glucose tolerance test (Hoenig et al., 2010). Dietary carbohydrate has been reported to cause gastrointestinal disturbances in cats due to its osmotic effect if the amount eaten exceeds the digestive capacity of the small intestine (Kienzle, 1994). Although diarrhea was only present on the day of the bolus, it may have contributed to the variability of the results herein. Our observation that leptin concentration in cats dosed with carbohydrate started increasing after 2.5 h postprandially may indicate the contribution of leptin on increasing the glucose uptake as well as the potential regulating effect of insulin on leptin. Postprandial leptin concentrations remained below baseline after fat ingestion in the present study, which is consistent with previous findings in lean and obese humans (Imbeault et al., 2001;
Poppitt et al., 2006). It has been suggested that the primary role of leptin in the regulation of energy homeostasis is a response to negative energy balance that leptin decreases during starvation, triggering an increased feeling of hunger (Havel, 2004). Protein had a weaker effect on postprandial leptin secretion compared to carbohydrate, indicating a reduced satiating effect of protein in cats. It is correlated with the postprandial ghrelin response to protein in the current study.

To apply this research to a more practical situation, response to three isoenergetic dry kibble diets that contained different macronutrient profiles were tested in the present study. From the control diet, which was based on a commercially available cat food, similar ingredients were used in different quantities to formulate a wide caloric distribution in terms of macronutrient content (approximately 50% of kcal ME from each macronutrient). We expected to observe similar postprandial responses to macronutrients in Study 2 and 3. The diet containing high carbohydrate content increased postprandial glucose in a similar manner, but did not lead to differences in baseline or postprandial insulin, ghrelin, or leptin concentrations. Cats fed the control and HC diets had a similar increase in postprandial glucose, but failed to increase insulin secretion. Farrow et al. (2002) reported a similar result. In that study, a high-carbohydrate diet resulted in a greater postprandial glucose AUC when compared to high-protein and high-fat diets in healthy non-obese cats, while insulin AUC only tended to be increased in cats fed a high-carbohydrate diet. Coradini et al. (2011) reported that a high-carbohydrate, low-protein diet resulted in higher postprandial glucose and insulin concentrations compared with a low-carbohydrate, high-protein diet in cats fed to maintain BW. The feeding of high-
carbohydrate diets has been suggested to increase the risk of developing diabetes in cats (Freskens et al., 1995). Hoenig et al. (2007) suggested that cats fed a high-carbohydrate, low-protein diet were more prone to develop obesity and insulin resistance compared to those fed a high-protein, low-carbohydrate with the same caloric intake, mainly because a high-protein diet led to greater heat production. In the present work, however, the heat production was not measured and the HP diet did not lead to differences in glucose or insulin AUC.

In the present study, body fat percentage, as measured by DEXA, was 14.1% fat in the cats studied. Because we were first interested in assessing feeding frequency and macronutrient responses in healthy non-obese cats, it must be noted that the dietary effects reported here may not be consistent with that of obese cats. For example, Hoenig et al. (2007) reported that obesity, but not dietary content, led to severe insulin resistance in cats and a marked decrease of glucose effectiveness, indicating that postprandial glucose and hormonal responses were affected more by body composition than dietary composition. Further research is needed to determine the effect of the macronutrient-rich test diets on appetite regulation in obese cats.

In conclusion, this study presents novel data regarding the effects of feeding frequency and dietary macronutrient composition on postprandial glucose, insulin, total ghrelin, and leptin concentrations in healthy non-obese adult cats. These data may provide a foundation for better understanding of the mechanisms of appetite regulation by dietary macronutrient manipulation. Even though circulating hormones were highly
variable, our data suggested that dietary macronutrients affected postprandial insulin, total ghrelin, and leptin secretions. Interestingly, dietary protein was observed to have a relatively weak effect on postprandial total ghrelin and leptin concentrations indicating a weak capacity on appetite regulation. Diets containing higher carbohydrate content increased blood glucose, but did not appear to impact appetite-regulating hormone concentrations in non-obese cats. Given the variability observed in the meal test study, increased numbers of animals are required in future studies to identify the impact of macronutrients on appetite. Moreover, identifying the relationship between dietary macronutrients and appetite regulation in obese or diabetic cats may be more meaningful and may aid in the development of weight-loss or diabetic diets. Further research is also needed to compare these responses in ad libitum-fed versus meal-fed cats.

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to be weaker for lipids but did not differ between proteins and carbohydrates in human subjects. Br J Nutr 104, 1406-1414.


Sanchez J, Cladera MM, Llopis M et al. (2010) The different satiating capacity of CHO and fats can be mediated by different effects on leptin and ghrelin systems. Behav Brain Res 213, 183-188.


### TABLES AND FIGURES

**Table 2-1.** Ingredient and chemical composition of test diets fed to cats

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>HF&lt;sup&gt;1&lt;/sup&gt;</th>
<th>HP</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ingredient, % (as-fed basis)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brewer’s rice</td>
<td>14.76</td>
<td>10.07</td>
<td>7.74</td>
<td>48.73</td>
</tr>
<tr>
<td>Chicken, whole carcass and parts</td>
<td>19.21</td>
<td>16.84</td>
<td>20.14</td>
<td>19.52</td>
</tr>
<tr>
<td>Poultry byproduct meal</td>
<td>12.43</td>
<td>10.29</td>
<td>17.02</td>
<td>6.00</td>
</tr>
<tr>
<td>Corn gluten meal, 60%</td>
<td>23.02</td>
<td>20.61</td>
<td>25.46</td>
<td>2.04</td>
</tr>
<tr>
<td>Whole yellow corn</td>
<td>6.88</td>
<td>6.47</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>12.54</td>
<td>11.64</td>
<td>13.15</td>
<td>4.71</td>
</tr>
<tr>
<td>Soy protein isolate</td>
<td>---</td>
<td>---</td>
<td>10.04</td>
<td>8.84</td>
</tr>
<tr>
<td>Fish meal</td>
<td>2.21</td>
<td>1.29</td>
<td>1.55</td>
<td>1.50</td>
</tr>
<tr>
<td>Tallow edible with vitamin E</td>
<td>7.00</td>
<td>18.50</td>
<td>2.50</td>
<td>5.50</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0.04</td>
<td>0.03</td>
<td>0.46</td>
<td>0.45</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.09</td>
<td>0.08</td>
<td>0.10</td>
<td>0.37</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>0.37</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>0.07</td>
<td>0.58</td>
<td>---</td>
<td>0.07</td>
</tr>
<tr>
<td>Phosphoric acid</td>
<td>0.63</td>
<td>0.56</td>
<td>0.67</td>
<td>0.64</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.55</td>
<td>0.79</td>
<td>0.62</td>
<td>0.69</td>
</tr>
<tr>
<td>Salt</td>
<td>0.07</td>
<td>0.17</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>Pea fiber</td>
<td>---</td>
<td>1.56</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Vitamin E, 50%</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Choline chloride, liquid</td>
<td>0.21</td>
<td>0.29</td>
<td>0.21</td>
<td>0.21</td>
</tr>
<tr>
<td>Mineral premix</td>
<td>0.18</td>
<td>0.16</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td>Vitamin premix</td>
<td>0.06</td>
<td>0.05</td>
<td>0.06</td>
<td>0.06</td>
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<tr>
<td><strong>Analyzed chemical composition (DM basis)</strong></td>
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<td></td>
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</tr>
<tr>
<td>DM, %</td>
<td>94.30</td>
<td>94.22</td>
<td>93.41</td>
<td>92.56</td>
</tr>
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<sup>1</sup> HF: High fat diet

---

72
<table>
<thead>
<tr>
<th>Nutrient</th>
<th>1HF</th>
<th>2HP</th>
<th>3HC</th>
<th>4HP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein, %</td>
<td>38.00</td>
<td>32.51</td>
<td>53.50</td>
<td>29.11</td>
</tr>
<tr>
<td>Acid-hydrolyzed fat, %</td>
<td>16.51</td>
<td>27.71</td>
<td>11.32</td>
<td>12.60</td>
</tr>
<tr>
<td>Total dietary fiber, %</td>
<td>3.66</td>
<td>5.16</td>
<td>3.20</td>
<td>2.53</td>
</tr>
<tr>
<td>Ash, %</td>
<td>8.12</td>
<td>6.89</td>
<td>6.90</td>
<td>5.66</td>
</tr>
<tr>
<td>Gross energy, kcal/g</td>
<td>5.23</td>
<td>5.68</td>
<td>5.35</td>
<td>5.01</td>
</tr>
<tr>
<td>ME&lt;sup&gt;2&lt;/sup&gt;, kcal/100 g DM</td>
<td>391.32</td>
<td>446.38</td>
<td>371.25</td>
<td>384.02</td>
</tr>
<tr>
<td>Nutrients on energy basis, g DM/1000 kcal ME</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude protein</td>
<td>97.11</td>
<td>72.83</td>
<td>144.11</td>
<td>75.80</td>
</tr>
<tr>
<td>Acid-hydrolyzed fat</td>
<td>42.19</td>
<td>62.08</td>
<td>30.49</td>
<td>32.81</td>
</tr>
<tr>
<td>Total dietary fiber</td>
<td>9.35</td>
<td>11.56</td>
<td>8.62</td>
<td>6.59</td>
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<tr>
<td>NFE&lt;sup&gt;3&lt;/sup&gt;</td>
<td>86.14</td>
<td>62.12</td>
<td>67.56</td>
<td>130.23</td>
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<td>Macronutrients on energy basis, % of ME</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude protein</td>
<td>34.0</td>
<td>25.5</td>
<td>50.4</td>
<td>26.5</td>
</tr>
<tr>
<td>Acid-hydrolyzed fat</td>
<td>35.8</td>
<td>52.8</td>
<td>25.9</td>
<td>27.9</td>
</tr>
<tr>
<td>NFE</td>
<td>30.2</td>
<td>21.7</td>
<td>23.7</td>
<td>45.6</td>
</tr>
</tbody>
</table>

<sup>1</sup>HF, high-fat diet; HP, high-protein diet; HC, high-carbohydrate diet.

<sup>2</sup>ME was calculated using modified Atwater values with the assumptions that protein, fat, and carbohydrate (nitrogen-free extract, NFE) provide 3.5, 8.5, and 3.5 kcal ME/g diet respectively (AAFCO, 2009).

