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DIETARY LYSINE:CALORIE RATIOS AND THEIR INFLUENCE ON NITROGEN METABOLISM AND DIGESTIBILITY IN MODERATELY OBESE MATURE DOGS

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ABSTRACT OF THESIS

DIETARY LYSINE:CALORIE RATIOS AND THEIR INFLUENCE ON NITROGEN METABOLISM AND DIGESTIBILITY IN MODERATELY OBESE MATURE DOGS

This experiment was conducted to determine if changing the amount of ideal amino acids (meaning the amount of amino acids necessary to supply all the animal's needs without excesses or deficiencies of any single amino acid), in relation to caloric intake will change nitrogen metabolism and weight loss in obese mature dogs. Information provided by this experiment can be used to formulate canine diets emphasizing weight loss in older animals.

Six moderately obese mature female crossbred hounds were fed diets varying in their ratio of lysine:calories (Lysine % : Mcal ME/g) (2.2, 3.0, and 3.8) in a 3 x 3 replicated Latin square design. Increasing the lysine:calorie of the diets linearly increased the amount of nitrogen absorbed. It did not, however, significantly affect blood chemistry values. Protein turnover exhibited a positive linear trend with increasing ratio and protein degradation showed a strong quadratic change with the lowest point of degradation occurring with the diet containing a 3.0 lysine:calorie ratio. Plasma urea and creatinine excretion demonstrated quadratic tendencies with the two highest values occurring with the diets containing lysine:calorie of 2.2 and 3.8, reflecting changes in muscle protein breakdown while nitrogen was retained in the body. Caloric restriction did not result in loss of lean mass as much as a loss of fat mass. By increasing the quality of protein fed as a percentage of caloric intake, lean muscle mass was conserved during periods of caloric restriction.

KEYWORDS: Nitrogen Metabolism, Nitrogen Digestibility, Canine Obesity, Lysine:Calorie, Protein Metabolism

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April 21, 2006

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THESIS

Trista Lynn Reeder

The Graduate School

University of Kentucky

2006

DIETARY LYSINE:CALORIE RATIOS AND THEIR INFLUENCE ON NITROGEN METABOLISM AND DIGESTIBILITY IN MODERATELY OBESE MATURE DOGS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science
at the University of Kentucky

By

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2006

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Introduction

Within the past few years people have become aware that their companion animals are becoming increasingly overweight. Obesity is a huge problem in animal health, contributing to problems and diseases including diabetes, decreased life expectancy, decreased overall quality of life, difficulty breathing, lethargy, cardiac problems, joint problems, and other similar maladies. With the popular trend of emphasizing weight loss in humans, it is not surprising that commercial dog food companies are taking an increasing interest in the health and weight management of companion animals. The goal of weight loss is to reduce the ratio of fat mass to lean muscle mass. While several different methods have been tried, few have been proven to reduce weight safely and effectively in dogs. Fewer still have focused on the loss of fat and the maintenance of body protein.

There have been very few studies of amino acid and protein metabolism in relation to weight loss in mature dogs. By monitoring protein turnover during periods of calorie restriction, weight loss can be attributed to fat, protein, or a combination of these. An increase in urea synthesis using a labeled amino acid protocol will show that the weight lost is due to an increase in protein oxidation, not solely fat degradation. By varying the amino acid composition in the diet, we should be able to reduce the amount of body protein degradation during times of caloric restriction. Therefore, the objective of the following study was to determine the effects of diets differing in dietary amino acid composition on nitrogen metabolism and muscle mass in mature, obese dogs during times of reduced caloric intake associated with weight loss.

Chapter Two

Literature Review

Background

Obesity is a very common health problem in today's companion animals. Estimates indicate the rate of obesity in dogs and cats can reach 25% (Edney 1986; Scarlett et al., 1994; Armstrong et al., 1996) or even 40% (Edney, 1986). Obesity has a number of causes including a lack of exercise, increased variety of palatable commercial dog foods and treats, and owner's lack of recognition of the pet being over weight. With such a variety of causes of obesity, there is no simple way to treat the problem and reduce an animal's body weight. The startling obesity rate gives rise to a long list of health implications including diabetes, osteoarthritis, increase in blood pressure, circulatory problems, and mammary tumors (Edney and Smith, 1986; Matthews et al., 1984; Rocchini et al., 1987; Joshua 1970; Alenza et al., 1998). Therefore, treating obesity is important in companion animals for increasing life expectancy as well as improving quality of life.

The goal of treating obesity is to make the animal lose weight at a slow and healthy rate. However, the primary loss of weight should be attributed to loss of fat mass without adversely affecting lean muscle mass. Most traditional weight reduction methods do not discriminate between loss of protein versus fat. Manipulating an animal's body composition to maintain lean muscle while losing fat requires that all protein and nutrient requirements be met through dietary or endogenous sources while allowing the body to use its fat stores for energy.

The issue of obesity is confounded when the subject of aging is introduced. As an animal ages, the occurrence of obesity increases. Factors that can lead to this include decreased activity, decreases in muscle mass, a decrease in protein synthesis and a reduction in steroidal hormones. More specifically, in humans, the excretion of urinary creatinine and total muscle mass decreases by 50% between the ages of 20 and 90 (Tzankoff and Norris 1978). After the age of 30, there is also a decrease in muscle density and an increase in intramuscular fat (Imamura et. al. 1983). In dogs, Kealy (1999) concluded that inadequate protein intake increases the rate of loss of lean body mass in aging animals while abundant protein reduces the loss. With the increasing

amount of knowledge about age related muscle wasting (sarcopenia) it is hoped that increased study of protein turnover in the mature companion animal can result in weight reduction while minimizing the loss of lean muscle mass.

Obesity

Obesity is the long-term outcome of an excess of energy intake over energy expenditure (Flatt 1995). There are many factors that can influence the body fat mass of an individual. Cuenot (1905) was the first to observe a genetic link to obesity in mice and Hetherington and Ranson (1940) were able to experimentally produce obesity in the rat by lesioning the ventromedial hypothalamus which causes an animal to lose control of food intake. Specific disorders of energy intake, energy expenditure, metabolic efficiency, adipocyte formation, appetite behavior, and possibly body weight "set point" have all been thought to interact with environmental factors to determine the body composition of obese individuals (Leibel et al., 1984).

Obese individuals can develop serious health problems associated with the increase in body fat. Humans have the propensity to develop the metabolic syndrome (simply defined as a metabolic disorder that encompasses many different maladies) which is associated with diseases such as hyperinsulinaemia, dyslipidaemia (a condition marked by abnormal concentrations of lipids or lipoproteins in the blood), type II diabetes mellitus, atherosclerosis, hypercoagulability, and hypertension (Das et al., 2004). In older humans, symptoms of the "frailty syndrome" (weight loss associated with muscle degradation, slowness, exhaustion, poor exercise tolerance, and weakness) can also be associated with obesity (Blaum et al., 2005). Such maladies in animals associated with excess body fat include: diabetes, fatigue, heart abnormalities, joint pain and damage, decreased life expectancy, decreased overall quality of life, difficulty breathing, and other similar dysfunctions. In a paired feeding experiment by Lawler et al. (2005), dogs were paired with litter mates and one was fed 25% less food than the other. The dogs on restricted intake lived significantly longer than their litter mates and were healthier overall. Obesity can also increase the risk of developing some forms of cancer (Deslypere 1995). There is a direct connection between overweight individuals and an impairment of physical, social, and mental well-being (Yan et al., 2004).

