Characterising protein and amino acid requirements and metabolism in humans, dogs and cell lines with stable isotope methods

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Abstract

This thesis assesses the amino acid stable isotope methods for measuring aspects of protein metabolism (amino acid oxidation, muscle protein synthesis and albumin synthesis), in dogs, humans and C2C12s. Minimally invasive methods are increasingly important in assessing protein metabolism particularly in companion animals and for vulnerable humans e.g. critically ill. Therefore, the assessment of the stable isotope methods to measure protein metabolism is important. In the dog model, methionine was used to refine the indicator amino acid oxidation (IAAO) technique to a non invasive 'free living' method. The results from this experiment produced a low variability between the 95% confidence interval surround the breakpoint value (0.33-0.36 g/1000kcal of methionine on a 95kcal/kgBW$^{0.75}$), suggesting a refinement in the IAAO method in dogs. In the human model, muscle and albumin FSR were used to assess the impact of the combined supplementation of whey protein (40g) with and without HMB (3g) in elderly males and females. The results indicated a significant increase in the muscle FSR (0.059 ± 0.015 vs. 0.078 ± 0.01 %/h^{-1}, p=0.0173) of elderly women supplemented with both whey protein and HMB and not in elderly males. The sexual dimorphism in elderly people suggests that the treatment in protecting muscle mass should take gender into account. In the C2C12, the rate of protein synthesis was compared when grown on animal and plant hydrolysates when leucine concentrations

were matched. There was no difference between the FSR when provided the different protein sources at a matched leucine concentration. These techniques have assessed the variety and capabilities of amino acid stable isotopes for assessing aspects of protein metabolism generally minimally invasively, however muscle protein synthesis *in vivo* requires more invasive measures.

Dedication

In loving memory of my late father, Nigel Lewis and grandfather, Donald Birch who supported me at the start of this journey but unfortunately never got to see it finish.

Acknowledgement

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1. Introduction

1.1. *Chapter synopsis*

This chapter will discuss the importance of protein nutrition in both dogs and humans, for maintenance, growth and as an energy source. It will also discuss the challenges that both species face in determining protein and amino acid requirements as they change across their lifetime. Finally, this chapter will outline the methods used to measure/determine protein requirements and turnover, with a focus on method development for improved future application, to begin to address some of the problems regarding each species such as the simplistic protein and amino acid requirements provided for dogs.

1.2. **Protein metabolism**

Protein is a vital macronutrient for sustaining mammalian life with deficiency in dietary protein leading to a condition called Kwashiorkor (Ibrahim *et al.*, 2017), more commonly identified in young growing animals where the demand for protein is the greatest. Sources of high levels of dietary protein can be found in both plants (soya, peas and beans) and animals (e.g. meat, milk and eggs), the type of protein source can be used as an indicator of a regions affluency (Gerbens-Leenes, Nonhebel and Krol, 2010).

Proteins consist of a combination of the 20 alpha amino acids (AA) in a sequence. AAs are categorised by the capability of the body to synthesize it at a level to sustain normal requirements for growth. Non-essential AA (NEAA) can be synthesised in abundance with no rate limiting effect on protein synthesis. Essential AA (EAA) cannot be synthesised and are rate limiting to protein synthesis. Conditionally essential AA (CEAA) can be synthesised but either not in sufficient amounts or is dependent on the synthesis of an EAA (Borman *et al.*, 1946; Reeds, 2000).

AAs can contain the following elements carbon, hydrogen, oxygen, nitrogen and sulphur, with the main structure being 3 carbon atoms bound in a line with the middle carbon having a variable R-group (unique to each AA) and a hydrogen and the end carbons an amine and carboxylic acid group. AA bind end to end to from a protein sequence. The order of the AA sequence is determined by the DNA sequence (Gale and Folkes, 1954) and post-transcriptional modification (Hecht and Woese, 1968). The AA positioning in a sequence determines the function of the protein in the form of chemical bonds between the AA folding of the polypeptide chain (Lewis, Momany and Scheraga, 1971). There are three main chemical bonds:

- **The peptide bond** – forms because of the interaction between the amine group and the carboxylic acid group of two adjacent AAs.
- **The hydrogen bond** - the weakest bond in a protein structure as it is due to the polarity of each AA. There are 11 AAs that can

either be a hydrogen donor or acceptor; 3 are solely donors (arginine, lysine, and tryptophan), 2 solely acceptors (aspartic acid and glutamic acid) and 6 can be both acceptors and donors (asparagine, glutamine, histidine, serine, threonine and tyrosine).

- **The disulphide bridge** – a bond between the sulphur atoms in two cysteine AAs arranged in proximity.

1.2.1 Protein absorption and digestibility

The bonds must be digested and absorbed for proteins to enter the body, the gastrointestinal tract plays a central role in digestion and absorption of protein; the mouth mechanically breaks down food, the stomach chemically digests protein (due the acidic conditions combined with pepsin), the small intestine for further proteolysis (via various peptidases) and absorption of AA/small peptides and the large intestine for fermentation and absorption.

Taste is also an important role that occurs in the mouth, this is important for the palatability of a diet. Due to a dogs inability to digest carbohydrates in the mouth, it has been suggested that they have a greater reliance on AAs to detect food preferences (Axelsson *et al.*, 2013). Although to the authors knowledge there has been no definitive experiment to determine if there is a compensatory effect in tasting amino acid rather than glucose in dogs.

The stomach is divided into three sections the cardiac, fundic and pyloric regions, with each section having different functions. The cardiac region is the non-glandular region (Kararli, 1995). The fundic and the pyloric

regions are glandular regions secreting hydrochloric acid, with the acidity maintained between pH 4 - 2 (Youngberg *et al.*, 1985). This acidity aids in the chemical breakdown of proteins, as the hydrochloric acid hydrolyses the peptide bonds within the proteins. In addition, the enzyme pepsin (pepsinogen nonactive form) is secreted and begins the enzymic hydrolysis of proteins and peptides (Erickson and Kim, 1990).

