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Effect of Dietary Protein Intake on Amino Acid Metabolism in Cats

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ABSTRACT

The domestic cat, an obligate carnivore, evolved eating a diet rich in protein. Their high protein requirement ensures an adequate supply of amino acids (AA) for metabolism even though AA are relied on for energy. However, there is little research examining AA use at intakes below their protein requirement. The first objective investigated AA kinetics using the [1-^{13}C]Leu precursor method in cats fed above, at, and below their protein requirement. The second objective measured hepatic protein synthesis using a modified D_2O method, which when in conjunction with Objective 1, allowed direct comparison of hepatic protein synthesis with whole-body synthesis. Domestic short-hair cats (n=18) from the Massey University Centre for Feline Nutrition were fed to maintain body weight on diets containing 7.5, 15, and 40% of metabolizable energy as crude protein (CP). After 3 wk, [^{13}C]Na bicarbonate and [$^{15}N_2$]Urea were infused into a cephalic vein while in the fed state. After 2 h, bicarbonate was switched to [1-^{13}C]Leu, and $^2H_20$ was administered as a subcutaneous bolus to label body water. Blood and breath were sampled after 0, 100, 110, 120, 440, 460, and 480 minutes after start of infusions. Deuterium enrichment in free and albumin-bound Ala was measured to estimate hepatic protein synthesis. Non-oxidative Leu disposal (representing protein synthesis) was not different among treatments. Leu rate of appearance in plasma (representing protein degradation) was nearly 1.5X greater in cats fed 7.5 and 15% CP ($P < 0.05$) than in those fed 40% CP. Leu oxidation was greatest in cats fed 40% CP ($P < 0.01$), but did not differ for cats fed at or below their requirement, despite oxidation in cats fed 15% CP tending to be 20% greater than those fed 7.5% CP. Results for urea production (representing net AA catabolism) mirrored those for Leu oxidation. Values for hepatic protein synthesis proved unreliable, possibly due to errors in administration of D_2O. To conclude, AA oxidation in cats fed below their protein requirement did not decrease compared to those fed at requirement. This suggests that the cat is unable to down regulate AA oxidation when protein requirements are low.

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"Anyone who does not believe in miracles, is not a realist"

– Audrey Hepburn

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LIST OF ABBREVIATIONS

AA	Amino acids
AAFCO	Association of American Feed Control Officials
AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
BCAA	Branched chain amino acids
BCATm	Mitochondrial branched chain aminotransferase
BE	The enrichment of expired carbon dioxide at a steady state during bicarbonate infusion
BW	Body Weight
CANZ	Companion Animals of New Zealand
CCK	Cholecystokinin
CO_2	Carbon Dioxide
CO_2PR	Carbon dioxide production rate
CP	Crude Protein
D_2O	Deuterium Oxide
DM	Dry Matter
DMF-DMA	Dimethylacetal-dimethylformamide
EAA	Essential amino acids
FFM	Free Fat Mass
FSR	Fractional synthetic rate

GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GCKR	Glucokinase regulator
GE	Gross energy
GK	Glucokinase
GKRP	Glucokinase regulatory protein
HCl	Hydrochloric acid
IE	Infusate enrichment
IR	Infusion rate
KIC	α-ketoisocaproic acid
Leu	Leucine
Leu flux	Leucine flux
Leu Ra	Leucine rate of appearance
ME	Metabolizable Energy
MPE	Mole Percent Excess
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin (pathway)
N	Nitrogen
Na	Sodium
NEAA	Non-essential amino acids
NH_4OH	Ammonium hydroxide
NOLD	Non-oxidative leucine disposal
NRC	National Research Council

OAT	Ornithine amino transferase
OM	Organic matter
P5C	Pyrroline-5-carboxylate
PE	Plasma enrichment at the plateau
PEPT1	Peptide co-transporter 1
PR	Production rate
RQ	Respiratory quotient
SAS	Statistical Analysis Software
SE	Standard Error
SEM	Standard error mean
SGLT1	Sodium glucose cotransporter-1
SIM	Selected ion monitoring
T0	Isotope ratio time point 0
TCA	Trichloroacetic acid
Te	Isotope ratio time point
USD	United States Dollars
V_d	Volume of distribution

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Domestic cats are obligate carnivores who require high amounts of protein to be supplied in their diet. This trait is due to metabolic and nutritional needs which have occurred during evolutionary adaptations, this means that a domestic cat's main energy sources are from protein and fat (Green et al., 2008 & Zoran, 2002). Omnivorous species rely on carbohydrates for energy; this is not true in regards to the domestic cat. The domestic cat requires 16% of metabolizable energy of high quality crude protein to meet their metabolic requirements (NRC, 2006). Their high protein requirement is due to their need to utilize amino acids (AA; Zoran, 2002) for energy and gluconeogenesis.

Although it is known that the domestic cat is a small carnivore; there is limited knowledge or research on the domestic cat's metabolism as a whole. Consequently, it is not fully understood how domestic cats regulate protein metabolism in response to different dietary levels. This study would be a follow-up on a study conducted by Wester et al., (2015) who concluded that the domestic cat ensures that AA demand is met by a high protein requirement combined with a low rate of whole-body protein synthesis. That study examined the domestic cat's protein metabolism at and above dietary protein requirements. However, protein metabolism when dietary protein was below requirements was not examined.

The domestic cat is unable to down-regulate enzymes involved in AA oxidation; the same is true for urea cycle enzymes (Rogers et al., 1977). Ultimately their lack of metabolic adaptations in these pathways result in a high loss of nitrogen; thus the domestic cat seems to have a high requirement of dispensable nitrogen (Green et al., 2008). Therefore, when the domestic cat is in a state of low protein intake, they are unable to regulate AA oxidation to conserve

nitrogen; thereby potentially entering negative protein balance. Data published by Green et al., (2008) supports this belief and states that the domestic cat is only able to regulate protein oxidation when protein intake is at or above requirements.

In a world where the cat is kept as a pet, they rely on their owners for meals and, therefore, to meet their nutritional and, thus, protein requirements. Furthermore, it is estimated that there are 25.4% of households are cat owners in the United States alone (American Veterinary Medical Association, 2018); while in New Zealand; over 41% of households own a domestic cat; a figure which shows that the number of cat owners in New Zealand is higher than in the United States itself (CANZ, 2020). In 2019, spending in the pet industry was approximately 75.38 billion USD; with around 42% of spending originating from pet food (American Pet Product Manufacturing Association, 2019). These values emphasize how owners are now committing to premium and super-premium products which are often nutrient rich. The rise in pet nutrition and owner dedication to a healthier cat diet increases the demand for research towards the cat's metabolism and their optimal dietary requirements.

1.2 PROBLEM STATEMENT

It is well-accepted that the cat is an obligate carnivore and, therefore, requires high levels of protein in their diet; animal tissue is therefore very complementing to the cat's diet. Although their protein intake is high, their rate of whole-body protein synthesis is lower than both omnivorous and herbivorous species (Russell et al., 2003); this is advantageous to the cat who requires the availability of AAs to be utilised in the gluconeogenic pathway for energy and glucose (Wester et al., 2015). Furthermore, although the cat is able to increase AA oxidation when protein intake is above requirements (Wester et al., 2015), minimal research has been published on cat AA oxidation when their dietary protein requirements have not been met.

Methods for assessing AA use (AA oxidation and protein synthesis) in the cat are time-consuming and invasive; with the requirement of catheterisation, infusions and repeated blood and breath sampling (Wester et al., 2015).

However, the whole-body [1-^{13}C]Leu precursor method used in in this thesis is able to directly measure AA use; as opposed to Green et al., (2008). This method can also be compared to Russell et al., (2002) whose method is dependent upon quantitative urine collection; which can be difficult in species such as the cat.

The measurement of hepatic protein synthesis through a less invasive procedure has been used for other animals in the past (MacDonald et al., 2013). As hepatic protein synthesis dictate whole-body rates, the use of the deuterium oxide method allows us to measure and compare hepatic protein synthesis with whole-body rates from the [1-^{13}C]Leu precursor method. The validation of this method for use in determining AA use in the cat has not yet occurred. Furthermore, the measuring of hepatic protein synthesis, if successful, may be used to approximate whole-body rates through an easier, less invasive procedure.

1.3 OBJECTIVES

The objective of this thesis was to fill a research gap and determine if amino acid oxidation decreases when dietary protein intake in the cat is below requirements. This study was designed to measure and evaluate the amount of AA being oxidised and used for protein synthesis, as well as to measure and estimate protein degradation. This was accomplished using the whole-body [1-^{13}C]Leu precursor method. Cats were also infused with [$^{15}N_2$] Urea from which urea production was measured and general AA catabolism was estimated. Additionally, our aim was to also test the validity of a minimally-invasive method, known as the deuterium oxide method, to determine hepatic protein synthesis in the cat.

CHAPTER 2: LITERATURE REVIEW

2.1 BACKGROUND

The domestic cat (*Felis catus*) is a small obligate carnivore belonging to the order of the Carnivora. The order of Carnivora is made up of nine surviving families and is grouped into two suborders: Feliformia (cat-like) and the Caniformia (dog-like). The order of Carnivora is not only composed of carnivores, but also contains omnivores and herbivores. By selectively eating non-healthy and phenotypically weak animals, carnivores are thought to maintain a healthy population of herbivores (Pendrago & Winkler, 2011)

The order Carnivora has a low rate of chromosomal evolution (Bush et al., 1977). The rate of chromosomal evolution, which is a means of measuring evolutionary changes is low in comparison to other orders. The lack of evolutionary changes is apparent when investigating the feline's dietary habits, especially in comparison to other members of the felidae family. Although the feline has become increasingly domesticated over time, there is a lack of major evolutionary changes when compared to other members of the same family. The feline has maintained similar metabolic and dietary patterns over time. The carnivores have a comparatively similar phenotype (Pendragon & Winkler, 2011), and, therefore, it is necessary to understand the feline's family's history in order to supply the cat with suitable dietary requirements.

In further support of the feline's low rate of chromosomal evolution, the instinctual dietary preference of the domesticated cat coincides with the macronutrient profile of cats found in the wild (Hewson-Hughes et al., 2011). The digestibility of raw-meat based diets has been compared in captive wild cats to the domestic cat. The objective was to study the total tract digestibility in three large exotic cats (African wildcats, jaguars and Malayan tigers) and compare these findings to the domestic cat when fed raw meat-based diets (Kerr et al., 2013). It was found that there were no differences observed between wild cats and domestic cats for total tract DM (dry matter), OM (organic matter), and GE

(gross energy) digestibility. However, the apparent total tract crude protein digestibility was higher in domestic and wild cats when compared solely to the Malayan tigers. This study highlights the lack of evolution in the cat's dietary habit and digestive physiology, as they are believed to have originated from their wild cat ancestors (Pendragon & Winkler, 2011).

The domestic cat is an obligate carnivore which means it requires a high amount of protein to be sourced from its diet. In fact, the cat is biologically adapted to a high protein diet, due to certain evolutionary pressures which further express their dietary requirements. This biological adaptation consists of physical, chemical and physiological factors or mechanisms which are highly specialized in the cat.

In the wild, the cat (such as the feral cat) obtains all its nutrients from prey. The diet of a feral cat provides us with a clear indication of the cat's natural diet which occurs through instinctual behaviours. For example, the "domestic mouse contains 55.8% of crude protein, 23.6% of crude fats and 8.8% of nitrogen free extract and 11.8% of ash on a dry matter basis" (Dierenfeld et al., 2002). This meal coincides with the composition of a high protein diet.

Today, the domestic cat is one of the most popular companion animals worldwide. In fact, globally there are approximately 600 million cats inhabiting homes (American Pet Product Manufacturing Association, 2007). Due to this popularity, a major shift in the cat's food source has now occurred. Instead of relying solely on hunting and catching prey, the cat's main source of food now comes directly from their human owners. It is, therefore, necessary that the commercially available pet foods meet all the nutritional needs of the pet cat. Furthermore, if owners give their cats improper diets, this may compromise the animal health and welfare of their pets, while also affecting urban ecology, as pets supplement their diet from nature (Hewson-Hughes, 2011).

2.2 THE CAT'S EVOLUTIONARY ADAPTATION AS A CARNIVORE

The cat has become increasingly domesticated since its first association with humans that was documented being in Cyprus, 9500 years ago (Driscoll et al., 2007). Although, the cat is generally found as a companion animal pet as opposed to a wild species, their dietary habits have remained highly carnivorous.

The domestication of the cat is rare in comparison to the domestication of other species such as agricultural animals like sheep, pig and poultry or animals associated with transport means, i.e., the donkey and the horse. The cat's association with humans began as a symbiotic (Crowley et al., 2020), as they fed on rodent pests. This is significant today as it portrays how the cat's natural diet was beneficial for humans and, therefore, why their dietary habits have not greatly evolved from being highly carnivorous. The highly carnivorous diet of the cat corresponds with its general anatomy and physiology which is adapted towards a lifestyle of predation.

2.2.1 THE DOMESTIC CAT'S GENERAL ANATOMY AND PHYSIOLOGY

The average domestic cat is approximately 3.6-4.5 kg, with a height of 23-25 cm and a length of 46 cm (not including the tail). The cat anatomy is similar to others from the Felidae family. These traits coincide with their natural instincts as hunters. These traits, which will be discussed in the following sections, include a strong flexible body, sharp retractable claws, impeccable hearing, night vision, and a strong sense of smell.

2.2.2 DIGESTIVE MECHANISTIC ADAPTATIONS

Cats are anatomically well-suited for predation (Christiansen, 2008) and, therefore, as carnivores they have many physical and anatomical adaptations which allow them to efficiently hunt, kill and digest their food. The domestic cat's jaw is specialized occupying a shortened mandible (lower jaw), enhancing the action of the temporalis muscle that closes the jaw (Hunter, 2005). This allows

for an increased bite force which is beneficial for the killing of prey. In fact, a cat will have a larger bite force than a dog will even if the jaw length is similar (Greaves et al., 1985). The domestic cat's jaw has no lateral movement. It does not require the use of sideways movement as the cat does not grind down food, but instead the cat consumes its food by the piercing and tearing of animal tissue. Additionally, the lack of lateral movement is advantageous for the cat because it would not be beneficial if the jaw were to "slip" whilst piercing and killing their prey.

As carnivores, the cat's teeth are also adapted for the piercing, shredding and tearing of animal tissue. The dental anatomy of the cat consists of molars, premolars, incisors and canines. However, although they have molars and premolars, these are not utilized for the grinding of carbohydrate material such as in other species. This is because, although they do aid in the grinding and chewing of food, they include no actual grinding surfaces. The number of molars and premolars also differs in carnivores such as the cat compared to other species. For example, the dog has a total of 16 premolars and 10 molars. There are four premolars on each side of the jaw, and four molars in the upper jaw, followed by six in the lower jaw (Clark et al., 1991). This can be compared to the cat which only has a total of 10 premolars and four molars, where six premolars are located in the upper jaw, two molars in the upper jaw, followed by four premolars in the lower jaw and two molars in the lower jaw (Clark et al., 1991). The different dentition between the dog and the cat emphasizes the difference in their strict natural diets, as the cat is an obligate carnivore. Furthermore, the last premolars and the first lower molars in the cat are collectively known as the "carnassial teeth". The cat has very sharp carnassial teeth which are utilized for the shearing of animal tissue. These carnassial teeth are specialized for the carnivorous animal. The incisors of the cat are responsible for grooming and the tearing of prey; they have a total of six incisors in the upper and lower jaw (Clark et al., 1991). Additionally, each jaw has one pair of canine teeth. These have a function in the actual grasping killing of the prey, and are very sharp to pierce the flesh of the animal.

2.2.3 GIT MORPHOLOGY AND ADAPTATION

The digestive compartments of a cat due to its carnivorous demeanor differ from other species such as omnivores and herbivores. Their digestive compartments have developed for the specific function of breaking down and successfully metabolizing a diet high in protein. The cat has a short small intestine that is only approximately three to six times their body length, or approximately 1.7m (NRC, 2006). As the cat's diet is composed of highly digestible food (mainly protein and limited carbohydrates) it does not require a long digestive tract as the breakdown of material in the natural cat diet is relatively easy. The cat is only able to effectively digest limited carbohydrates, such as carbohydrates which have already been broken down during food cooking processes. For example, cats readily digest cooked starch, but are less capable of digesting sucrose (Buffington, 2008). Therefore, a short digestive tract is suitable for the cat as the digestion of proteins are fast in comparison to the digestion of a range of carbohydrates.

The transit time of the small intestine is the time taken for food to pass through this area in the gastro-intestinal tract. The small intestine of the cat has a relatively short transit time of approximately 2.5 hours (Chandler et al., 1999). This can be compared to the dog's small intestine transit time of approximately 3.5 hours (NRC, 2006). The short transit time can be explained by the relatively large stomach of the cat, as the stomach's volume accounts for a total capacity of two to three times of the entire digestive system (Slatter, 2003). The cat's stomach accounts for a large proportion of its digestive system. This may be because a large proportion of protein digestion occurs here, and protein accounts for a large percent of their natural diet. Whereas, small intestine digestion is usually limited due to their natural diet consisting of only a small amount of carbohydrates. This, therefore, means that the large stomach capacity is of benefit to the cat.

Furthermore, it is the stomach acidity of the cat (pH ~1-2) which facilitates the efficient digestion of protein (NRC, 2006). Like other mammals, carnivores

require a stomach with a more acidic environment due to the need to activate specific enzymes required for the proper digestion of protein. For example, pepsinogen needs to be converted to its active form of pepsin and this requires an acidic environment with a pH of approximately 2-4 (Beasley et al., 2015). Pepsin activation is greater after the ingestion of collagen (which is found in the tissues of the cat's prey) and, therefore, its activity is highly suitable for the digestion of proteins sourced from the cat's diet (NRC, 2006).