Obese individuals do have options. There are several ways by which body fat can be reduced. Surgical procedures such as liposuction and gastric bypass offer humans fast results without the individual changing their lifestyle. Gastric bypass has proven effective in morbid obesity, but long term effects vary. One follow up study found that a majority of the people who underwent the gastric bypass surgery felt it was beneficial, however, several people experienced problems with continuing vomiting, "plugging up" (digesta stops moving past the surgery site), and some died (Mitchell et al., 2001). However, in animal practice, more practical and less invasive methods of body fat reduction are available and include increasing caloric expenditure through exercise and decreasing caloric intake through restriction, coupled with supplementation with various nutrients.

Weight Loss

For many years weight loss has been studied in companion animals. Such methods include: caloric restriction through fiber enriched diets or direct diet restriction, increased caloric expenditure through exercise, and changes in diet composition including starch alteration and supplementation with additives such as carnitine, vitamin A, and chromium (Sunvold 2000). The most basic form of dietary manipulation for weight loss is the reduction of caloric intake or increase in caloric expenditure. By simply reducing the amount of calories an animal consumes or increasing the number of calories used, the body is forced to find other means to meet the daily caloric requirements for maintenance, and the body will begin to mobilize stores of fat.

If the period of caloric restriction is too severe or too long, muscle protein will also be broken down for energy as needed for necessary bodily functions. If specific parameters are not measured, there is no conclusive way of monitoring the composition of weight loss during periods of caloric restriction. In one experiment (Laflamme et al., 1997) dogs were fed various restricted diets with treatments of 100%, 75%, 60% or 50% of their maintenance energy requirements. The researchers concluded that as caloric restriction increased, the amount of weight lost increased linearly. Although there was weight loss across all groups, whether the weight loss was due solely to fat was not mentioned.

Another form of caloric restriction in companion animal weight management uses high fiber diets. The purpose of the high fiber diet is to allow the animal to consume enough food to satiate itself while reducing the total number of digestible calories being ingested, since fiber has little or no caloric value for non-ruminant monogastrics. These diets typically contain 10-20% crude fiber or 20-40% total dietary fiber (Sunvold 2000). Cellulose, peanut hulls, or soybean mill run are the primary sources of fiber used in these diets (Sunvold 2000). However, these diets can cause excessive fecal output, increased microbial gas production and increased frequency of defecation, (Sunvold 2000) which can adversely affect quality of life for animal and owner.

Weight loss can be achieved by caloric restriction, and by increased caloric expenditure such as exercise. However, severely obese individuals appear to have difficulty losing body weight when the only lifestyle change is increasing exercise. When Björntorp and colleagues (1973) focused on eight overly obese individuals and subjected them to six months of physical training at ad libitum dietary intake, it was noted that overall, the subjects failed to lose any body weight because of an increase in mean body cell mass and a decrease in mean body fat. However, on an individual basis, some decreased body fat, some remained constant and some even increased body fat. The authors concluded that exercise had little to no effect on appetite regulation and the subjects continued to take in more calories than could be burned by exercise.

A common alteration to companion animal diets is a change in the type of starch and carbohydrates being used as an energy source. It has been found that starch can account for upwards of 50% of an animal's daily caloric needs (Sunvold 2000). By altering the type of starch in the diet or the ratio of starch to protein, researchers may be able to improve insulin resistance, reduce muscle catabolism, alter glycemic responses, reduce weight and control diabetes in dogs. In one study (Diez et al., 2002) two experimental diets were compared: a high protein, low starch diet (test diet) and a low protein, high starch diet (commercially available weight-reduction diet). The dogs' intake was determined before the trial by ad libitum feeding the commercial diet and averaging daily intake. After adaptation, intake was restricted to 90% of initial intakes. Each time body weight reached a plateau for 2 weeks, the diet was further restricted by an additional 10% until ideal body weight was attained. To determine the fat mass of the dogs before and

after the experiment, total body water and thereby fat free mass was estimated using a deuterium injection. Fat mass was determined by difference in body weight and fat free mass. While both groups lost significant weight, dogs fed diets with high crude protein and low starch lost more fat mass than muscle mass when compared to the commercial diet. These data demonstrate that the proportion of starch to protein in the diet can affect substrate utilization within the body during caloric restriction.

Supplementation with chromium, Vitamin A, and carnitine has also been studied in conjunction with weight reduction. Vitamin A supplementation in dog and cat diets caused a reduction in lipid deposition (Kumar et al., 1999; Scarpace et al., 2000). When caloric intake was increased to exceed maintenance energy requirements the addition of Vitamin A reduced fat deposition compared to a control group without supplemental Vitamin A. While it has not been studied as a weight reduction supplement, retinoic acid, a derivative of Vitamin A, may help induce the expression of the gene for uncoupling protein (UCP), which is responsible for the thermogenic effect of brown adipose tissue (Pairault et al., 1987). Increased expression of UCP can increase thermogenesis, enhancing caloric expenditure and possibly fat loss. In acute administration, retinoic acid could also be useful in reducing leptin levels in the blood as well as inhibiting the differentiation of preadipocytes into mature adipocytes (Hausman et al., 2001). The multiple actions of retinoic acid can decrease lipogenesis and enhance lipolysis to decrease fat mass.

Carnitine, an essential cofactor for lipolysis in fatty acid metabolism, transports fatty acids into the mitochondria of the cell for β-oxidation. Carnitine was found to reduce weight and body fat in dogs while maintaining lean muscle mass. When supplemented in feline diets, carnitine increased the rate of fatty acid oxidation and increased the percentage of weight loss in calorie restricted diets when compared to an unsupplemented calorie-reduced control (Center et al., 1997; Sunvold et al., 1998; Sunvold et al., 1999; Center et al., 2000).

While there are many different methodologies and theories on weight loss, none have been completely exhaustive in their research on the effect of weight loss on body composition and overall animal health. Research preformed by Diez et al. (2002) has shown that the addition of excess protein during caloric restriction allowed 80% of the

body weight lost in dogs to be attributed to fat. However, it has yet to be determined if an increase in essential amino acids at a lower level of protein intake will have a similar effect.

Body Composition with Weight Loss

With some dietary weight loss, there is a loss of both adipose tissue as well as lean muscle mass. It is preferred that the loss occur in adipose tissue, not lean muscle mass. Bahadori et al. (2005) found that humans consuming a low-fat high-carbohydrate diet lost an average of 15% of fat mass and only 5% of lean muscle mass in comparison to a moderate-fat, moderate-carbohydrate diet. This demonstrates that specific alteration of the diet can preserve lean body mass while significantly decreasing fat mass. However, as age becomes an additional factor when evaluating weight loss, it is even more important to retain lean muscle mass since protein loss is a natural result of aging, also referred to as sarcopenia. As humans age, muscle mass accounts for less than 20% of the whole-body protein turnover compared to 30% in young adults (Young et al., 1982).

Sarcopenia is not wholly irrevocable. One experiment preformed by Dardevet et al. (2002) found that when old rats were fed a leucine supplemented meal, muscle protein synthesis was stimulated. A second experiment by Arnal et al. (2002) showed a similar increase in protein synthesis in old rats when a pulse feeding pattern (80% of the daily protein requirement fed at one meal) was implemented. This feeding pattern also improved protein retention in elderly women, however, it had no effect on young women (Arnal et al., 1999; Arnal et al., 2000). Further experiments in elderly humans demonstrated that exogenous amino acids can stimulate protein synthesis (Volpi et al., 1998). An experiment in horses found that old and young horses alike benefited from the addition of supplementary amino acids (Graham-Thiers and Kronfeld 2005). By the end of the trial, both age groups had an increase in muscle mass, increase in overall body weight, and lower body condition scores in comparison to the unsupplemented group. With these findings across species, it leads one to believe that the canine model would follow a similar pattern and feeding an excess of balanced amino acids to older dogs on a weight reduction program may counter not only the natural sarcopenia associated with old age but also any protein loss associated with reduced caloric intake.