The enzymatic breakdown of protein continues in the small intestine, where proteins are digested by bile enzymes such as trypsin and chymotrypsin in the duodenum (the first section; Gass *et al.*, 2007). The jejunum (the middle section) is a transition section from predominantly digesting to absorbing proteins. When most dietary proteins are broken down into either individual AAs, dipeptides or tripeptides between the stomach and the jejunum. In the ileum (the final section) absorption of dietary AAs occurs, where they are transported to the liver via the hepatic portal vein to be distributed throughout the body.

The absorption of AAs is not 100%, therefore any undigested peptides and proteins pass to the large intestine, where they are utilised by the gut microflora and provide AA that can also be absorbed and utilised by the host (Nasset and Ju, 1961). Any remaining protein is excreted in faeces.

1.2.2 Structure and function of protein

In the body, proteins are expressed in all cells and they have a number of uses that include;

1) **structural/postural,** being key components in the formation of collagen in bone, tendon, ligaments, and skin, actin skeletons within cells.
2) **locomotion/mechanical,** as the mechanism for contraction of cardiac, smooth, and skeletal muscles involve specific protein-protein interactions.
3) **enzyme signalling pathways,** as either signalling molecules or to amplify the signalling cascade.
4) **transport proteins,** such as haemoglobin, transporting O_2 and albumin, which transports fatty acids and drugs throughout the body or into cells through the phospholipid bilayer e.g. Glut4, AA transporters
5) **to aid in the chemical digestion,** of food material as enzymes e.g. alpha amylase breaks down the 1-4 carbon bond in starch molecules into glucose molecules.
6) **Communication,** as protein hormones in an autocrine, paracrine or endocrine manner between cells, tissues and organs e.g. insulin, its main role is to regulate blood glucose levels.
7) **a source of energy,** through oxidative phosphorylation, (1g of protein produces 4Kcal of energy), excess amino acids for protein synthesis or during energy deficiency proteins and amino acids can be converted into energy.

1.2.3 The dynamic process of protein turnover

Once the AA have been absorbed in the body it mixes with the respective "AA pools" where they are part of the dynamic process of protein turnover. This cycle involves new proteins being synthesised from AA and older proteins are being degraded back to AA. The net protein balance of the two sources is determined by measuring the rate of protein synthesis compared to the rate of protein breakdown.

One of the major protein pools to measure protein turnover in the body is skeletal muscle. This tissue is an important organ both functionally (for mobility) and metabolically, as an amino acid and glucose reservoir (Meyer *et al.*, 2002) and contains the largest proportion of protein in the body (50-75%; Bergstrem *et al.*, 1974; Frontera and Ochala, 2015). As it contains the greatest proportion of protein in the body, skeletal muscle protein can therefore act as a temporary buffer for proteins throughout the body, providing AA substrate for other organs such as skin, brain, heart, and liver (Felig *et al.*, 1969; Biolo, Zhang and Wolfe, 1995; Wolfe, 2006). Therefore, the assessment and regulation of skeletal muscle protein turnover through the changes in muscle protein synthesis (MPS) and breakdown (MPB) are important. When the net MPS is greater than the net MPB over prolonged periods e.g., days, then the animal is undergoing skeletal muscle hypertrophy resulting in an increase in muscle mass. This is common in either young growing animals or as the result of resistance type exercise. Conversely when the net daily MPB is greater than the net MPS the muscle is undergoing atrophy, resulting in

a decrease in skeletal muscle mass. This can be observed in older animals or when the muscle has not been used (disuse atrophy; Rennie *et al.*, 2010).

1.1.1.1. Protein synthesis

Protein synthesis is the formation of a sequence of AAs translated from a specific mRNA (messenger ribonucleic acid) that has been transcribed from a section of DNA (deoxyribose nucleic acid; Ross, 1995). The translation of mRNA to a protein sequence requires ribosomes to temporarily bind tRNA (transport ribonucleic acid), a molecule that contains an anticodon at one end and an AA at the other (Drabkin and RajBhandary, 1998)**.** The anticodon is a sequence of 3 RNA nucleotides on the tRNA that temporarily bind to 3 RNA nucleotides in the mRNA sequence referred to as a codon (Drabkin and RajBhandary, 1998). These anticodons correspond to a specific AA, there are 61 possible combinations for anticodons for the 21 AAs found in proteins, with the remaining 3 combinations of anticodons being indicators for stop codons in the mRNA and the end of a protein (Berg and Brandl, 2021). As there are more anticodon combinations than AAs found in proteins, most AAs have more than one anticodon sequence. Although, methionine is one of the few AAs (tryptophan being the other) with one anticodon associated with it, this anticodon is also the first to bind to the mRNA sequence, so methionine is at the start of each protein sequence (Drabkin and RajBhandary, 1998).

1.1.1.2. Protein breakdown

Protein breakdown is a process where a protein is reverted to individual AAs, this mechanism is constantly occurring as it plays an important role in the body (Tipton, Hamilton and Gallagher, 2018). This is to remove excess/unnecessary peptides from the body, recycling any unaltered AA into new proteins, to oxidise AA as an energy source and methylated AA being excreted (Tipton, Hamilton and Gallagher, 2018). The three main mechanisms to break proteins down are:

1) **Ubiquitin proteasomal pathway** – the protein is tagged by 4 ubiquitin molecules signalling a 26S Proteasome to catabolise the protein (Murton, Constantin and Greenhaff, 2008).
2) **Autophagy** – non-selective breakdown of cytoplasmic content using lysosomes, this method being more prominent when the body is in disease or starvation states (Mizushima, 2007).
3) **Calpain Ca^{2+}-dependent cysteine proteases** – a group of selective proteases active by calcium, found in high concentrations in the z line of muscle tissue (Busch *et al.*, 1972; Kumamoto *et al.*, 1992).