Physical mechanisms which occur in response to protein uptake are the main reason why the cat's stomach is very acidic. Firstly, gastrin release occurs due to nerve impulses in the vagal nerve which in turn occurs in response to senses such as sight, smell, taste and physical chewing of food. Gastrin is released by the G cells in the stomach and causes the parietal cells to release HCl (Hydrochloric acid). Once HCl is released, H^+ concentration increases, lowering the pH level of the stomach. Generally, in non-carnivorous species when the stomach pH begins to fall, the release of gastrin is inhibited in response to the negative feedback due to the high H^+. Additionally, when a non-carnivorous animal ingests food, buffers are released into the stomach in response to the increasing H^+. Thereby these two mechanisms cause the pH of the stomach to eventually increase. In comparison, a low pH (high acidic) environment is maintained in the cat even during ingestion of a meal. This primarily occurs due to a high protein content present in the cat's food. As mentioned, the cat has a highly acidic stomach (pH ~2-4) which is maintained to facilitate the proper breakdown of proteins through the activation of specific enzymes. Therefore, the higher the protein content in the food then the greater the secretion of HCl, thus, the lower the pH and the more acidic the stomach environment (Sjaastad et al., 2010).

2.2.4 GIT FUNCTION IN DIGESTION

The gastrointestinal system mainly has a chemical role in digestion. This is due to the production of enzymes and absorptive processes which help to break down and metabolise several nutrients.

2.2.5 DIETARY ABSORPTION AND UTILIZATION

As in many species, dietary absorption and utilization in the cat varies in regards to the type of macronutrient. Protein, lipids and carbohydrates are all absorbed and utilized through different mechanisms in the cat's body.

2.2.5.1 CARBOHYDRATE

Although sugars are not a natural source of energy in the cat; they have been reported to be highly digestible by the cat (Kienzle,1993c). However; it is common knowledge that the cat lacks the proper gene coding for the "sweet receptor" (*Tas 1r2*) (Li et al., 2005; Li et al., 2006) and, therefore, have no preference or acknowledgement of this sense. However; different types of sugars are found in commercial pet food diets. Therefore, the digestion of these sugars in the cat is important in terms of understanding full carbohydrate digestion and metabolism in the cat gastrointestinal system.

There are two main types of sugars; disaccharides and monosaccharides. Disaccharides are sugars composed of two monosaccharides held together by a glycosidic linkage. Disaccharides are known as digestible carbohydrates as they require the hydrolysis by gastro-intestinal enzymes in order to be effectively metabolized (NRC, 2006). Disaccharides are broken down into monosaccharides by their corresponding enzyme. They are then capable of diffusing into the small intestine through processes explained in the next section: *Monosaccharides*. The presence and activity of the enzymes responsible for metabolizing disaccharides (known as disaccharidases) is very important for understanding carbohydrate metabolism in the cat. In this respect, the activities of lactase, maltase, sucrase, and trehalase have all been studied in the cat.

Maltase catalyzes the hydrolysis of maltose to the simple sugar glucose that can then be absorbed through the intestine. The maltase activity in the cat is present in the pancreatic tissue (Kienzle, 1993d) and the jejunum (the middle section of the small intestine) (Hore & Messer, 1968). Although it is true that

maltase activity in the cat is relatively low, this activity has been shown to increase from the duodenum to the ileum in the adult cat (Kienzle, 1993d).

Sucrase catalyzes the hydrolysis of sucrose to its monosaccharide constituents and allows them to be easily absorbed through the intestinal wall. Even when no sucrase activity is found in the pancreatic tissue of the cat (Kienzle, 1993), low traces of sucrase activity are found in the small intestine showing a similar pattern to maltase as its activity increases from the duodenum to the ileum (Kienzle, 1993d). However, the overall ability of cats to digest and use sucrose is limited (Morris, 2002).

Although trehalase activity is found in many mammalian species (Sacktor, 1968), it is absent in the cat (Hore & Messer, 1968). Trehalase's function is to catalyze the hydrolysis of trehalose. Trehalose is the major blood sugar of insects (Sacktor, 1968) and it is also present in plants (Hore & Messer, 1968). This seems fitting for the domestic cat who although may often hunt and consumes insects; this only makes up a small proportion of their total nutrient intake; therefore such enzyme activity would be "energy-wasting".

Lactase activity has also been examined in the cat. It is responsible for catalyzing the breakdown of lactose. As in other mammalian species, this enzyme level is highest at birth and declines after weaning (Hore & Messer, 1968). This is because newborn mammals are highly adapted to utilize milk, but changes in their diet occur with age and growth. "Lactase decreases approximately 10-fold between the period of birth and at six weeks of age" (Washabau & Day, 2013). However, in adulthood lactase activity remains relatively low. The low levels of lactase during adulthood are no surprise due to several findings of the cat being regarded as "lactose intolerant" (Batchelor et al., 2011; Kirk et al., 2000).

The decrease in lactase activity is found in many mammalian species. However, it is also true that in some individuals or even species, LPH (lactase) expression is high throughout their entire life period (Grand et al., 2003). This means that there are some populations which express high lactase activities for

prolonged periods. The decrease in lactase activity in relation to age is due primarily due to differences in gene regulation in individuals or populations (Grand et al., 2003), it is, therefore, plausible to say that the same may be true for the cat species.

Additionally, although the pattern of lactase activity from birth to adulthood is similar to other mammalian species, it is still relatively low in comparison. For example, lactase in the adult cat is 1.6- to 3.1-fold lower than lactase found in the dog (Batchelor et al., 2011). Again, it is a species difference in the enzyme activity which accentuates the cat's biological adaptations to their natural high protein diet.

Monosaccharides are simple sugars which include glucose, fructose, and galactose. Monosaccharides are readily absorbed by the cat small intestine and, therefore, do not require hydrolysis by gastro-intestinal enzymes (NRC, 2006). Hence, they are known as absorbable carbohydrates.

In terms of glucose absorption, detection of glucose in the lumen activates a "reflex pathway" (Sjovall et al., 1984). This reflex pathway uses a sodium dependent transport mechanism which is accompanied by sodium independent simple diffusion (NRC, 2006). The mechanism by which glucose absorption occurs through the brush border membrane of the small intestine is, therefore, similar to many other species. It is important to note that galactose absorption occurs through this similar mechanism (NRC, 2006).

The sodium glucose cotransporter-1 (SGLT1) is the correct name for the transport system used to carry glucose and galactose into the enterocyte (Arthur et al., 2014). This transport system is sodium dependent and, therefore, cannot operate in the absence of sodium. The process of glucose and galactose using SGLT1 is as follows:

1. The transporter is initially facing the lumen and then binds a sodium ion
2. A conformation change occurs allowing the binding of either a glucose or a galactose molecule

3. Glucose or galactose binds successfully and the transporter then rearranges itself so that the areas binding glucose/galactose and sodium are now moved inside of the cell
4. Sodium dissociates into the cytoplasm causing glucose/galactose to also be dissociated
5. The transporter then returns back to its initial position.

Although this co-transport system is found in many species, it differs in the cat. The cat is unable to upregulate intestinal glucose transport in response to a high carbohydrate meal (Batchelor et al., 2011). Instead the level of SGLT1 expression and its activity remains steady. The steady and low activity of SGLT1 in the cat intestine may be due to its sufficiency in absorbing the little carbohydrate which is found in the cat's natural diet (Batchelor et al., 2011). The cat has no need for high levels of direct glucose absorption from the diet as it relies on gluconeogenesis and fat for its energy demands. Therefore, the systems involved in those processes are up-regulated instead.

2.2.5.2 PROTEIN

The stomach is one of the first sites of protein digestion. This is discussed in detail in *Section 2.2.3*. Protein digestion continues in the small intestine, specifically the duodenum. Here, AA and polypeptides stimulate the secretion of cholecystokinin (CCK) from the intestinal mucosa. CCK then stimulates pancreatic enzymes to be released into the duodenum, where they are activated. The two major pancreatic proteases are chymotrypsin and trypsin. These pancreatic enzymes further cleave polypeptides into tripeptides, dipeptides, and individual AA.

Dipeptides, tripeptides and individual AA can be absorbed into the enterocyte by either facilitated diffusion or active transport via either Na^+ or H^+ co-transporters (Frenhani & Burini, 1999). Dipeptides and tripeptides are absorbed into the enterocyte by H^+/peptide co-transporter 1 (PEPT1) (Qandell et al., 2009). This transporter is Na^+ independent and relies upon H^+ movement and the driving force of the transmembrane electrochemical gradient. Once inside enterocytes, peptides are hydrolyzed by peptidases to form free AA. Amino acids

on the other hand, are absorbed into the enterocytes in a manner which is similar to that of monosaccharides. Firstly, there are at least four Na^+-dependent AA transporters located on the luminal plasma membrane. The process of AA absorption by AA transporters is as follows:

1. The transporter is initially facing the lumen and then binds to a Na ion
2. A conformation change occurs allowing the binding of an AA
3. Binding of an AA to the transporter causes rearrangement so that the areas bound to the AA and Na^+ are moved into the cell
4. Na^+ dissociates into the cytoplasm causing the AA to also dissociate
5. The transporter then returns to its initial orientation.

Nonetheless, there are several AA transporters expressed on the enterocytes which are substrate specific for different AA. Amino acid transport systems include: SLC1A1, SLC6A19, SLC6A20, SLC6A6, SLC36A1. These amino acid transport systems use secondary active transport. The SLC1A1 transporter, a basic AA transporter, has a high affinity for glutamate and aspartate. The AA transport system SLC6A19, also known B^0AT1 (broad neutral AA transporter 1), transports neutral AA, such as tryptophan (Bröer, 2009), phenylalanine (Bröer, 2008), tyrosine, methionine, valine, leucine and isoleucine. This transporter has a high affinity for large neutral AA and a lower affinity for smaller AA. The Na-dependent IMINO transporter system known as SLC6A20 (or SIT1) has a high affinity for proline. The AA transporter system SLC6A6, also known as BETA, has a high affinity for beta-alanine and taurine. The PAT1 amino acid transporter, also known as SLC36A1, transports AA such as glycine, alanine, and proline. Furthermore, there is also an AA transporter known as SLC3A1/SLC7A9 (or rBAT/b^0AT) that transports lysine, arginine and the resorption of cysteine using facilitative transport co-transport with a positive charge, and counter-transport with neutral AA. Lastly, the AA transporter SLC1A5, also known as ASC, transports alanine, serine and cysteine via an AA exchange.

2.2.6 METABOLIC ADAPTATION

The cat has metabolic adaptations suitable for their dietary habits. One of the main metabolic adaptations in the cat suited to a high protein and a low carbohydrate diet is the internal physiological preference to the gluconeogenic pathway. There are adaptations in the enzyme activity of the cat's liver (Verbrugghe et al., 2012) towards a diet low in carbohydrates and high in protein.

Hepatocytes, cells found within the liver, produce enzymes which are responsible for metabolism of glucose. Glycolysis is a series of reactions resulting in the breakdown of glucose into pyruvate. Although this process alone results in the generation of energy (two Adenosine triposphate molecules (ATP), pyruvate can enter the citric acid cycle where more ATP is generated.

The cat liver has some evolutionary adaptations in its enzyme activity. Glucokinase (GK), an enzyme involved in the first "rate-limiting" step of glycolysis, catalyzes the reaction of glucose to glucose-6-phosphate. In many studies, glucokinase activity in the cat is absent (Tanaka et al., 2005; Verbrugghe et al., 2012; Schermerhorn, 2013).

Although the cat lacks GK activity; the enzyme hexokinase is present (Schermerhorn, 2013). Hexokinase catalyzes the same reaction as GK. It is present in the cat liver in relatively small amounts (Verbrugghe et al., 2012) and has a lower Km than GK. The lower Km of hexokinase means that it has a higher binding affinity for glucose when it is present in low concentrations. This, however, may also result in a slower rate of glucose clearance after a meal high in carbohydrates (Villaverde & Fascetti, 2014). Most importantly, glucokinase only works at high glucose concentrations. The cat is not adapted to a high glucose diet and, therefore, regular activity of this enzyme is not needed.

The lack of GK activity in the cat is not in response to inhibitory signals from glucokinase regulatory protein (GKRP) because the cat lacks GKRP (Hiskett et al., 2008). In most mammals, GKRP binds to GK inhibiting its activity (Hiskett

et al., 2008). Interestingly, the lack of GK activity in the cat is not in response to inhibitory signals from GKRP because the cat lacks GKRP (Hiskett et al., 2008). Instead the lack of activity in the cat may be attributed to a reduced GK mRNA expression which occurrs in response to the lack of Glucokinase Regulator (GCKR) gene expression (Farrelly et al., 1999). It appears that the lack of permissive effects of GCKR expression on GK, likely influences the lower GK activity in the cat liver (Hiskett et al., 2008). This information again emphasizes the way in which the cat has adapted over time to an animal derived diet; and how many biological adaptations have limited their ability to metabolize carbohydrates efficiently. Additionally, the cat's lack of the GCKR expression to regulate GK protein in the liver, demonstrates how the cat has limitations for glucose metabolism compared to other animals. For example, the glucokinase in the rat's liver was shown to be mainly inhibited through this regulatory protein (Van Schaftigen et al., 1992).

Glycogen synthetase is an enzyme responsible for the conversion of glucose into glycogen. Glycogen serves as the main form of glucose storage. The activity of glycogen synthetase is suggested to be relatively low in the cat (Ballard, 1965). Ballard (1965) found that the incorporation of glucose into glycogen was very minimal for the cat (1.49 μmoles/g/2h). This was also measured in the rat (50.7 μmoles/g/2h) and dog (25.7 μmoles/g/2h) allowing for comparisons to be made (Ballard, 1965). The low level of glycogen synthetase activity in the cat may be due to the cat's adaptation to a carnivorous diet. The cat meets its need for glucose through gluconeogenesis. As glucose from absorption is normally minimal, there is usually no "excess" glucose to be stored. Additional glucose is not generally stored as glycogen, but is instead stored as energy in fat (Ettinger, 2009).

Furthermore, in the cat, enzymes in other metabolic pathways attributed to glucose metabolism are upregulated. The cat liver shows increased activity in pyruvate carboxylase, fructose-1,6-biphosphatase, and glucose-6-phosphatase (Washizu et al., 1999; Tanka et al., 2005). These are essential gluconeogenic enzymes in the cat which are responsible for rate limiting steps in gluconeogenesis. The activity of these particular gluconeogenic enzymes is

higher compared to other species, such as the dog. Tanaka et al. (2005) measured the rate of activities of many enzymes involved in glucose metabolism and reported that the dog had significantly lower gluconeogenic enzyme related activity in comparison to the cat. The cat's fructose-1,6-bisphosphatase activity was found to be 102 ± 10 nmol/min per mg protein, compared to the dogs' activity which was only 41 ± 10 nmol/min per mg protein. The activity of glucose-6-phosphatase also differs between the cat and dog. The cat's activity was found to be 345 ± 55 nmol/min per mg protein, while the dog's was found to be 197 ± 33 nmol/min per mg protein. The increased activity of these gluconeogenic enzymes suggests that the cat liver is adapted to produce glucose through gluconeogenesis (Verbrugghe et al., 2012).

One adaptation by cats to a diet high in protein and low in carbohydrate is a need to produce their own glucose from non-glucose precursors. Gluconeogenesis allows cats to use normally plentiful AA to produce enough glucose to meet their body's energy requirements. It is the pathway of gluconeogenesis which provides a link between the metabolism of glucose and the metabolism of protein.

The domestic cat has a post prandial increase of blood glucose following the ingestion of a high carbohydrate diet (Farrow et al., 2012). Additionally, this post prandial response continues for up to 12 hours in the domestic cat compared to two hours in the dog (Hewson-Hughes et al., 2011). The domestic cat has a reduced capacity to decrease blood glucose concentrations after the ingestion of a high carbohydrate meal. The cat's slower rate of absorption and clearance may be due to delayed gastric emptying (Coradini et al., 2014). For example, the average gastric emptying time for the cat may be as high as 24 hours. This can be compared to the dog, who has a gastric emptying time of five to ten hours (Miyabayashi et al., 1984). Their reduced capacity to respond to blood glucose levels are further emphasized by their delayed time to peak blood glucose concentrations (Farrow et al., 2012).

The intake of a meal high in carbohydrates does not lead to a decrease in gluconeogenesis in the cat (Verbrugghe & Hesta, 2017). Instead, if the consumption of a meal high in carbohydrates occurs which leads to an excess amount of digestible carbohydrates, these then provide a substrate for microbial fermentation to occur. The stimulation of short chain fatty acids such as propionate occurs in response to increased amounts of digestible carbohydrates in the hindgut. In turn, propionate can be converted into glucose and absorbed which can therefore regulate blood glucose levels and downregulating gluconeogenesis (Verbrugghe et al., 2009).

There is evidence that gluconeogenesis in the cat is a constitutive process, regardless whether the meal ingested is a regular carnivorous meal (high in protein) or not. Rogers et al., (1977) found that the activity of hepatic enzymes involved in gluconeogenesis are not adaptive to different protein levels and are always active or "switched on"; this was compared to the activity of gluconeogenesis in the omnivorous rat who had lower levels of hepatic enzyme activity. Furthermore, Eisert (2011) argued that the cat's large brain and thus high glucose demands means that the cat has an obligatory increased need for endogenous gluconeogenesis. In order to meet the brains high metabolic demands, gluconeogenesis needs to occur irrespective of nutritional state. Both points supported by Rogers et al., (1977) and Eisert (2011) give evidence that the gluconeogenesis in the cat is constitutive so that endogenous glucose needs can be met. (Verbrugghe et al., 2009).

2.3 DIETARY NUTRIENTS RECOMMENDED FOR THE CAT

There has been a great deal of research involved in determining the appropriate dietary recommendations for the domestic cat due to the large pet population. However, dietary nutrient recommendations vary in the cat. The variation occurs due to differences in the physiological state of the individual. The recommended allowance for adult cats at maintenance is shown below in *Table 2.1*.