Ideal Amino Acid Composition

Amino acids are the building blocks of protein. There are twenty common amino acids, nine of which are considered essential in the dog, meaning they cannot be synthesized by the body in amounts necessary for normal physiological function and must be supplied in the diet. The ten essential amino acids for dogs are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Rose and Rice, 1939; Milner, 1979; Brown, 1989). Signs of essential amino acid deficiency include: hyperammonemia, unthriftness, loss of appetite, vomiting, acid-base imbalance, poor protein synthesis, and other symptoms. Deficiencies in total protein intake can also have detrimental effects on an animal. Reduced protein turnover secondary to inadequate protein intakes can result in decreased immune competence and increased incidence of infection and disease (Wannemacher et al., 1966; Young et al., 1990; McMurray 1999). Subclinical protein deficiencies in dogs can also result in increased body fat and a reduction in lean body mass (Wakshlag et al., 2003). Therefore, a correct balance of amino acids along with adequate dietary protein intake are essential for life and normal metabolism.

Imbalances can be caused by several factors, including incomplete knowledge of animal nutrition, processing, storage, and instability resulting in loss of nutrients, and errors in feed formulation (Hilton, 1989). Broiler chickens fed cereal grain diets with very high crude protein but limiting in essential amino acids failed to grow to their maximum potential until the diet was supplemented with the deficient amino acids (D'Mello, 1993). In another experiment, broiler chicks were fed moderately to severely imbalanced mixtures of amino acids devoid of lysine; the severity of growth retardation was proportional to degree of imbalance of amino acids, and even when the imbalanced diets were supplemented with lysine, growth was still significantly below the control group with balanced amino acids (D'Mello, 1993).

While the dogs in our experiment are fully mature in comparison to the growing chicks, we use the concept of the "ideal" amino acid ratio for dogs to balance the amino acid requirements at maintenance. We define the "ideal" ratio as the balance of amino acids that meet the animal's requirements without exceeding or limiting any one amino

acid. Therefore, the ideal ratio is based on lysine being set at 100% of the dog's requirement and every other amino acid is formulated as a ratio to lysine (Baker et al., 1991).

Measuring Protein Turnover

Proteins in the body are continually synthesized and degraded in a process called protein turnover. By measuring protein turnover and nitrogen metabolism in adult dogs, muscle degradation associated with excess weight loss can be monitored. When loss of fat mass is of interest, lean muscle mass and protein turnover should be quantified to insure that weight loss is due exclusively to fat and not lean muscle mass. Measuring lean muscle mass is also an indirect means to measure fat mass when direct measurement is not practical or easily attainable.

Most experiments examining protein requirements for dogs have used the nitrogen balance technique, which measures the difference between nitrogen ingested and nitrogen excreted. This method, however, does not differentiate between changes in internal nitrogen metabolism (synthesis, oxidation, and breakdown) in response to altered intake (Humbert et al., 2001). To measure nitrogen flux and allow for calculation of protein breakdown, synthesis, and amino acid oxidation, isotopic amino acids must be used. Common isotopes are ^{15}N glycine, ^{13}C leucine, ^{14}C leucine, and labeled phenylalanine. Markers can be continuously, intravenously, infused or dosed as a single oral bolus for both methods and the excretion of labeled urinary nitrogen is measured.

In addition to dietary protein being excreted as urea, protein from muscle degradation is also found in the urea pool. Since the single dose end-product method assumes that the oxidized end products will be excreted quantitatively in the urine (Wessels et al., 1997), by measuring the difference between the amount of label ingested and the amount excreted in the urine, the amount of labeled nitrogen incorporated into the body tissues can be determined.

Protein turnover can then be calculated by dividing the total amount of nitrogen in the urine by the fraction of the total ^{15}N dose excreted. Recovery fractions of ^{15}N are determined by the amount of ^{15}N excreted over the total marked amino acid dose given. Therefore total protein turnover can be calculated as $Q = d/G$, where Q is protein turnover

in g N/day, d is the rate of urea N excretion in urine, and G is the fractional recovery of ^{15}N in the urine.

Whole body protein turnover (Q) can then be fractionated into whole body protein synthesis (PS) and protein degradation (PD). This relationship can be described in the formula: Q which is equal to PD + N absorbed = PS + N excreted in the urine. Protein retention can be determined by subtracting the value calculated for protein degradation from the value for protein synthesis (Wessels et al., 1997). A change in the amount of protein degradation or synthesis will respond to dietary changes and therefore affect protein turnover. This relationship allows researchers to determine the difference in weight loss due to lean muscle mass or body fat.

There are two ways in which to monitor protein turnover in an adult animal. One method is the direct technique, in which a constant infusion of a labeled amino acid is administered intravenously and tissue samples are taken to determine the amount of marker being deposited into tissues. The second technique administers a single oral dose of ^{15}N glycine and measures ^{15}N urea excretion in urine. The end product method assumes that a certain amount of the glycine will be taken up by the body and incorporated into the tissues, while the remaining glycine will be metabolized and excreted. Since tissue protein content is not constant and the process of anabolism and catabolism are continuous, excretion of glycine can be used to determine by difference how much tracer the body incorporated into the tissues. The rate at which body protein is being turned over in a short period of time can be determined by rate of tracer excretion after the initial glycine is removed from the system.

^{15}N is the most frequently used stable isotope for nitrogen and has been determined to be a safe isotope to use in humans as well as animals. The method of a single dose of marker assumes that the labeled nitrogen is not recycled, nitrogen metabolism remains constant throughout the study, the labeled amino acid is a valid tracer for total amino nitrogen, and there is a single pool of metabolic nitrogen (Fern et al., 1981). Using a single dose tracer is simple, non-invasive, and has been shown to give consistent results in both man and ruminant animals when determining protein turnover (Fern et al., 1981; Wessels et al., 1997).

Indicators of Protein Turnover

Quantitative measurement of protein turnover requires administration of an isotopic marker to the subject. However, for estimating specific degradation of skeletal muscle (the most represented tissue in fat-free mass; Wang et al., 1992), creatinine and 3-methylhistidine are commonly used indicators.

Following peptide bond synthesis, actin and myosin in the skeletal muscle are methylated and 3-methylhistidine is formed as a result of methylation of histidine residues (Young et al., 1978). During intracellular breakdown of these proteins, 3-methylhistidine is released and excreted into the urine without any further chemical or metabolic change. Due to its direct excretion into the urine, 3-methylhistidine can be considered proportional to the total muscle mass in a steady-state body (Young et al., 1978). There are several different ways by which 3-methylhistidine can be experimentally measured. Virgili et al. (1994) first derivitized the compound with fluorescamine and fluorometrically measured the abundance with reverse-phase high pressure liquid chromatography. Chevalier et al. (2003) also derivitized their samples, however, they used othophthalaldehyde followed by mercaptopropionic acid and then high pressure liquid chromatography.

Creatinine is used as a measure of muscle degradation; this metabolite is derived from the catabolism of phosphocreatine, a molecule contained primarily in the muscle (Narayanan and Appleton, 1980). Creatinine in urine and serum (or plasma) is often used as both an index of the total amount of muscle mass contained in a body under steady-state conditions (Heymsfield et al., 1983) as well as an index of renal function and glomerular filtration rate (Perrone et al., 1992). Creatinine is an almost ideal glomerular filtration marker because it is not protein bound, is freely filtered, is not metabolized by the kidney, and is physiologically inert (Perrone et al., 1992).