<sup>3</sup>Nitrogen-free extract (DM basis) was calculated using the following equation: (100 - crude protein - acid hydrolyzed fat - total dietary fiber - ash).
Table 2-2. Blood glucose, insulin, total ghrelin, and leptin concentrations of cats dosed with water, lard (Fat), canned chicken (Protein), or maltodextrin (Carbohydrate) (Study 2).^{1}

<table>
<thead>
<tr>
<th>Item</th>
<th>Water</th>
<th>Fat</th>
<th>Protein</th>
<th>Carbohydrate</th>
<th>Pooled SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline concentration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>81.9</td>
<td>83.6</td>
<td>81.7</td>
<td>80.0</td>
<td>3.1</td>
<td>0.78</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>78.4</td>
<td>74.6</td>
<td>75.2</td>
<td>70.2</td>
<td>15.8</td>
<td>0.98</td>
</tr>
<tr>
<td>Ghrelin (ng/mL)</td>
<td>8.5</td>
<td>9.9</td>
<td>9.2</td>
<td>9.3</td>
<td>1.1</td>
<td>0.45</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
<td>0.3</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>2IAUC_{0-6h}</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dL × h)</td>
<td>13.6^a</td>
<td>10.4^a</td>
<td>17.0^a</td>
<td>114.2^b</td>
<td>10.0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Insulin (pmol/L × h)</td>
<td>24.1^a</td>
<td>61.2^a</td>
<td>31.6^a</td>
<td>300.7^b</td>
<td>39.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Ghrelin (ng/mL × h)</td>
<td>3.9</td>
<td>2.7</td>
<td>2.5</td>
<td>1.0</td>
<td>1.5</td>
<td>0.60</td>
</tr>
<tr>
<td>Leptin (ng/mL × h)</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
<td>0.4</td>
<td>0.2</td>
<td>0.39</td>
</tr>
<tr>
<td><strong>DAUC_{0-6h}</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dL × h)</td>
<td>23.0^a</td>
<td>24.7^a</td>
<td>19.1^ab</td>
<td>1.0^b</td>
<td>8.3</td>
<td>0.08</td>
</tr>
<tr>
<td>Insulin (pmol/L × h)</td>
<td>204.5^a</td>
<td>153.1^ab</td>
<td>131.3^ab</td>
<td>44.7^b</td>
<td>49.6</td>
<td>0.10</td>
</tr>
<tr>
<td>Ghrelin (ng/mL × h)</td>
<td>3.1</td>
<td>12.3</td>
<td>5.4</td>
<td>8.6</td>
<td>3.4</td>
<td>0.19</td>
</tr>
<tr>
<td>Leptin (ng/mL × h)</td>
<td>1.2</td>
<td>1.3</td>
<td>1.8</td>
<td>0.8</td>
<td>0.4</td>
<td>0.14</td>
</tr>
</tbody>
</table>

{1} Significantly different from fat (a) or from carbohydrate (b),\(P < 0.05\).
Table 2-2. (cont.)

<table>
<thead>
<tr>
<th></th>
<th>NAUC_{0-6h}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL × h)</td>
<td>-9.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Insulin (pmol/L × h)</td>
<td>-199.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ghrelin (ng/mL × h)</td>
<td>0.8</td>
</tr>
<tr>
<td>Leptin (ng/mL × h)</td>
<td>-0.9&lt;sup&gt;xy&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are means, n=12.

<sup>2</sup>AUC: areas under curve; IAUC: incremental AUC; DAUC: decremental AUC; NAUC: net AUC.

<sup>a,b,c</sup>Means within a row with unlike superscript letters are significantly different (\(P \leq 0.05\)).

<sup>x,y,z</sup>Means within a row with unlike superscript letters are tended to be different (\(P \leq 0.10\)).
Table 2-3. Blood glucose, insulin, total ghrelin, and leptin concentrations of cats fed control, high-fat (HF), high-protein (HP) and high-carbohydrate (HC) diets (Study 3)\(^1\)

<table>
<thead>
<tr>
<th>Item</th>
<th>Con</th>
<th>HF</th>
<th>HP</th>
<th>HC</th>
<th>Pooled SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline concentration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>88.9</td>
<td>88.6</td>
<td>87.5</td>
<td>89.7</td>
<td>4.6</td>
<td>0.91</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>84.3</td>
<td>65.5</td>
<td>71.7</td>
<td>86.8</td>
<td>23.4</td>
<td>0.58</td>
</tr>
<tr>
<td>Ghrelin (ng/mL)</td>
<td>6.7</td>
<td>6.4</td>
<td>6.1</td>
<td>6.7</td>
<td>0.6</td>
<td>0.52</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>4.3</td>
<td>4.1</td>
<td>4.0</td>
<td>4.3</td>
<td>0.3</td>
<td>0.40</td>
</tr>
<tr>
<td>(^2)IAUC(_{0-6h}) Glucose (mg/dL × h)</td>
<td>59.9 (^a)</td>
<td>29.1 (^b)</td>
<td>29.9 (^b)</td>
<td>50.3 (^ab)</td>
<td>10.2</td>
<td>0.03</td>
</tr>
<tr>
<td>Insulin (pmol/L × h)</td>
<td>174.9</td>
<td>101.0</td>
<td>192.2</td>
<td>162.5</td>
<td>62.9</td>
<td>0.52</td>
</tr>
<tr>
<td>Ghrelin (ng/mL × h)</td>
<td>4.8</td>
<td>1.6</td>
<td>0.9</td>
<td>1.2</td>
<td>2.0</td>
<td>0.50</td>
</tr>
<tr>
<td>Leptin (ng/mL × h)</td>
<td>0.4</td>
<td>0.7</td>
<td>0.4</td>
<td>0.9</td>
<td>0.3</td>
<td>0.43</td>
</tr>
<tr>
<td>(^2)DAUC(_{0-6h}) Ghrelin (ng/mL × h)</td>
<td>2.9</td>
<td>3.7</td>
<td>4.1</td>
<td>3.1</td>
<td>1.2</td>
<td>0.87</td>
</tr>
<tr>
<td>(^2)DAUC(_{0-6h}) Leptin (ng/mL × h)</td>
<td>1.1</td>
<td>1.4</td>
<td>1.7</td>
<td>1.3</td>
<td>0.4</td>
<td>0.65</td>
</tr>
<tr>
<td>NAUC(_{0-6h}) Ghrelin (ng/mL × h)</td>
<td>2.0</td>
<td>-2.2</td>
<td>-3.2</td>
<td>-1.9</td>
<td>2.6</td>
<td>0.48</td>
</tr>
<tr>
<td>NAUC(_{0-6h}) Leptin (ng/mL × h)</td>
<td>-0.8</td>
<td>-0.7</td>
<td>-1.3</td>
<td>-0.4</td>
<td>0.5</td>
<td>0.60</td>
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</table>
Table 2-3. (cont.)

<table>
<thead>
<tr>
<th></th>
<th>IAUC&lt;sub&gt;0-12h&lt;/sub&gt;</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL × h)</td>
<td>145.8&lt;sup&gt;ace&lt;/sup&gt;</td>
<td>83.9&lt;sup&gt;bce&lt;/sup&gt;</td>
<td>69.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>149.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.2</td>
<td>0.03</td>
</tr>
<tr>
<td>Insulin (pmol/L × h)</td>
<td>256.6</td>
<td>278.0</td>
<td>347.0</td>
<td>188.8</td>
<td>97.8</td>
<td>0.92</td>
</tr>
<tr>
<td>Ghrelin (ng/mL × h)</td>
<td>5.5</td>
<td>2.9</td>
<td>2.0</td>
<td>3.2</td>
<td>2.2</td>
<td>0.70</td>
</tr>
<tr>
<td>Leptin (ng/mL × h)</td>
<td>0.7</td>
<td>1.2</td>
<td>0.6</td>
<td>1.6</td>
<td>0.6</td>
<td>0.54</td>
</tr>
<tr>
<td>DAUC&lt;sub&gt;0-12h&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ghrelin (ng/mL × h)</td>
<td>6.8</td>
<td>7.1</td>
<td>9.4</td>
<td>6.0</td>
<td>2.9</td>
<td>0.83</td>
</tr>
<tr>
<td>Leptin (ng/mL × h)</td>
<td>2.7</td>
<td>3.9</td>
<td>3.4</td>
<td>3.4</td>
<td>0.9</td>
<td>0.82</td>
</tr>
<tr>
<td>NAUC&lt;sub&gt;0-12h&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ghrelin (ng/mL × h)</td>
<td>-1.2</td>
<td>-4.2</td>
<td>-7.4</td>
<td>-2.8</td>
<td>4.0</td>
<td>0.69</td>
</tr>
<tr>
<td>Leptin (ng/mL × h)</td>
<td>-2.0</td>
<td>-2.8</td>
<td>-2.8</td>
<td>-1.7</td>
<td>1.3</td>
<td>0.90</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are means, n=12.

<sup>2</sup>AUC: areas under curve; IAUC: incremental AUC; DAUC: decremental AUC; NAUC: net AUC.

<sup>a,b,c</sup>Means within a row with unlike superscript letters are significantly different (P ≤ 0.05).
Figure 2-1. Incremental changes in blood glucose (A), insulin (B), total ghrelin (C), and leptin (D) of cats fed two meals (●) or four meals daily (▲) in Study 1.
Figure 2-1. (cont.)

Values are incremental change from baseline means. (A) Pooled SEM = 3.5, Treatment $P = 0.008$, Time $P < 0.0001$, Treatment $\times$ Time $P = 0.05$. (B) Pooled SEM = 13.99, Treatment $P = 0.06$, Time $P < 0.0001$, Treatment $\times$ Time $P = 0.27$. (C) Pooled SEM = 1.6, Treatment $P = 0.93$, Time $P = 0.38$, Treatment $\times$ Time $P = 0.91$. (D) Pooled SEM = 0.4, Treatment $P = 0.0001$, Time $P = 0.28$, Treatment $\times$ Time $P = 0.69$. Symbol indicates that means differ at the time points specified, * $P \leq 0.05$, † $P \leq 0.10$. 
Figure 2-2. Incremental changes in blood glucose (A), insulin (B), total ghrelin (C), and leptin (D) of cats fed water (●), lard (Fat, ■), canned chicken (Protein, ▲), and maltodextrin (Carbohydrate, ◊) in Study 2.
Figure 2-2. (cont.)

Values are incremental change from baseline means. (A) Pooled SEM = 3.4, Treatment $P < 0.0001$, Time $P = 0.02$, Treatment $\times$ Time $P = 0.0002$. (B) Pooled SEM = 17.1, Treatment $P < 0.0001$, Time $P = 0.08$, Treatment $\times$ Time $P = 0.06$. (C) Pooled SEM = 0.9, Treatment $P < 0.0001$, Time $P = 0.04$, Treatment $\times$ Time $P < 0.0001$. (D) Pooled SEM = 0.1, Treatment $P < 0.0001$, Time $P < 0.0001$, Treatment $\times$ Time $P = 0.0002$. Symbol indicates that means differ at the time points specified, $^* P \leq 0.05$, $^† P \leq 0.10$. 
ABSTRACT: Metabolomics assays have been widely used in humans for dietary assessment and identification of biomarkers for food intake and diseases. However, the application of metabolomics to feline nutrition has been very limited. The objective of this study was to characterize the feline blood metabolome and identify how it changed in response to dietary macronutrient composition. Twelve neutered male adult domestic cats were fed four nutritionally complete diets [control (CD), high-fat (HF), high-protein (HP), high-carbohydrate (HC)] at amounts to maintain ideal body weight and body condition score. Cats were randomly allotted to diets in a replicated 4 × 4 Latin square design experiment for a total of 64 d (four 16-d periods). Overnight fasted plasma samples were collected in the morning of d 16 and subjected to liquid/gas chromatography and mass spectrometry. Principal component analysis showed that metabolite profiles of cats fed HP, HF, and HC dietary regimes formed distinct clusters. Gamma-glutamylleucine, 3-hydroxyisobutyrate, and 3-indoxyl sulfate were identified by random forest analysis to distinguish cats fed the three macronutrient-rich diets. Cats fed the HP diet had a metabolite profile associated with decreased nucleotide catabolism, but increased amino acid metabolism and ketone bodies, indicating a greater use of protein and fat for energy. Cats fed the HP diet had a significant increase in metabolites associated with gut microbial metabolism. Cats fed the HF diet had metabolites indicative of increased lipid metabolism, including free fatty acids, monoacylglycerols, glycerol-3-
phosphate, cholesterol, ketone bodies, and markers of oxidative stress. In conclusion, macronutrient-rich diets primarily altered markers of amino acid and lipid metabolism, with little change in markers of carbohydrate and energy metabolism. Moreover, the HP diet influenced metabolites originating from gut microbial metabolism.