2.3.1 PROTEIN REQUIREMENTS

Animals utilize protein as a source of nitrogen and AA that are needed for growth, synthesis and maintenance. The demand for high protein in the cat is essential. Comparing their requirement to other mammalian species enables us to visualize just how important protein is for the cat. Basically, the cat is an obligate carnivore and, therefore, has the biological need and/or drive to ingest animal tissue which is high in protein and AA.

2.3.1.1 TOTAL PROTEIN REQUIREMENTS

As seen in *Table 2.1* the recommended total protein allowance for the cat is 200 g of crude protein per kg of dry matter. A cat's high protein requirement may be emphasized when in comparison to an omnivorous species, such as the dog. For growth, the cat requires 20% more protein in the diet compared to the one required by the dog (293 g CP/MJ required for the growing cat vs. 230 g CP/MJ in the growing dog) (Legrand-Defretin, 1994). The difference becomes even greater during comparison of protein requirements at maintenance in the adult. For maintenance, the cat requires nearly 100 g/MJ more protein in their diet when compared to the dog (Legrand-Defretin, 1994).

Table 2.1: Nutrient Requirements of Adult Cats for Maintenance (National Research Council, 2006).

Nutrient (in grams)	Recommended Allowance (g per kg of DM)
Crude Protein	200.0
Amino Acids	
Arginine	7.7
Histidine	2.6
Isoleucine	4.3
Methionine	1.7
Methionine & Cystine	3.4
Leucine	10.2
Lysine	3.4
Phenylalanine	4.0
Phenylalanine & Tyrosine	15.3
Threonine	5.2
Tryptophan	1.3
Valine	5.1
Taurine	0.4

Note: DM, dry matter.

2.3.1.2 AMINO ACID REQUIREMENTS

Amino acids are the building blocks of protein. The cat has a total of 10 essential amino acids (EAA) plus taurine. These are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine and taurine. It should be noted that taurine is not an alpha-amino acid, however, it is an indispensable amino acid in the cat. The cat also has a total of eight non-essential amino acids (NEAA) which include, alanine, aspartic acid, asparagine, glutamic acid, glutamine, proline, glycine and serine.

Non-essential AA and essential AA are usually classified as whether they can be synthesized in the body. Essential AA, which cannot be made by the body, are needed to be supplied directly from the diet. Generally, NEAA are not needed in the diet as the body itself is able to synthesize these using EAA. However, this is not true in the cat. The cat requires an external supply of NEAA acids in order to provide its body with the correct nutritional requirements, and to meet metabolic demands for gluconeogenesis and the urea cycle. When any of these AA are reduced or removed from the purified diet then voluntary food intake will decrease and body weight loss will occur (NRC, 2006).

2.3.1.2.1 ARGININE METABOLISM

Arginine is an amino acid which is classified as an “essential” AA in the cat, yet a “nonessential” AA in the human. In fact, if the cat has a single arginine-free meal then this results in detrimental health effects such as ammonia toxicity, as soon as 2-5 hours later (Legrand-Defretin, 1994); this alone signifies the importance of arginine in the metabolism in a species, such as the cat, who has a high intake of protein and consequently requires the removal of ammonia from the body on regular occasions. Most of the time, a cat will not eat an arginine deficient diet because it won’t be able to get rid of the ammonia from a protein-rich diet.

The inability of the cat to synthesize levels of arginine to meet their requirement is due the lack of activity in three distinct enzymes found in the enterocytes and the hepatocytes (Levillain et al., 1996).

Ornithine amino transferase (OAT) catalyzes the reaction of glutamate to ornithine. Activity of OAT is much lower in cat mucosa in comparison to the rat (Burger & Rivers, 2009). In fact, the activity of OAT in the rat is about 80% higher than in the cat (Little, 2015).

Pyrroline-5-carboxylate synthetase is an enzyme which is responsible for driving the reaction of L-glutamate to pyrroline-5-carboxylate (P5C). While glutamate is ultimately converted into ornithine, it is first converted to pyrroline-5-carboxylate. This step is therefore fundamental in the biosynthesis of L-ornithine and, therefore, L-arginine. Rogers & Phang (1985); reported that “the activity of pyrroline-5-carboxylate synthetase in the intestinal mucosa of cats compared to that of rats is only 18% as high per gram of mucosa and only 5% as high per kilogram body weight”. The activity of this enzyme is extremely low in the cat which signifies that the synthesis of adequate levels of ornithine will also be low.

Ornithine is a non-proteinogenic AA which acts as a precursor in the biosynthesis of arginine. If the concentration of available ornithine is low or down regulated, the ability to produce arginine will also be diminished. The cat is only able to synthesize limited amounts of ornithine due to the low activity of pyrroline-5-carboxylate synthetase (Rogers & Phang, 1985). The lack of ornithine synthesis means that the cat must rely heavily on dietary arginine to produce the ornithine required for the urea cycle. Insufficient ornithine then leads to depletion of the urea cycle, causing ammonia toxicity.

Although not fully understood, Rogers & Phang (1985) reported the activity of carbamoyl phosphate synthase to be low in the cat. Carbamoyl phosphate synthase catalyzes the first committed step in the biosynthesis of arginine. Therefore, if the activity of this enzyme is low, arginine synthesis will also be low. The same also applies to OAT, and pyrroline-5-carboyxlate synthetase as mentioned above.

2.3.1.2.2 TAURINE METABOLISM

It is a common misconception that the cat is unable to synthesize taurine from cysteine. However, the cat is able to produce taurine, although in inadequate amounts as a result of limited biosynthetic capability (Markwell & Earle, 1995). Before domestication, the cat obtained taurine from muscle in their prey. However, most commercially available diets are supplemented with taurine in order to meet the cat's requirement. This is because the processing of pet food has been found to severely limit taurine bioavailability (Earle & Smith, 1991). Therefore, the supplementation of taurine compensates for losses which may occur due to heat treatment (and fibre content from grain products) on processed diets. Taurine deficiency may lead to severe health problems such as retinal degeneration, poor reproductive performance, developmental abnormalities and dilated cardiomyopathy (Markwell & Earle, 1995).

According to Morris & Rogers (1992), "dry diets containing 1200 mg taurine per kg of dry matter and canned diets containing 2500 mg taurine per kg of dry matter", would meet much more than the requirements of the average domestic cat. However, as mentioned, the supplementation of high amounts of taurine is essential to compensate for losses during processing.

The rate-limiting step in taurine biosynthesis is the decarboxylation of cysteine sulfinic acid to hypotaurine by cysteine decarboxylase. However, in the cat, the activity of cysteine decarboxylase is relatively low in comparison to non-carnivorous species (Knopf et al., 1978). The hepatic cysteine decarboxylase activity is 200-300 μmol/min lower in the cat compared to the rat (de la Rosa & Stipanuk, 1985). The low activity of cysteine decarboxylase in the cat provides evidence that this step in the pathway is downregulated and, therefore, leads to a lower rate of taurine biosynthesis.

Another factor in the cat which limits the taurine biosynthetic pathway is the low activity of cysteine dioxygenase (Markwell & Earle, 1995). This enzyme

is responsible for catalyzing the initial reaction of cysteine (from the diet) to cysteine sulfinic acid.

Another reason for the dietary requirement of taurine in the cat is their inability to conserve taurine in response to depletion. A major role for taurine is in the formation of bile salts from bile acids through a process known as conjugation. In many mammals during periods of taurine depletion, glycine is instead used for this reaction. However, taurine is used exclusively for bile salt formation in the cat who has an inability to conserve taurine in bile salt production (Markwell & Earle, 1995). Once bile salts are formed, they are released into the small intestine and are reabsorbed and then returned to the liver. Per contra, bile salts may be deconjugated by enteric bacteria before they can be reabsorbed, and, therefore, disrupt this cycle (Markwell & Earle, 1995). Deconjugation releases taurine from bile acids and leads to it being either further metabolized by enteric bacteria, or excreted. It is this disruption and the consequential unavailability of taurine which leads it to being lost from the body's taurine pool (Morris & Rogers,1992). Due to the cat's inability to use glycine as a conjugate for bile salt formation, taurine requirement increases in response to factors that stimulate bile salt production. The cat is only capable of producing taurine to a very limited extent and, therefore, any losses which may occur can be detrimental and result in deficiencies.

Once again, when compared to an omnivorous species such as the dog, the cat's high necessity for AA can be emphasized. The assurance that the AA requirements are met is especially critical during the developmental period of an animal. This is because the animal is still maturing and during these phases the body critically requires efficient nutrients so that it can thrive. This can be further emphasized as taurine deficiency in the developing cat can result in delayed growth or it may be even stop growth all together (Hayes, 1982). The kitten is also more susceptible to other taurine deficiency related issues such as retinal degeneration, and dilated cardiomyopathy.

2.3.1.2.3 BRANCHED CHAIN AMINO ACIDS

Branched chain amino acids include valine, leucine and isoleucine. In addition to being essential amino acids, branched chain amino acids are involved in nutrient signalling, stimulation of protein synthesis (via the mTOR pathway), decreasing protein degradation, insulin secretion, and CNS (central nervous system) control of feed intake and energy balance (Herman et al., 2010; Holeček, 2018). First pass metabolism of branched chain amino acids in the liver is minimalcompared to other amino acids, with the main site for BCAA catabolism occurring in muscle tissue. In fact, BCAA accounts for over 50% of splanchnic output of amino acids following a high protein meal (Wahren et al., 1976). The lack of BCAA metabolism in the liver is due to low hepatic activity of the enzymes required for initial BCAA metabolism, mitochondrial branched chain aminotransferase (otherwise known as BCATm) (Holeček, 2018). Catabolism of BCAA in liver is catalysed by branched chain keto acid dehydrogenase (BCKD), forming branched chain alpha keto-acid (BCKA) and ammonia (Huston et al., 2005). Any ammonia produced is scavenged into the urea cycle for safe excretion via the kidney.

Branched chain amino catabolism in muscle tissue begins with reversible transamination which involves the transfer of the amino group to alpha-ketoglutarate which generates a corresponding BCKA and glutamate. There are two ways in which this amino group is transferred to the liver for urea synthesis. The first method is known as the glucose-alanine cycle whose main role is to transport the amino group generated by BCAA catabolism in the muscle to the liver. The second method is by the formation of glutamine from addition of another amino group to glutamate. Glutamine can be taken up by the liver, intestines, and kidney for further metabolism.

2.3.1.3 PROTEIN TURNOVER

Protein turnover in the cat (as a carnivore) differs from that of omnivores or herbivores. Protein turnover is the continuous replacement of protein in the body and is defined as the balance between protein synthesis and protein

degradation. Whole-body protein synthesis in a cat is seems to be lower than in omnivores or herbivores (Russell et al., 2003; Wester et al., 2015). Generally, what can be seen in animals is their ability to effectively regulate protein turnover in response to different feed intakes. For example, during fasting, whole-body protein degradation exceeds the rate of whole-body protein synthesis, however, protein degradation and synthesis both decrease, at least 24 to 36 hours since the last meal, to conserve protein (Arnal et al., 1987). Protein turnover in the cat is shown to adapt to levels of dietary protein above its requirement. Russell et al., (2003) reported that oxidation increases as protein intake above requirement increases. In contrary, when given low protein diets, cats are unable to adapt and face a negative nitrogen (N) balance (Green et al., 2008). Therefore, suggesting that the cat in fact does not adapt well to a change in diet below requirement.

Unlike the cat, omnivores and herbivores are able to successfully adapt to changes in protein intake (Verbugghe & Bakovic, 2013). For example, the concentration of urea cycle enzymes is greater in sheep fed a high protein diet when compared to sheep given a low protein diet thereby suggesting that the sheep (who is a herbivore) is able to adapt to changes in protein intake effectively (Payne & Morris, 1969). The same is true in chicks, who are omnivores. Muramatsu et al., (1987) reported that alterations in both protein synthesis and protein degradation occurred in response to changes in dietary protein intakes in the chick.

2.3.1.4 WHY THE CAT HAS A GREATER PROTEIN REQUIREMENT

The cat has a greater protein requirement than omnivores and herbivores. This is because the cat not only relies on AA for protein synthesis, but also as a source of energy. Amino acids can be used for energy in the liver and limited other tissues by being oxidized, or some can be converted to glucose via gluconeogenesis and be used throughout the body. An animal that consumes a diet containing little carbohydrate requires gluconeogenesis to meet glucose demands for energy. The high hepatic gluconeogenic enzyme activity found in cats also supports use of AA to meet energy (glucose) demands.

Unlike other species, the cat is unable to reduce AA oxidation below maintenance levels to conserve AA in response to a diet insufficient in protein. "The lack of adaptation to dietary protein intake in activities of aminotransferases for NEAA and urea cycle enzymes results in an elevated loss of nitrogen, regardless of dietary protein content" (Wester et al., 2015). In short, cats lack proper adaptations to regulate their protein dietary intake due to their inability to regulate AA enzyme activity. A diet high in protein supplies the cat with sufficient AA which cannot be manufactured at a rate sufficient to meet its requirement (NRC, 1986).

2.3.2 ESSENTIAL FATTY ACIDS

Lipids (fats) are an important component in the cat diet. Dietary fats provide a source of energy, while also supplying the cat with essential fatty acids which are not synthesized by the body itself (NRC, 2006). According to AAFCO (2014), despite the lack of a true requirement for crude fat, recommendations have been given as crude fat provides essential fatty acids, is a carrier of fat-soluble vitamins and also adds caloric density to foods. It has been shown that the cat requires some fatty acids in their diet, these are considered essential fatty acids, also known as long chain polyunsaturated fatty acids (Rivers et al., 1975)

The cat is unique in regards to the metabolism of fatty acids. As seen in other mammals, cats require a dietary supply of linoleic acids and alpha-linoleic acids. However, unlike humans, the cat also requires a dietary source of arachidonic acid due to their limited ability to synthesize arachidonic acid from linoleic acid potentially due to limited delta-6 desaturase (Bauer, 2006) and delta-5 desaturase (Rivers et al., 1975) activity.

The minimum adult maintenance requirements of crude fat is recognized at 9% dry matter (DM). Additionally, the minimum recommendation of linoleic acid is 0.6% DM and the minimum requirement of arachidonic acid is 0.02% DM.

Fat is an excellent source of energy in many species. However, in the cat, the inclusion of lipids in the diet is additionally a means of increasing the palatability and, therefore, approval of a meal (Zoran, 2002).

2.4 THE GENERAL COMPOSITION OF COMMERCIALLY AVAILABLE FEEDS FOR THE CAT

Due to domestication, the cat now resides in a household environment where it is not usually able to obtain sufficient hunted food, therefore, it relies upon its owner to provide it with its nutritional requirements. Pet owners themselves rely upon researchers and pet food manufacturers to produce diets which meet the correct nutritional recommendations of the animal. These physiological and dietary recommendations have been set by the National Research Council.

Pet food manufacturers produce different forms of cat diets which are available for sale to owners. These diets range from being wet-based canned diets, kibbled dry diets, and lastly raw diets. The base ingredients and their proportions differ among these diets. For example, wet-based diets contain up to 30% carbohydrates in total while dry-based diets may contain up to 60% of carbohydrates in total (de-Oliveira et al., 2008). Common carbohydrates which are included in the cat's commercial diet are cassava flour, brewers rice, maize, sorghum, peas and lentils.

Many of the commercial pet food companies include cereals or purified starch to construct a meal which is cat friendly and easily digestible. For example, starch gelatinization occurs during processing. As a result of this, the crystalline structure is disrupted, water is absorbed, swelling occurs and digestive enzymes such as amylase become more accessible to the starch molecule (Gibson & Alavi, 2013). As processing enables increased accessibility by pancreatic amylases, the cat is able to effectively break down the starch. This then increases the total tract apparent digestibility of the feed (de-Oliveria et al., 2008).

Additionally, heat treatment is commonly used in commercial diets to extend shelf life, improve palatability and to attain a preferred physical shape and form (Hendriks et al., 1999). Differences in the processing methods may not only affect carbohydrate digestibility, but also the protein digestibility, and, therefore, AA content of the feed.

Different commercial pet food companies process their pet food using different methods. The actual AA composition of dietary protein is a substantial index of the food's nutritional value. However, not all the protein found in the food is able to be digested and, therefore, those AA that make up the protein cannot be utilized. This is from heat during cooking and processing denaturing the protein, inducing the cross-linking of amino acids, or causing Maillard reaction products to form.

The digestibility of AA is very often lowered due to processing methods. The National Research Council (NRC, 2006) outlined four main ways how it occurs:

1. Excessive heat. This induces cross-linkage of AA and, therefore, yield proteins resistant to digestion.
2. Heat treatment in the presence of reducing sugars results in the Maillard reaction and amino acid availability is reduced.
3. Exposing proteins to alkali solutions may racemize L-amino acids and/or form poorly digestible compounds, such as lanthionine.
4. Oxidative damage may occur if the protein is stored with polyunsaturated lipids. The oxidative products of lipids react with AA, such as, methionine, tryptophan and histidine, reducing their bioavailability

The lower AA digestibility due to processing methods must be taken into account when formulating a diet. Allowances must be made for digestibility, so that AA requirements are met effectively. For example, the NRC (2006) assumes that the availability of nitrogen and AA in commercial diets is 80-90 % and this figure is used to base recommendations and requirements.

In general, the dietary composition of commercially available cat diets differs due to the type of feed, this being dry, wet or raw. Wet diets, for example,

usually contain 30% carbohydrate, while dry diets consist of as much as 60% carbohydrate (Carciofi et al., 2008).

2.4.1 CONTROVERSY OVER LEVEL OF PROTEIN IN DOMESTIC CATS DIETS

There are several studies surrounding the recommended protein requirements in the cat diet. However, requirement levels found in studies have not always agreed. For example, AAFCO (2019) states the minimum protein allowance in adult cat food to be 26%. However, Laflamme & Hannah (2012) report that the AAFCO recommendations are set too low, and suggested that the protein requirement is instead between 30 to 40% of the diet DM. They suggested that high variability exists due to the methodology used to estimate the cat's protein requirements. More specifically, a discrepancy exists when determining protein requirements through nitrogen balance compared to using the maintenance of lean body mass as an indicator (Laflamme & Hannah, 2012).