Experimental quantification of creatinine can vary depending on sample type, available equipment, and preference. Several experiments have used Jaffe's reaction method and measured the amount of creatinine in a sample using spectrophotometry as described in Bonsnes et al. (1945). Some experiments require the removal of noncreatinine chromogens for analysis. This can be achieved by enzymatic digestion or adsorption with porous aluminum silicate clays such as Fuller's earth and Lloyd's reagent before

being subjected to the Jaffe reaction and again measured spectrophotometrically (Miller et al., 1938). Other experiments have used kinetic alkaline picrate assays (Bowers, 1980), high pressure liquid chromatography (Lim et al., 1978), ion exchange chromatography (Weatherburn et al., 1978), mass fragmentography (Bjorkhem et al., 1977), and several enzymatic methods. For the purposes of this experiment, urine and plasma creatinines were determined using the method by Toro et al. (1975). Picric acid and heat to precipitate any proteins in solution and sodium hydroxide is added to the filtrate to generate a yellow/orange color which is measured spectrophtometrically.

By utilizing both measures of muscle breakdown, we can determine if the dogs are losing muscle mass while on caloric restriction or if weight loss/weight maintenance is due to the repartitioning of fat to fat free mass. An increase in either metabolite in the urine during restriction will indicate an increase in muscle degradation. Changes in plasma creatinine levels will indicate muscle breakdown as well as any renal abnormalities in response to the experimental diets.

Conclusion

By investigating nitrogen metabolism, energy balance, fat mass metabolism and protein turnover, obesity in companion animals can be better understood and treated. Weight loss in companion animals is becoming increasingly important to reduce health risks such as diabetes, heart dysfunction, shortness of breath, fatigue, and a general decrease in the quality of life. It is crucial, especially in older animals, to view weight loss in such a way that allows the animal to lose adipose tissue while still maintaining lean muscle mass.

To avoid excess protein degradation during periods of weight loss, it is imperative that the animal be supplied with enough essential amino acids to maintain normal protein synthesis. By determining the different amounts of protein used by the animal during weight loss using a marked amino acid tracer, a proper diet can be formulated to include all the necessary amino acids reducing the possibility of a protein deficiency during times of restriction as well as counteract body protein degradation due to sarcopenia and caloric restriction.

Chapter Three

Introduction

With the public becoming increasingly interested in their own dietary intake, it is of no surprise that the same people are taking an increased interest in their pets' diets. Between 20-40% of dogs and cats are becoming increasingly obese (Sunvold, 2000) due to overfeeding, lack of exercise, and supplementation with table food. With the growing need for weight control, the application of diets for weight reduction has become common in the pet food industry. The goal of such diets is to reduce body weight, however, the optimum goal would be to reduce body fat while maintaining body protein. One tool that may aid in this concept is manipulation of the lysine:calorie ratio in the diet.

So far, most of the research on lysine:calorie ratios has been in growing and finishing pigs and the effect of these ratios on back fat as analyzed by ultrasound (Smith et al.,1999). Although it may not be wholly accurate to compare growing animals to maintaining animals, such studies can form a basis for comparing the deposition of fat and muscle. Back fat decreased at the optimal lysine:calorie ratio in each treatment while average daily gain increased, demonstrating muscle, not fat, was being accreted. This leads to the concept of the ability to alter muscle and fat composition through variations in lysine:calorie.

By looking at the effects of varying lysine:calorie in mature moderately obese dogs on a calorie restricted diet, our goal was to directly determine the impact of lysine:calorie on nitrogen absorption and retention. The ultimate goal was to allow the dogs to lose fat as opposed to lean muscle mass. We hypothesized that increasing lysine:calorie, and therefore the percentage of total calories from protein, would induce fat mass loss while maintaining muscle protein during periods of caloric restriction. The objective of this experiment was to evaluate nitrogen and protein metabolism during periods of caloric restriction and determine if nitrogen and protein metabolism is affected by the proportion of calories from protein in mature moderately obese dogs.

Materials and Methods

Dogs. Six mature female crossbred hounds (24.5 $\pm$ 2.8 kg BW) were used to evaluate nitrogen metabolism and protein turnover during nutrient restriction.

The dogs were located in the Division of Laboratory Animal Research Facility at the University of Kentucky (Lexington, Kentucky) and were cared for as specified in IACUC protocols. Dogs were housed in an environmentally controlled room at 20-25˚C with a light:dark cycle of 12:12. For a majority of the experiment, the dogs were housed in cages measuring 1x1.5 meters with a slotted floor and access to a 1x2 meter outside run with concrete flooring during the day. During periods of total urine and fecal collection the dogs were housed in 1x1 meter cages with slotted floors. Each cage was cleaned twice daily and during periods of total excreta collection, feces and urine were removed three to four times daily. Dogs were exercised for a minimum of 30 minutes a day except during confinement for urine and fecal collections.

Feeding and Treatments. The amino acid, ingredient, and chemical compositions of each treatment is presented in Tables 3.1, 3.2 and 3.3. The diets were prepared at the Hills Science and Technology Center in Topeka, Kansas. Each diet was formulated with the ideal amino acid profile as described by Baker and Czarnecki-Maulden (1991) and in accordance with the Association of American Feed Control Officials (2002) nutrient guide for dogs and balanced to meet maintenance requirements. The ideal amino acid profile for the dogs is defined as the amount of amino acids necessary to meet the animal's dietary needs without any excess or limiting amino acids. Differences in the three treatments resulted from varying lysine:calorie to give ratios of 2.2, 3.0, and 3.8 (%Lysine : Mcal ME/g). Each day food was weighed and divided into two equal portions and fed at 0700 and 1900 in stainless steel bowls. Each dog was allowed 12 hours to consume the food and following that time any remaining orts were weighed and recorded. Throughout the experiment, food samples were taken daily and composited for nutrient content analysis.

Sampling. Each experimental period was 28 days in length with a 7 day adjustment period to the new diets in order to avoid gastric upset. On days 1-14 of each period the dogs were fed ad lib to keep them approximately 20% over ideal body weight. During days 15-28 diets were reduced to 75% of maintenance energy as determined by calculating maintenance energy ($145kcal*kgBW^{0.67}$) at ideal body weight (NRC, 1985).

The final six days of the experiment (days 23-28) dogs were confined for urine and fecal collection.

On the first day of fecal collection by 0700, all dogs were placed in cages half the size of their original pens. Fecal and urine output was collected for the next 6 days at meal time and samples were placed into labeled containers. Urine samples were collected every 12 hours via catch pans into vessels containing 5 mL 6 N H_3PO_4 and a light spray of water was used to wash down the catch pan for complete urine collection. The urine sample was then divided into two equal parts. Half of the sample was kept for ^{15}N urea analysis and total urea excretion, while the other half was composited for the period and analyzed for total nitrogen output. Feces was removed 2-3 times per day, placed in labeled plastic bags, composited for the period and stored frozen for further analysis.

At 0700 on day 25 of the experiment the dogs were all given an oral dose of ^{15}N-glycine (Cambridge Isotope Laboratory. Andover, MD) at 5 mg glycine/kg BW to monitor nitrogen metabolism. Subsequent urine collections were analyzed for excreted ^{15}N present in urea.

Blood samples were taken on days 22 and 28, three hours after the morning feeding. Venous blood was drawn from a foreleg and 5 cc were placed in a vacutainer containing heparin and placed on ice. The vacutainer tubes were then centrifuged at 5,000 x g for 15 minutes and the plasma was transferred into storage vials and frozen until further analysis

Analyses Following collection, fecal samples were thoroughly mixed and stored frozen until analysis. Feed was ground using a conventional (Hamilton-Beach ®, Mexico) 14-speed blender, composited, and stored at room temperature. Dry matter was determined in fecal and whole feed samples by the difference in sample weight before and after drying in a 55˚C forced air oven. Samples were determined dry when samples reached a constant weight. Ground feed samples were dried overnight in a 100˚C vacuum oven till samples reached a constant dry weight.