INTRODUCTION

Obesity is the most common nutritional disorder in domestic cats and is a key risk factor for insulin resistance and diabetes mellitus (Rand et al., 2004). Similar to humans (Larson-Meyer et al., 2006; Reinehr et al., 2004; Savoye et al., 2007), obesity leads to insulin resistance but weight loss increases insulin sensitivity (Feldhahn et al., 1999). Therefore, macronutrient intake may contribute to weight management and the development of insulin resistance. Cats are obligatory carnivores, yet most dry commercial cat foods include high amounts of digestible carbohydrate and fat because of cost, manufacturing considerations, or palatability. Macronutrient intake is thought to be a risk factor for the development of insulin resistance and diabetes in cats, but this idea is controversial.

Recent research suggested that healthy cats fed a low-protein, high-carbohydrate diet (protein: 24% of ME, fat: 33%, carbohydrate: 33%) were more prone to develop obesity and insulin resistance compared to those fed a high-protein, low carbohydrate diet (protein: 39% of ME, fat: 33%, carbohydrate: 21%) consuming the same caloric take (Hoenig et al., 2007). High-fat, low-carbohydrate diets (protein: 33% of ME, fat: 64%, carbohydrate: 3%) was shown as being more likely than a low-fat, high-carbohydrate diet
(protein: 33% of ME, fat: 9%, carbohydrate: 57%) to induce weight gain and a corresponding increase in insulin in healthy cats (Backus et al., 2007). However, Verbrugghe et al. (2010) reported a negative effect of a high protein intake (45% vs. 28% ME) on insulin sensitivity, which corresponds with the highly gluconeogenic nature of amino acids in healthy cats. Further studies are needed to elucidate the discrepancies from these studies to obtain a better understanding of how metabolism is altered by macronutrient intake in cats.

Metabolomics analytical platforms may allow an increased understanding of metabolism by providing a global metabolite profile of organisms. Merging metabolomics to nutritional sciences allows investigation of the complexity of interactions of all of the nutrients within complex biosystems. In humans, the application of nutritional metabolomics identified biomarkers of dietary protein, specific meat, or vegetable intakes (Rasmussen et al., 2012; Stella et al., 2006; O'Sullivan et al., 2011; Xu et al., 2010). The biomarkers of a high meat intake have been associated with several diseases, including hypertension, type II diabetes, cardiovascular disease, and cancer (Cross et al., 2007; Stolzenberg-Solomon et al., 2007; Cross et al., 2006; Verhoef et al., 2005; Koeth et al., 2013). Stella et al. (2006) applied a nuclear magnetic resonance spectroscopy (NMR)-based metabolomics approach to characterize the urinary metabolite profiles of vegetarian, low-meat, and high-meat diets in twelve healthy men. In addition to identifying potential biomarkers of different macronutrient intakes, they reported an alteration of the bacterial metabolism in response to diet. These studies provided a foundation required for personalized diet and nutrition forecasting.
Compared with the rapid development and application of nutritional metabolomics in humans, little is known about the influence of dietary macronutrient composition on the global metabolite profiles in domestic cats. The objective of this study was to use a high-throughput global metabolomics platform to characterize the feline plasma metabolome and identify the differences in the fasted plasma metabolome of cats fed high-protein, high-fat, or high-carbohydrate diets compared to a control diet with a balanced macronutrient profile. Using a holistic metabolomics approach in the present study, we may identify signatures indicative of specific dietary macronutrient intakes and metabolic status that may increase our understanding of the development of obesity, insulin resistance, and other diseases.

MATERIALS AND METHODS

Animals

A total of twelve healthy adult, neutered, male domestic shorthair cats [4 yr of age; 4.56 ± 0.16 kg body weight (BW); 4.5-5.5 body condition score (BCS) on a 9-point scale] were used in this study and housed in the animal facility of the Edward R. Madigan Laboratory at the University of Illinois (Urbana, IL). Cats were group-housed in the same room (26.67 m² × 3 m) most of the day but were individually housed in cages (0.61 m × 0.61 m × 0.61 m) to access diets from 8 am to 9 am and from 8 pm to 9 pm each day. The room was environmentally controlled (20 °C) with a 16 h light: 8 h dark cycle. Cats were provided access to various toys and scratching poles and socialized with each other and humans for behavioral enrichment. The protocol for this present study was reviewed and
approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

**Diets**

Response to four isoenergetic diets that contained different macronutrient profiles were tested: 1) control diet [CD diet: approximately 33% kcal metabolizable energy (ME) from each macronutrient], 2) high-fat diet (HF diet: approximately 50% kcal ME from fat), 3) high-protein diet (HP diet: approximately 50% kcal ME from protein), and 4) high-carbohydrate diet (HC diet: approximately 50% kcal ME from carbohydrate). Dietary ingredient and chemical composition of the 4 test diets are presented in Table 2-1 (Chapter 2).

To apply this research to a more practical scenario, the control diet was a typical commercial cat dry food. Based on the control diet, similar ingredients were used in different quantities to formulate a wide caloric distribution in terms of macronutrient content (approximately 50% of ME from each macronutrient). Test diets were extruded kibble diets based on milled brewer’s rice, poultry by-product meal, corn gluten meal, whole yellow corn, whole wheat, soy protein isolate, and fish meal. Diets were formulated to meet all nutrient recommendations provided by the Association of American Feed Control Officials (AAFCO, 2009) and were manufactured at Nestlé Purina PetCare Product Technology Center (St. Louis, MO).

Cats were offered two meals daily at 8 am and 8 pm. Prior to the trial, food intake was individually determined by calculating the daily maintenance energy requirement of
lean domestic cats (100 kcal × BW kg\(^{0.67}\)) (National Research Council, 2006) and was adjusted to maintain ideal BW. Cats were weighed weekly and food intake was adjusted to maintain BW (± 5% ideal BW) and BCS throughout the study. Water was available ad libitum at all times.

**Experimental design**

Twelve healthy lean adult male cats were used in a replicated 4 × 4 Latin square design for a total of 64 d (four 16-d periods). Cats were randomly allotted to one of the four test diets listed in Table 2-1 (Chapter 2). In each period, after a 15-d adaptation phase, overnight fasted blood samples were collected on d 16 before the morning meal was provided to measure global metabolite profiles.

**Blood collection and plasma preparation**

After a 12-h fast, a total of 1.5 mL of blood from each cat was collected via jugular or saphenous catheters at 8 am. Blood was immediately transferred into a pre-cooled Vacutainer tube (#367835, Becton, Dickinson, and Company, Franklin Lakes, NJ) containing EDTA and centrifuged at 1000 x g at 4 °C for 10 min. After centrifugation, plasma was collected into its respective cryovial and stored at -80 °C prior to overnight shipment on dry ice to the metabolomics facility (Metabolon Inc, Research Triangle Park, NC).

**Chemical analyses**

The four test diets were analyzed for proximate components (Table 2-1 in Chapter...
2). Diet subsamples were collected and ground using a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) through a 2-mm screen and dry ice in preparation for chemical analyses. Diet samples were analyzed for dry matter (DM) and organic matter (OM) according to Association of Official Analytical Chemists (AOAC, 2006). Crude protein was measured using a Leco Nitrogen/Protein Determinator (model FP-2000, Leco Corporation, St. Joseph, MI) according to Association of Official Analytical Chemists (AOAC, 2006). Fat concentrations were determined by acid hydrolysis according to the American Association of Cereal Chemists (AACC, 1983) followed by ether extraction (Budde, 1952). Total dietary fiber (TDF) was determined according to Prosky et al. (1992). Gross energy was measured using a bomb calorimeter (Model 1261, Parr Instrument Co., Moline, IL).

Sample preparation

Upon receipt of the fasted plasma samples, each sample was accessioned into the Laboratory Information Management System (LIMS; Metabolon Inc) to track sample handling, tasks, and results. All samples were maintained at -80°C until time of analysis. The sample preparation process was performed using the automated MicroLab STAR® system (Hamilton Company, Salt Lake City, UT). Recovery standards were added prior to the first step in the extraction process for quality control (QC) purposes. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions, one for analysis by LC/MS and one
for analysis by GC/MS. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent, and then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS, as described previously (Boudonck et al., 2009).

**GC/MS and LC/MS/MS analysis**

Biochemical profiling was performed using multiple platform (LC and GC) mass spectrometry technology as described previously (Boudonck et al., 2009). LC/MS was based on a Waters Acquity UPLC (Waters Corporation, Milford, MA) coupled to an LTQ mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) equipped with an electrospray ionization source and linear ion-trap mass analyzer. The sample extract was split into two aliquots, dried, then reconstituted in acidic or basic LC-compatible solvents. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient-eluted using water and methanol both containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5 mM ammonium bicarbonate. The MS analysis alternated between MS and data-dependent MS/MS scans using dynamic exclusion.

The samples destined for GC/MS analysis were re-dried under vacuum desiccation for a minimum of 24 h prior to being derivatized under dried nitrogen using bistrimethyl-silyl-trifluroacetamide (BSTFA). The derivatized samples for GC/MS were
analyzed on a Thermo Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer operated using electron impact ionization. The GC column was 5% phenyl and the temperature ramp was from 40° to 300 °C over a 16 min period. The instrument was tuned and calibrated for mass resolution and mass accuracy on a daily basis.

**Metabolite identification**

Metabolites were identified by comparison to library entries of purified standards or recurrent unknown entities. Identification of known chemical entities was based on comparison to Metabolon’s reference library entries of purified standards. Currently, more than 1000 commercially available purified standard compounds have been acquired and registered into Metabolon LIMS for distribution to both the LC and GC platforms for determination of their analytical characteristics. The combination of chromatographic properties and mass spectra gave an indication of a match to the specific compound or an isobaric entity. Additional entities were identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard or by classical structural analysis.

**Data normalization**

Raw area counts for each metabolite in each sample were normalized to correct for variation resulting from instrument inter-day tuning differences. For each metabolite, the raw area counts were divided by its median value for each run-day, therefore setting the medians equal to 1 for each day’s run. This correctly preserves all of variation among the samples, yet allows metabolites of widely different raw peak areas to be compared.
directly on a similar graphical scale. Missing values were assumed to result from areas falling below limits of detection. For each metabolite, the missing values were imputed with the observed minimum after the normalization step.

**Statistical analyses**

Principal component analysis (PCA) was performed using all named metabolites to provide a simultaneous comparison of metabolite changes with relation to diets. Then for the purpose of classification, random forest (RF) analyses (Cross et al., 2006) were performed to give an estimate of how well individuals can be classified in the dataset into each dietary group (Breiman, 2001). To determine which variables (metabolites) make the largest contribution to the classification, a “Mean Decrease Accuracy (MDA)” measure is computed. The MDA is determined by randomly permuting a variable, running the observed values through the trees, and then reassessing the prediction accuracy. If a variable is important to the classification, the prediction accuracy will drop after such a permutation. Thus, the RF analysis provides an importance rank ordering of metabolites. The top 30 metabolites were reported.

For each metabolite, ANOVA contrasts and one way ANOVA with cats as a random effect were performed following log transformation to identify metabolites that differed significantly among diets. A probability of $P \leq 0.05$ was considered significant and $P \leq 0.10$ was considered a trend. For significance testing, multiple comparisons were compensated for using false discovery rate (FDR) calculations (Benjamini and Hochberg, 1995), and FDRs were estimated using the q-value method (Storey and Tibshirani, 2003).
Statistical analyses were performed using the program “R” (http://cran.r-project.org/) and JMP (SAS Inst. Inc., Cary, NC: http://www.jmp.com).