The main mechanism for muscle protein breakdown is the ubiquitin proteasomal pathway as it degrades actin and myosin, the most abundant proteins in muscle and the body. However this mechanism works complimentary to the autophagy and calpain mechanisms, as the ubiquitin proteasomal pathways are only able to breakdown actin and

myosin when not in the actomyosin complex (Solomon and Goldberg, 1996; Du *et al.*, 2004).

1.1.1.3. The role amino acids on basal levels of protein synthesis

Basal levels of protein synthesis are determined by the availability of AA's, each AA must be at a sufficient level in the body/tissues to support the rate of protein synthesis. Otherwise if an individual EAA is lower than the required level for protein synthesis then the rate of protein synthesis is restricted by the availability of the most limiting AA (Kumta, Elias and Harper, 1961) i.e., if lysine is at 80% then the rate of protein synthesis is restricted to 80% due to the lower level of lysine. This theory is Liebig's law of the minimum (Alexander N Gorban *et al.*, 2011), originally applied to soil mineral composition and plant growth. All excess AAs are either converted into different metabolites or oxidised (Pencharz and Ball, 2003) for energy.

1.1.1.4. Amino acids in energy metabolism

When AA's are in excess they are oxidised to form energy, phosphates are the "currency" for energy, when they are transferred from an adenosine molecule; adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP). ATP is formed in the electron transport chain within the mitochondria through oxidative phosphorylation. Macronutrients (proteins, fats, and carbohydrates) are used to fuel the reaction via the Krebs/TCA

(tricarboxylic acid) cycle (figure 1.1). AAs (components of protein) can enter the Krebs cycle via 6 different roots depending on the AA either pyruvate, Acetyl CoA, alpha-ketoglutarate, succinyl CoA, fumarate, and oxaloacetate. AAs in excess to the current demand for protein synthesis and other metabolic pathways in which they are precursors, can be oxidised through the Krebs cycle to produce ATP and CO_2, the product of energy metabolism, and generate the precursors for producing energy in the body NADH and FADH.

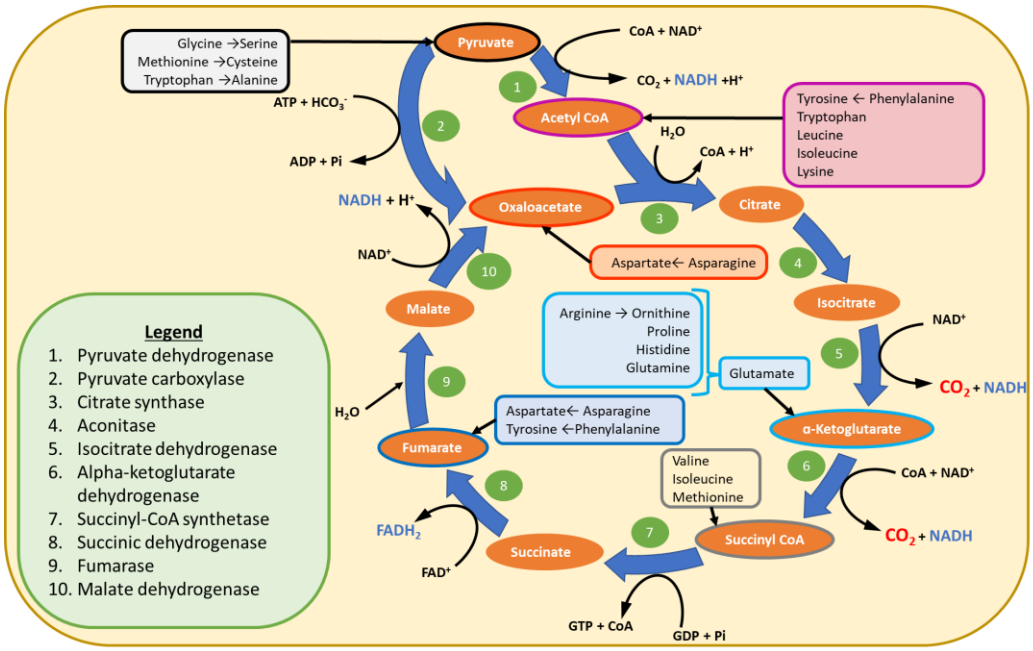


Figure 1.1: *A diagram of the Krebs cycle. Based upon* (Rezaei *et al.*, 2013)

In the Krebs cycle (figure 1.2) the primary products regarding energy production are the coenzymes NADH (Nicotinamide adenine dinucleotide) and $FADH_2$ which are reduced from their oxidised form of NAD^+ and FAD^{2+}. They are a product when Acetyl CoA, α-ketoglutarate, succinyl CoA and oxaloacetate are formed, when an electron is donated

to reduce NAD+/FAD+ forms of the coenzymes. These coenzymes undergo oxidative phosphorylation to produce adenosine triphosphate (ATP) and revert to their oxidised form (NAD^+ and FAD^+).

1.3 Protein metabolism in dogs

The first assessment protein requirements to maximise lifespan in dogs performed in the early part of the 19th century by Magendie (1816 cited by NRC, 2006). In this study dogs were fed with either olive oil or sugar with and without protein and it was concluded that the dogs maintained a healthier state for a longer period with protein rather than without it. The exclusion method for determining nutritional requirements, involves simply removing or limiting a particular AA (or indeed any nutrient) and measuring the growth rate or lack thereof – a simple but effective method to identify essential protein requirements, indeed a similar method was adopted to identify the essential AAs in dogs over 100 years later (Stekol, 1935).