Most commercial pet food companies incorporate high levels of carbohydrate into their meals. Carbohydrate-rich ingredients such as grains, potatoes and legumes are incorporated into commercial pet foods. Most importantly, a certain level of starch is necessary for proper processing of these diets, especially dry kibbled diets (Verbrugghe & Hesta, 2017). As mentioned, the amount of carbohydrate varies between the different types of commercial diets (by 30-60%) (Carciofi et al., 2008). Therefore, although this is also true for canned wet diets, kibbled dry diets contain a greater amount of carbohydrate. It is essential that the processing requirements are met for the correct development of a commercially available feed. It is important that any commercial diet provides the necessary nutrients while also remaining palatable as well as "fresh" or durable on the store shelves. However, many of the commercial pet food companies which include carbohydrate, construct a meal which is cat friendly and easily digestible. For example, processing causes starch to gelatinise and be accessible to digestive enzymes, such as amylase (Gibson & Alavi, 2013).

2.4.2 DISCUSSION ON “BIOLOGICALLY APPROPRIATE” DIETS FOR CATS

Biologically appropriate diets are designed to reflect the quality and composition of foods which cats have evolved to eat (Freeman et al., 2013). Biologically appropriate diets are mostly raw meat-based diets which have grown in popularity over the recent years due to claims of nutritional superiority. However, the American Animal Hospital Association, American Veterinary Medical Association, and the Canadian Veterinary Medical Association have all released statements asserting that raw or undercooked animal protein sources should not be included in the cat’s diet (Freeman et al., 2013). Like any concept, there are both pros and cons. In the recent years, the natural pet food market in the United States has grown steadily. This increase can be seen from US$2.0 billion in 2008 to $3.9 billion in 2012 (Buff et al., 2014).

The benefits of feeding a raw meat-based diet were manifested from the concept of cats being obligate carnivores and feeding predominantly on animal tissue in the wild. This, therefore, leads pet owners to believe that these diets are biologically more suited to their pets. Furthermore, many health benefits for the cat fed a raw meat-based diet have been proposed. Among them are improvements in skin and coat health, increased energy, an improved immunity, and fewer allergies, arthritis, pancreatitis, dental issues and parasites (Freeman et al., 2013).

On the contrary, raw meat-based diets may contain additional bacterial and parasitic pathogens which may be harmful to the animal. This was investigated in 2018 by van Bree and colleagues. Thirty-five commercial diets were analysed from 8 different brands. Results indicated that *E. coli*, listeria and salmonella were among the pathogens isolated from the feed, among these were also parasites such as toxoplasma. This is of large concern due to the potential of gastrointestinal diseases in the companion animal. Additionally, some pathogens may be transmitted to the owner by handling pet food or the pets themselves and can pose a health risk to pet owners.

Furthermore, Schlesinger & Joffe (2011) report that there is no published data on level 1, 2 or 3 of nutritional risk or benefit on the feeding of raw-based diets to the cat. Level 1 studies are the best standard and basically include systematic reviews of multiple studies or randomized control clinical trials. Next, there are level 2 which include systematic reviews of cohort studies. Then, level 3 studies which are systematic reviews of case control studies. Lastly, level 4 and 5 studies in which level 4 includes poor quality case series; while level 5 studies include an expert outlook or assumptions based upon research or theory. There are only level 4 and 5 studies on the feeding of raw-based diets to cats. As mentioned, level 4 studies are poorly controlled, while level 5 studies are based on "expert" opinions. A level 4 study (survey) was performed by Laflamme et al., (2008) which consisted of pet owners characterising the health of their pets (e.g., coat condition). This study failed to take into account factors such as differing perceptions and different styles of raw-based meals (home-cooked vs. store bought).

Interestingly, Scott et al., (1961) also reported that kittens fed on a raw-based diet had a "thick-lustrous coat and shining eyes". It was further reported that although these kittens appeared healthy in appearance, they showed signs of osteoporosis and thyroid hyperplasia. However, a Siamese cat who had been fed on a raw-based diet with minimal supplementation was examined post-mortem. This cat had a history of lameness, but otherwise appeared healthy (e.g., excellent coat condition). The examination showed abnormalities in bones and the thyroid gland similar to that observed in the kittens fed the raw-based diet. This study concluded that it is essential that the cat diet include the correct supplementation of nutrients because, although it may appear that the cat is in excellent health, underlying problems such as calcium deficiency may not be so apparent and may lead to severe osteoporosis.

The rise in the interest of "biologically appropriate diets" not only signifies the increased feeding of raw-meat based, high protein diets, but it also implies the increased interest into grain-free cat diets. In fact, the purchase of grain-free cat food in the United States has more than tripled from 4% to 15% from 2012 to 2016 alone (American Pet Product Association, 2018).

Grain can be readily incorporated into dry cat foods. Common grains found in cat food include: wheat, maize, oats, barley and rice. Motivation for "grain-free" diets is the perception that these diets are lower in carbohydrate and allergens; however, it has been reported that this cannot be guaranteed (Prantil et al., 2017). Furthermore, food allergens are not common in the cat (Guilford et al., 2001). When food allergies do occur, they are most commonly related to animal-based products such as beef, chicken and fish (Mueller et al., 2016), as opposed to plant-based ingredients.

2.5 MEASUREMENT OF PROTEIN SYNTHESIS, DEGRADATION AND OXIDATION

Protein synthesis is a process by which AA are joined together in a specific order as required by the mRNA sequence. Protein degradation is the breakdown of proteins into AA. In the cat, these AA may be used for energy (oxidized directly or by first being converted to glucose via gluconeogenesis), as a substrate for protein synthesis, or in any other metabolic process that may require them.

2.5.1 [1-^{13}C]LEUCINE METHOD TO MEASURE WHOLE-BODY LEUCINE KINETICS

The whole body [1-^{13}C]Leu precursor method with [$^{15}N_2$]urea infusion can be used to determine the rates of protein synthesis, degradation, and oxidation (Wester et al., 2015). It is essential that the correct method be chosen in order to fully investigate AA metabolism and, thus, protein metabolism in the cat. This method has been utilized widely in animal and human studies, however, it has rarely been used to determination protein turnover in cats or other obligate carnivores. This method is ethical and relatively noninvasive in comparison to other methods. Other benefits include its relative simplicity and convenience. For example, a single experiment can be used to generate a full range of results at once (i.e., protein synthesis, degradation and AA oxidation itself; Wester et al., 2015).

2.5.2 [$^{15}N_2$]UREA TO MEASURE UREA PRODUCTION AS AN INDEX OF AMINO ACID CATABOLISM

Urea kinetics, specifically urea synthesis measured using [$^{15}N_2$]urea used as a tracer, determines the total rate of AA catabolism. Use of ^{15}N as the tracer allows the method to be used at the same time as the [1-^{13}C]Leu precursor method, thus, values for overall amino catabolism can be measured at the same time as Leu kinetics. This method is based on deamination when AA are catabolized. It is only an indirect estimate of protein degradation and is very dependent on quality and amount of dietary protein. This method to measure urea kinetics is efficient and straight forward in that no urine or faeces sampling is needed; an alternative method which is open to error.

2.5.3 THE D_2O METHOD TO MEASURE HEPATIC PROTEIN SYNTHESIS

The theory behind the D_2O method is similar to other precursor:product labelling methods (Gasier et al., 2010). In summary, once a flooding dose of D_2O is administered, the deuterium from water rapidly equilibrates across body water pools and is transferred to the nonessential AA alanine when it is synthesised. This labelled alanine is then used to label albumin as it is synthesised in the liver, which can then be recovered from plasma samples at the end of the experiment.

The D_2O method offers several advantages over other methods which are used for determining protein synthesis. The advantages of the D_2O method were described by Dufner et al. (2005). Briefly, there are three main advantages. Firstly, if the labelling of a free AA equilibrates rapidly with body water prior to incorporation into a newly synthesized protein, the precursor labelling (i.e., body water) can be determined with a high degree of certainty (Dufner et al., 2005). Secondly, the use of the D_2O method allows for protein assessment to occur with minimal intervention (i.e., no surgical insertion of catheters, no constant infusions or breath samples, and minimal blood sampling). Lastly, when compared to other isotopes, D_2O is relatively inexpensive. This, alongside its long half-life in body water, means that it is a desirable isotope for studying the synthesis of proteins with a slow turnover rate (Dufner et al., 2005).

CHAPTER 3: MATERIALS AND METHODS

Cats were fed at maintenance for three weeks on diets containing 7.5, 15, or 40% of ME as CP, after which AA and urea kinetics were measured using infusions of [1-^{13}C]Leucine (Leu) and [$^{15}N_2$]Urea, respectively.

3.1 DESIGNED FEED COMPOSITIONS FOR THE TRIAL

Food was offered at 250 kJ/kg BW for cats fed 40% ME as CP. Previous research (personal communication, T. Wester) indicated that cats fed at and below their protein requirement require higher energy intakes. The cats fed 7.5, 15% ME as CP were offered 350 and 300 kJ/kg BW, respectively. Amount of feed offered to each cat was adjusted as necessary to maintain BW.

3.1.1 INGREDIENTS USED

The ingredients chosen for the trial were based on previously formulated diets by Wester et al., (2015). These ingredients were chosen on their basis of their nutritional value.

Chicken mince was the source of protein for the diets used in this trial, because it has a high protein content while also being a leaner and a highly digestible type of meat. Chicken mince can also easily be mixed with other ingredients and formulated into a palatable diet. Chicken is also rich in vitamins and minerals. The chicken mince used in this trial was sourced in its raw form from the local Pak'n'Save supermarket in Palmerston North. Further processing of the chicken mince occurred at the Massey University Pilot Plant and is described in *section 3.1.2.*

Cellulose (Avicel, Hawkins Watts, Auckland, NZ) was the source of dietary fibre for each diet used in this trial. Cellulose is an ingredient which is frequently added to commercial pet foods to increase food volume and its positive effects

on faecal quality (Prola et al., 2010). The cellulose used in this trial was in a powered form.

Inulin (Orafti Synergy 1, Benuo, Belgium) was another source of dietary fibre in the diets in this trial. However, inulin is known to be a "prebiotic" which is commonly used in commercial pet foods to "improve gut microbial ecology and faecal quality" (Flickinger & Fahey, 2002). The inulin used in this trial was also in a powdered form.

Starch is a complex carbohydrate which was added to all diets in the trial. Starch is one of the most commonly used carbohydrates in the pet food industry and is known to be essential for the proper formation of kibble as it provides the kibble with its needed structural integrity. The starch used in this trial was in a raw powdered form.

Dripping was the primary source of fat used in each diet in this trial. Dripping was chosen as a fat source as it is sourced from animal fat; contains the same amount of saturated fat as butter and does not have any unwanted additives. Before use, the dripping was placed in the microwave in a glass Pyrex jug for 30 seconds, or until it was soft enough to fully mix into each diet. The dripping used in this trial was sourced from both Pak'n'Save supermarket and New World supermarkets in Palmerston North.

Premix (Dog and Cat Premix, Image Holdings Ltd., Auckland, NZ) was a source of vitamins and minerals for the diets in this trial. The addition of premix provided the cat with a more balanced diet and aimed to ensure that their vitamin and mineral intakes reached requirements. Premix is frequently added to commercial diets in accordance with AAFCO regulations; that is until the minimum requirement of vitamins and minerals is reached. The premix used in this trial was in a powdered form.

Powdered chicken stock concentrate was used to increase the acceptance of the diets. Minimal chicken stock was added to the diet (less than 1%), and it

was sourced from the local New World supermarket in Palmerston North. The brand used in this trial was Maggi.

3.1.2 PALATABILITY TESTING

Prior to commencing the feeding trial period, the palatability of the assigned diets were tested on the assigned cats accordingly. Cats which did not eat a diet were either switched to a different treatment or excluded from the trial. If a majority of the cats did not accept diet, then the diet composition was changed.

An error initially occurred when calculating the correct amount of premix needed for the diets. Initially it was suggested that the cat's diet would need approximately 3-5% of premix. However, this error was made notable during the initial feed testing period when all cats found each treatment unpalatable. It was later found that the amount of premix needed was only approximately 0.5%. The amount of premix to be added was then adjusted as needed. The diets then increased in palatability with only a few minor problems which were then corrected through the addition of chicken stock or jelly.

Chicken stock was sprinkled over each cat diet to further increase its palatability. Jelly was sourced from Chefs canned diet from the Massey Feline Unit. The addition of jelly increased the diets palatability. This was especially true for the 7.5% diet which was otherwise crumbly and dry. The addition of jelly was made in accordance to each individual's taste and not to every individual diet, or any particular treatment. Finding the correct balance of chicken stock and jelly to enhance palatability proved to be tricky overall.

It is important to note that laboratory analysis of the jelly showed that the dry matter percentage was 6.8 and therefore the added jelly was mostly water; making no significant difference to the final diet compositions.

Furthermore, any cat who lost more than 15% of their body weight during the trial, was immediately removed. Any individuals removed were successfully replaced.

Once each treatment was palatable to the cats, the feeding treatment was accepted for use in the experiment.

3.1.3 FINALISED FEED COMPOSITIONS

The finalised feed compositions per 100 g are shown in *Table 3.1.* The 7.5% diet produced was a crumbled dry-kibbled diet texture. This diet contained 7.5% of ME as CP. The 15.0% diet produced was moderately wet, though sticky. This diet contained 15% of ME as CP. The 40.0% diet produced was very wet and also resembled a wet-canned diet. This diet contained 40% of ME as CP.

Table 3.1: Finalised feed compositions per 100g, as-fed.

Ingredient (per 100g)	7.5% Protein	15% Protein	40% Protein
Chicken Mince	35.0	61.5	92.7
Cellulose	1.5	1.5	1.5
Inulin	1.5	1.5	1.5
Starch	31.8	9.2	0.9
Dripping	29.7	25.8	3.0
Premix	0.5	0.5	0.4

3.1.4 FEED MIXING AND PROCESSING

The first step in feed mixing and processing begun in the Pilot Plant at Massey University. Here, the raw chicken mince was subdivided into 500g bags that were vacuum sealed before cooking in order to maintain freshness throughout the trial. Once the chicken was subdivided, they were placed in a hot water bath at 70°C for 15-20 minutes. After this period, the chicken was observed. Once the internal temperature of the chicken was approximately 75°C then the

chicken was determined to be cooked. They were then stored at -20°C until required.

3.1.5 DESCRIPTION OF CATS AND CARE

Eighteen neutered male domestic short-haired cats were selected from the closed colony located at the Feline Nutrition Centre at Massey University in Palmerston North. Cats were selected to be even-tempered when handled, within a healthy weight, and middle-aged. Selected cats were aged 3.48 ± 0.30 years old (mean ± SE) and weighed 3.9 ± 0.12 kg (mean ± SE). Each cat participant was randomly allocated to one of the three treatments available in this experiment.

Generally, the cats are housed in colony cages at the Feline Nutrition Centre at Massey University. Each large cage housed approximately 9-10 cats. However, during the feeding trials, the cats were housed in individual metabolic cages (measuring ~0.7 m^3). The cats were kept in isolated cages so that feed intake could be monitored during the trial. The cats were kept separate from the regular colony but were kept in visual and aural contact of the other cats.

The cats were taken out of their individual metabolic cages for one to two hours per day. During this time, the cats were taken to a common area in which they were able to socially interact with other cats and humans during the trial period. This area also provided the cats with exercise and recreational facilities. In addition, the cats were provided with toys in their individual metabolic cages.

The individual metabolic cages were cleaned daily or as required over the entire feeding trial period.

This entire trial was approved by the Massey University Animal Ethics Committee (MUAEC 16/124) prior to the beginning of the experiment. As mentioned, the cats body weights were monitored regularly. Cats were weighed Monday, Wednesday and Friday each week using an electronic scale. If an

individual showed a decrease in weight of more than 15% of their BW then they would be removed from the trial straight away.

3.1.6 FEEDING ROUTINE

The cats were provided with *ad libitum* access to water. Diets were prepared each morning prior to feeding. All of the cats were offered their experimental diets for 21 days. The cats were fed their treatments in the morning at approximately 0800h daily. Refusals were measured.

The diets were mixed and processed manually by students at Massey University. The diets were made fresh using the ingredients described in *section 3.1.2* every morning at approximately 0830 in the weekday and 0930 in the weekend. The ingredients were weighed out and then thoroughly mixed by hand.

The cats were offered their individually calculated amount of food daily in order to maintain their body weight and daily energy requirements.

3.2 CATHETERIZATION

After three weeks on treatment diets, food was removed at approximately 2100h to allow the cat to be food deprived overnight in preparation for the catheterization procedure. The catheterization was performed by a trained veterinarian from Massey University in Palmerston North. The veterinarian was assisted by either a qualified veterinary nurse or a research technician at the Massey University Feline Unit.

The cats had two intravenous catheters inserted under anesthesia the day before infusions. In order to induce short-term anesthesia, the cats were given a single intravenous dose of ketamine. Once the cats were catheterized, they were allowed to fully recover from effects of anesthesia and were offered their day's ration. The catheter used for the blood sampling was placed into the vena cava via a medial saphenous vein. The catheter used for the infusion was placed in

the cephalic vein. The catheters were kept in place for approximately 24 to 36 hours. This was ensured by the use of Elizabethan collars (product no. 273320; Jørgen Kruuse A/S) which restricted the cats movement so that they were not able to pull out the catheters after insertion. All infusions and blood sampling occurred the day following catheterization.

3.3 FEEDING ON THE DAY OF THE INFUSION

On the day of the infusion, cats received 1/3rd of their daily food ration divided in hourly aliquots throughout the infusion period to ensure cats are maintained at a fed state. After the infusion period, the cats were offered ad libitum access to the regular cat colony diet.