RESULTS

Diet composition and macronutrient intake

Nutrient composition and energetic distribution of the four test diets is given in Table 2-1 (Chapter 2). Ingredients selected in the test diets are commonly used in the pet food industry, with the control diet being formulated similar to a commercially available cat food. The HF diet is characterized by a 52.8% energy contribution from the supplementation of tallow and fat from other ingredients, and a lower contribution from protein (25.5%) and carbohydrate (21.7%). The HP diet had a 50.4% energy contribution from protein sources, including corn gluten meal, chicken, and poultry byproduct, wheat flour, and soy protein isolate, and had a lower contribution from fat (25.9%) and carbohydrate (23.7%). The HC diet contributed a 45.6% energy from carbohydrate, mainly from brewer’s rice, and had a lower contribution from fat (27.9%) and protein (26.5%).

Average food intake, energy intake, and macronutrient intake of cats fed the four test diets is given in Table 3-1. Average food and energy intake were not different ($P > 0.10$) among diets. Diets were highly palatable by all cats (no food refusals) and no gastrointestinal symptoms or diarrhea issues were observed throughout the entire trial.
**Dietary clusters in metabolite profiles**

In the present study, our dataset comprised a total of 289 named metabolites. Using information from those metabolites, initial analysis of the dataset was conducted using PCA analysis (Figure 3-1). The first two principal components accounted for 24.02% of the total variation (PC1: 13.33% and PC2: 10.69%). The PCA plot showed a clear and complete separation of the HC diet from the HP diet, while the HF dietary group clustered between these 2 dietary groups. Cats fed the HP diet were mainly located in the negative region of principal component (PC) 1 and cats fed the HC diet were mainly present in the positive region of PC1. Cats fed the control diet were situated among three macronutrient-rich dietary groups.

To identify the significant metabolites responsible for the clustering patterns, RF analysis was performed using three comparisons (HC vs. HP; HC vs. HF; and HF vs. HP). As shown in Figure 3-2A, 3-hydroxyisobutyrate was the top-ranked metabolite, which made the largest contribution to the classification of cats into the HC or HP diet group with 100% predictive accuracy. If 3-hydroxyisobutyrate was removed from the dataset, the predictive accuracy would drop approximately 12.8% (MDA in Figure 3-2A) accordingly, indicating the importance of this metabolite to predict the macronutrient intake of cats. Interestingly, by comparing the top 30 metabolites from the 3 RF plots in Figure 3-2, gamma-glutamylleucine, 3-hydroxyisobutyrate, and 3-indoxyl sulfate were identified to distinguish cats fed the three macronutrient-rich diets. These three metabolites in the fasted plasma showed the same pattern, with higher concentrations in
cats fed the HP diet compared to cats in the HF diet group, and lower concentrations in
cats fed the HC diet compared to the HF diet (Figure 3-3).

**Metabolite profiles associated with the HP diet**

The HP diet had widespread effects on the fasted plasma metabolite profiles associated with amino acid metabolism, including increased phenylalanine and tyrosine; valine, leucine, and isoleucine; threonine; asparagine; urea cycle and proline; dipeptide and gamma-glutamyl peptide (Table 3-2). Plasma metabolites associated with BCAA metabolism (Table 3-2) were increased in cats fed the HP diet, especially leucine and isovalerylcarmitine that had more than a 2-fold change compared to cats fed the HC diet. Moreover, the metabolites associated with protein catabolism, 3-methylhistidine and trans-4-hydroxyproline, were less abundant in cats fed the HP diet. Cats fed the HP diet had elevated BHBA in plasma compared with cats fed the control and HC diet (Table 3-2) and decreased abundance of nucleic acid metabolites including N1-methyladenosine, N1-methylguanosine, 5-methylcytidine, and 2’-deoxycytidine compared with cats fed the HF and HC diet.

**Metabolite profiles associated with the HF diet**

Cats fed the HF diet had metabolite profiles indicative of increased glutathione (GSH) metabolism [cysteine-glutathione disulfide and oxidized GSH (GSSG)], tocopherol metabolism (α-tocopherol and γ-tocopherol) and monohydroxy fatty acids (2-hydroxydecanoic acid and 2-hydroxypalmitate) (Table 3-2). In addition to the increase in oxidative stress metabolites, cats fed the HF diet had increased concentrations of
numerous plasma lipid metabolites, such as cholesterol, long chain fatty acids, dicarboxylates, glycerolipids, and BHBA (Table 3-2).

**Metabolite profiles associated with the HC diet**

The HC diet had little effect on abundance of metabolites involved in carbohydrate metabolism. Compared with effects of the HP diet, the HC diet had a negative effect on amino acid metabolism (Table 3-2), with the abundance of amino acids (BCAA and aromatic) and their gamma-glutamyl peptides both being decreased. However, several metabolites (kynureinate and indolepropionate) associated with tryptophan metabolism were more abundant in cats fed the HC diet. Several metabolites related to oxidative stress (cysteine-glutathione disulfide, GSSG, and monohydroxy fatty acids) remained at a lower level compared to those in the HF dietary group.

**Gut microbiome-derived metabolites associated with dietary intervention**

An alteration of plasma metabolites associated with gut microbial metabolism in response to different dietary macronutrient intake was observed. These metabolites were represented in Figure 3-4. Cats fed the HP diet showed greater concentrations of 3-indoxyl sulfate, phenylalanine, tyrosine, hippurate, phenylacetylglycine, phenol sulfate, phenylpropionylglycine, cinnamoylglycine, 4-hydroxyphenylacetate, 4-ethylphenylsulfate, catechol sulfate, and equol sulfate compared to cats fed the HF diet. Plasma from HC group resembled HP plasma with respect to increased abundance of polyphenolic compounds (equol sulfate and 4-ethylphenylsulfate). In addition, cats fed the HC diet had an elevated concentration of indolepropionate.
DISCUSSION

Cats, as obligatory carnivores, rely on high-protein animal tissue to meet their specific nutritional requirements in the wild and are metabolically adapted to a lower glucose utilization and higher protein metabolism (MacDonald et al., 1984). However, due to the transformation of lifestyle and the composition of many commercial diets, the cat’s diet was changed from high-protein, low-carbohydrate, prey typical for feral cats (e.g., domestic mouse: 55.8% crude protein, 23.6% crude fat, 8.8% nitrogen free extract and 11.8% ash, DM basis; Dierenfeld et al., 2002) to a relatively low-protein, high-carbohydrate commercial diet. Some think these diets contribute to the recent increase in incidence of obesity, insulin resistance, and type II diabetes in domestic cats (Slingerland et al., 2009; O'Sullivan et al., 2011). The results of food preference experiments showed that cats had a clear preference for diets high in protein, fat, and low in carbohydrate (Hewson-Hughes et al., 2011; Fekete et al., 2005). Research testing diets with different dietary macronutrient composition may have implications for feline health and welfare. To build a foundation in this area of research, the present study tested the effect of 3 different macronutrient-rich diets on the circulating metabolite profiles of cats.

The application of metabolomics to study the effect of dietary intervention on feline metabolism has been very limited. To our knowledge, only one study (Allaway et al., 2010) has used a high-throughput metabolomics strategy in cats. In that study, the change in the fasted plasma metabolome from a simple nutritional intervention (3.85% additional glucose) in cats after a 2-week feeding period was evaluated. They reported
44% of plasma metabolites had a significant change when glucose was added to the basal diet. However, PCA analysis did not show distinct clustering based on dietary groups.

To characterize the changes of metabolites profiles as a function of diets, healthy cats were fed the test diets for 15 days. The HP diet had widespread effects on the fasted plasma metabolite profiles associated with amino acid metabolism. Silva and Mercer (Silva and Mercer, 1985) suggested that the amount of amino acid-based gluconeogenic precursors was not affected by the cat’s protein intake. Russell et al. (2002) and Green et al. (2008) both proved that cats can adapt to a wide range of protein intake by increasing protein oxidation when fed a HP diet, but were unable to fully adapt to low dietary protein concentrations. Complete oxidation of amino acid carbons occurs when they are ultimately converted to acetyl-CoA, which is oxidized to CO2 and H2O via the citric acid (TCA) cycle and mitochondrial electron transport system. However, metabolites associated with the TCA cycle did not change in the HP dietary group or other groups which may indicate that protein oxidation was not affected by the diets, and energy from protein oxidation was not a predominant energy source for cats in the fasted state.

Branched-chain amino acids (BCAA) are an important energy source when glucose is depleted as an energy source. BCAA can be catabolized to contribute carbon skeletons to TCA cycle for energy production. BCAA can be derived from either dietary uptake or protein degradation. In this study, plasma metabolites associated with BCAA metabolism (Table 3-2) were increased in cats fed the HP diet compared to cats fed the HC diet, especially leucine and isovalerylcarnitine. Moreover, 3-methylhistidine, a
marker of muscle catabolism (Elia et al., 1981; Long et al., 1981), and trans-4-
hydroxyproline, a collagen catabolite, were less abundant in cats fed the HP diet. Altogether, these results may suggest that increased protein consumption may slow the muscle protein catabolism. Leucine was reported to activate mTOR signaling, which inhibits protein degradation in the liver and muscle (Nakashima et al., 2007; Rhoads and Wu, 2008). Additionally, Yasuda et al. (2011) reported that arginine had a strong insulinotropic effect, whereas leucine and alanine had weaker insulinotropic effects in healthy adult cats. However, it must be noted that fasted circulating concentrations of BCAA often are often associated with increased obesity, insulin resistance and type II diabetes (Huffman et al., 2009). Newgard et al. (2009) reported that supplementation of a HF diet with BCAA reduced food intake and body weight, but caused resulted in insulin resistance in rats. They also characterized the blood metabolite profiles of obese versus lean humans and revealed a BCAA-related metabolic signature that was suggestive of increased catabolism of BCAA and correlated with insulin resistance (Newgard et al., 2009). Using a metabolomics approach to compare the metabolite changes of obese, diabetic, and lean cats fed a HP diet may be more meaningful and may aid the development of weight-loss and diabetic diets.

Three-hydroxybutyrate (BHBA) is a ketone body that can be generated from fatty acid β-oxidation or from catabolism of ketogenic amino acids. Cats fed the HP diet had elevated BHBA in plasma compared with cats fed the control and HC diet, indicating a greater use of fat for energy in a fasted state. In humans, when carbohydrate intakes are very low, a ketogenic state occurs because of the reduced glucose availability that results
in increased production of ketone bodies from fat reserves (Volek and Westman, 2002). Thus, a high-protein ketogenic diet often is used for weight loss in humans (Freedman et al., 2001). However, it must be noted that ketoacidosis is a complication of diabetes mellitus in both humans and pets, which is caused by an absolute or relative deficiency of insulin in the body (Stojanovic and Ihle, 2011).

Nucleic acid metabolites are products of DNA/RNA catabolism. In aged humans, the increased concentration of degradation products of purine metabolism in plasma may reflect an increased cellular nucleic acid turnover owing to inflammation or cellular necrosis (Lawton et al., 2008). The decreased abundance of nucleic acid metabolites in cats fed HP diet may suggest that unlike cats fed HC and HF, which may have greater postprandial oxidative stress response (Gregersen et al., 2012), cats fed HP may be less likely to undergo oxidative stress and have a lower risk of acute inflammatory diseases or tissue inflammation.

Cats fed the HF diet had metabolite profiles indicative of increased GSH metabolism, tocopherol metabolism, and free fatty acid oxidation (Table 3-2). These metabolites are markers of oxidative stress and highly associated with redox balance regulation. GSH is the major non-enzymatic defense system against reactive oxygen species (ROS) in the cytoplasm. It is responsible for reducing disulfide bonds in cytosolic proteins by serving as an electron donor. In this process, GSH is converted to its oxidized form, GSSG, providing cells with a reduced environment and protecting against oxidative stress (Aslund and Bbeckwith, 1999; Meister and Tate, 1976). In our study, the increased
level of cysteine-glutathione disulfide and GSSG in cats fed HF may suggest that high fat intake induces ROS generation, leading to an oxidized redox status in cats. Additionally, free fatty acids are more prone to be oxidized into hydroxylipids under oxidative conditions. Increased 2-hydroxydecanoic acid and 2-hydroxypalmitate concentrations are also indicative of increased oxidation status in cats fed the HF diet.