1.3.1 Dietary protein and amino acids minimum requirement levels in dogs

In adult dogs there are 9 essential AAs; histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Rose and Rice, 1939;). Deficiencies in an essential AA can lead to weight loss, inappetence and eventually death (Rose and Rice, 1939). In puppies, arginine is also classed as an essential AA, as a low arginine diet causes a decreased food intake, increased vomiting and

hyperammonaemia, resulting in a decreased bodyweight (Rose and Rice, 1939; Ha, Milner and Corbin, 1978; Czarnecki, Hirakawa and Baker, 1985). The current requirement levels for all the AA's are provided by the NRC, these requirement levels are either, all measured in beagles or the data is artificially transposed to if the dog was a beagle although the experiment behind the calculation to define the requirement level is not defined.

The primary governing body for nutritional requirements in dogs, the NRC, provides EAA requirements for four of a dog's life stages; 4-14 weeks, 14+ weeks adult and bitches in late gestation. However, the research behind the recommendations for AA requirements appears incomplete and a more rigorous and comprehensive evaluation is required. Most studies determining the requirement levels in dogs, were conducted in the 1980's (Table 1.1 for EAA). These studies detected requirements in pre-adult dogs, in humans EAA requirements are greater in children than adults (i.e. BCAA requirement 147-192mg/kgBW in children (Mager *et al.*, 2003) and ~79mg/kgBW in adults (Kurpad *et al.*, 2006)). Therefore, defining adult requirements where the original data was from young animals, then reducing the values to compensate for age related differences leads to potential inaccuracies in requirement levels. Although it is clear that the minimum requirement levels have come from these papers, it is unclear where the origin of the calculations to compensate for the requirement differences in older dogs. Also the studies used to define requirement levels (see Table 1.1) are under powered with treatment groups being as low as 2 dogs for two of the

studies (Ha, Milner and Corbin, 1978; Czarnecki, Hirakawa and Baker, 1985), in these studies no power calculations were stated to define the numbers so it is unclear if one was used. However, it is understandable for the low number of dogs in each treatment due to the longevity of the studies, with compliance and cost having an impact on long-term animal experiments. There was no cross over analysis in these studies due to the longevity of the experiment to gain the desired measures (growth rates and nitrogen balance) in young dogs.

Amino acid	MRRL (g/100g of the diet)	Technique	Breed	Age range	Number of dogs in each diet group	Reference
Arginine	>0.56	GR	Labrador retriever	puppies	2	(Ha, Milner and Corbin, 1978)
	0.28	GR + NB	Pointer	mature	3	(Burns, Milner and Corbin, 1981a)
	<0.4	GR	Pointer	11 wks.	2	(Czarnecki and Baker, 1984)
Histidine	0.21	GR + NB	Beagle	6-7 wks.	5	(Burns and Milner, 1982)
Isoleucine	0.4	GR + NB	Beagle	11-13 wks.	6	(Burns, Garton and Milner, 1984)
Leucine	0.65	GR + NB	Beagle	10-12 wks.	5	(Burns, Garton and Milner, 1984)
Lysine	0.577	GR + NB	Beagle	2-6 wks.	5	(Milner, 1981)
Threonine	0.52	GR + NB	Beagle	14-15 wks.	5	(Burns and Milner, 1982)
Tryptophan	0.17	GR + NB	Beagle	6-7 wks.	5	(Burns and Milner, 1982)
	0.16	GR	Pointer	6-10wks	8	(Czarnecki and Baker, 1982)
	0.12-0.16			10-12wks		
	≤0.12			12-14wks		
Phenylalanine w/o tyrosine	0.8	GR + NB	Beagle	5-6 wks.	6	(Burns, Garton and Milner, 1984)
w/ tyrosine	0.32	GR + NB	Beagle	5-6 wks.	5	(Burns, Garton and Milner, 1984)
tyrosine	0.28	GR + NB	Beagle	5-6 wks.	5	(Burns, Garton and Milner, 1984)
Valine	0.43	GR + NB	Beagle	11-12 wks.	5	(Burns, Garton and Milner, 1984)
Methionine w/o cysteine	0.39	GR +NB	Beagle	8-16 wks.	5	(Burns and Milner, 1981)
with cysteine	0.21	GR+NB	Beagle	8-16 wks.	4	(Burns and Milner, 1981)

***Table 1.1**: The known and recognised by the NRC 2006 amino acid requirements in dogs, with the techniques, breeds, ages and number of animals used. MRRL- minimum recommended requirement level GR-Growth Rate, NB- Nitrogen Balance*

This table demonstrates that, with one exception (on sulphur amino acid requirement levels; Sanderson *et al.*, 2001), these requirements have remained unchanged from the 1980's based on the imprecise nitrogen balance technique. This contrasts with AA requirements in other species (i.e. humans) where alternative techniques (such as IAAO) with improved accuracy and understanding are now used for the assessment of AA requirements (Wooding *et al.*, 2017). Furthermore, the suggested reduction in energy intake requirements being reduced from 130kcal/kgBW$^{0.75}$ to 95kcal/kgBW$^{0.75}$ for dogs (Bermingham *et al.*, 2014), also may be used to suggest that the AA requirements also need updating. Updated requirements should of course consider breed size and age, including mature and older dogs, for which no current recommendations are available. However, before this can be done, the techniques to assess AA requirements need to be optimised in dogs to ensure they limit burden and provide data for AA requirement levels that are accurate, robust and safe.

1.1.1.5. Protein turnover in dogs

There are few studies that assess either protein synthesis or breakdown in dogs, these studies are used more as method development studies for human protein turnover studies rather than determining exact synthesis and breakdown rates in dogs. For example research by Biolo *et al.*, (1994) simultaneously measured muscle protein synthesis and breakdown in a post absorptive state, it identified a muscle fractional synthetic rate (0.318±0.109 %/hr) and fractional breakdown rate (0.454

±0.116 %/hr) in dogs with two different stable isotope tracers (^{15}N lysine and $^{2}H_5$ phenylalanine), however the dogs were mongrels, anesthetized and no dietary information was provided. The sole purpose of the study was to compare the stable isotopes capability to measure MPS and MPB simultaneously, if the authors were more selective with dog breed and provided more information regarding the diet, then a greater wealth of scientific information could have been collected to advance both fields of research (dog protein metabolism and stable isotope).