3.4 THE [1-^{13}C]LEUCINE TRACER METHOD

The [1-^{13}C]leucine tracer method is considered the “method of reference” when determining whole-body protein synthesis, protein breakdown and protein oxidation (Wagenmakers, 1999). This procedure followed was the same as the method performed by Wester et al., (2015). This occurred after the baseline blood and breath samples were taken. Briefly, primed continuous infusions of [^{13}C]Na bicarbonate [7.5 µmol/kg BW and 10 µmol/(kg BW · h); ^{13}C 99%; Cambridge Isotope Laboratories] were administer via the cephalic vein catheter. The [^{13}C]Na bicarbonate was infused from 0 to 120 minutes (min) to determine basal carbon dioxide production rate and prime the ^{13}C bicarbonate pools. This brought carbon dioxide from [1-^{13}C] leucine oxidation into an isotopic equilibrium. At 120 min, the [^{13}C]Na bicarbonate infusion ended and a primed continuous infusion of [1-^{13}C]Leu [10 µmol/kg BW and 10 µmol/(kg BW · h); 1-^{13}C 99%; Cambridge Isotope Laboratories] was administered until 480 min to measure whole-body Leu flux, rate of Leu disposal, and appearance of $^{13}CO_2$. *See Figure 3.5*.

The isotopic solutions were prepared in 0.9% saline and infused at the approximate rate of 6 mL per hour.

3.5 COLLECTION OF BLOOD AND BREATH SAMPLES

The animals were handled regularly and were accustomed as much as possible to the anesthetic breathing masks. This was done in advance to minimize any distress or bad sampling which could have occurred during the infusion and sampling period. During both blood and breath sampling, the cats were held by a technician.

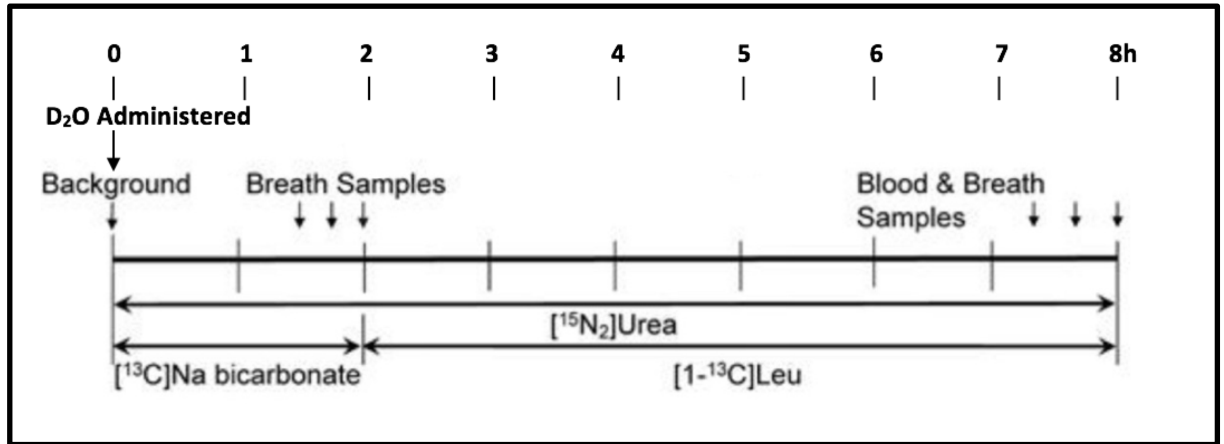
The collection of blood and breath samples occurred using the same method performed by Wester et al., (2015). Briefly, the collection of blood and breath samples occurred the day of the infusions. Baseline blood and breath samples were initially taken the morning of the experiment. *See Figure 3.5*. Each blood sample taken was approximately 2.5 mL. A total of approximately 10 mL of blood was sampled from each cat on the day of the infusion.

Breath samples were collected using an anesthetic mask which was connected to a 500 mL rebreathing bag fitted with a one-way valve so that only expired air was able to enter the bag. Breath samples were taken to obtain expired CO_2 samples. Once breath samples were collected, they were transferred from the re-breathing bag into evacuated 12 mL glass tubes and stored at room temperature until needed for analysis.

Blood samples were taken from the catheter inserted via the medial saphenous vein so that blood was collected from the vena cava. Once collected, the blood samples were placed into individual glass tubes containing sodium heparin (to prevent blood clot formation). They were then placed on ice until centrifuged at 3000 x *g* at 4°C. Plasma was harvested and stored at -20°C until required for analysis.

In addition to baseline samples, more blood and breath samples were collected throughout the infusion period. breath samples were taken at 100, 110, 120, 440, 460, and 480 minutes to measure the enrichment of expired carbon dioxide, with blood sampled at 440, 460, and 480 minutes to measure ^{13}C enrichments of Leu and α-ketoisocaproic acid (KIC) and $^{15}N_2$ enrichments of urea.

Figure 3.1: Protocol for infusion and sampling.



Modified from Wester et al., (2015)

The cats were monitored throughout the infusion periods to ensure that they did not harm themselves or the experimental equipment. Once final blood and breath samples were collected, the infusion lines, Elizabethan collars, and catheters were all removed. The cats were re-monitored for a short period to ensure that there was no bleeding from the catheter insertion sites. They remained in their individual metabolic cages over-night and then transferred back into their original colony pens.

3.6 THE INFUSION OF [$^{15}N_2$] UREA

The Infusion of [$^{15}N_2$] urea occurred following the same method used by Wester et al., (2015). Briefly, [$^{15}N_2$]urea (250 μmol/kg BW and 25 μmol/(kg BW · h); $^{15}N_2$ 98+%; Cambridge Isotope Laboratories] was administered via the cephalic vein catheter. The infusion of [$^{15}N_2$]urea was administered from 0 to 480 minutes to measure urea production (general AA catabolism). *See Figure 3.5*.

3.7 THE SUBCUTANEOUS ADMINISTRATION OF DEUTERIUM OXIDE

The primary purpose of the D_2O administration was to test whether a method could be used to measure rate of hepatic protein synthesis and then to compare that value with the one for whole-body protein synthesis obtained using [1-^{13}C]Leu.

In this method, isotonic D_2O was administered at a rate of 10 mL/kg BW by subcutaneous injection before the start of the initial intravenous infusions (subcutaneous injection of ≥ 60 mL of isotonic fluid is performed routinely for fluid therapy). *See Figure 3.5.*

The theory behind this method is that deuterium from water is transferred to newly synthesised alanine. This labelled alanine is then used to synthesise albumin in the liver (the major hepatic export protein) and it can then be recovered in plasma sampled at the end of the experiment.

3.8 CHEMICAL ANALYSES

Sample preparation and analysis for stable isotopes in the cat plasma were performed at AgResearch, Palmerston North under the supervision and guidance of the metabolomics team. The methods used for chemical analyses was based upon the work of Previs et al., (2004).

3.8.1 PROTOCOL FOR PREPARATION AND ANALYSIS OF PLASMA SAMPLES FOR [^{13}C]-LABELLED LEU AND KIC, AND [^{15}N]-LABELLED UREA DETERMINATION VIA GC-MS

Intracellular leucine is reversibly converted into α-ketoisocaproic acid (KIC) and is the only source of KIC. Therefore, KIC enrichment accurately reflects intracellular leucine enrichment compared to the measurement of leucine itself. Measurement of $^{15}N_2$ urea assisted with the calculation of protein breakdown and synthesis.

[^{13}C]-labelled Leu and KIC, and [^{15}N]-labelled urea, were measured in cat plasma. The keto group of KIC was converted to the methyloxime derivative to increase stability, followed by derivatisation of the acid with *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide. Leucine was also derivatised to produce the tert-butyl(dimethylsilyl) (TBDMS) protected product. This method provided simultaneous determination of the derivatised, isotopically labelled

leucine and KIC. Urea was also derivatised using this method, however due to its much higher abundance it was measured in a separate GC-MS analysis run.

Plasma samples were stored at –80°C and thawed at room temperature. 200 μL of plasma was transferred into a 2 mL Eppendorf tube, to which was added 800 μL acetonitrile. The sample was vortexed (5 s) and centrifuged (12,000 × *g*, 4°C, 20 min). The supernatant was transferred into a 1 mL V-shaped Pierce Reacti-Vial^{TM}. The sample was dried under N_2 at 70°C for 30 min. 50 μL methoxyamine hydrochloride (2.5% w/v in dry pyridine, made fresh prior to use) was added, and the vial was capped and heated at 70°C for 30 min. The sample was cooled to room temperature and then treated with 50 μL *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide, followed by 50 μL acetonitrile. The vial was capped and heated at 70°C for 30 min. The sample was then cooled to room temperature and transferred into a 200 μL limited-volume insert in a GC vial for immediate GC-MS analysis. For each sample, two GC-MS runs were undertaken.

3.8.1.1 GC-MS ANALYSIS FOR KIC AND LEU

KIC and leucine were detected with selected ion monitoring (SIM) of the $[M–57]^+$ and $[M+1-57]^+$ ions (loss of *t*-butyl), corresponding to the unlabelled and $[^{13}C]$-labelled derivatised compounds, respectively. For KIC, *m/z* = 216.10 and 217.10; for leucine, *m/z* = 302.15 and 303.15 (*Table 3.2*).

3.8.1.2 GC-MS ANALYSIS FOR UREA

The second run employed a high inlet split flow and targets urea. Urea was detected with SIM of the $[M-57]^+$, $[M+1-57]^+$, $[M+2-57]^+$ and $[M+3-57]^+$ ions, corresponding to the unlabelled, $[^{15}N]$-labelled and double-$[^{15}N]$-labelled derivatised urea compounds, respectively. For these compounds, *m/z* = 231.15, 232.15, 233.15 and 234.15, respectively *(Table 3.3)*.

Table 3.2: GC-MS parameters for detection of KIC and Leu.

GC conditions		MS conditions	
Column oven	60-280°C over 18.33 min	Ion source temperature	200°C
Carrier gas pressure	87.6 kPa	Interface temperature	250°C
Carrier gas total flow	28.4 mL/min	Solvent cut time	7.5 min
Column flow	1.21 mL/min	Acquisition mode	Q3 SIM
Purge flow	3.0 mL/min	Q3 SIM settings for KIC	8-10.5 min; event time = 0.05 s; *m/z* = 216.10, 217.10; Q3 res. = high
Linear velocity	37.2 cm/sec	Q3 SIM settings for Leu	11–14 min; event time = 0.05 s; *m/z* = 302.15, 303.15; Q3 res. = high
Split ratio	20		

Table 3.3: GC-MS parameters for detection of Urea.

GC conditions		MS conditions	
Column oven	60-280°C over 18.33 min	Ion source temperature	200°C
Carrier gas pressure	87.6 kPa	Interface temperature	250°C
Carrier gas total flow	101.0 mL/min	Solvent cut time	8 min
Column flow	1.21 mL/min	Acquisition mode	Q3 SIM
Purge flow	3.0 mL/min	Q3 SIM settings for urea	10-12 min; event time = 0.05 s; *m/z* = 231.15, 232.15, 233.15, 234.15; Q3 res. = high
Linear velocity	37.2 cm/sec		
Split ratio	80		

3.8.1.3 GC-MS DATA ANALYSIS

Peak areas for the key KIC, leucine and urea ions mentioned above were extracted and exported to Excel using the Shimadzu post run analysis software. Duplicate measurements of the T0 time point were used to calculate the standard deviation of the isotope measurement. The isotope ratios were calculated by dividing the M+1 peak area by the M peak area.

Mole percent enrichments were then calculated by the following formula:

MPE= Te – Mean T0 x 100 Te – Mean T0 +1

Where MPE is mole percent enrichment; Te is the isotope ratio time point and T0 represents the isotope ratio time point 0.

3.8.2 PROTOCOL FOR PREPARATION AND ANALYSIS OF PLASMA SAMPLES FOR FREE AND BOUND ALANINE DETERMINATION VIA GC-MS

Subjects given deuterium oxide incorporate deuterium-labelled species in their metabolism. Relevant to this work, a rapid exchange occurs between hydrogen in body water and alanine. The subject's albumin synthesis rate was a proxy for their hepatic protein synthesis rate, and the former can be inferred from the ratio of isotopic enrichment of alanine in plasma that is either free or albumin-bound.

3.8.2.1 FREE ALANINE FROM PLASMA

The materials used were as follows: 2 mL Eppendorf tubes; 8 mL Kimax glass tubes; 1 mL V-shaped vials; heated nitrogen stream sample concentrator; vortex; centrifuge set at 4 °C; GC vials and 200 μL limited-volume inserts.

The chemicals used were as follows: Methanol; 0.1 M HCl; 4 M Ammonium hydroxide (NH_4OH); MilliQ water; cation-exchange resin; dimethylacetal-dimethylformamide (DMF-DMA); acetonitrile.

3.8.2.1.1 PLASMA EXTRACTION AND DERIVATIZATION

First, 1.8 mL of methanol was added to 200 μL plasma in a 2 mL Eppendorf tube. This was then vortexed for 5 s and centrifuged (20 min, 14,000 × rpm). The supernatant was transferred to an 8 mL Kimax glass tube and reduced to dryness under a constant stream of nitrogen at 60°C. Next, 0.5 mL 0. 1M HCl was added to the residue, which was then added to the preconditioned cation-exchange resin.

The cation-exchange resin comprised a bed of 1 cm Dowex AG 50W-X8 H-form resin. The resin was preconditioned by washing with 0.1 M HCl, and stored in 0.1 M HCl ready for addition to a 2 mL polyethylene tube fitted with a Teflon frit. Because air may prevent the bed from draining under gravity, a slight pressure of air was applied from above (e.g., with a Pasteur pipette bulb) to force liquid to permeate through the frit, and thereafter no problem occurred. All cation-exchange steps were performed under gravity elution. Prior to sample addition, the residual acid was drained to be level with the resin bed surface. The sample was added slowly, avoiding resuspension of the cation-exchange bed. The sample was drained to the surface of the bed prior to addition of water, which was carefully performed to avoid disturbance of bed.

The resin was washed with 1 mL MilliQ water. The resin was eluted with 2 mL 4 M NH_4OH, and the eluant was collected into an 8 mL Kimax tube. The sample was reduced to dryness under nitrogen at 95°C. The residue was redissolved in 0.1 mL 0.1 M HCl, and transferred into a 1 mL V-shaped vial. The sample was reduced to dryness under nitrogen at 95°C. Once dry and cool, the sample was treated with 50 µL DMF–DMA and 50 µL acetonitrile, then heated at 100°C for 30 min. The mixture was then cooled, and transferred into a 200 µL limited-volume insert in a GC vial. The sample was analysed via GC-MS, monitoring selectively for ions *m/z* = 143, 144, 158 and 159.

3.8.2.2. BOUND ALANINE FROM PLASMA ALBUMIN

The materials used were: 2 mL Eppendorf tubes; 8 mL Kimax glass tubes; 1 mL V-shaped vials; heated nitrogen stream sample concentrator; vortex; centrifuge set at 4 °C; ice; GC vials and 200 µL limited-volume inserts.

The chemicals used were: Ethanol; 6 M HCl; 0.1 M HCl; 4 M NH_4OH; 20% (w/v) Trichloroacetic acid (TCA); 5% (w/v) TCA; MilliQ water; cation-exchange resin; dimethylacetal–dimethylformamide (DMF-DMA); acetonitrile.

3.8.2.2.1 PLASMA EXTRACTION, HYDROLYSIS AND DERIVATIZATION

First, 1 mL 20% (w/v) TCA (Tricholoroacetic acid), prechilled on ice, was added to 200 µL plasma in a 2 mL Eppendorf tube, vortexed for 5 s and centrifuged (10 min, 14,000 × rpm). The supernatant was discarded, and the pellet resuspended in 1 mL 5% (w/v) TCA (prechilled on ice). The mixture was centrifuged (10 min, 14,000 × rpm). The supernatant was discarded, and the pellet resuspended in 1 mL ethanol. The mixture was centrifuged (10 min, 14,000 × rpm). The supernatant was transferred into an 8 mL Kimax glass tube, while the pellet was resuspended in 1 mL ethanol and centrifuged again (10 min, 14,000 × rpm). The supernatant was combined with the previous ethanol extract in the 8 mL Kimax glass tube, and reduced to dryness under a constant stream of nitrogen at 70°C. The residue was redissolved in 0.1 mL 0.1 M HCl, and transferred into a 1 mL V-shaped vial. 0.5 mL 6 M HCl was added, and the vial was capped and heated for 24 h at 100°C. The mixture was reduced to dryness under a constant stream of nitrogen, and the residue was redissolved in 0.5 mL 0.1 M HCl, then added to the preconditioned cation-exchange resin. The resin was washed with 1 mL MilliQ water. The resin was eluted with 2 mL 4 M NH_4OH, and the eluant collected into an 8 mL Kimax tube. The sample was reduced to dryness under nitrogen at 95°C. The residue was redissolved in 0.1 mL 0.1 M HCl, and transferred into a 1 mL V-shaped vial. The sample was reduced to dryness under nitrogen at 95°C. Once dry and cool, the sample was treated with 50 µL DMF–DMA and 50 µL acetonitrile, then heated at 100°C for 30 min. The mixture was then cooled, and transferred into a 200 L limited-volume insert in a GC vial. The sample was analysed via GC-MS, monitoring selectively for ions *m/z* = 143, 144, 158 and 159.

3.8.2.3 GC-MS DATA ANALYSIS

The *m/z* 143 and 144 corresponded to the unlabelled and single-deuterium-labelled alpha-carbon fragments of alanine (loss of the alpha-carbon methyl group), respectively, while *m/z* 158 and 159 corresponded to the unlabelled and labelled alanine ions, respectively (which may be labelled at the

alpha or beta position). An unknown compound with an ion of *m/z* 144 eluted immediately prior to alanine, so the peak at *m/z* 144 was difficult to integrate with accuracy. Ions *m/z* 144 and 159, corresponded to the deuterated species, eluted slightly ahead (~600 ms) of the unenriched ions *m/z* 143 and 158. Alanine's retention time was approximately 12.85 min.