Both α-tocopherol (the active form of vitamin E) and its isoform, γ-tocopherol, can stop the production of ROS formed when fat undergoes oxidation. As fat-soluble antioxidants, they can be incorporated into biological membranes, which protect them from oxidative damage (Schaffer et al., 2005). The tallow used in the HF diet was stabilized with vitamin E (Table 2-1 in Chapter 2). Although the concentration of vitamin E in the test diets was not analyzed, it is likely that the increased plasma tocopherol concentrations were due to the vitamin E (in tallow) in the HF diet.

There are numerous studies suggesting that oxidative stress is associated with the pathogenesis of insulin resistance (Evans et al., 2002; Furukawa et al., 2004; Bashan et al., 2009). Excessive production of ROS can cause oxidative damage in itself, in addition to inducing the production of several secondary by-products as the result of lipid peroxidation, such as 4-hydroxy-2-nonenal (4-HNE). A recent study reported that 4-HNE impaired insulin signaling and thoroughly diminished the insulin-stimulated glucose uptake in mouse skeletal muscle (Pillon et al., 2012). Backus et al. (2007) reported that high dietary fat, but not carbohydrate, induced weight gain and a congruent increase of in insulin in cats. The decreased insulin sensitivity may be explained by the increased
oxidation status in cats fed high dietary fat.

In addition to the increase in oxidative stress metabolites in cats fed the HF diet, the increase in plasma cholesterol has a high correlation with the high prevalence of obesity and diabetes in dogs (Jeusette et al., 2005). Plasma long-chain fatty acids were found to be associated with the degree of obesity in humans (Scaglioni et al., 2006). The increase in plasma glycerol concentration was reported during the early phase of human obesity (Le Stunff and Bougneres, 1992). Taken together, the HF diet had widespread effects on the abundance of many lipid metabolites and oxidative stress metabolites. Therefore, it may increase the risk of feline obesity and diabetes mellitus.

In the present study, we observed an alteration of plasma metabolites associated with gut microbial metabolism in response to different dietary macronutrient intake. Botsford and Demoss (1972) reported enteric bacteria express tryptophanase, which can convert tryptophan to indole-containing compounds. Although cats fed the HP diet had increased plasma amino acid concentrations, tryptophan was not increased, possibly due to the metabolism of dietary tryptophan by gut bacteria. This was also consistent with a recent study in mice (Wikoff et al., 2009), where germ-free mice had 1.7 fold higher serum tryptophan concentrations than conventional mice. In contrast, metabolites associated with tryptophan metabolism (kynurenate and indolepropionate) were more abundant in plasma from cats fed the HC diet. These results suggest that dietary macronutrient composition influenced blood metabolites originating from gut microbial metabolism in healthy adult cats, particularly the HP diet. These results may have been
due to substrate availability and/or alteration of gut bacterial species. Interestingly, an alteration of gut microbial metabolism has been strongly linked to obesity in humans (Turnbaugh et al., 2006; Calvani et al., 2010). Everard et al. (2013) reported that administration of Akkermansia muciniphila, a gut bacteria resided in the mucus layer, to mice fed a HF diet reversed the HF diet-induced inflammation, fat-mass gain, and insulin resistance. Thus, further research is needed to study the effects of dietary intervention on the relationships between host and gut microbial metabolism and to develop dietary strategies for the prevention or treatment of obesity and other metabolic disorders.

A high-throughput metabolomics approach was used to characterize blood metabolite profiles of cats fed various dietary macronutrient concentrations. Cats fed HP, HF, and HC diets formed distinct metabolite profiles and three potential biomarkers, gamma-glutamylleucine, 3-hydroxyisobutyrate, and 3-indoxyl sulfate, were identified to distinguish cats fed the three macronutrient-rich diets. Our study revealed that cats fed the HP diet had decreased metabolites associated with nucleotide catabolism, but increased metabolites associated with amino acid and fat metabolism for energy, as well as increased metabolites associated with gut microbial metabolism. Cats fed the HF diet had increased metabolites associated with lipid metabolism and oxidative status. In conclusion, macronutrient-rich diets primarily altered markers of amino acid and lipid metabolism, with little change in markers of carbohydrate and energy metabolism. Further studies to investigate the plasma metabolite profiling after a postprandial challenge with different macronutrients may be required to reveal the diet effects on feline metabolic status.
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### Table 3-1. Energy and macronutrient intake of cats fed test diets

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<tr>
<th>Item</th>
<th>CD</th>
<th>HF</th>
<th>HP</th>
<th>HC</th>
</tr>
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<tr>
<td>Food intake, g/d</td>
<td>51.5</td>
<td>46.2</td>
<td>54.4</td>
<td>54.3</td>
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<tr>
<td>Energy intake, kcal/d</td>
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<td>206.1</td>
<td>202.0</td>
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<td>Protein intake, g/d</td>
<td>19.6</td>
<td>15.0</td>
<td>29.1</td>
<td>15.8</td>
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<td>Acid-hydrolyzed fat intake, g/d</td>
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<td>12.8</td>
<td>6.2</td>
<td>6.8</td>
</tr>
<tr>
<td>NFE (digestible carbohydrate) intake, g/d</td>
<td>17.4</td>
<td>12.8</td>
<td>13.6</td>
<td>27.1</td>
</tr>
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</table>
Table 3-2. Diet-associated changes in cat plasma metabolite profiles

<table>
<thead>
<tr>
<th>Metabolic pathway</th>
<th>Metabolite</th>
<th>Mean values</th>
<th>Group effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD²</td>
<td>HC</td>
<td>HF</td>
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<tr>
<td>Amino acids metabolism</td>
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</tr>
<tr>
<td></td>
<td>Tyrosine</td>
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<td>0.72</td>
</tr>
<tr>
<td></td>
<td>3-(4-hydroxyphenyl)lactate</td>
<td>1.08</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>4-hydroxyphenylpyruvate</td>
<td>1.18</td>
<td>0.72</td>
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<td></td>
<td>4-hydroxyphenylacetate</td>
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<td></td>
<td>Phenylacetylglycine</td>
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<td>Phenylacetylglutamine</td>
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<td>Phenylpropionylglycine</td>
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<td>3-phenylpropionate</td>
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<td>Phenol sulfate</td>
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<td>Kynurenate</td>
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<td>3-indoxyl sulfate</td>
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<td>Metabolic Pathway</td>
<td>Value 1</td>
<td>Value 2</td>
<td>Value 3</td>
</tr>
<tr>
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<td>Indolepropionate</td>
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<td>3-methyl-2-oxovalerate</td>
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Carbohydrates metabolism

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Lipid metabolism

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<td>2-hydroxystearate</td>
<td>2-hydroxypalmitate</td>
<td>Sebacate (decanedioate)</td>
<td>Azelate (nonanedioate)</td>
<td>Octadecanedioate</td>
<td>Undecanedioate</td>
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<td>------------------------------------</td>
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<td>Undecanedioate</td>
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<td>3-hydroxybutyrate (BHBA)</td>
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<td>0.69</td>
<td>1.28</td>
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<td>Cofactors and vitamins</td>
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Table 3-2. (cont.)

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<tr>
<th>Metabolite</th>
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<th>HP</th>
<th>HC</th>
<th>p-Value</th>
<th>p-Value</th>
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<td>0.96</td>
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<td>0.86</td>
<td>1.39</td>
<td>0.76</td>
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<td>Hippurate</td>
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<td>&lt; 0.001</td>
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<td>Catechol sulfate</td>
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<td>&lt; 0.001</td>
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<td>&lt; 0.001</td>
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<td>1.25</td>
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<td>4.68</td>
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1For each metabolite, the mean value is the group mean of re-scaled data to have a median equal to 1.

2CD, control diet; HF, high-fat diet; HP, high-protein diet; HC, high-carbohydrate diet.
Figure 3-1. Principal component analysis score plot derived from plasma metabolic profiles for 12 healthy adult cats

Samples from each cat fed different test diets are represented in the plot and are colored according to diets as follows:  ❄ Control diet;  🌿 High-carbohydrate diet;  🌝 High-fat diet;  🍔 High-protein diet.
**Figure 3-2.** Classification of cats fed the high carbohydrate (HC), high-fat (HF), and high-protein (HP) diets using random forest analysis of metabolic profiles

(A)
Figure 3-2. (cont.)

(B)
The metabolites rank according to their contribution to the classification of 12 cats into different macronutrient-rich diet. The top 30 metabolites are listed on the y-axis in decreasing order of importance. The mean decrease in accuracy for each metabolite is plotted on x-axis. Symbols in the plots are colored by super pathway as follows: Amino acid; Cofactors and vitamins; Lipid; Nucleotide; Peptide; Xenobiotics. Insets show the prediction accuracy of the separation of diets. (A) Classification of cats fed HC versus HP diets; (B) Classification of cats fed HC versus HF diets; (C) Classification of cats fed HF versus HP diets.
Figure 3-3. Box plots of metabolites altered by macronutrient-rich diets

(A) gamma-glutamylleucine  
(B) 3-hydroxyisobutyrate  
(C) 3-indoxyl sulfate

The x-axis shows the diet group, including control (CD), high-carbohydrate (HC), high-fat (HF), and high-protein (HP). The y-axis shows the relative normalized intensity for each metabolite. In the box plots, the top and the bottom of the box represent the 75th and 25th percentile, respectively, and the top and the bottom bars represent the entire distribution of the data points excluding “extreme” points that are represented by “◦”. The median value is represented by the black line “-” in the box and the mean value is represented by the symbol “+” in the box.
Figure 3-4. Box plots of gut microbiome-derived metabolites in response to the test diets

(A) 3-indoxyl sulfate (B) Indolepropionate
(C) Phenylalanine (D) Tyrosine
Figure 3-4. (cont.)

(E)  

(F)  

(G)  

(H)
Figure 3-4. (cont.)

(I) cinnamoylglycine

(J) 4-hydroxyphenylacetate

(K) 4-ethylphenylsulfate

(L) catechol sulfate
Figure 3-4. (cont.)