Samples that were stored frozen were allowed to reach room temperature before analysis. Fecal, ground feed, and urine samples were placed in ceramic boats and combusted for total nitrogen content using the LECO CN2000 (St. Joseph, MI) nitrogen analyzer. After thawing, individual urine samples, urine composites, and plasma samples

were analyzed for urea content using the Technicon Auto-Analyzer (Bran + Luebbe, Buffalo Grove, IL; Marsh et al. 1965).

Creatinine content of urine composites and plasma samples were determined by deproteinizing 0.5 ml of plasma or an equal amount of a 1:100 dilution of urine with 0.04M picric acid. The sample was then heated in a 100˚C boiling water bath, filtered, developed with 2.5M Sodium Hydroxide and the absorbance of the filtrate measured at 520 nm on the UV-VIS spectrophotometer (Shimadzu Scientific Instrument Inc. Columbia, MD) (Toro and Ackermann, 1975).

Labeled nitrogen enrichment of the individual urine samples was determined by combustion isotope ratio mass spectrometer. After determination of the urea content of the samples, the urine was centrifuged for 10 minutes at 1,000 x g at 4˚C. An aliquot of urine containing 100 μm of urea was mixed with 4.0 ml of water, pH was adjusted to 2.5, and then it was poured onto a cation exchange column. The first 5 ml of filtrate was discarded and then 20 ml of deionized H_2O was added to the column the eluent collected. The samples were then dried overnight in a 60˚C oven and reconstituted with 2ml 0.1 M pH 7.0 phosphate buffer. Following reconstitution, 100ul of the sample was placed in a 25 ml Erlenmeyer flask with 3 ml of 0.1 M pH 7.0 phosphate buffer. Two 0.6 cm filter paper disks were suspended from the stopper and 5 ul of 2.5 M $KHSO_4$ was added to trap ammonia. Urease was then added and the flask was immediately stoppered. The flasks were incubated for 20 minutes at 25˚C in a Dubnoff shaking incubator. Two hundred microliters of 3 N NaOH was injected through the stopper into the flask and the flasks were shaken for an additional hour and then allowed to stand for 24 hours. The stoppers were then removed and placed in a desiccator containing an open container of concentrated H_2SO_4 and allowed to dry for an additional 24 hours. When the filter papers were dry, they were placed in an opened Sn cup and the cup was folded around the filter papers in preparation for analysis. Samples were then sent to the University of California, Davis Stable Isotope Facility and placed in a PDZ Europa Anca Sample Preparation Unit to be analyzed for ^{15}N using a PDZ Europa 20-20 Isotope Ratio Mass Spectrometer.

Blood chemistry values were determined by sending plasma samples on dry ice to Hill's Pet Nutrition Clinical Laboratory where they were thawed and analyzed using a Boehringer Mannheim Hitachi 912 Automatic Analyzer.

Calculations and Statistics

Nitrogen turnover was calculated using the equation:

$$Q, \text{ protein turnover, g N/d} = \frac{\text{d, rate of Urea excretion (g/d) in urine on d 26, 27, 28}}{\text{G, fractional recovery of } ^{15}\text{N from } ^{15}\text{N-Gly in urine urea}}$$

Protein degradation was calculated as PD = Protein turnover (Q) – N absorbed. Protein synthesis was calculated as PS = Q – N excreted in urine. Nitrogen retention is then calculated as the difference between protein synthesis and protein degradation.

Most of the data were analyzed as a replicated 3X3 Latin Square using the General Linear Models procedure of SAS (1989). The experimental unit was dog, the model included age, dog (age), period (age), and treatment, and the error was residual error mean square.

For blood data collected on day 22 and 28, the Proc Mixed Procedure of SAS (1989) was used that added day and treatment by day were included in the model. The model included the fixed effect of treatment. Dog, day, and period were included as random effects. Means were separated using polynomial contrasts for linear effects of the lysine:calorie ratio. Differences were considered significant when $P < 0.05$

Results

There were some side effects when the experimental diets were fed for longer than three weeks. One dog exhibited diarrhea while on the diets while some of the other dogs experienced symptoms including excessive shedding on the tail, dry skin, dry scaly patches of skin and sores on the tail along the vertebrae. Two dogs found the diets unpalatable when fed ad libitum as the study progressed as they stopped consuming as much as they had previously. All dogs appeared to suffer from excessive shedding while on the experimental diets, however, this did not appear to affect the overall health or comfort of the dog. None of the symptoms appeared to be related to a particular lysine:calorie and could have been related to the nutrition restriction imposed by the study. There were no age affects for any variables measured in this experiment.

Because restricted intakes were adjusted to ideal body weight, there were no differences in dry matter intake (Table 3.4). There was no major change in total body weight over time, despite a tendency for a linear decrease in body weight with increasing

lysine:calorie ratio (P=0.09, Table 3.6). Body weight change over the second week of restriction was quadratic (P = 0.03) with the highest weight loss occurring with the diet containing a lysine:calorie of 3.0. Differences in body weights were expected during the restriction period, but the loss of weight during these short periods of restriction was not great enough to be considered statistically significant, however total weight lost increased by 20% from the lowest to the higher lysine:calorie diets.

Because we chose to maintain similar profiles of essential amino acids between diets, nitrogen intake (Table 3.5) increased linearly with increasing lysine:calorie (P < 0.0001). Apparent nitrogen absorption increased quadratically as lysine:calorie increased (P = 0.01). Excreted nitrogen in the feces and urine did not differ across treatments. Nitrogen retained by the dogs demonstrated an increasing linear tendency across treatments (P = 0.13). The percentage of nitrogen absorbed remained the same between diets, however, the percentage of nitrogen retained tended to be lower (linear P = 0.16) with the diet containing a ratio of 2.2 in comparison to the other two diets.

Data from one of the dogs was omitted for the determination of protein metabolism due to erroneous values. Urea excretion and fractional ^{15}N recovery were similar across all treatments (Table 3.6). Protein synthesis rate did not demonstrate any change across treatments. Protein degradation demonstrated strong quadratic tendencies (P = 0.06) with the lowest rate of degradation being found in the diet containing a lysine:calorie of 3.0. Protein turnover, as determined by ^{15}N-Urea excretion, tended to increase quadratically (P = 0.07) with increasing lysine:calorie.

Urine creatinine, urea excretion (Table 3.7), and most blood chemistry values (Table 3.8) did not change between treatments. Most plasma values did not change between days nor was there an interaction between treatment and day. Only five dogs were used for the blood chemistry analysis due to hemolysis contaminating all the plasma samples of one of the dogs. Plasma urea concentrations demonstrated quadratic changes with increasing lysine:calorie (P = 0.04) with the lowest value observed with the 3.0 lysine:calorie. Quadratic tendencies were also observed for plasma creatinine concentrations (P = 0.06) with the lowest values occurring with the 3.0 diet. Plasma potassium decreased linearly (P = 0.02) while the ratio of sodium to potassium increased linearly (P = 0.03) with increasing lysine:calorie. Total blood protein demonstrated a

treatment by day effect (P = 0.02). On day 22, the 3.0 lysine:calorie had the highest blood protein concentration (P = .04), while on day 28, the diet containing a ratio of 2.2 had the highest blood protein concentration. The only variable to change across days was alkaline phosphatase (P = 0.002), which decreased during the restriction period.

Discussion

The purpose of this study was to determine the effects of differing dietary lysine:calorie on nitrogen metabolism, maintenance of lean muscle mass, and weight loss in mature, moderately obese dogs. Studies in rats, horses, and humans have found that changing the percentage of calories from dietary protein can have benefits such as maintenance of lean muscle mass, an increase in the fat mass loss and decreased lean muscle mass loss during a weight-reduction program with amino acid supplementation and an increased percentage of calories from protein (Das et al. 2004; Diez et al. 2002; Graham-Theirs and Kronfeld 2000; Laflamme and Hannah 2005; Tremblay et al. 2002; Volpi et al. 1998; Wakshlag et al. 2003; Wessels et al. 1997; Williams et al 2001). In older canines, maintenance of muscle mass becomes increasingly important as muscle wasting becomes more prevalent with age. Kealy (1999) noted that an inadequate supply of protein increases loss of lean muscle mass. Because obesity is also common in older animals, maintaining lean muscle mass while losing fat mass becomes a primary focus. Data from this experiment will improve diet formulation to maximize the amount of fat mass lost while minimizing lean muscle loss.