3.8.2.4 METHOD EVALUATION

Two samples of plasma were prepared and analysed, as well as 5 µg of an alanine reference standard (5 µL of 1 mg/mL alanine in 0.1 M HCl). Reference standards were used to aid in the identification of unknown compounds, and to help characterize column properties.

For derivatisation, each sample was reduced to dryness under nitrogen at 95°C. Once dry and cool, the sample was treated with 50 µL DMF-DMA and 50 µL acetonitrile, then heated at 100°C for 30 min. The mixture was then cooled, and transferred into a 200 µL limited-volume insert in a GC vial. The sample was analysed via GC-MS, monitoring selectively for ions *m/z* = 143, 144, 158 and 159 *(Table 3.4).*

As shown in *Figure 3.2*, an unknown compound with an ion of *m/z* 144 eluted immediately prior to alanine, the integration of *m/z* 144 is inaccurate, and thus the calculated MPE (mole percent excess) for alanine's alpha-carbon proton was likely to be misleading. The contaminant was only observed in plasma samples, and may have been glycine or a related compound.

Alanine's total MPE appeared to be readily measurable for both free and bound alanine. As expected, the level of enrichment observed for albumin-bound alanine was significantly lower than free alanine (*Table 3.5*). It is important to note that due to poor separation of contaminant species prior to alanine, only alanine's total MPE was reported. Additionally, there were low levels of bound-alanine enrichment and therefore, replicate GC-MS sample analysis were performed (samples were run twice).

Table 3.4: GC–MS parameters for detection of Alanine (Ala).

GC conditions		MS conditions	
Column	SH-Rtx-5ms (30 m, 0.25 mm id, 0.25 µm df)	Detector voltage	0.2 kV relative to tuning result
Column oven	Initial temp 60°C held for 2 min; ramp 5°C/min to 150°C; ramp 40°C/min to 280°C, held 5 min	Ion source temperature	200°C
Injection temperature	250°C	Interface temperature	250°C
Carrier gas pressure	60.6 kPa	Solvent cut time	5 min
Carrier gas total flow	24.8 mL/min	Acquisition mode	Q3 SIM
Column flow	1.04 mL/min	Q3 SIM settings for alanine	10-14 min; event time = 0.05 s; m/z = 143.1, 144.1, 158.1, 159.1; Q3 res. = high
Purge flow	3 mL/min		
Linear velocity	37.2 cm/sec		
Split ratio	20[1]		

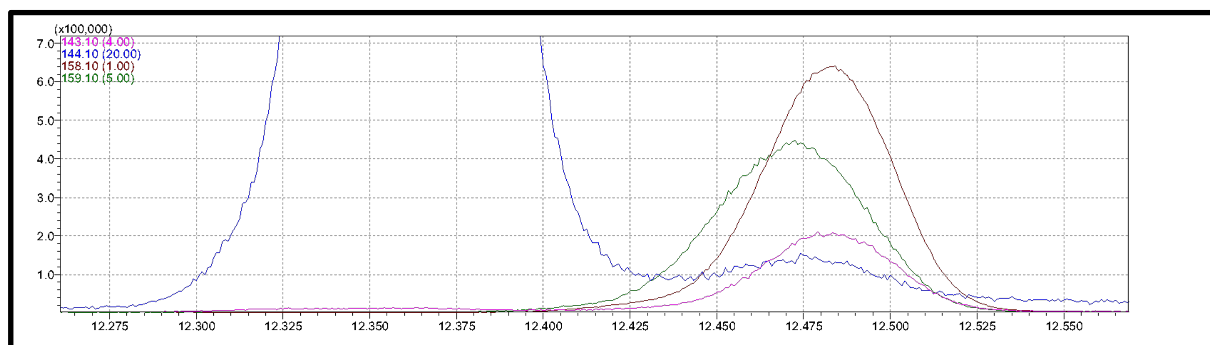
[1]Potentially room to adjust split from 1:20 to 1:40-80 to reduce signal.

Table 3.5: Calculated enrichments relative to unlabelled standard.

Sample	Alanine alpha-carbon MPE (mole percent excess)	Alanine total MPE[1]
Plasma, free alanine #1	5.32	6.01
Plasma, free alanine #2	4.58	7.35
Plasma, albumin-bound alanine #3	0.449	0.316
Plasma, albumin-bound alanine #4	1.21	0.296

[1] Total MPE calculated from *m/z* 159/158 ratios relative to unenriched standard. Includes enrichment associated with both alpha and beta carbons.

Figure 3.2: GC-MS Chromatogram of plasma free alanine and albumin-bound alanine.



3.9 LABORATORY ANALYSIS OF FEED SAMPLES

During the feeding period, feed samples of each of the diets were collected in corresponding, individual zip-lock bags. These were then stored in a -3°C freezer until they were sent for analysis to the Massey University, Nutrition Laboratory. These feed samples were analyzed for their dry matter, protein and for AA content on an as-fed basis.

3.9.1 DRY MATTER

The dry matter was determined using the AOAC (2005), Official Method of Analysis 930.15/925.10.

Briefly, approximately 2 grams of the feed samples were weighed into a dish. The sample was then dried in an oven at 135 ± 2°C for two hours. The dish was then covered and then removed from the oven. It was then transported to a desiccator, and cooled. The final mass was then taken. This method is commonly used in food industries.

For the method 925.10. Briefly, approximately 2 grams of the feed samples were weighed into a dish. The sample was then dried in a ventilated oven at 130 ± 3°C for one hour. The dish was then covered and removed from the oven. Again, the sample was then transported to a desiccator and cooled to room temperature. The final mass was now taken. This method is widely used in animal feed industries.

3.9.2 CRUDE PROTEIN

Firstly, nitrogen was determined using the AOAC (2005), Official Method of Analysis 968.06 Dumas method. Briefly, a known amount of feed sample was combusted in an 800 to 900ºC chamber with a stream of oxygen and carbon dioxide, water and nitrogen gases were released. The gases were then passed through potassium hydroxide aqueous solution, so that carbon dioxide and water could be removed. Moisture free gases were then passed through a heated copper catalyst under helium removing the oxygen. This changed NO_x to N_2. The nitrogen content was now determined by a thermal conductivity detector.

In order to determine the crude protein of the sample, the total nitrogen content was multiplied by 6.25. This calculation is represented below:

% N x 6.25 = % Crude Protein/Protein

3.9.3 AMINO ACID

The AA content in the diets was determined using the AOAC (2005), Official Method of Analysis 994.12. Briefly, a known amount of feed sample was exposed to HCl hydrolysis to release the free AA. This was followed by RP HPLC separation using AccQ Tag derivatization, this allowed the free AA to be separated chromatographically and thus quantified.

3.10 DETERMINATION OF BREATH AND BODY WATER ENRICHMENTS

The determination of enrichment of $^{13}CO_2$ in expired air was performed by the Waikato Stable Isotope Unit (University of Waikato, Hamilton, New Zealand) by using a Dumas Elemental Analyzer (Europa Scientific ANCA-SL) interfaced to an isotope-ratio MS (IRMS; Europa Scientific Tracermass; manufactured by Europa Scientific Ltd.).

Enrichment of ^{2}H in plasma samples at 0 and 440 min was determined using IRMS (Iso-trace, Chemistry Department, University of Otago, Dunedin, New Zealand).

3.11 CALCULATIONS

Whole body protein turnover was calculated using the standardized state-steady equations as used by Wester et al., (2015) and described by Vann et al., (2000).

All calculations were established upon the following principal:

Flux = Synthesis + Oxidation = Breakdown + Intake

Assuming that the cats are at a nitrogen equilibrium, the inflow of the leucine entering the plasma free pool is equal to the outflow of leucine from this pool.

According to this model, inflow comes from leucine which is absorbed from the diet in the fed state (this is calculated from the leucine content of the diet x rate of dietary intake). Inflow also comes from leucine which is released from body protein which is broken down (Leu Ra).

Transamination of leucine (primarily in muscle) results in the enrichment of KIC in plasma, this is equal to leucine intracellular enrichment in muscle; this is used as the metabolic precursor pool.

The enrichment of KIC plasma does not account for first-pass metabolism upon absorption where the liver and gut receive unlabelled leucine; and intracellular precursor enrichment is not the same as in muscle.

The outflow of leucine from the free AA pool (intracellular precursor pool, as estimated by KIC enrichment) consists of leu oxidation (measured as expired $^{13}CO_2$) and the synthesis of body protein (which is estimated as Leu NOLD).

Non-oxidative Leucine disposal (NOLD) was calculated as follows:

NOLD = Leucine Flux – Leucine Oxidation

Leu Flux was calculated as follows:

Leucine Flux = [([1-^{13}C]leucine IE/[1-^{13}C]KIC PE) -1] x [^{13}C]leucine IR

Where IE represents infusate enrichment, PE is the plasma enrichment at plateau and IR indicates the infusion rate during the leucine infusion.

Leu oxidation was calculated from measuring the amount of ^{13}C appearing in carbon dioxide from [1-^{13}C] KIC, the labelled precursor substrate. The calculation was performed as follows:

Leucine Oxidation = (CO_2PR x $^{13}CO_2E_I$)/[1-^{13}C]KIC PE

Where PR represents the production rate and E_I indicates the ^{13}C enrichment in breath at a steady rate during the infusion of leucine.

The total carbon dioxide production rate was estimated from the 2 hour [^{13}C]Na bicarbonate infusion as follows:

CO_2PR = [(NaH$^{13}CO_3$IE/$^{13}CO_2$BE)-1] x NaH$^{13}CO_3$IR

Where BE indicates the enrichment of the expired carbon dioxide at a steady state during the bicarbonate infusion.

The rate of appearance of urea (urea flux) was calculated as follows:

$$\textbf{Urea Flux} = [([^{15}N_2]\ \text{Urea IE}/[^{15}N_2]\text{Urea PE}) - 1] \times [^{15}N_2]\text{Urea IR}$$

Where IE represents infusate enrichment, PE is the plasma enrichment at plateau and IR indicates the infusion rate during the urea infusion.

The infusion of [$^{15}N_2$] urea and the measurement of the rate of appearance of urea (urea flux) in plasma allowed the estimation of the total AA catabolism.

The rate of whole-body protein synthesis and protein breakdown was calculated by using the mean Leu content (6.7%) of whole-body protein as reported by Reeds & Lobley, (1980).

Whole-body protein synthesis was calculated as follows:

Protein Synthesis = (NOLD x 0.046866)

Protein breakdown was calculated as follows:

Protein Breakdown = (Leu Ra x 0.046866)

Protein balance was determined as the difference NOLD – Leu Ra.

Protein balance was calculated as follows:

Protein Balance = Protein Synthesis – Protein Breakdown

The rate of hepatic protein synthesis was calculated as follows:

Hepatic Protein Synthesis = [(Change of protein-bound alanine enrichment with time) / (Average free alanine enrichment)] x 100

Lean mass was calculated in order to determine if the rate of hepatic protein synthesis was feasible. This occurred through the following steps:

Total body water was determined using similar methods as Leung, (2021) and Speakman, (2001).

Firstly, the total amount of D_2O administered was calculated as follows:

$$\textbf{\% concentration of deuterium in injectate} = \left[\frac{\textbf{deuterium solution (g)}}{\textbf{deuterium solution (g) + saline solution (g)}} \right]$$

Then, the total amount of D_2O administered was calculated as follows:

Total amount of D_2O administered = (Initial D_2O – Residual D_2O) x 0.998

Where 0.998 is the correction for purity of the D_2O solution.

Thirdly, the volume of distribution was calculated as follows:

$$V_d = \frac{\text{total amount of } D_2O \text{ administered} \times 1000}{(\text{Deuterium abundance (ppm) in enriched plasma} - \text{deuterium abundance in baseline plasma})}$$

Next, total body water was calculated as V_d divided by 1.04.

This accounted for isotopic exchange of deuterium with non-water hydrogen in proteins, carbohydrates and fats (Leung, 2021).

Lastly, fat free mass (otherwise known as lean body mass) was calculated as follows:

$$\text{Fat Free Mass (kg)} = \frac{\text{Total body water}}{0.713}$$

Where 0.713 is the hydration constant for lean mass in dogs as used by Leung (2021) and Burkholder & Thatcher (1998).

3.12 STATISTICAL ANALYSES AND MODELS USED

Data was statistically analysed in accordance with the statistical model used by Wester et al., in 2015. The statistical model was a completely random design with 1-factor ANOVA by using the general linear models procedure of SAS 9.4 (SAS Institute) with cat as an experimental unit. Differences were deemed significant when the model F test was $P<0.05$. If this was concluded, then the means were compared using a least significant difference test.

The model used in this trial is described by the following equation:

$$Y_{ij} = \mu + \alpha_i + e_{ij}$$

Where:

Y_{ij} : j^{th} observation in the ith treatment group

μ : a general mean

α_i : random effect of the ith treatment group

e_{ij} : random residual error

CHAPTER 4: RESULTS

4.1 CAT INTAKE AND BODY WEIGHT

The body weight (BW) of cats fed 7.5, 15, and 40% of their ME requirements as CP for three weeks was monitored daily. There were no differences in BW changes between the 7.5 and 15, or the 15 and 40% CP diets (*Table 4.1*). However, a difference was seen between the 7.5 and 40% CP diet ($P < 0.007$) where cats fed 7.5% CP lost 389g and cats fed above their requirement gained 17g.

Table 4.1: The mean BW of cats fed 7.5, 15, and 40% of their ME requirements as CP for three weeks.

	Diet, % of ME as CP			
	7.5	**15**	**40**	**SEM**
Initial BW, g	4139	3891	4041	231
Final BW, g	3750	3816	4058	332
Difference, g	-389^{a}	-75.0^{a,b}	17^{b}	110

Note: BW, body weight; CP, crude protein; ME, metabolizable energy.

^{a,b} Means within a row with different superscripts are different, $P < 0.007$.

4.2 THE COMPOSITION OF EXPERIMENTAL DIETS

The composition of the 7.5, 15 and 40% diets varied as expected (*Table 4.2*). The 40% CP diet had a higher numerical level of all calculated nutrients than both the 7.5 and 15% CP diets. “Jelly,” from a commercial canned cat food used as a flavour enhancer in diets, was mostly water (6.8% DM) and very low in CP.

Table 4.2: The composition of experimental diets and jelly.

	Diet, % of ME as CP			
	7.5	**15**	**40**	**Jelly**
DM%	71.8	55.6	34.8	6.8
ME, kJ/100gDM	2657	2930	3625	-
CP, %DM	8.3	18.4	44.0	4.2
Intake needed to maintain BW, kJ/kg BW	350	300	260	-

Note: BW, body weight; CP, crude protein; DM, dry matter; ME, metabolizable energy.

4.3 THE AMINO ACID COMPOSITION OF EXPERIMENTAL DIETS

As expected, the AA composition of the experimental diets differed greatly (*Table 4.3*). The 40% CP diet had the highest amounts of AA. This was followed by the 15% CP diet and the 7.5% CP diet. These can be directly compared to the NRC (2006) requirements. The percentage of requirements were also calculated (*Table 4.4*). The zinc concentration of the experimental diets was calculated based on diet formulation; these can be compared directly to the NRC requirements, *this will be specifically discussed in Section 7.7*. The percentage of requirements were also calculated (*Table 4.4).* The AA intake of the experimental diets also differed *(Table 4.5);* with the 40% CP diet having the highest amounts of AA intake, followed by the 15% CP diet and the 7.5% CP diet.

Table 4.3: The amino acid and zinc composition of experimental diets on a dry matter basis.

	Diet, % of ME as CP			
Nutrient (mg/100mg)	**7.5**	**15**	**40**	**NRC (2006) Dietary Requirement (g/100mg DM)**
Asparagine/Aspartic Acid	0.69	1.46	3.61	-
Threonine	0.32	0.66	1.74	0.52
Serine	0.29	0.58	1.47	-
Glutamine/Glutamic Acid	1.14	2.24	5.40	-
Proline	0.29	0.62	1.52	-
Glycine	0.33	0.78	1.72	-
Alanine	0.42	0.90	2.25	-
Valine	0.38	0.81	2.08	0.13
Methionine	0.18	0.40	1.07	0.17
Isoleucine	0.33	0.72	1.84	0.43
Leucine	0.58	1.25	3.16	1.02
Tyrosine	0.24	0.51	1.31	-
Phenylalanine	0.30	0.65	1.61	0.40
Histidine	0.24	0.51	1.20	0.26
Lysine	0.64	1.40	3.51	0.34
Arginine	0.46	1.05	2.53	0.77
Zinc	23.5	23.5	18.8	7.4

Note: CP, crude protein; ME, metabolizable energy.

Table 4.4: Percentage of NRC (2006) requirement for amino acids and zinc supplied by experimental diets.

	Diet, % of ME as CP		
Nutrient (% of requirement)	**7.5**	**15**	**40**
Asparagine/Aspartic Acid	-	-	-
Threonine	61.5	127	334.6
Serine	-	-	-
Glutamine/Glutamic Acid	-	-	-
Proline	-	-	-
Glycine	-	-	-
Alanine	-	-	-
Valine	292.3	623.1	1600
Methionine	106	235.3	629.4
Isoleucine	76.7	16.74	427.9
Leucine	56.9	122.5	309.8
Tyrosine	-	-	-
Phenylalanine	75.0	162.5	402.5
Histidine	92.3	196.2	461.5
Lysine	188.2	411.8	1032.4
Arginine	59.7	136.4	328.6
Zinc	317.6	317.6	254

Note: CP, crude protein; ME, metabolizable energy.

Table 4.5: The calculated amino acid intake from the experimental diets on a dry matter basis.