For each metabolite, a significant dietary group effect exists: $p < 0.05$ and $q < 0.05$. The x-axis shows the diet group, including control (CD), high-carbohydrate (HC), high-fat (HF), and high-protein (HP). The y-axis shows the relative normalized intensity for each metabolite. In the box plots, the top and the bottom of the box represent the 75th and 25th percentile, respectively, and the top and the bottom bars represent the entire distribution of the data points excluding “extreme” points that are represented by “○”. The median value is represented by the black line “-” in the box and the mean value is represented by the symbol “+” in the box.
CHAPTER 4: EFFECTS OF FEEDING FREQUENCY AND DIETARY WATER CONTENT ON VOLUNTARY PHYSICAL ACTIVITY IN HEALTHY ADULT CATS

ABSTRACT: Low physical activity has been identified as a major risk factor for the development of feline obesity and diabetes. This study aimed to evaluate the effects of increased meal frequency and dietary water content on voluntary physical activity in cats fed to maintain body weight (BW). Ten adult lean neutered male cats were used in two tests, both crossover studies composed of a 14-d adaptation period, followed by a 7-d measurement of physical activity from d 15 to d 22 using Actical activity collars. Cats were group-housed under a 16 h light: 8 h dark cycle for most of the day, except for times when they were individually housed in cages to access their diet. In Experiment 1, the difference in voluntary physical activity among cats fed one, two, four, or a random number of meals per day were tested in a $4 \times 4$ Latin square design in 4 individual rooms. In Experiment 2, the effect of increasing dietary water content on voluntary physical activity was tested in a cross-over design including a 5-d phase for fecal and urine collection from d 22 to d 27. Cats were randomly assigned to two rooms and fed a dry commercial diet with or without added water (70% hydrated) twice daily. Activity levels were expressed as ‘activity counts’ per epoch (15 sec). In Experiment 1, average daily activity level for one-meal-fed cats was lower than four-meal-fed ($P = 0.004$) and random-meal-fed ($P = 0.02$) cats, especially during the light period. The activity level of cats during the dark period was greater in one-meal-fed cats compared with cats fed two meals ($P = 0.008$) or four meals ($P = 0.007$) daily. Two-h food anticipatory activity
(FAA) prior to scheduled meal times for one-meal-fed cats was lower ($P < 0.0001$) than the multiple-meal-fed cats. In Experiment 2, average daily activity level of cats fed the 70% hydrated diet tended to be higher ($P = 0.06$) than cats fed the dry diet, especially during the dark period ($P = 0.007$). Two-h FAA prior to the afternoon meal for cats fed the 70% hydrated diet was lower ($P < 0.05$) than cats fed the dry diet. Cats fed the 70% hydrated diet had greater daily fecal ($P = 0.008$) and urinary ($P = 0.001$) outputs and lower ($P < 0.001$) urinary specific gravity compared to cats fed the dry diet. In conclusion, increased feeding frequency and dietary water content, without changing energy intake or dietary macronutrient composition, appear to promote physical activity, which may aid in weight maintenance in cats.

**INTRODUCTION**

The latest nationwide survey conducted by the Association for Pet Obesity Prevention (2012) reported that 55% of cats were classified as overweight or obese by their veterinarians. Feline obesity mainly results from an energy imbalance, either excessive food intake or insufficient physical activity. The primary weight loss strategy is through energy restriction to achieve negative energy balance. However, the high incidence of unsuccessful weight loss or subsequent weight regain may not only be due to a lack of owner compliance (Kienzle and Berglert, 2006), but also may result from a mass-adjusted decrease in energy expenditure in cats during energy restriction (Villaverde et al., 2008). To date, many dietary strategies have been developed to reduce obesity incidence by restricting food intake, however, strategies to increase physical activity are lacking.
Increased feeding frequency has been suggested to manage a healthy BW. To manage feline weight loss, owners often are instructed to offer the daily food ration in several meals (2-4) throughout the day rather than in a single meal (German and Martin, 2008). However, very little research has been conducted to elucidate the mechanisms involved. Other than feeding frequency, dietary water content also may influence the physical activity of cats. Data from a recent study (Cameron et al., 2011) reported that reducing dietary energy density via manipulation of dietary water content increased voluntary physical activity level in cats fed ad libitum. The body mass in that study, however, changed over time. Therefore it was not clear whether the decreased physical activity was due to alterations in BW or dietary water content.

The purpose of this study was to evaluate the effects of feeding frequency and dietary water content on voluntary physical activity in cats. We hypothesized that by increasing feeding frequency and dietary water content, without altering the energy consumed per day, an increased activity from food seeking or anticipation would result in an altered activity pattern and increase overall activity. These results may aid in developing weight management strategies for cats.

MATERIALS AND METHODS

Two experiments were conducted to determine the physical activity level of adult cats fed a varied 1) meal frequency or 2) dietary water content. The protocol for these experiments was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.
**Animals**

A total of ten healthy adult neutered male domestic cats (initially 6 yr; 4.59 ± 0.42 kg BW; 4.5-5.5 BCS on a 9-point scale) were used in these two tests. Cats were group-housed in rooms most of the day, but were individually housed in cages (0.61 m x 0.61 m x 0.61 m) to access diets in the animal facility of the Edward R. Madigan Laboratory at the University of Illinois (Urbana, IL). Rooms were environmentally controlled (20 °C) with a 16 h light: 8 h dark cycle (light was on at 6 am and off at 10 pm). Cats were provided access to various toys and scratching poles for behavioral enrichment.

Cats were fed at amounts to maintain BW and body condition score (BCS, 4-6 on a scale of 9) throughout the study. Prior to the experiments, food intake to maintain BW was determined by calculating the daily maintenance energy requirement of lean domestic cats (ME requirement (kcal) = 100 × BW kg\(^{-0.67}\)) (NRC, 2006) and by using previous feeding records. Cats easily consumed all meals within the 1 h time periods in their cage. Cats were weighed and assessed for BCS weekly, and food intake was adjusted accordingly every week. Water was available ad libitum throughout all experiments.

**Experimental Design**

**Experiment 1.** This study aimed to determine the physical activity level of adult cats with varied feeding frequency. An unequal replicated 4 x 4 Latin square design consisting of 84 d (four, 21-d periods) was used. Each period included a 14-d adaption phase, followed by the measurement of physical activity from the morning of d 15 to the morning of d 22. Cats were randomly assigned to 4 rooms (2 rooms had 3 cats/room; 2 rooms had 2
cats/room) and remained in the same room throughout the experiment. Cats were fed a commercial dry kibble diet (Whiskas® Adult Meaty Selection®, Chicken & Turkey Flavors, Mars, Inc., Franklin, TN) in one of four treatments: 1) One meal daily (100% of the daily intake was offered at 8 am); 2) Two meals daily (50% of the daily intake was offered at 8 am and 8 pm); 3) Four meals daily (25% of the daily intake was offered at 8 am, 12 pm, 4 pm, and 8 pm); and 4) Random feeding frequency (1, 2, or 4 meals per day were fed randomly throughout the 21-d period so that cats did not develop a regular feeding behavior/pattern). The commercial diet fed in the present study contained a maximum of 12% moisture and 4% crude fiber, and a minimum of 35% protein and 13% fat. Cats were maintained on a strict housing schedule, being individually housed in cages with access to diet from 8 to 9 am, 12 to 1 pm, 4 to 5 pm, and 8 to 9 pm each day. For the other 20 h, cats were group-housed in rooms to allow for voluntary physical activity without human interference.

**Experiment 2.** This study aimed to determine the physical activity level changes of adult cats when water was added to a standard commercial dry kibble feline diet. A cross-over design consisting of 52 d (two 26-d periods) was used. Both periods included a 14-d adaptation phase, a 7-d phase for voluntary physical activity measurement from the morning of d 15 to the morning of d 22, and a 5-d phase for fecal and urine collection from the morning of d 22 to the morning of d 27. Cats were randomly assigned to 2 rooms (5 cats/room) and remained in the same room throughout the study. The diet was based on a commercially dry kibble diet (Royal Canin Adult Fit 32, Mars, Inc., Rolla, MO): maximum 8% moisture (basal hydration), minimum 32% protein and 13% fat, and maximum 5.8% crude fiber. Cats were fed the Royal Canin Adult Fit 32 diet without (dry
diet) or with added water (contained 70% water; 70% hydrated diet). For this treatment, tap water was added to the kibble 1 h before feeding to allow water absorption into the kibble and minimize evaporation. The kibble maintained its normal structure after water absorption. Cats were fed twice daily at 9 am and 3 pm. Each cat was provided the same amount of food (dry weight) throughout the study. Half of the daily intake was offered to cats at each feeding time. Cats were group-housed in the rooms for 22 h/d and were individually housed 2 h/d in cages at feeding times (9-10 am and 3-4 pm).

**Physical activity level assessment**

Voluntary physical activity levels were evaluated using Actical activity monitors (Mini Mitter, Bend, OR), which has been validated for use in research cats (Lascelles et al., 2008). The monitor was programmed before the first day of each activity measurement period. Physical activity was assessed for seven consecutive days. Monitors were worn around the neck from 8 am on d 15 to 8 am on d 22 of each period. The data were collected and analyzed using the receiver and Actical software. To control for variability, cats wore the same monitor for each period of the study. The Actical software analyzed the data compiled by the monitor and converted it into arbitrary numbers referred to as ‘activity counts’. Activity levels are expressed as activity counts per epoch (epoch length = 15 sec). Values represent the mean epoch activity count over 7 d, during the selected hours (light hours, dark hours, and average throughout the entire day).

**Food Anticipatory Activity**

Food anticipatory activity (FAA) in this study was expressed as the ratio of the activity in the 2 h before the availability of food (scheduled feeding times) divided by
total 24-h activity and expressed as a percentage. Total daily FAA was calculated as the sum of the FAAs prior to the scheduled feeding times.

Sample collection and analysis

From d 22 to 27 of each period in Experiment 2, total fecal output from each cat was collected, weighed, scored, and stored at -20 °C until further analyses. For each cat, fecal DM was determined according to AOAC (2006). Fecal moisture content values were calculated using this equation: 100% - fecal DM (%). Scoring was conducted using a 5-point scale as follows: 1 = hard, dry pellets; 2 = dry, well-formed stools; 3 = soft, moist, formed stool; 4 = soft, unformed stool; and 5 = watery, liquid that could be poured.

Urine samples were analyzed for total volume and specific gravity using the Reichert Rhino clinical handheld VET360 refractometer (Reichert Technologies, Depew, NY) to indicate urinary concentration and body hydration status.

Statistical Analysis

All data were analyzed using the MIXED procedure of Statistical Analysis Systems 9.3 (SAS Institute Inc., Cary, NC). Feeding frequency (Experiment 1) or water content (Experiment 2) was tested as the main fixed effect. Random effects of cat (nested within room) and period were included in the model. When food intake was analyzed as the main fixed effect, random effects of cat (nested within room), period, and week were included in the model. Treatment means were calculated using the LSMEANS statement and means were separated using the PDIFF statement of PROC MIXED. A probability of $P < 0.05$ was considered significant and a trend considered $P < 0.10$. 
RESULTS

Experiment 1

The total average physical activity (activity count/epoch) for one-meal-fed cats was lower than four-meal-fed ($P = 0.004$) and random-meal-fed ($P = 0.02$) cats (Table 4-1). The same patterns and treatment differences in activity were observed during the light period (Table 4-1). The average physical activity level of cats during the dark period, however, was greater in one-meal-fed cats compared with cats fed two meals ($P = 0.008$) or four meals ($P = 0.07$) daily (Table 4-1). This led to a lower ($P = 0.03$) light: dark ratio for one-meal feeding than multiple-meal feeding (Table 4-1). Total daily FAA for one-meal-fed cats was lower ($P < 0.001$) than the multiple-meal-fed cats (Figure 4-1). This mainly resulted from the lower FAA for one-meal-fed cats before the second, third, and fourth meals compared with the multiple-meal-fed cats. However, cats fed only one meal at 8 am had higher FAA than those fed multiple meals daily before the morning meal (Figure 4-1). As the daily activity patterns show in Figure 4-2, although the activity peak for one-meal-fed cats occurred at the same time as multiple-meal-fed cats (during the 8 am meal), peaks of activity for one-meal-fed cats during the other meals were delayed when compared with four-meal or random meal-fed cats. Interestingly, peaks of activity for two-meal fed cats were also delayed at 12 pm and 4 pm meals, when compared with four-meal or random meal-fed cats. Cats fed four meals and random meals daily had similar daily activity patterns (Figure 4-2).
Experiment 2

Compared with cats fed the dry diet, those fed the 70% hydrated diet tended to have increased ($P = 0.06$) total average physical activity (Table 4-2). During the dark period (from 10 pm to 6 am), cats fed the 70% hydrated diet had increased ($P = 0.007$) activity compared with those fed the dry diet (Figure 4-3). This tended to result in a lower ($P = 0.058$) light: dark ratio for cats fed the 70% hydrated diet than those fed the dry diet (Table 4-2). Two-h FAA prior to the afternoon meal was lower ($P = 0.049$) for cats fed the 70% hydrated diet than cats fed the dry diet, but there was no difference in FAA prior to the morning meal nor the total daily FAA (Figure 4-4). As shown in Figure 4-4, the daily activity curve for cats fed the 70% hydrated diet was above the curve for cats fed the dry diet during most hours of the day. In addition to the peaks of activity during the two meal times, there was an additional peak of activity from 5 to 9 pm for cats fed the 70% hydrated diet (Figure 4-3).