1.3.2 Dog breeds and the impact on protein metabolism

Dogs are a unique species as they encompass a large range of breed sizes with large variations in bodyweight (ideal bodyweight <5kg to >100kg) and morphology (e.g muscle conformations in greyhound and Staffordshire bull terrier; Webster, Hudson and Channon, 2014), this is prior to assessing any potential physiological differences. Also it is common for dogs from the developed world to be neutered therefore this adds 2 additional groups when comparing any impact gender has on physiology. In addition, research surrounding protein metabolism in dogs is limited, with older papers assessing the impact of protein metabolism in dogs being useful as methodological papers but not for improving the understanding of the metabolic processes in dogs. Therefore, information regarding impacts on protein metabolism venture into speculation or the transference of research from other species.

Protein requirements vary with age, young animals require diets that contain a greater dietary protein content than adult animals to meet the demands of growth. The range of bodyweights and body compositions associated with dogs adds an additional complexity to growth rates and thus protein requirements. It has been shown that larger dog breeds have a greater growth rate (gBW/day) than smaller breeds (figure 1.2; Hawthorne *et al.*, 2004). This means that smaller dog breeds reach maturity faster than larger breeds, <6months compared to >1.5yrs respectively (Hawthorne *et al.*, 2004). Thus smaller dogs have a shorter period of time on a high protein puppy and adolescent food than a larger dog. There has been no research to determine if this impacts negatively, but as it is just to meet nutritional needs it is unlikely that the length of time on a high protein diet would have a negative effect. Large breed dogs require a nutrient dense diet to enable them to maintain the extremely high growth rate (Dämmrich, 1991; Meyer and Zentek, 1991; Nap *et al.*, 1991). Therefore, the larger breeds such as the Great Dane require a different energy calculation (200kcal/$BW^{0.75}$ rather than 130kcal/$BW^{0.75}$) and as all nutrients in dog foods are formulated “per 1000kcal” this then ensures that the dogs have sufficient nutrients to meet their daily requirements to support growth and maintain mass in adulthood. This point was used to highlight how protein metabolism could be different in two dogs of the same age but of different breeds.

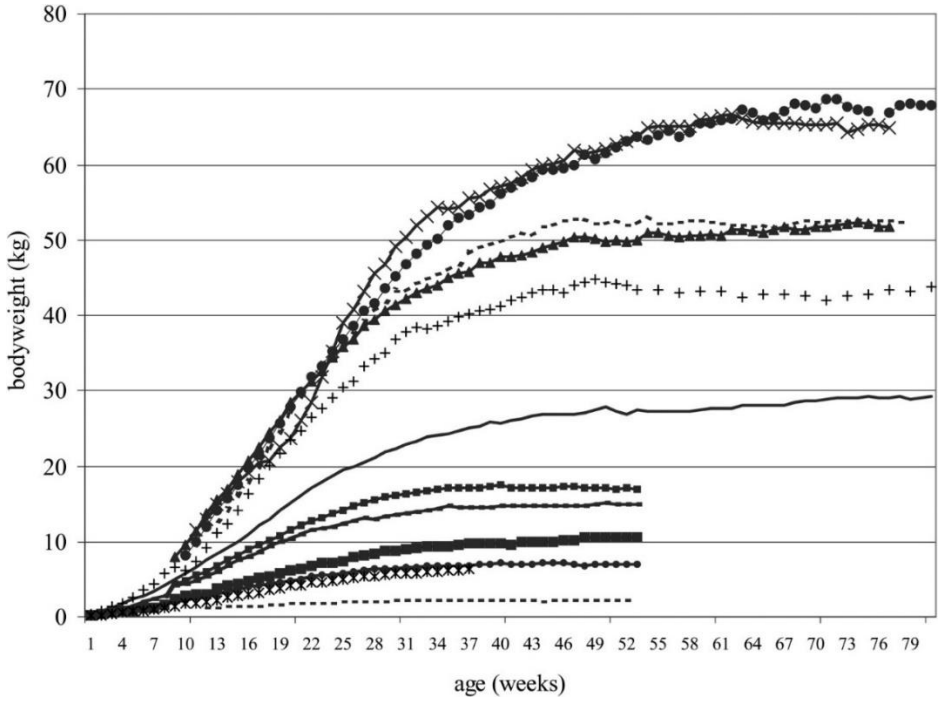


Figure 1.2: *Mean growth curves for 12 breeds of dogs: English Mastiff (×), St. Bernard (•), Irish Wolfhound (----), Great Dane (▲), Newfoundland (+), Labrador Retriever (—), Beagle (▪), English Springer Spaniel (▪▪▪), Cocker Spaniel (▪▪), Miniature Schnauzer (•••), Cairn Terrier (×), and Papillon (----). (Hawthorne et al., 2004)*

Although it has been suggested that larger breeds age have a shorter lifespan than smaller breeds, with an earlier indicator for aging being rapid weight loss (Speakman, van Acker and Harper, 2003). Beagles older than 8 years old have less lean body mass than beagles younger than 8 years old (Hall *et al.*, 2015). Whereas larger and giant breed dogs show signs of skeletal muscle atrophy at 7.5 and 9 yrs respectively (de A. Braga, G. F. Padilha and M. R. Ferreira, 2016). Therefore it would seem that muscle atrophy occurs between 7.5-9 yrs old in medium to giant dogs, suggesting that differences in skeletal muscle atrophy might not be the reason why the lifespan in breed sizes differs. However this area of research has not been properly analysed therefore further investigation is required to measure changes in muscle mass throughout

a dogs life as well as breed specific differences that impact health span and lifespan.