Changes in body weight were not apparent until week 2 of the restriction period, where a quadratic effect (P = 0.03) could be seen as the weight loss associated with the diet containing a 3.0 lysine:calorie was greater than the other two diets. However, weight loss during week 1 was substantial , and overall, total weight loss tended to be significant (linear P = 0.09), with animals consuming the 3.0 and 3.8 diets both losing 1.14 kg over a 14 day period whereas animals consuming the 2.2 diet only lost 0.95 kg. The higher proportion of calories from protein resulted in a 20% greater weight loss than the 2.2 diet. Presumably this loss could be maintained over a longer restriction period. Thus, the 3.0 and 3.8 treatments could improve weight loss in comparison to the 2.2 diet.

Contrary to our hypothesis, nitrogen and protein metabolism exhibited little change with varying proportions of calories from protein. As the lysine:calorie increased, total intake of nitrogen increased because the diets were chosen to maintain similar essential amino acid profiles. Thus nitrogen intake increased linearly because of the increase in the total amount of supplemented dietary amino acids. By maintaining similar amino acid profiles across the diets, any secondary effects or deficiencies as a result of limiting amino acids could be eliminated. However, increasing nitrogen intake with increasing lysine:calorie may have reduced the ability to observe small differences in total nitrogen metabolism.

Apparent nitrogen absorption by the gastrointestinal tract was calculated as the difference between nitrogen intake and nitrogen excreted in feces. Excess absorbed nitrogen is excreted in the urine, thus calculated nitrogen absorption does not solely reflect the true nitrogen metabolism taking place with varying lysine:calorie, but instead is a measure of the GI tract's ability to absorb increasing amounts of nitrogen. Apparent nitrogen absorption (g) increased with increasing lysine:calorie, however, the percentage of nitrogen absorbed as a function of total nitrogen intake did not differ among treatments. Nitrogen intake increased at nearly the same rate as nitrogen absorption and fecal N excretion did not differ among treatments. These results are similar to a study by Williams et al. (2001) using dogs, who found that increasing protein concentrations in an almost isocaloric diet linearly increased nitrogen intake and total N absorption (g). In the present study, urinary nitrogen excretion did not differ across treatments. Because most excess nitrogen is excreted in the urine as urea, the rate of urea excretion closely reflects true nitrogen metabolism and was similar across all treatments with a mean of 9.94 ± 0.56 g/day. Any excess protein and amine group the body does not use for protein synthesis, or is not transaminated, or does not recycle to the GI tract is excreted as urea. Because daily urea excretion was similar across treatments, dietary protein in excess of maintenance protein requirements was likely being utilized for protein accretion. Specifically, amino acid supplementation in diets 3.0 and 3.8 supplied amino acids that resulted in a tendency for an increase in N retention and thus the use of the amino acids for protein accretion. However, when calculated as the difference between protein synthesis and protein degradation, there was no significant changes in protein accretion

across treatments. This could be a result of a small sample size or errors associated with nitrogen metabolism methodology. Since one of the dogs used in this study yielded erroneous data for the protein turnover calculations (2-2.5 times other dogs), it is not unreasonable to assume that the addition of additional dogs would give more consistent results.

Nitrogen balance studies can be invaluable when used in conjunction with protein metabolism experiments, however, because of the relatively small differences in retained nitrogen for a mature dog fed near maintenance, finding differences can be difficult. This is why we chose to study N metabolism in the moderately obese dog during a period of restriction. We hypothesized that the nutrient restriction would allow us to differentiate between the differing lysine:calorie diets and many of the tendencies supported this idea. However, the small changes may require a greater number of dogs to definitely answer the question. Furthermore, it is often very difficult to completely separate different types of nitrogen excretion due to methodology. Hair in fecal and urine analysis can be skimmed or gently removed, but the possibility for contamination still exists. As dogs groom themselves they ingest hair that can not be accounted for as nitrogen intake, but will incidentally be accounted for in fecal nitrogen. Since most nitrogen data are calculated as differences, the error becomes distributed across all data values.

The similarities in urine nitrogen excretion, protein accretion and urea excretion across treatments suggests the dogs were able to acclimate to an increase in nitrogen intake by utilizing supplemental essential amino acids for energy or protein accretion. As the proportion of calories from dietary protein (and therefore the amount of available amino acids) increased, the excess amino acids beyond protein maintenance were likely being used for protein accretion as nitrogen excretion was fairly constant. This overall trend in the data suggests that the concept of formulating diets such as these merits further study in the mature dog.

Additionally, the amount of nitrogen retained across all treatments was not different from zero (mean 1.32g ± 0.8). While the percentage of nitrogen retained demonstrated a tendency for a linear increase with increasing lysine:calorie ($P=0.16$), these data suggest that the highest diets met the requirements for maintenance while the lowest diet seemed to be slightly below maintenance and the additional amino acids in the higher protein

diets could promote additional protein synthesis. Furthermore, total fecal and urine nitrogen excretion as well as markers of muscle degradation did not change with 2.2 compared to 3.0 and 3.8, demonstrating that while the body was not able to accrete protein, it was able to maintain existing muscle mass during caloric restriction and weight loss.

Protein synthesis was determined using ^{15}N Glycine as a marker for protein turnover. Calculated protein synthesis of dogs was not different across treatments. Because the dogs were on a reduced caloric intake, it is possible that the supplemental amino acids were being utilized for tissue maintenance because excretion of urea did not increase with the greater N intake and absorption. Further experimentation with a wider range of ratios will provide a more precise point at which the optimum nitrogen retention can be achieved and protein synthesis may be stimulated without wasting excess amino acids. Given the consistent percentage of nitrogen absorbed by the gut, the maximum capacity for N absorption was not reached in the present study.

Mean protein degradation in dogs consuming diets with lysine:calorie of 2.2 and 3.8 was 2.88 g N/day. In contrast, mean protein degradation in dogs consuming the 3.0 lysine:calorie diet was only 0.10 gN/day. Similarly, Williams et al. (2001) fed three different amounts of protein using a basal, isocaloric diet and the mid-ranged diet (which had a lysine:calorie of about 2.8) showed the greatest decrease in protein degradation by providing an apparently ideal amount of essential amino acids. The absolute differences in protein degradation rates are not as important as the relative changes. Because so many of the variables for protein metabolism are calculated by difference, and with the small number of dogs employed, we should not expect these treatments to induce a 28-fold change in protein degradation. However, based on these results and others (Williams et al., 2001) we could expect the intermediate treatment to produce the lowest protein degradation. This lysine:calorie (2.8-3.0) may be the optimum lysine:calorie diet to feed dogs with a greater propensity for muscle wasting as it may slow the degenerative process of sarcopenia by maintaining muscle mass by decreasing muscle degradation. Similar results have been found in humans when the interactions of protein intake and muscle degradation and aging were examined. Volpi et al. (1998) found that infusing essential amino acids in elderly individuals stimulated protein synthesis. While the

supplemental amino acids used in this experiment were dietary, comparisons between the two experiments can still be made. Because protein degradation is calculated as the difference between protein turnover and nitrogen absorbed, the drastic decrease in degradation for lysine:calorie 3.0 is a result of a slight decrease in the amount of nitrogen absorbed. When compared to the other two diets, protein degradation was substantially reduced while protein synthesis remained the same, and thus for this lysine:calorie ratio, muscle mass is maintained by a decrease in protein degradation. Surprisingly, urine creatinine and urea excretion (g/d) did not change between treatments, despite a substantial reduction in protein degradation for the 3.0 lysine:calorie. However, these data do support the evidence that supplemental amino acids may be utilized for protein synthesis rather than deaminated for excretion. Because of the lack of change in protein synthesis across treatments, combined with the high percentage of N retention associated with the two highest lysine:calorie diets and the lack of increased nitrogen excretion in urine or feces, these data suggest that the supplemental amino acids may be utilized for protein synthesis rather than being deaminated for energy and the N excreted as urea.