Despite no differences in fecal score, cats fed the 70% hydrated diet had greater ($P = 0.008$) daily fecal output and tended to have greater fecal moisture ($P = 0.063$) and daily dry fecal output ($P = 0.095$) compared to cats fed the dry diet. In addition, cats fed the 70% hydrated diet had greater daily urinary volume ($P = 0.001$) and lower ($P < 0.001$) urinary specific gravity than those fed the dry diet.

DISCUSSION

The incidence of obesity and type II diabetes mellitus in domestic cats has rapidly increased in recent decades. In addition to the prevalence of highly palatable commercial pet foods, the sedentary indoor lifestyle and inappropriate feeding strategies (e.g., the
amount and frequency of food provision) also largely contribute to obesity, insulin resistance and diabetes in domestic cats (Rand et al., 2004; Prahl et al., 2007; Slingerland et al., 2009). This study aimed to quantitatively evaluate the effects of two different feeding strategies, namely increased meal frequency and increased dietary water content, on voluntary physical activity by using accelerometer-based activity monitors. For the first time using objective measurements, our data showed that increasing meal frequency and dietary water content increased voluntary physical activity levels in cats fed to maintain BW.

Although cats fed four meals and a random number of meals daily had greater physical activity than cats fed once daily, it did not differ from those fed twice daily. These results were consistent with our previous study (Deng et al., 2011) in which two-meal and four-meal feeding patterns were not different. In addition, similar daily activity patterns were observed in both studies, with one-meal-fed cats having the highest activity peak prior to the 8 am meal, but lower and delayed peaks of activity during the other meal times compared with multiple-meal-fed cats. It must be noted that housing conditions in the previous study (all cats housed in one room) were different than the present study that housed cats with different treatments in different rooms. This difference indicated that even though the interactions among cats from different treatments might attenuate the effects of feeding frequencies on activity, it was not the key factor affecting activity. Bradshaw and Cook (2006) conducted a multivariate analysis of variance of the behavior profiles to study the effects of cat characteristics at feeding occasions. They did not observe significant behavior differences due to the presence of another cat or a dog in the household.
When animals are meal-fed at scheduled times, increased periods of activity prior to meal delivery occur, a response referred to as FAA. This anticipatory activity has been observed in several species, including rodents (Van der Harst et al., 2003), mink (Vinke et al., 2004), and horses (Peters et al., 2012). For the first time using quantitative measures, the present study demonstrated the occurrence of FAA in domestic cats. Interestingly, the daily FAA (a total of 8 h/d) in all cats fed varied meal frequencies contributed to 51 to 65% of total daily activity (24 h). Daily FAA for one-meal-fed cats was lower than the multiple-meal-fed cats (Figure 4-1). Those results indicate that by increasing meal frequency, an increased overall activity results from increased FAA.

Another interesting finding in this study was that cats on a fixed feeding schedule appear to quickly learn their feeding times, with increased FAA prior to their scheduled meal time, but much less at other times when placed in their individual cages (Figure 4-2, one-meal fed cats at 8 am, two-meal fed cats at 8 am and 8 pm, four-meal fed cats at 8 am, 12 pm, 4 pm and 8 pm). Because activity peaks at the other time points were delayed (Figure 4-2, one-meal fed cats at 12 pm, 4 pm and 8 pm, two-meal fed cats at 12 pm and 4 pm), it appears to indicate that the increased physical activity during those time points may have been mainly due to the human interactions prior to being placed in their cages. FAA is known to be a circadian behavior that occurs when food availability is restricted to a short temporal window in the day. The suprachiasmatic nucleus (SCN) is the site of the light-entrainable pacemaker, which is principally entrained to light/dark cycles. However, it is not clear whether the SCN is the locus of the food-entrainable pacemaker to control FAA. Neural and/or hormonal signaling systems between the gut and the brain appear to be involved in FAA (Davidson, 2009). Ghrelin and leptin are important
hormones coordinating behavioral activity rhythms around the timing of food availability (Bodosi et al., 2004; Martinez-Merlos et al., 2004). Blum et al. (2009) reported that FAA is reduced in ghrelin receptor-mutant mice. Moreover, feeding frequency can alter secretion and daily fluctuation of ghrelin and leptin in humans (Chapelot et al., 2006; Solomon et al., 2008). Previous studies in our laboratory demonstrated that cats fed varied meal frequencies altered leptin secretion (Deng et al., 2011). In addition to ghrelin and leptin, other hormones such as corticosterone (Honma et al., 1984), melatonin (Feillet et al., 2008), insulin (Carneiro and Araujo, 2009), and orexins (Mieda et al., 2004) have also been suggested to be involved in meal anticipatory behavior. Those hormones were not measured in the present study, but would be of interest in the future.

Cameron et al. (2011) was the first to report a significant increase in physical activity levels of cats fed a low-energy diet with 40% water added compared to cats fed a dry (0% water added) diet. Despite having no differences in energy intake, gut transit time, or energy digestibility, cats fed the dry diet gained more weight than those fed the 40% hydrated diet. The authors suggested that one possible contributor to the reduced body mass gain in cats fed the 40% hydrated diet was increased physical activity and energy expenditure. Given their study design, it was not clear whether the increased physical activity was due to the decreased BW or increased dietary water content. In our second experiment, we aimed to investigate whether increasing dietary water content could increase voluntary physical activity in cats fed to maintain BW. We hypothesized that increased water content without changing energy intake or dietary macronutrient composition would influence gut fill and body hydration status, resulting in increased voluntary physical activity. We observed increased voluntary physical activity in cats fed
the 70% hydrated diet, with greater activity throughout the day, but especially during the dark period compared with cats fed the dry diet. The mechanism responsible for this observation requires further investigation.

Water, which is often neglected in companion animal nutrition, is an important component of the food, contributing to volume, sensory, and other characteristics. Because water consumption is usually associated with greater “gut fill” leading to the effects on satiation, the impacts of consuming water on cognitive, anticipatory and sensory responses also appear to be involved (Welch, 2011). However, Carbannel et al. (1994) reported that altering dietary energy density via manipulations of dietary water content did not alter satiety in humans. In the present study, the FAA was very similar between cats fed the 70% hydrated diet and the dry diet when represented as a percentage of total activity. Although the FAA prior to the afternoon meal appeared to be lower (as a percentage) in cats fed the 70% hydrated diet, this may be largely due to the overall increased daily physical activity level in those cats. There was no difference in the total daily FAA or the FAA prior to the morning meal. Therefore, the increased physical activity in cats fed the 70% hydrated diet was not due to the change of FAA. An additional peak of activity during 5 to 9 pm for cats fed the 70% hydrated diet was observed in this study. It is likely that the cats fed the hydrated diet may have increased activity for urination and defecation, which were corroborated by the fecal and urinary characteristics in this study (Table 4-3). Because the increase in activity, however, appears to be more than a greater number of trips to the litter box, more research in this area is justified.
A limitation we had with this study was that drinking water intake of individual cats could not be accurately measured due to inadequate facilities. Therefore, water was provided ad libitum throughout the study. We observed that cats fed the 70% hydrated diet had greater total fecal output, fecal moisture content, and total urinary volume compared to cats fed the dry diet, which indicates a higher total water intake in cats fed the 70% hydrated diet. The excreted water through feces and urine on the 70% hydrated diet was calculated to be less than the water added to the dry diet. Cats typically do not drink much water naturally, receiving much water from their prey. Thus, feeding canned food or a raw meat diet to cats, which typically contain in excess of 75% moisture, will likely increase total water consumption compared to feeding dry foods (Burger et al., 1980). Moreover, cats fed the 70% hydrated diet had lower urinary specific gravity than those fed the dry diet. These results may indicate an alteration in hydration status even though all of the cats were well hydrated. Further research is needed to address the effect of hydration status on voluntary physical activity in cats.

In conclusion, our data suggest that increased meal frequency without changing energy intake or dietary macronutrient composition, promoted overall physical activity by increasing the FAA of cats. Increased dietary water content also increased daily physical activity, but appeared to occur by increasing the physical activity level after meals. These results may be used to develop new weight management strategies in controlling feline obesity. Further studies are needed to elucidate the mechanisms by which meal frequency and dietary water content influence FAA and total physical activity in cats.
LITERATURE CITED


Cameron KM, Morris PJ, Hackett RM et al. (2011) The effects of increasing water content to reduce the energy density of the diet on body mass changes following caloric restriction in domestic cats. J Anim Physiol Anim Nutr 95, 399-408.


Van der Harst JE, Baars A, & Spruijt BM (2003) Standard housed rats are more sensitive to rewards than enriched housed rats as reflected by their anticipatory behaviour. Behav Brain Res 142, 151-156.


### Table 4-1. Physical activity levels of cats fed one, two, four, or a random number of meals daily

<table>
<thead>
<tr>
<th>Items</th>
<th>Activity counts/epoch$^2$</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One meal</td>
<td>Two meals</td>
<td>Four meals</td>
</tr>
<tr>
<td>Total average</td>
<td>13.81$^a$</td>
<td>15.05$^{ab}$</td>
<td>16.66$^b$</td>
</tr>
<tr>
<td>Light average</td>
<td>17.77$^a$</td>
<td>20.56$^{ab}$</td>
<td>22.70$^b$</td>
</tr>
<tr>
<td>Dark average</td>
<td>6.08$^b$</td>
<td>4.03$^a$</td>
<td>4.57$^a$</td>
</tr>
<tr>
<td>Light: dark ratio</td>
<td>3.63$^a$</td>
<td>6.13$^b$</td>
<td>5.51$^{ab}$</td>
</tr>
</tbody>
</table>

$^1$Data were compiled by activity collars by Actical software. Activity levels were defined as arbitrary units referred to ‘activity counts’.

$^2$The unit for activity levels is presented in activity counts per epoch (epoch length = 15 s).

$^a$-$^c$Means with unlike superscripts letters differ, $P < 0.05$; $n = 10$ cats per treatment.
Table 4-2. Physical activity levels of cats fed dry or 70% hydrated diets

<table>
<thead>
<tr>
<th>Items</th>
<th>Activity counts/epoch[^2]</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry diet</td>
<td>70% hydrated diet</td>
<td></td>
</tr>
<tr>
<td>Total average</td>
<td>25.26</td>
<td>34.33</td>
<td>7.79</td>
</tr>
<tr>
<td>Light average</td>
<td>31.74</td>
<td>41.35</td>
<td>9.97</td>
</tr>
<tr>
<td>Dark average</td>
<td>12.31</td>
<td>20.30</td>
<td>3.83</td>
</tr>
<tr>
<td>Light: dark ratio</td>
<td>2.84</td>
<td>2.19</td>
<td>0.30</td>
</tr>
</tbody>
</table>

[^1]Data were compiled by activity collars by Actical software. Activity levels were defined as arbitrary units referred to ‘activity counts’, n = 10 cats per treatment.