Other breed differences with dogs include their muscle and fat content for example, the greyhound, husky and the Staffordshire bull terrier are lean muscular breeds whereas Labradors, King Charles Cavalier Spaniels, Beagles and Cocker Spaniels are more susceptible to an increased weight gain and adipose deposition (German, 2010). However, there are also differences in muscle composition and distribution between the greyhound, husky and the Staffordshire bull terrier, as their muscles have evolved for different phenotypic traits (Pasi and Carrier, 2003). This may have an impact on the overall protein requirements in these breeds.

1.4 Protein metabolism in humans

The general mechanisms surrounding protein metabolism in dogs and humans are very similar. With mechanism of absorption, digestion and protein turnover being similar between the two species and as previously stated protein metabolism experiments in dogs were used as proof of concept experiments before the experiments were conducted in humans. The main difference between the research in dogs and humans is there is a greater wealth of research conducted in humans than in dogs and therefore greater understanding of the various fields e.g. nutrition.

1.4.1 Dietary protein sources

In humans there are two main dietary protein sources that are utilised: plant and animal. The ratio of dietary protein from animal and plant sources differs throughout the world. The simplistic view of protein intake can be measured via continent (Berrazaga *et al.*, 2019), where African and Asian diets contain mainly plant-based protein and European, American and Oceanic diets contain animal-based protein. The limitation to this assessment is that it the general overview has the potential to be misrepresentational for country and individual specific intake. Previously, an increased animal protein intake has been identified as representative of wealth and a symbol of affluence (Gerbens-Leenes, Nonhebel and Krol, 2010). However in modern times, animal protein consumption compared against per capita income the figure forms an inverted U-shaped, with a decline in the consumption of animal protein at both high and low income people (Andreoli *et al.*, 2021) and plant protein intake showing the inverse. Therefore at high income households at least factors other than wealth influence the choice of dietary protein in humans, these include: spiritual (ingestion of any of the animal is being a compromise of “spirituality and inflaming animal passions”), nutritional (plant-based diets being seen as superior and healthier), ecological (animal production systems being harmful to the environment) and moral (violating animal rights; Beardsworth and Keil, 1993).

1.4.2 The effects of different protein sources on human health

The intake of different protein sources result in different macronutrient intake profiles, vegetarians and vegans consume a lower proportion of protein and total fat and a greater proportion of carbohydrate than people who eat an omnivorous diets (Table 1.2). In addition, omnivorous diets contain more total and saturated fats whereas vegetarian diets contain a greater proportion of polyunsaturated fats (Wilson and Ball, 1999). The greater total and saturated fats intake in those consuming omnivorous diets increase the risk of cardiovascular disease (Nestel *et al.*, 2021) by increased low density lipoproteins and total cholesterol levels which are strongly associated with an increased risk of cardiovascular disease (Geneva:FAO/WHO, 2008). Similarly, although obesity (which is itself an independent risk factor for cardiovascular disease; Powell-Wiley *et al.*, 2021), in its simplest terms, is the result of a positive energy imbalance (i.e., intake>expenditure), the higher fat consumption with animal-based protein diets may also contribute to this given the energy density of fat (i.e., 9kcal/g vs. 4kcal/g for carbohydrate and protein; Southgate and FAO, 1981) and its relative lack of satiating effects (Boyd *et al.*, 2003). As such, a plant based diet is more beneficial to human health than a diet comprising animal protein sources; however, the reduction in relative protein intake in diets for vegetarians and vegans may negatively impact multiple organ systems.

Papers	Gender	Age range	Diet (numbers in study)	Protein intake (g)	Percentage of energy intake		
					Protein	Fat	Carbohydrate
(Ball and Bartlett, 1999)	Females	18-45	Omnivorous (24) Vegetarian (50)	66.7±16.3 54.1±14.7	17.5 14.1	37.2 34.1	34.3 51.8
(Wilson and Ball, 1999)	Males	20-50	Omnivorous (25) Vegans (10)	108 81	17.3 12.4	33.5 28.2	44.9 59.1
(Larsson and Johansson, 2002)	Males	17.5±1	Vegan (15) Omnivores (15)	72 ± 13 117 ± 21	10±1 15±1.7	27±2.6 29±4.2	63±3.2 55±4
	Females	17.5±1	Vegan (15) Omnivores (15)	55±17 80±18	10±1 14±1.3	24±5.4 29±3.8	66 ± 5.3 55±4
(Clarys *et al.*, 2014)	Males and Females	20-69	Vegans (104) Omnivores (155) Vegetarian (573)	82±39 122±45 93±37	14±4 15±3 14±3	25±8 36±7 31±7	57±8 44±8 51±8
(Clarys *et al.*, 2013)	Male and female	18+	Vegetarians (69) Omnivores (69)	66.4±18.4 79.3±23.5	13±2.2 15.2±3.1	29.8±6.6 33.8±7.6	55.2±7.3 48.4±7.4
(Karabudak, Kiziltan and Cigerim, 2008)	Female	29±8.8 27.4±6.6	Vegetarian (26) Omnivores (26)	58.5±28.6 68.2±24.96	13.4±3.67 15.7±2.79	35.8±7.6 35.6±6.5	49.7±9.1 48.1±6.4
(Knurick *et al.*, 2015)	Males and Females	19-50	Omnivores (27) Vegans (28)	97±47 69±29	18.4 13.3	- -	- -

Table 1.2: *Papers comparing the protein intake, percentage of protein, fat and carbohydrate of omnivorous, vegan and vegetarian in males and females*.

In summary, the reduced fat intake associated with plant-based diet may offer benefit in terms of cardiac health and weight management. The reduction in total dietary protein in a plant-based diets and the composition of dietary proteins will likely have a detrimental impact on skeletal muscle; an organ of known importance for whole-body health (Wolfe, 2006).