These results conflict with the results found by Williams et al. (2001). Williams et al. (2001) determined that the increase in amount of dietary protein resulted in an increase in protein synthesis. Williams et al. (2001) did not use a calculated lysine:calorie as high as the one used in this experiment (3.3 in comparison to 3.8), however, they used the same ^{15}N-glycine method for calculating protein turnover as in our experiment, thereby allowing for direct comparisons. The dogs in the study by Williams et al. (2001) were on a positive plane of nutrition in comparison to the dogs in the present study who were calorically restricted. Dogs on a positive plane of nutrition may have additional protein synthesis since all maintenance requirements have been met and additional dietary protein can be used to accrete body protein. However, when energy is limiting, a decrease in protein degradation does not require additional energy but can still positively affect protein turnover.

Turnover of protein is defined as either the difference between protein synthesis and N excreted in the urine or as the difference between protein degradation and N absorption. Protein turnover was variable in this experiment, with the highest rate of turnover at the lysine:calorie of 3.8. This is likely due to the higher rate of protein synthesis taking place

with this treatment, and is likely a result of excess supplemental essential amino acids. Protein synthesis and degradation are normal, constantly occurring processes, however they can be influenced by both protein and energy intake. If the animal's energy requirements are met, it does not require the break down of endogenous protein or dietary protein to meet energy needs and excess amino acids supplied can be utilized for protein accretion. Our study utilized a diet with a lysine:calorie of 3.8, which was comparatively high compared with the 3.3 ratio used by Williams et al. (2001). However, Williams et al. (2001) also noted that the diet containing the highest proportion of calories from protein increased protein synthesis in both young adult and geriatric dogs. These results are in agreement with the tendencies seen in our experiment. While we did not note a significant change across diets, the magnitude of protein synthesis increased with the highest ratio. The lowest rate of turnover occurred with the diet containing a ratio of 3.0 as a result of decreased protein degradation as discussed previously. Diet supplementation with essential amino acids may have resulted in a decrease in protein degradation because the greater supply of dietary protein during periods of restriction prevented utilization of muscle protein stores for energy. Further excesses of dietary amino acids can increase protein synthesis, as observed by an increase in the magnitude of protein synthesis with the 3.8 diet. These results are in disagreement with Salter et al. (1990), who reported increases in protein synthesis and degradation for protein deficient pigs supplemented with essential amino acids, but because protein synthesis was greater than protein degradation, a net increase in protein accretion was observed. In the current study, there were changes in protein degradation with changing proportions of dietary protein similar to those found in the experiment preformed by Salter et al. (1990), however this did not result in a significant change in protein accretion.

To evaluate any possible negative consequences of the increased protein intake during energy restriction, we evaluated blood chemistries on two separate days during the restriction period. Days 22 and 28 of the experimental period were chosen to compare values from the end of the first week of restriction, after the body had time to adjust to the initial reduction in gut fill and caloric decrease, to blood taken at the end of the second week of restriction, when the body may have been altering substrates for energy use and tissue maintenance. Most blood chemistry values did not change during our

study, regardless of treatment. This was not unexpected as it was hoped that a short restriction period and changes in dietary protein would not affect most electrolyte and metabolite concentrations enough to cause significant physiological changes to possibly cause harm to the animal. Potassium levels decreased (P = 0.02) with increasing lysine:calorie, however, this change may not have been biologically significant as they fell within the normal range for the dog (15.56-16.81 mg/dl). The sodium:potassium increased linearly with increasing lysine:calorie because of the decrease in plasma potassium but these changes also were not biologically significant.

Plasma alanine aminotransferase and alkaline phosphatase were measured as indicators of normal liver and hepatobiliary system function. Plasma alanine aminotransferase and alkaline phosphatase concentrations were not affected by treatment on either day 22 or 28, but values on day 28 were lower than those on day 22 (P = 0.05). However, the average plasma alanine aminotransferase still fell within the normal physiological range for the dog (40.12 and 35.39 U/L respectively). Alkaline phosphatase was also within physiological range of the dog. Alkaline phosphatase is used to measure the integrity of the hepatobiliary system and to indirectly monitor the flow of bile in the small intestine. The decrease in alanine aminotransferase and alkaline phosphatase between day 22 and 28 may be reflective of the body's adjustment to decreased intake during the restriction period and thus decreased bile production in response to reduced gut fill.

Plasma urea concentrations changed quadratically (P = 0.04) with increasing concentrations with the middle ratio (3.0) having the lowest concentrations. This quadratic effect could be a result of the lowest dietary ratio (2.2) requiring the body to breakdown endogenous protein for energy and thereby creating more ammonia dumping into urea. In contrast, the highest dietary ratio of lysine:calorie (3.8) might have resulted in too much protein in the diet, and coupled with a greater protein turnover, may have caused an increase in plasma urea concentrations.

Plasma creatinine concentrations demonstrated a quadratic tendency within both days of the experiment (P = 0.06). Plasma creatinine decreased then increased with increasing lysine:calorie, coupled with the percentage of nitrogen retained, suggests that the 2.2 diet provides just under the amount of supplemental amino acids needed to maintain muscle

mass. Protein turnover is a normal bodily function, thus there will always be some basal synthesis and degradation. Changes in protein degradation can be indirectly monitored by plasma creatinine excretions. The quadratic tendency exhibited by creatinine is similar to the quadratic effect seen in protein degradation.

Conclusion

Protein intake during weight loss has been studied in several species. Our nitrogen retention results suggest that increasing lysine:calorie ratio may positively affect protein metabolism. Protein turnover and protein degradation were also influenced by varying proportions of calories from protein. Lysine:calorie can alter protein and nitrogen metabolism during weight loss while maintaining or decreasing the degradation of lean muscle mass. Because increasing the proportion of calories from protein affects protein turnover and protein degradation, it may be beneficial to older animals suffering from excess protein degradation during periods of caloric restriction.

Implications

It has been stated that increasing the percentage of calories from protein can help maintain muscle mass during weight loss. The results of this experiment imply that if all essential amino acids are in adequate supply in the diet, diets with a greater proportion of calories from protein can reduce muscle degradation during periods of caloric restriction. Our optimum diet for decreasing protein degradation was the diet containing 3.0 lysine:calorie. The results from this experiment can greatly improve regimes for companion animals affected by high rates of protein degradation, especially for older animals that have a propensity for muscle loss. Diet regimens such as these may improve animal health and longevity.