	Diet, % of ME as CP			
Nutrient (mg)	**7.5**	**15**	**40**	**SEM**
Asparagine/Aspartic Acid	3.49	6.80	15.89	0.47
Threonine	1.62	3.07	7.66	0.22
Serine	1.47	2.70	6.47	0.19
Glutamine/Glutamic Acid	5.77	10.43	23.77	0.71
Proline	1.47	2.89	6.69	0.20
Glycine	1.67	3.63	7.57	0.22
Alanine	2.13	4.19	9.90	0.29
Valine	1.92	4.19	9.16	0.27
Methionine	0.91	1.86	4.71	0.13
Isoleucine	1.67	3.35	8.10	0.23
Leucine	2.94	5.82	13.91	0.40
Tyrosine	1.22	2.37	5.77	0.17
Phenylalanine	1.52	3.03	7.09	0.21
Histidine	1.22	2.37	5.28	0.16
Lysine	3.24	6.52	15.45	0.45
Arginine	2.33	4.89	11.14	0.33

Note: CP, crude protein; ME, metabolizable energy.

4.4 LEU AND UREA RESULTS

4.4.1 LEU KINETICS

The Leu flux in cats which were fed the 40% diet (above protein requirements) was only 15% more than the cats fed the 15% CP diet, and almost 30% more than the cats fed the low protein diet (7.5% diet; *Table 4.6*). There were no differences in the Leu flux between the 7.5 and 15, or the 15 and 40% CP diets. However, a difference was seen between the 7.5 and 40% CP diets ($P < 0.05$).

The Leu rate of appearance (Leu Ra) differed ($P < 0.05$) when comparing the 40% diet to the 7.5 and 15% CP diets. No differences were seen between the 7.5 and 15% CP diets.

Leu oxidation in cats which were fed the 40% CP diet was just over twice as much as those fed the 7.5% CP diet ($P < 0.05$). However, no difference ($P > 0.05$) was seen when comparing the 15% CP diet with the 7.5 or 40% CP diets.

The ratio of Leu oxidation to Leu flux was calculated. This showed just over a third more of the Leu flux was oxidized by cats fed the 40% CP diet when compared to the 7.5% diet ($P < 0.05$). The same was true when comparing the 40% CP diet to the 15% CP diet ($P < 0.05$). There was no difference between the ratio in the 7.5 and 15% CP diets.

No difference in leu NOLD (non-oxidative Leu disposal) was seen across any of the treatments despite how values were expressed.

4.4.2 CALCULATION OF PROTEIN TURNOVER

Protein turnover was estimated using the Leu kinetic data (*Table 4.7*). Protein breakdown was approximately 1.7 times slower in cats fed the 40% CP diet than in cats fed below their protein requirement ($P<0.05$). Protein breakdown was also 1.6 times lower in 40% CP diets than in cats fed the 15% CP diet ($P<0.05$). Differences in protein synthesis among the diets were not significant regardless how the values were expressed. Protein oxidation was over 2.1- and 1.6-times greater ($P<0.01$) in cats fed the 40% CP diet compared to cats fed the 7.5 and 15% CP diets, respectively. No differences in protein oxidation were seen between the 7.5 and 15% diet. Cats fed the 40% CP diet were in a positive protein balance (5.5 g/d). However, cats fed below and at their protein requirement were in a negative protein balance at -2.8 and -0.7 g/d, 7.5 and 15% CP, respectively. Differences in protein balance were seen between the 40% CP diet and those fed 15% and 7.5% CP diets ($P<0.01$).

Table 4.6: Leu kinetic in cats fed 7.5, 15, and 40% of their ME requirement as CP.

	Diet (% of ME as CP)			
Item	**7.5%**	**15%**	**40%**	**SEM**
Leu flux				
μmol/h	520.8[a]	617.6[a,b]	726.4[b]	46.75
μmol/(kg BW · h)	145.3[a]	161.7[a,b]	179.3[b]	8.92
μmol/(kg $BW^{0.75}$ · h)	198.7[a]	225.7[a,b]	254.1[b]	12.65
Leu NOLD				
μmol/h	370.5	415.7	404.3	36.19
μmol/(kg BW · h)	106.8	109.6	99.6	8.22
μmol/(kg $BW^{0.75}$ · h)	141.5	152.7	141.2	11.32
Leu Ra				
μmol/h	431.0[a]	430.7[a]	286.1[b]	47.42
μmol/(kg BW · h)	120.5[a]	111.9[a]	68.9[b]	9.56
μmol/(kg $BW^{0.75}$ · h)	164.5[a]	156.5[a]	98.2[b]	42.23
Leu oxidation				
μmol/h	153.2[c]	192.2[c]	328.8[d]	29.18
μmol/(kg BW · h)	39.7[c]	49.9[c]	81.0[d]	6.36
μmol/(kg $BW^{0.75}$ · h)	58.6[c]	69.9[c]	114.8[d]	9.00
Leu oxidation:Leu flux				
	0.30[a]	0.31[a]	0.46[b]	0.04

Note: BW, body weight; CP, crude protein; ME, metabolizable energy; NOLD, nonoxidative leu disposal; Ra, rate of appearance.

[a,b] Means within a row with different superscripts are different, $P < 0.05$.

[c,d] Means within a row with different superscripts are different, $P < 0.01$.

Table 4.7: Protein turnover in cats fed 7.5, 15, and 40 of their ME requirement as CP calculated from Leu kinetics.

	Diet (% of ME as CP)			
Item	**7.5%**	**15%**	**40%**	**SEM**
Protein synthesis				
g/d	17.4	19.5	18.9	1.69
g/(kg BW · d)	5.0	5.1	4.7	0.38
g/(kg $BW^{0.75}$ · d)	6.6	7.2	6.6	0.54
Protein breakdown				
g/d	20.2^{a}	20.2^{a}	13.4^{b}	2.26
g/(kg BW · d)	5.6^{c}	5.2^{c}	3.2^{d}	0.45
g/(kg $BW^{0.75}$ · d)	7.7^{a}	7.3^{a}	4.6^{b}	0.67
Protein balance				
g/d	-2.8^{c}	-0.7^{c}	5.5^{d}	1.25
g/(kg BW · d)	-0.6^{a}	$-0.1^{a,}$	1.4^{b}	0.34
g/(kg $BW^{0.75}$ · d)	-1.1^{c}	-0.2^{c}	2.0^{d}	0.47
Protein oxidation				
g/d	7.2^{c}	9.0^{c}	15.4^{d}	1.38
g/(kg BW · d)	1.9^{c}	2.3^{c}	3.8^{d}	0.30
g/(kg $BW^{0.75}$ · d)	2.7^{c}	3.3^{c}	5.4^{d}	0.42

Note: BW, body weight; CP, crude protein; ME, metabolizable energy.

[a,b] Means within a row with different superscripts are different, P < 0.05.

[c,d] Means within a row with different superscripts are different, P < 0.01.

4.4.3 UREA PRODUCTION RATE

Urea production in cats fed 40% CP was 2.3 times greater than in cats fed 7.5% CP diet (P<0.01; *Table 4.8*). The urea production in cats fed the 40% CP diet was also 1.8 times greater than in cats fed 15% CP diet (P<0.01).

Table 4.8: Urea production in cats fed 7.5, 15 and 40% of their ME requirement as CP.

	Diet (% of ME as CP)			
Item	**7.5%**	**15%**	**40%**	**SEM**
Urea production				
µmol/min	22.08[a]	30.18[a]	55.54[b]	3.28
µmol/(kg BW · min)	6.1[a]	7.9[a]	13.9[b]	0.86
µmol/(kg $BW^{0.75}$ · min)	8.4[a]	11.0[a]	19.6[b]	1.15

Note: BW, body weight; CP, crude protein; ME, metabolizable energy.

[a,b] Means within a row with different superscripts are different, $P < 0.01$.

4.5 HEPATIC PROTEIN SYNTHESIS RESULTS

The hepatic protein synthesis in cats fed below their protein requirement (7.5% CP) was 1.5 times greater than in cats fed 15 and 40% CP (P<0.05; *Table 4.9*). No differences in hepatic protein synthesis were seen between cats fed 15 and 40% CP.

Table 4.9: Hepatic protein synthesis in cats fed 7.5, 15 and 40% of their ME requirement as CP.

	Diet (% of ME as CP)			
Item	**7.5%**	**15%**	**40%**	**SEM**
Fractional synthetic rate, %/h	1.20 [a]	0.78 [b]	0.79 [b]	0.19

Note: CP, crude protein; ME, metabolizable energy.

[a,b] Means within a row with different superscripts are different, $P < 0.05$

4.6 BODY COMPOSITION RESULTS CALCULATED FROM D_2O DATA

The body composition was calculated to check the reliability of D_2O data (*Table 4.10*). No significant differences were found when comparing body composition values (fat free mass) across the diets. Moreover, values of 100% fat-free mass suggest results are erroneous as fat is present in all animals, for example, as an essential component of cell membranes.

Table 4.10: Body composition in cats fed 7.5, 15 and 40% of their ME requirement as CP.

	Diet (% of ME as CP)		
Item	**7.5%**	**15%**	**40%**
Fat-free mass/kg	3.78	3.75	3.91
% Fat-free mass	102.10	98.80	98.90

Note: CP, crude protein; ME, metabolizable energy.

CHAPTER 5: DISCUSSION

The first objective investigated AA kinetics using the $[1\text{-}^{13}C]$Leu precursor method in cats fed above, at and below their protein requirement. Results of the study demonstrated that domestic cats modulate protein turnover when fed above their protein requirement by increasing rates of AA oxidation and catabolism. The ratio of Leu oxidation to Leu flux did not reduce when domestic cats were fed below their protein requirement. This suggests that the cat is therefore not utilising their AA more efficiently when fed below their protein requirement. The cat is not able to adapt to protein intake below their requirement, however, it is unknown if this is only transiently or how this occurs. Long-term feeding studies and adjustments in essential and non-essential AA profile should be performed in the domestic cat to fully investigate the cat's ability to adjust to a diet below their protein requirement.

The second objective measured hepatic protein synthesis using a modified D_2O method. Doing so would enable our results from whole-body rates to be compared to hepatic protein synthesis rates estimated by this method. Hepatic protein synthesis was significantly higher in cats fed below protein requirements. No differences were found between cats fed moderate and high protein diets. As the method used to estimate hepatic protein synthesis was a newly adapted method for cats; it was difficult to compare the results to cats or other carnivorous species. Regardless, values from hepatic protein synthesis proved unreliable, perhaps due to errors in the subcutaneous administration of D_2O.

Cats in this study were closely monitored to ensure that no distinct weight loss occurred. However, weight loss in an animal fed a novel diet may be inevitable. Any cats that did lose weight, were properly maintained to ensure that no health issues or pro-longed weight loss could occur. As a result, two cats had to be replaced in this study. Furthermore, as the replacement of these two cats occurred during the feeding trial period, this therefore had no effect on our results.

The diet analysis for the compositions of our experimental diets were as expected based on the formulation. The AA concentrations in each diet met or exceeded NRC (2006) requirements. Many AA were present in amounts far exceeding their requirements, however, this should not been seen as concerning. Firstly, it is important to note that the NRC requirements are minimum requirements. Secondly, cats are relatively insensitive to AA imbalances or toxicity (Morris 2002; Roger et al., 1998). And thirdly, AA profiles in the diets are that of minced chicken, which is a natural source of high-quality protein and therefore has a good AA composition.

5.1 COMPARISON OF WHOLE-BODY RESULTS WITH PUBLISHED REPORTS

Whole-body results have been reported in the cat previously.

Protein synthesis did not differ across protein levels as represented by NOLD. This was expected as the cat, who has a high protein intake and a low to zero carbohydrate intake, relies on a lower rate of protein synthesis to ensure that there is an abundance of AA free for brain glucose demands, gluconeogenesis and energy (Wester et al., 2015).

Overall, our results did not align with a similar experiment done by Russell et al., (2003). Russell et al., (2003) found that protein synthesis showed a significant increase in a high protein diet (70% of ME as CP) when compared to a moderate protein diet (20% of ME as CP) in the cat. It should be noted that Russell et al., (2003) also used a different method and instead used the [^{15}N] glycine end-product method. It has been reported that when the ^{15}N-glycine end-product method is compared to the [1-^{13}C]leucine precursor method, there is more variability in the results (Duggleby & Waterlow, 2005).

Compared to other herbivorous and omnivorous species, a well-fed cat's whole-body protein synthesis is at the lower end of the range (Russell et al., 2003). In the sheep, it is reported that at maintenance, the whole-body protein synthesis is 15.7 g/kg $BW^{0.75}$ per day (Reeds & Harris, 1981). Pacy et al. (1994)

reported whole-body protein synthesis for the human at maintenance to be 12.6 g/kg $BW^{0.75}$ per day. This can be compared to our results which indicated whole-body protein synthesis in the cat to be 7.2 g/kg $BW^{0.75}$ per day when dietary protein intake was at 15%.

When compared to other small carnivorous species such as the mink, whole-body rate of protein turnover for the domestic cat is much lower. The mink, although a cat-sized carnivore, belongs to the Mustelidae family. Similar to the domestic cat, the mink is said to use dietary protein as a source of metabolic energy (Verbrugghe & Bakovic, 2013). Species differences are pronounced when examining resting metabolic rate of both the cat and the mink. The mink has a resting metabolic rate of nearly twice as much of the resting metabolic rate found in the cat. Higher resting metabolic rate means a greater protein turnover and explains the differences which are seen between the mink and the domestic cat (Wester et al., 2015).

Whole-body protein turnover differences in the domestic cat may be attributed to physiological differences. The domestic cat has a shorter gastrointestinal tract than omnivores and herbivores. For example, the domestic cat's small intestine is 1.7m in length, and their large intestine is 0.4m in length (NRC, 2006), whereas the human's small intestine is approximately 6.5m in length (Wagner et al., 2018), and their large intestine is approximately 1.5m in length (Kiela & Ghishan, 2016). Since protein synthesis occurs at a high rate in the gut, the short gastrointestinal tract of the cat may aid in reducing overall whole-body protein uptake, as proposed by Russell et al. (2003).

Protein synthesis has also been shown to be sensitive to dietary changes in previous studies (Russell et al., 2003). However, our study showed no significant relation between different dietary levels and the synthesis of protein. This is in agreement with Wester et al., (2015) who reported no significant changes in protein synthesis in the cat with different dietary protein intakes. As mentioned, the domestic cat, being an obligate carnivore, consumes a high protein diet. However, it appears that regardless of the dietary protein levels in their food, the rates of protein synthesis do not increase, this is advantageous for

an animal who is said to rely on AA for energy and/or gluconeogenesis (Wester et al., 2015). This is because without increasing the synthesis of proteins with the abundance of AA; they are instead leaving these AA free to be utilised for energy and gluconeogenesis.

Like protein synthesis, protein degradation (also known as protein breakdown) is sensitive to dietary changes. Our findings showed a 40% decrease in protein breakdown when comparing low and medium protein diets to high protein diets. This is in contradiction to results obtained by Russell et al. (2003) whose values in cats fed 20% and 70% of their ME of CP showed a 47% increase in protein breakdown as dietary protein content increased. Whole-body protein degradation will increase if dietary protein cannot meet AA requirements. The quality (the ability of a protein to supply all the needed AA in the correct balance) is more important than the dietary CP level. When dietary protein is high, AA concentration is also increased; if AA requirements are met, protein breakdown is inhibited (Waterlow, 1999). This seems logical for a species such as the cat who relies on proteins for energy as well as fuelling other bodily processes. For the cat to be constantly breaking down body proteins into AA may be wasteful when AA concentrations are already high.

The results from the current work for protein degradation were similar to those found in other species such as the rat. Yoshizawa et al. (1997) concluded that protein degradation in rats decreased in response to feeding because protein degradation is regulated by the release of insulin and dietary factors. However, it should be noted that Yoshizawa et al. (1997) mainly took into account muscle protein synthesis instead of whole-body reports. However, a similar pattern has been shown in the hepatocytes of the rat (Hopgood et al., 1977). Furthermore, species related differences may also occur, and although AA do have an inhibitory effect on protein degradation in the cat, this is much less prominent than in the rat (Silva & Mercer, 1996). Furthermore, a significant difference may have been found when comparing protein degradation from the low protein diet to the medium protein diet had the trial extended longer than three weeks; these small insignificant differences may have resulted in differences in protein mass (body composition) and body weight. The same can be said for protein balance.

Protein balance was not maintained for cats being fed diets low or moderate in protein, whereas protein balance was positive for cats fed a diet high in protein. Although protein balance was seen to decrease when minimum protein requirements were not; there was only significant differences in protein balance when comparing the low and moderate protein diets to the high protein diet. It is commonly understood that cats have a high requirement for dispensable Nitrogen (Green et al., 2008); if this is not met, then a negative protein balance is observable.

Urea production occurs in response to the degradation of AA, therefore serving as an estimate for AA catabolism. In our study, we used the [$^{15}N_2$] urea infusion method to quantify rates of urea production. Urea production showed a similar trend to previous studies done by Wester et al., (2015), and Russell et al., (2003). As dietary protein intake increased, the rate of urea production increased. However, only significant differences were found when comparing the 40% diet to the 7.5% or 15% diet.

Although essential AA are usually not reflective of the cat's high protein requirement (Rogers & Morris, 1979) it may be important to investigate this further to see if the deficiencies have any effect or if the essential AA deficiencies were existent. Cats have 10 essential AA (plus taurine). However, not all AA concentrations were assessed; therefore, I am unable to report if there were deficiencies in the diets and if the lack thereof (deficiency) made the data and dietary proteins unreflective. However, a cat's high protein requirement has been said to be due to their high requirement of dispensable nitrogen, therefore, it appears that the differences in our data may be due to differences in our methodology.