1.4.3 Digestibility scoring

The quality of dietary protein is dependent on the AA content, AA availability and AA digestibility, as such dietary proteins are graded were by a system that used to be called the: Protein Digestibility-Corrected Amino Acid Score (PDCAAS, see equation below; Schaafsma, 2000).

$$\text{PDCAAS} = \frac{\text{mg of Limiting AA in 1g of test protein}}{\text{mg of the same AA in 1g of reference protein}} \times \text{True faecal digestibility} \times 100$$

This value is representative of dietary proteins quality via the limiting AA of the protein in relation to that AA requirement for age and gender combined with the true faecal digestibility of the protein source (Schaafsma, 2000). However there are limitations to this method, that include: faecal digestibility is assessed rather than ileal digestibility, so both AA utilised by intestinal microbiota and microbial AAs excreted into the large intestine are incorporated into the score (Gaudichon *et al.*, 2002; Schaafsma, 2012). Plant proteins have lower scores than animal proteins, primarily due to increased digestibility of animal protein sources (Tomé, 2013), a factor that needs to be considered when comparing the

impact of different protein sources on physiological processes (i.e., MPS).

As well as the variability in the digestibility depending on the protein source the rate of digestion also differs depending on the dietary protein. This is important as it can impact the rate AAs enter the bloodstream. A protein with a fast digestive transit time (e.g., whey) can cause an acute hyper aminoacidemia, the increase of plasma AA concentration results in an increase in protein synthesis and oxidation (Boirie *et al.*, 1997; Pennings *et al.*, 2011) whereas a slowly digested protein (e.g., casein) are less likely to cause acute aminoacidemia, although will inhibit protein breakdown (Boirie *et al.*, 1997; Pennings *et al.*, 2011).

1.4.4 The effect of age on protein metabolism

Throughout a person's life protein metabolism changes, this is most evident in muscle. A healthy adult skeletal muscle is in dynamic equilibrium, where there is a balance between MPS and MPB, hence muscle mass is maintained. Although muscle mass changes throughout the lifespan, in children MPS is greater than MPB so muscle mass increases, adolescents muscle mass is greatly increased until early adulthood (30yrs), this is when dynamic equilibrium occurs (Metter *et al.*, 1999; Moore, 2019). Conversely if the dynamic equilibrium is altered, such that muscle protein breakdown predominates (via reductions in MPS and/or increases in MPB), skeletal muscle atrophy will occur; as can be seen with advancing age, physical inactivity, poor diet due to

inadequate protein nutrition (Wilkinson, Piasecki and Atherton, 2018) and underlying disease (e.g., in cancer cachexia; Oliveira and Gomes-Marcondes, 2016). As a persons age increases to 50 years old, MPS begins to decrease causing muscle mass to decrease (Tzankoff and Norris, 1977).. Although not only muscle mass decreases with age, the total concentration of albumin in the body was lower in elderly people, 4.2 compared to 4.6g/day for old and young respectively (Gersovitz *et al.*, 1980). Although the rate of albumin synthesis in a post prandial state is not altered by age (Gersovitz *et al.*, 1980; Barle *et al.*, 1997; Thalacker-Mercer *et al.*, 2007), therefore the reduction may be due to albumin FSR decreasing with age in a fasted state (Caso *et al.*, 2007), this would lower the total concentration of albumin per day.

1.1.1.6. Sarcopenia

When skeletal muscle atrophy is prolonged it can lead to sarcopenia (Cruz-Jentoft *et al.*, 2019), this is “a progressive and generalised skeletal muscle disorder that is associated with increased likelihood of adverse outcomes including falls, fractures, physical disability and mortality” (Cruz-Jentoft *et al.*, 2019). It has been estimated that 5-13% of elderly people have sarcopenia between 60-70 years old and 11-50% over 80 years old (Castillo *et al.*, 2003; von Haehling, Morley and Anker, 2010; Yamada *et al.*, 2013; Kurose *et al.*, 2020). To exemplify to magnitude of this condition, this equates to approximately 250,000-1,000,000 men and 300,000-1,300,000 women in the UK alone according to population data from the Office for National Statistics (Office for National Statistics [ONS],

2021). The average annual healthcare costs for people with sarcopenia is £2707 (Pinedo-Villanueva *et al.*, 2019), therefore the estimated annual healthcare cost ranges from £1.5-6.2 billion attributable to sarcopenia alone. In addition to the detrimental impact of sarcopenia, older adults are also more likely to have underlying health problems and non-communicable disease such as advanced cancer and heart failure that also result in further losses of skeletal muscle mass (i.e., cachexia (von Haehling and Anker, 2010)). Cachexia is reported to present in 60-80% of cancer patients (although this does vary greatly based on site of primary tumour) and 5-15% of those with chronic heart failure (von Haehling and Anker, 2010), further exemplifying the need for muscle mass maintenance in those individuals at risk of both sarcopenia and chronic diseases associated with cachexia. Also frail elderly women, have an increase the rate of skeletal muscle catabolism compared to healthy women of the same age, when protein intakes are matched (Chevalier *et al.*, 2003)

Age related sarcopenia is a multifactorial condition with several features that are associated with the condition, these include:

1) **Reduction in physical activity**
2) **Reduction in nutrient, especially protein, intake**
3) **Reduction in anabolic responses**
4) **Changes in hormone concentrations**

Although each of these factors may reduce appetite with advancing age, they may also act independently to reduce protein intake and as such negatively impacting skeletal muscle mass, for many older individuals it will be a combination of these factors that impact their dietary intake. Therefore, the treatment for delaying sarcopenia for any period of time may also be multifactorial.