Table 3.1 Ideal Canine Amino Acid Profile (Baker and Czarnecki-Maulden 1991)

Amino Acid	Ideal AA:Lysine
Lysine	1.00
Methionine+Cystine	0.64
Tryptophan	0.22
Threonine	0.67
Arginine	0.71
Isoleucine	0.57
Valine	0.75
Leucine	1.00
Histidine	0.29
Phenylalanine+Tyrosine	1.00

Table 3.2 Ingredient composition of Lysine:Calorie diets fed to dogs[a]

	Formulated Lysine % : Mcal ME/g		
Ingredients, %	2.2	3	3.8
Rice, Brewer's	43.69	39.81	41.61
Corn starch	23.00	26.30	23.09
Low-ash poultry meal	13.94	13.50	13.48
Choice white grease	7.55	7.55	7.55
Corn gluten Meal	4.50	4.50	4.50
Soybean oil	2.00	2.00	2.00
Cellulose	2.00	2.00	2.00
Potassium Chloride	1.00	1.00	1.00
Pal enhancer	1.00	1.00	1.00
Dicalcium Phosphate	0.71	0.71	0.71
Iodized salt	0.25	0.25	0.25
Choline chloride	0.17	0.17	0.17
Calcium carbonate	0.11	0.11	0.11
Vitamin premix	0.10	0.10	0.10
Mineral premix	0.05	0.05	0.05
Magnesium Oxide	0.03	0.03	0.03
L-Lysine	--	0.43	0.77
DL-Methionine	--	0.16	0.31
L-Threonine	--	0.16	0.31
L-Tryptophan	--	0.09	0.13
L-Valine	--	0.06	0.30
L-Isoleucine	--	0.03	0.16
L-Histidine	--	--	0.11
L-Tyrosine	--	--	0.27

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

Table 3.3 Analyzed chemical composition of Lysine:Calorie diets on a dry matter basis.

	Formulated Lysine : Calorie		
Item	***2.2***	***3.0***	***3.8***
Dry Matter %	92.95	93.45	93.20
Crude Protein	18.00	18.65	19.95
Crude Fat	13.60	13.18	13.76
Ash	3.99	3.99	3.99
Crude Fiber	1.72	1.61	1.72
Alanine	1.17	1.23	1.19
Arginine	0.98	1.11	1.03
Aspartate	1.43	1.50	1.45
Cystine	0.24	0.24	0.24
Glutamate	2.70	2.92	2.78
Glycine	1.24	1.28	1.27
Histidine	0.34	0.41	0.43
Isoleucine	0.53	0.72	0.69
Leucine	1.51	1.66	1.52
Lysine	0.90	1.21	1.50
Methionine	0.36	0.48	0.59
Phenylalanine	0.75	0.82	0.77
Proline	1.19	1.10	1.18
Serine	0.83	0.82	0.84
Threonine	0.68	0.82	0.98
Tyrosine	0.47	0.48	0.68
Valine	0.71	0.92	1.02

Table 3.4. Dry matter intake and body weight change in dogs fed varying lysine:calorie ratios during caloric restriction.

	Treatment Lysine:calorie				Contrasts	
	2.2	3.0	3.8	SEM	Linear	Quadratic
Average DM Intake (g/day)	190	192	192	0.901	0.18	0.22
BW d15-21 (kg lost)	0.80	0.80	0.95	0.215	0.32	0.55
BW d22-28 (kg lost)	0.15	0.34	0.19	0.108	0.60	0.03
Total Change (kg lost)	0.95	1.14	1.14	0.144	0.09	0.28

Table 3.5. Nitrogen metabolism during nutrient restriction in dogs fed varying ratios of lysine:calorie

	Treatment Lysine:calorie				Contrasts	
	2.2	3.0	3.8	SEM	Linear	Quadratic
N intake (g)	26.53	28.38	29.48	0.214	<0.0001	0.21
N feces (g)	2.59	2.66	2.97	0.202	0.23	0.64
N urine (g)	23.93	23.76	24.51	0.794	0.62	0.65
N absorbed (g)	23.94	25.72	26.51	0.113	<0.0001	0.01
N retained (g)	0.004	1.95	1.99	0.801	0.13	0.37
% N Absorbed	90.17	90.60	89.90	0.683	0.79	0.53
% N Retained	-0.05	6.96	6.66	2.993	0.16	0.35

Table 3.6. Protein Metabolism during caloric restriction in dogs fed varying ratios of lysine:calorie

	Treatment Lysine:calorie				Contrasts	
	2.2	3.0	3.8	SEM	Linear	Quadratic
Urea Excretion(g/day)	9.62	10.01	10.20	0.558	0.51	0.89
Protein Synthesis (gN/day)	2.77	2.16	4.64	1.042	0.28	0.30
Protein Degradation (gN/day)	2.81	0.10	2.95	0.8484	0.91	0.06
Protein Turnover (gN/day)	26.34	25.42	28.95	0.745	0.07	0.07
^{15}N Fractional Recovery	0.37	0.39	0.36	0.013	0.60	0.11

n = 5

Table 3.7. Urine Creatinine values for dogs fed varying lysine:calorie ratios

	Treatment Lysine:calorie				Contrasts	
	2.2	3.0	3.8	SEM	Linear	Quadratic
Urine Creatinine (mg/dl)	203.6	198.6	208.5	16.78	0.8439	0.7281
Creatinine Excretion (g/day)	0.988	0.995	1.11	0.070	0.2766	0.5669

Table 3.8. Blood Chemistry profile of dogs fed varying lysine:calorie ratios

	Day 22			Day 28					
	Treatment Lysine:calorie			Treatment Lysine:calorie				Contrasts	
Item, mg/dl	2.2	3.0	3.8	2.2	3.0	3.8	SEM	Linear	Quadratic
Glucose	101.50	104.50	101.00	100.17	101.67	99.25	3.837	0.99	0.60
Urea	44.16	42.30	42.18	43.62	40.26	44.64	2.604	0.69	0.04
Creatinine	1.24	1.01	1.09	1.21	1.04	1.08	0.0670	0.08	0.06
Sodium	378.57	391.98	380.55	383.54	380.48	378.18	4.435	0.74	0.17
Potassium	16.81	16.30	15.56	16.54	16.30	16.34	0.289	0.02	0.86
Chloride	420.72	433.69	425.43	417.17	417.17	418.55	5.116	0.68	0.38
Calcium	9.33	9.72	9.22	9.63	9.35	9.41	0.196	0.32	0.36
Phosphorus	3.55	3.28	2.89	2.95	3.31	3.74	0.201	0.99	0.85
Total Protein[a]	5670	6080	5590	5950	5750	5770	138.4	0.19	0.04
Total Bilirubin	0.73	0.87	0.73	0.90	0.83	0.89	0.1121	0.99	0.65
Triglycerides	84.33	94.67	84.17	105.17	103.50	92.56	11.573	0.57	0.39
Cholesterol	224.67	238.67	217.39	227.50	220.17	211.33	14.992	0.12	0.11
Magnesium	1.98	2.05	1.94	2.07	1.97	2.04	0.0503	0.53	0.99
Albumin	3480	3630	3260	3600	3520	3450	107.4	0.09	0.15

[a]TreatmentxDay effect ($P < 0.05$)
n=5

Table 3.8 Blood Chemistry profile of dogs fed varying lysine:calorie ratios. (continued)

	Day 22			Day 28						
	Treatment Lysine:calorie			Treatment Lysine:calorie				Contrasts		
Item, U/L	2.2	3.0	3.8	2.2	3.0	3.8	SEM	Overall	Linear	Quadratic
Alanine Aminotransferase	35.83	46.00	38.53	34.17	37.83	34.17	7.163	0.4104	0.5854	0.2541
Alkaline Phosphatase[ac]	56.50	63.00	49.14	54.83	55.00	42.14	11.793	0.1403	0.1597	0.1122
Ratios										
Urea:Creatinine	36.41	42.05	38.87	36.60	38.41	43.19	2.155	0.3132	0.1627	0.5814
Albumin:Globulin	1.62	1.48	1.59	1.53	1.58	1.48	0.060	0.2780	0.1954	0.4092
Sodium:Potassium	38.67	41.33	41.50	39.50	39.83	39.61	0.732	0.0323	0.0293	0.1186

n=5
[a]Day effect ($P < 0.05$)
[b]TreatmentxDay effect ($P < 0.05$)
c, Quadratic within day 28 ($P<0.02$)

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