5.2 RATE OF AA OXIDATION AT DIFFERENT PROTEIN INTAKES

5.2.1 RATE OF OXIDATION BELOW PROTEIN REQUIREMENT

Protein oxidation in relation to low dietary protein levels is comparable to a study by Green et al., (2008) who also measured protein oxidation in relation to protein levels at or below requirements. As mentioned, Green et al., (2008) studied the protein oxidation in cats fed diets with 7.5% of ME as CP, 14.2% of ME as CP, 27.1% of ME as CP, and 49.6% of ME as CP. Their findings indicated that the cat was able to regulate AA oxidation given that their minimum protein requirements were met; when cats were fed below their minimum protein requirements, protein oxidation exceeded intake (Green et al., 2008). Although protein oxidation was shown to decrease by approximately 15-20% in our study when comparing cats fed at maintenance to cats fed below maintenance; protein oxidation did not exceed intake, and no overall significant difference was found.

As mentioned, protein oxidation did not significantly decrease further from 15% of ME as CP to 7.5% of ME as CP. This may represent a level for the minimum need for AA that are used for gluconeogenesis, and the amount of glucose needed to meet metabolic glucose requirement. This would then suggest that gluconeogenesis is a constitutive process in cats; this can be verified by the fact that the cat is unable to downregulate hepatic enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK) in the liver when protein intake is low or high (Rogers et al., 1977). As the cat relies on a high protein diet, it seems logical that gluconeogenesis would be continuously produced so that the cat is able to successfully meet their metabolic demands.

Furthermore, this experiment also compared protein oxidation above requirement and found that protein oxidation increased with a higher dietary protein intake. Protein oxidation when dietary protein is above requirement was significantly higher than protein oxidation below and at requirement. A higher protein oxidation in a higher protein diet is expected due to the lack of storage capacity for AA that are not required for protein synthesis.

Protein oxidation in both moderate and high protein diets were similar to those reported by previous studies (Green et al., 2008, & Russell et al., 2002). As dietary protein levels increase, protein oxidation increases due to the body's degradative pathway in response to excess AA (Jackson, 1999).

The overall method used to determine the rate of oxidation below protein requirement differed to the method used by Green et al. (2008), who used indirect calorimetry as opposed to directly measuring AA use. The use of [1-^{13}C]Leu directly allowed us to grasp a complete picture of AA kinetics.

The indirect calorimetry method makes assumptions on fuel oxidation based on RQ (respiratory quotient). In reality, the RQ value fluctuates depending upon the protein and fat mixture which is being oxidised. In addition, there are physical limitations which may affect the RQ value; inaccurate volume measurements due to a leak in the equipment may cause the volume of the gas to be incorrectly measured (Gupta et al., 2017). Stress or agitation during the measurement of gas exchange is also known to affect the RQ (Gupta et al., 2017), this is something that would be evident in the domestic cat.

Furthermore, the quantitative urine collection used by Green et al. (2008) to estimate protein metabolism is open to error. In fact, the best manner to obtain a contaminant-free urine sample from the domestic cat is through cystocentesis; followed by the "free-catch" method which is better for qualitive purposes (Kurien et al., 2004). However, Green et al. (2008) did not collect urine using these two methods, and instead collected urine out of the container/litter. This method, although less stressful and non-invasive, may result in incorrect collection of the sample (i.e., spills, or cats urinating outside of the desired area), or incorrect results due to contamination. Even through proper collection of urine the risk of contamination must be acknowledged (Yadav et al., 2020).

Differences in the rate of oxidation below protein requirement in our study compared to published data (Green et al., 2008) may be due to a shorter treatment period. We allowed an adaptive period of a minimum of 21 days for the domestic cat and their new diet. Once, the domestic cat was adapted to this diet

we began the infusion period. As our adaptation period is shorter; it may be argued that the domestic cat may be able to regulate AA oxidation when dietary protein content is below requirement for a certain period of time. For example, this is evident in the emperor penguin, who is also a carnivore, and who undergoes long-term fasting. During the first stage of fasting (phase I), protein catabolism decreases; this is followed by phase II known as protein sparing. During phase I and II, the rates of AA oxidation are low when there is stored fat to supply energy, however, when the preset threshold of minimum body fat is reached, protein oxidation increases (phase III) (Robin et al., 1988). The same can therefore be argued in the domestic cat. Although a longer adaptation period may seem advantageous; the examination of a shorter adaptation period may suggest that there is a threshold for cats to adapt to a low protein diet for a certain period of time. In the future, this may be worth examining as it introduces new research questions as to what this time threshold may be, and why it may occur in the domestic cat as there is limited knowledge on the appropriate time for a trial feeding period for the cat in regard to protein metabolism.

5.3 COMPARISON OF WHOLE-BODY RATE OF SYNTHESIS VERSUS HEPATIC SYNTHESIS

Hepatic protein synthesis was estimated by calculating the fractional synthetic rate. The fractional synthetic rate is defined as the rate at which a labelled AA precursor is incorporated into protein (Cross et al., 2020). When D_2O is administered, the deuterium from the water rapidly equilibrates across body water pools and is transferred to newly synthesized alanine. This labelled alanine can then be incorporated into albumin (a major hepatic export protein) and can then be recovered in the plasma at the end of the experiment.

The liver and the gut accounts for 25% of whole-body protein synthesis (Stoll et al., 1998). McNurlan & Garlick (1981) reported that although tissues such as the liver and the gut make up only a small proportion of protein mass; their high rates of fractional synthesis suggest that these tissues account for a notable component of whole-body protein synthesis. In fact, McNurlan & Garlick (1981)

estimated a high of 43% of whole-body protein synthesis occurred in the liver and gut of the rat. Overall, it is hypothesized that protein synthesis in tissues such as the liver, which alone is one of the most important organs for synthesizing proteins, may also dictate whole body rates.

Both AA availability and insulin modulate protein metabolism (Wester et al., 2015). However, there are key differences in how insulin and AA availability can interact and stimulate protein synthesis in different tissues. For one, the ability of insulin to stimulate skeletal muscle protein synthesis is shown to decline with age. Whereas the ability of AA to stimulate skeletal muscle protein synthesis in tissues does not (Davis et al., 2003). Hepatic protein synthesis on the other hand, is not stimulated by insulin alone. Instead, hepatic protein synthesis is mainly modulated by AA concentration (Davis et al., 1998). Additionally, feeding (especially an increase in the intake of AA) stimulates hepatic protein synthesis; regardless of age or physiological state (Davis et al., 2003).

Like whole-body protein synthesis rates, and skeletal muscle protein synthesis; hepatic protein synthesis is also affected by nutritional status. However, different adaptions exist after food deprivation and refeeding. Mosoni et al., (1996) reported that total and skeletal muscle protein synthesis was not stimulated by 6 h refeeding. In fact, total and skeletal muscle protein synthesis are further delayed allowing for AA to be used for other tissues such as the liver. This is further supported by the finding that hepatic protein synthesis was rapidly stimulated after refeeding (Mosoni et al., 1996). Overall, this shows that there is an adaptation in protein synthesis rates in response to nutritional status; and that priority is given to metabolism pathways in the liver.

5.3.1 COMPARISON OF HEPATIC SYNTHESIS RATE TO PUBLISHED REPORTS

Eisenstein & Harper (1991) found that when protein levels were below requirement in the rat; the rate of hepatic protein synthesis was also limited. They attributed the low rate of hepatic protein synthesis due to a diminished rate of AA

supply. The same has been seen in humans. Our results indicated hepatic protein synthesis was highest when dietary protein was low. This seems strange when it would be assumed that protein synthesis rates are lowest when there are limitations on dietary protein intake, and thus a limited AA supply.

A previous study by Raggio et al. (2007) found that hepatic protein synthesis was maintained when protein supply decreased in the lactating dairy cow. It appears that the lactating dairy cow was able to prioritize hepatic protein synthesis due to their physiological demands to produce milk; even when protein intake itself was low. The maintenance of their high metabolic demands for AA (and protein synthesis) seemed to occur through an increase in the net hepatic removal of phenylalanine, which could then be used to maintain export synthesis, as well as hepatic protein synthesis (Raggio et al., 2007). Overall, the lactating dairy cow displays physiological adaptations in place for when hepatic protein synthesis may be at risk of being compromised. In other words, their hepatic metabolism seems to be highly adaptable.

In the rat, increased protein intake results in increased AA availability which in turn results in an increase in hepatic protein synthesis. However, it is shown that this occurs at a translational level. The increased AA availability, specifically leucine, promotes ribosomal protein mRNA translation in the liver via the S6K1 signaling pathway, leading to an increase in hepatic protein synthesis (Anthony et al., 2001). The opposite has also been reported, when AA availability is low (for example, in a low protein diet), protein synthesis in the liver is decreased.

It could be hypothesized that the domestic cat, who has a high metabolic demand for a protein-rich diet, may not have an adaptable hepatic system for protein metabolism, which allows hepatic protein synthesis to decrease during periods of limited protein intake. Afterall, our whole-body results for protein synthesis showed no significant difference for protein synthesis for both protein intakes both above and below requirement. If hepatic protein synthesis was to increase significantly in response to a high protein diet, then limited AA would be left for other important metabolic processes in the liver such as gluconeogenesis.

It would seem desirable that hepatic protein synthesis in the cat would decrease if dietary protein was low; allowing the anabolic fate of AA open for gluconeogenesis instead of hepatic protein synthesis itself. However, a carnivorous species such as the cat, is used to a high intake of protein and may not have adaptations for a low protein diet. This is the case for whole-body protein oxidation, where our results showed that the cat was not able to reduce protein oxidation when faced with a low protein intake.

Overall, our results do not fit well with the theory. However, this is suspected to be due to an error in our procedure in our trial. Not only were we not able to attain reasonable values for hepatic protein synthesis using the D_2O method; but we were also not able to withdraw rational values for the domestic cat's body composition. If our initial procedure of D_2O administration was successful, then rational values to measure body composition would have been available. Therefore, it can be speculated that an error occurred due to human error causing incomplete subcutaneous administration of D_2O to the patients. This speculation is further emphasized by the enrichment data used to calculate body composition. The plasma deuterium enrichment is unusually low in cats with unreliable data. Consequently, indicating that not all the isotope was administered.

The D_2O method relies upon subcutaneous administration through the injection of a bolus injection of deuterium oxide. Subcutaneous administration in a species such as the cat is easily susceptible to error. The domestic cat may react in this stressful situation through excessive movement thereby causing leakage from the injection site, or incorrect administration (i.e., the fluid not entering the subcutaneous tissue at all). Of course, it is possible to increase the domestic cat's tolerance to subcutaneous injection (Cooley et al., 2018), and all cats used were handled regularly, we were unable to increase their tolerance to this procedure in an ethical manner. Therefore, most cats found this situation more stressful than others. Ultimately, leaving room for error during the procedure and/or administration of D_2O; which is again, supported by the irrational body composition values in the cat.

CHAPTER 6: LIMITATIONS AND RECOMMENDATIONS

The findings of this study have to be seen in light of the following limitations that may have potentially impacted the findings as well as the ability to effectively answer the research questions.

6.1 LACK OF PREVIOUS RESEARCH AND KNOWLEDGE

There is limited peer-reviewed research available to provide a solid foundation for the understanding of the domestic cat's protein metabolism as a whole, let alone when focused during periods of low dietary protein intake. Although there is limited research on the cat species, protein metabolism research on the cat itself can be quite limiting. Moreover, there have been only two published reports regarding the domestic cat's AA oxidation below requirement.

There are also limited published scientific studies available for comparison with regard to the hepatic protein synthesis of cats.

6.2 INGREDIENT COMPOSITION

The initial diet composition chosen for the diets, presented a challenge at the beginning of the feeding trial because it was not accepted during palatability testing. When calculating the amount of premix needed for the diets, it was first estimated that the cats required 3-5% of premix. However, once cats found the diet unpalatable during testing; it was then determined that only 0.5% of premix was needed in each diet. The ingredient compositions of each diet were then changed accordingly.

6.3 THE USE OF PALATABILITY ENHANCERS

The domestic cat has a reputation of being a “picky eater”. Like the human who relies on aroma, taste and appearance to determine the palatability of their food, the domestic cat also uses similar factors. However, in the cat it is the AA taste system which dominates, as the cat has evolved to consume a feed with the correct ratio of protein to fat. This causes great difficulty when offering subjects a diet low in protein. To overcome this factor, palatability enhancers were used. Initially, Marmite was used but it was not able to compensate for other factors, such as unsuitable feed texture perhaps. A powdered chicken stock concentrate was tried and found to be more successful. However, as the palatability issues continued, ‘jelly’ from Chefs wet, canned diet was sourced and used as needed. The protein composition of the jelly was not determined by analysis until after the infusion period, but it was found that the jelly had a very low CP (4.2% CP, DM basis) and 6.8% DM, so the actual amount of CP supplied was miniscule.

It is recommended that all aspects of the diet are taken into account when considering dietary composition and protein levels. This will prevent any unwanted changes in protein levels across the diets due to estimation errors regarding palatability enhancers and their effects on diet compositions. Overall, palatability issues need to be accounted for when preparing experimental and purified diets for cats and adjustment to experimental diets may take longer than expected.

6.4 SHORT FEEDING TRIAL PERIOD

Time is usually a limiting factor in dietary clinical trials. However, time limitation in our study seemed to bring forth new research questions which may be answered in a more longitudinal study. For example, we allowed an adaptive period of a minimum of 21 days for the domestic cat and their new diet. It would be beneficial to study the AA metabolism of the cat for a longer period of time. As mentioned in our discussion *section 5.2.1*, it may be argued that the domestic cat is able to regulate their AA oxidation when dietary protein intake is low for a

certain period of time, as is the case in other carnivorous species (Robin et al., 1988). However, to fully analyse this would require a longer feeding trial period. On the other hand, feeding a cat a diet low in protein for extended periods of time may lead to extensive periods of negative protein balance and subsequently cause a catabolic state in the cat; thereby leading to excessive loss of lean BW and thereby leading to removal of the subject from the trial.

6.5 THE SUBCUTANEOUS ADMINISTRATION OF D_2O

Deuterium oxide was administered through a subcutaneous (SC) injection in the cat. In theory, this is ideal as the cat, having a scruff, seems like a good candidate for SC administration due to the larger area of subcutis when compared to non-scruff species such as the human. However, in this circumstance, the subcutaneous administration resulted in a greater inter-individual variability in dose (McDonald et al., 2010); as made evident by the dodgy body composition values.

Additionally, in this current study a large volume of D_2O (10 mL/kg BW) was administered subcutaneously which may have resulted in unwanted leakage out of the injection site due to pressure from the distended skin.

A recommendation for future trials wishing to test this method in a species such as the domestic cat, would be to instead administer D_2O via a catheter. Additionally, after administration of the D_2O, flushing the catheter with saline to ensure complete administration of the D_2O would be beneficial. Furthermore, if a large volume was to be administered then this would need to be administered slowly over a few minutes as opposed to the administration of a bolus. Administration by catheter would have decreased inter-individual variability while also decreasing the overall risk of incomplete administration and therefore resulted in more reliable values for hepatic protein synthesis.

6.6 ASSESSING THE CONCENTRATION OF MINERALS IN THE CAT DIET

Like the essentiality of AA, the cat also has a requirement for essential minerals in their diet. The prepared diets of this study, were not deficient in any important minerals due to the addition of premix (Dog and Cat Premix, Image Holdings Ltd., Auckland, NZ). In fact, the prepared diets of this study exceeded NRC (2006) requirements. However, as scientists are essentially preparing “home-made diets” for research trials with their experimental or purified diets, care must be taken. Even a minor contributor to the diet may influence health or results. For example, zinc deficiency has been shown to result in reduced rate of hepatic protein synthesis in the rat liver (Kimball et al., 1995). This emphasizes the importance of fully analysing the concentration of essential mineral concentrations on the cat’s diet. Additionally, it is not uncommon for cat owners to produce their own diets for their pets. In fact, a survey by Dodd et al., (2020) developed a questionnaire with over 3500 participants from English-speaking countries and found that over 60% of pet owners included home-prepared foods in their pet’s diet. Additionally, they found that 6% of these pet owners solely fed their cats home-prepared foods. As home-prepared meals have not been efficiently analysed for their nutritional components; it is not unheard of for these meals to be deficient and not meet NRC requirements. For example, Larsen et al., (2012) examined the nutritional composition of home-prepared diets for cats and dogs with chronic renal disease and found that at least over 50% of these home-prepared diets did not meet the recommended allowances made by the NRC. For example, choline, zinc, calcium, and selenium in the home-prepared diets were commonly below the recommended allowance provided by the NRC (Larsen et al., (2012).

CHAPTER 7: CONCLUSION

This study used the same method used by Wester et al., (2015); the [1-^{13}C]Leu precursor method alongside the infusion of [$^{15}N_2$]Urea to estimate whole-body protein metabolism and protein turnover in the domestic cat. Although Wester et al., (2015) examined AA and urea kinetics at and above protein requirement; our study took this a step further and observed metabolism changes below protein requirement. It was found that protein synthesis showed no significant difference in response to different dietary protein intakes; this is in agreement with a previous study by Wester et al., (2015). Protein oxidation from a moderate to a low protein diet showed no significant differences; indicating that the domestic cat was unable to adapt their AA oxidation when protein sources were scarce. This fits in well with past studies by Green et al. (2008); who reported that although the cat is able to adapt to different protein intakes above their requirement, this was not the case when protein requirements were not met. This observation is important for animal nutritionists, veterinarians and pet owners due to the domestication of the cat who heavily relies on commercial diets selected by their owners. It is important to understand the domestic cat's biological needs so that they are able to thrive in a healthier state without limited or a disproportionate amount of essential nutrients.

This study also addressed another technique which has been modified to measure hepatic protein synthesis in animals. As hepatic protein synthesis is a major determinant of whole-body protein synthesis; these values can be compared. Unfortunately, our results did not agree with any published reports. Upon further investigation, we were able to find errors which may have occurred during our trial. Mainly, a failure occurred in the subcutaneous administration of D_2O which resulted in incomplete injection of deuterium oxide thereby rendering our results inconclusive and unreliable. However, this just emphasizes the importance of proper administration.

CHAPTER 8: REFERENCES

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