[^2]The unit for activity levels is presented in activity counts per epoch (epoch length = 15 s).
Table 4-3. Effects of increasing dietary water content on fecal and urinary characteristics

<table>
<thead>
<tr>
<th>Items</th>
<th>Dry diet</th>
<th>70% hydrated diet</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fecal characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fecal score</td>
<td>1.8</td>
<td>2.0</td>
<td>0.1</td>
<td>0.247</td>
</tr>
<tr>
<td>Daily fecal output, g</td>
<td>20.1</td>
<td>25.1</td>
<td>2.8</td>
<td>0.008</td>
</tr>
<tr>
<td>Fecal moisture content, %</td>
<td>56.6</td>
<td>60.2</td>
<td>1.4</td>
<td>0.063</td>
</tr>
<tr>
<td>Daily dry fecal output, g</td>
<td>8.7</td>
<td>9.9</td>
<td>0.8</td>
<td>0.095</td>
</tr>
<tr>
<td><strong>Urinary characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily urinary volume, ml</td>
<td>52.0</td>
<td>79.4</td>
<td>4.78</td>
<td>0.001</td>
</tr>
<tr>
<td>Urinary specific gravity</td>
<td>1.053</td>
<td>1.032</td>
<td>0.003</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

\(^1 n = 10\) cats per treatment.
Figure 4-1. Food anticipatory activity (FAA) of cats fed one, two, four, or a random number of meals daily.

FAA was expressed as the ratio of the activity in the 2 h before the availability of food (scheduled feeding times) divided by total daily activity and expressed as a percentage, including average FAA over 7 d from 6 to 8 am, 10 am to 12 pm, 2 to 4 pm, and 6 to 8 pm. Total FAA was calculated as the sum of the four individual FAA. a-dLeast squares means ± pooled SEM with unlike superscripts letters differ, $P < 0.05$; $n = 10$ cats per treatment.
Figure 4-2. Effects of feeding frequency on daily activity patterns in healthy adult cats

A 16 h light: 8 h dark cycle was used in the animal facility. The dark period is represented by the grey area in the graph. Cats were maintained on a strict housing schedule, being individually housed in cages for access to diet from 8 to 9 am, 12 to 1 pm, 4 to 5 pm, and 8 to 9 pm each day which is shown as thick lines on the x-axis. For the other 20 h, cats were group-housed in the same room to allow for voluntary physical activity without human interference. Values are average activity counts/h over 7 d, n = 10 cats per treatment.
A 16 h light: 8 h dark cycle was used in the animal facility. The dark period is represented by the grey area in the graph. Cats were maintained on a strict housing schedule, being individually housed in cages for access to diet from 9 to 10 am and 3 to 4 pm each day, which are shown as thick lines on the x-axis. For the other 22 h, cats were group-housed in the same room to allow for voluntary physical activity without human interference. Values are average activity counts/h over 7 d, n = 10 cats per treatment.
Figure 4-4. Food anticipatory activity (FAA) of cats fed dry or 70% hydrated diets

FAA was expressed as the ratio of the activity in the 2 h before the availability of food (scheduled feeding times) divided by total daily activity and expressed as a percentage, including average FAA over 7 d from 7 to 9 am and 1 to 3 pm. Total FAA (C) was calculated as the sum of the two FAA. Values are means ± SEM. *Differ \( P < 0.05 \) between two treatments; \( n = 10 \) cats per treatment.
Cats, as obligatory carnivores, adapt to higher dietary protein and lower dietary carbohydrate compared to humans and dogs, which has been attributed to their unique protein and carbohydrate metabolism. Cats prefer to utilize protein as an energy source to maintain blood glucose, and they have a limited ability to spare protein utilization by using carbohydrate instead (MacDonald et al., 1984). This preference for dietary protein is a consequence of the lack of regulation of the aminotransferases of dispensable N metabolism and of the urea cycle enzymes (Morris, 2002). The potential problem of using dietary carbohydrate in cats is due to the limited capacity of their gastrointestinal tract to digest and absorb carbohydrate and of the liver to process glucose (Verbrugghe et al., 2012).

Cats have a high incidence of obesity likely due to the transformation of lifestyle and food sources. On one hand, the cat’s diet was changed from a high-protein, low-carbohydrate prey to commercial diets that typically contain high carbohydrate concentrations. This increase in carbohydrate intake from commercial diets has been blamed for the increase in feline obesity. Although commercial cat diets generally exceed the protein recommendation of cats (NRC, 2006; AAFCO, 2009), the protein intake is still much lower than the typical feline prey consumed in the wild. On the other hand, being transformed from wild animals to companion animals, cats no longer live outdoors but have a sedentary indoor lifestyle, which leads to a decreased physical activity. These changes in cats’ lifestyle have been thought to be responsible for the recent increase in the incidence of obesity and obesity-induced diseases.
For years, although feline obesity has drawn many people’s attention, and weight-loss diets have become more popular in the marketplace, the number of pet obesity-related claims still is increasing steadily over the last few years. There is a limited number of metabolic, endocrine, and behavior studies conducted in cats to elucidate the relationship between dietary macronutrient composition and the development of feline obesity. Some of these studies have yielded inconsistent results. Therefore, a more comprehensive study in this area is very much needed. The availability of ELISA or RIA analytical methods for measuring many hormone concentrations and the advent of metabolomics techniques for characterizing a global metabolite profile allow further investigations into feline metabolism and appetite regulation. By using accelerometer-based activity monitors, physical activity has been quantitatively measured in cats (Lascelles et al., 2008), which provides the opportunity for researchers to evaluate the effect of different feeding strategies on the physical activity of cats.

Six studies were designed to address two primary research objectives: 1) to evaluate how dietary macronutrient composition influences circulating metabolites and appetite-regulating hormones in healthy cats; and 2) to investigate whether different feeding strategies promotes voluntary physical activity in healthy cats.

The objective of the first study was to monitor daily fluctuation of circulating glucose, insulin, total ghrelin, and leptin concentrations in cats fed 2 or 4 meals per day. Cats fed 4 meals daily had less variable concentrations of glucose and insulin compared to cats fed 2 meals, indicating the benefit of increasing feeding frequency on preventing large daily glucose and insulin fluctuations. In addition, cats fed 4 meals daily had greater leptin incremental area under the curve (AUC) 0-24h compared to cats fed 2 meals. Leptin...
stimulates energy expenditure (Kalra et al., 2003), which may result in the inverse relationship between people’s habitual frequency of eating and BW reported in several epidemiological studies reviewed by Bellisle et al. (1997). To manage feline weight loss, owners often are instructed to offer the daily food ration in 2-4 meals throughout the day rather than in a single meal or to be fed ad libitum (German and Martin, 2008). This study is the first to reveal the mechanism of this feeding strategy in cats.

The objectives of the second study were to determine the acute response of a single macronutrient dose on postprandial glucose, insulin, total ghrelin, and leptin concentrations by using a macronutrient bolus test in healthy cats. Circulating hormone data were highly variable and indicated changes due to dietary macronutrients. This was not the first to report that a carbohydrate bolus increased glucose and insulin incremental release. Similar results were reported in previous studies in dogs (Lubbs et al., 2010) and cats (Hoenig et al., 2010). We reported that protein load showed the weakest ghrelin response in cats compared to fat and carbohydrate loads. This finding is inconsistent with previous human studies in which protein induced a prolonged postprandial ghrelin suppression (Al Awar et al., 2005; Bowen et al., 2006; Tannous dit El Khoury et al., 2006). Moreover, protein had a weaker effect on postprandial leptin secretion compared to carbohydrate, indicating a reduced satiating effect of protein in cats. Taken together with the weak effects of protein on ghrelin and leptin, our results are consistent with the feeding preference experiments that demonstrated that cats had a clear preference for diets high in protein (Fekete et al., 2005; Hewson-Hughes et al., 2011). Therefore, a HP diet is not recommended for ad libitum feeding due to its weak capacity on appetite control in cats. Additionally, we reported the postprandial leptin secretion in cats dosed
with carbohydrate tended to increase compared to protein and fat, which may indicate the contribution of leptin on increasing the glucose uptake as well as the potential regulating effect of insulin on leptin.

The objective of the third study was to evaluate the effects of three isoenergetic diets rich in protein, fat, or carbohydrate (vs. a control diet with balanced macronutrients) on fasting and postprandial glucose, insulin, total ghrelin, and leptin concentrations in healthy cats. We reported that cats fed control and high-carbohydrate (HC) diets had greater glucose incremental AUC compared to the high-fat (HF) and high-protein (HP) diets. To our knowledge, no one has tested the effect of a single macronutrient or a macronutrient-rich diet on postprandial ghrelin and leptin concentrations in cats. We did not observe differences in postprandial ghrelin and leptin responses, mainly due to the variability observed in this meal test study.

The objective of the fourth study was to characterize blood metabolite profiles of cats fed various dietary macronutrient concentrations using a high-throughput metabolomics approach. This study revealed that macronutrient-rich diets primarily altered markers of amino acid and lipid metabolism, with few changes in markers of carbohydrate and energy metabolism. We reported that cats fed the HP diet had decreased metabolites associated with nucleotide catabolism, but increased metabolites associated with amino acid and fat metabolism for energy, as well as increased metabolites associated with gut microbial metabolism. Cats fed the HF diet had increased metabolites associated with lipid metabolism and oxidative status. These findings re-validated that cats, as true carnivores, have their idiosyncratic metabolism responsible for their unique nutrient requirements. Interestingly, three potential biomarkers, gamma-glutamylleucine,
3-hydroxyisobutyrate, and 3-indoxyl sulfate were identified in this study to distinguish cats fed the three macronutrient-rich diets.

The objectives of the fifth and sixth studies were to evaluate the effect of two different feeding strategies (feeding frequency and dietary water content) on voluntary physical activity in cats fed to maintain BW. Our data suggested that increased meal frequency promoted overall physical activity by increasing the food anticipatory activity (FAA). Increased dietary water content also increased daily physical activity, but appeared to occur by increasing the physical activity level after meals. Interestingly, the daily FAA (a total of 8 h/d) in all cats fed varied meal frequencies contributed 51 to 65% of total daily activity (24 h), indicating the importance of food-associated behavior on cats’ physical activity.

Based on the data presented in this thesis and the existing literature, we suggest the following dietary composition and feeding strategies for weight loss or weight maintenance in cats. Restricted-energy meal feeding is recommended, offering the daily ration in 4 individual meals rather than a single meal to promote physical activity. Increasing dietary water content by feeding a wet diet or by adding additional water to a dry diet also increased daily physical activity and is recommended. Although dietary formulations may vary, dry kibble weight-loss diets should contain no more than 3250 kcal/kg metabolizable energy, containing less than 10% of the diet from fat, up to 8% ash, 35-45% protein, and 15-30% NFE (digestible carbohydrate). The recommended total dietary fiber concentration ranges from 15-20% for weight loss and 6-15% for weight maintenance.
In conclusion, the body of research presented here clarifies the effects of dietary macronutrient and feeding frequency on physiological and physical outcome variables in healthy adult cats. This research provides insight into the impact of manipulating dietary macronutrient composition, especially protein and carbohydrate, on blood glucose, insulin, ghrelin, and leptin postprandial responses, the influence of dietary macronutrients on blood metabolite profiles in the fasting state, the effect of increasing feeding frequency on glucose and hormonal response as well as voluntary physical activity level, and the effect of adding water to the dry diet on cats’ physical activity. The data presented in this dissertation are expected to aid in the development of weight-loss or maintenance programs including optimal dietary regions and appropriate feeding strategies for cats. Further research is necessary to determine the metabolic and endocrine changes in obese or diabetic cats.

**LITERATURE CITED**