1.4.5 The effect of dietary protein intake on protein metabolism

Dietary protein stimulates MPS acutely (per meal), 48g of whey protein induces a maximum stimulatory effect on MPS through increased myofibrillar and sarcoplasmic protein fractional synthetic rates (FSR) 45 minutes after ingestion. The FSR remained increased for 60-90 minutes and returned to basal rates afterwards regardless of the availability of plasma EAAs (Atherton, Etheridge, *et al.*, 2010). This suggests that intrinsic mechanisms determined that the muscle was ‘full” and the ‘excess’ amino acids were then diverted towards oxidation pathways. These results echo findings from an earlier study whereby a constant infusion of amino acids, rather than a single dose, was given. A 6 hour infusion increased MPS for 30-120 minutes of the infusion with the remainder of the infusion the rate of MPS returned to baseline (Bohé *et al.*, 2001). The threshold for the mechanism appears to be when dietary whey protein exceeds 20g (Witard *et al.*, 2014). A supplementation of 20g of whey protein increased the rate of albumin synthesis to a maximum with further supplementation having no impact on the rate of

albumin synthesis (Moore et al., 2009). Others have suggested that protein intake has no impact on the rate of daily albumin synthesis (Thalacker-Mercer *et al.*, 2007; Tjiong *et al.*, 2007). However in these studies small hourly meals were provided rather than bolus doses in the aforementioned studies where dietary protein increased albumin FSR (Bukhari et al., 2015; Jackson et al., 2001; James and Hay, 1968; Moore et al., 2009). It has been suggested that the rate of albumin synthesis increases in response to the acute ingestion of protein, approximately 30 minutes into the postprandial period (Caso *et al.*, 2007). However, if the protein is dosed as small intermittent feeds, this response at the end of the entire feeding window (i.e. after 5 hours of feeds every 1 hour) is not apparent (Thalacker-Mercer *et al.*, 2007; Tjiong *et al.*, 2007). This could suggest a process that is similar to the aforementioned “muscle full” effect (Atherton, Etheridge, *et al.*, 2010).

Increased protein intake increased whole body protein synthesis and breakdown increased in a post absorptive state in young males when dietary protein is increased to 1.5 g/BW/day from 0.6g/BW/day, once acclimatised to the dietary protein level for 6 days (Motil *et al.*, 1981). However, when fed at hourly intervals, whole body protein synthesis and oxidation are increased but protein breakdown is decreased (Motil *et al.*, 1981). In addition, the consumption of a very low protein diet for 8 days reduced whole body synthesis, oxidation and breakdown (Motil *et al.*, 1981).

Also it has been recommended to increase elderly dietary protein requirement to between 1.2-1.6 g/kgBW (Lancha *et al.*, 2017), to increase MPS and protect muscle mass. As a protein intake of 1.6g/kgBW in elderly people for 11 days increased whole body protein synthesis, breakdown and leucine oxidation (Campbell *et al.*, 1995) and when measured over a 10 week period lean mass increased (Moore *et al.*, 2015). Also when 20g of whey protein is fed in combination with vitamin D (800IU) appendicular lean mass increased (<0.2kg) following 13 weeks of supplementation (Jürgen M. Bauer *et al.*, 2015). Conversely, when dietary protein is reduced 1.2-0.8g/kgBW for 10 weeks appendicular lean mass is decreased (Mitchell *et al.*, 2017), with similar being seen in the daily rate of albumin synthesis (40% decrease), when dietary protein is reduced (40g/day) for a week(Jackson *et al.*, 2001). However the long term effect of protein supplementation on MPS has been suggested to be limited, as over a 2 year period there was no difference in skeletal muscle mass between protein supplementation (30g of protein in 250ml skimmed milk) and control group (2.1g protein in 250ml skimmed milk) in healthy elderly women (Zhu *et al.*, 2015).

1.4.6 Specific protein sources and the impact on protein metabolism

The common dietary protein used to assess the impact on protein metabolism, is whey protein. This protein is one of two proteins derived from cows' milk, with the other being casein. It is commonly used in protein supplementation studies as it has been found to acutely stimulate

both muscle (Moore *et al.*, 2009; Atherton, Etheridge, *et al.*, 2010a; Pennings *et al.*, 2011; Witard *et al.*, 2014; Nabuco *et al.*, 2018) and whole-body protein synthesis (Boirie *et al.*, 1997). This is due to whey protein having a fast rate of absorption over the space of 30 minutes as it causes an acute hyper aminoacidemia (Boirie *et al.*, 1997) coupled with high in EAAs including branch chain amino acid and leucine (Pennings *et al.*, 2011).

In addition, beef fed at 113g (30g protein) has been shown to stimulate rates of mixed MPS in males and females of both young (35±3 yrs), middle age (41±8 yrs) and elderly (>65yrs) adults (Symons *et al.*, 2007, 2009). However Robinson *et al,* (2013) claimed that beef protein provided at 113g (approximately 24g of protein) does not increases myofibrillar protein synthesis, but is increased by 170g of beef (approximately 36g of protein) in elderly men (59yrs old). Although the protein content at 113g of beef in Robinson *et al,* (2013) is lower than that of Symons *et al* (2007, 2009) therefore beef protein needs to be greater than 30g to stimulate MPS. Pork has also been shown to increase MPS when provided at 36g of protein (170g of pork) in healthy adults but not with overweight (BMI: 27.1±0.5) or obese (BMI: 35.9 ±1.3; Beals *et al.*, 2016).

As mentioned in above different protein sources require different concentrations to stimulate MPS, predominantly animal proteins contain a greater concentrations of EAA than plant proteins (Table 1.3), therefore a greater amount of plant material is required to achieve the same dietary

protein concentration and thus stimulate MPS to the same extent, with evidence for this in both humans (Kouw *et al.*, 2021) and rats (Norton *et al.*, 2009). Also dietary animal protein intake has been associated with a greater increase in lean mass than plant protein (Lim *et al.*, 2021). As well as a positive correlation between muscle mass and animal protein intake in sedentary and moderately active middle aged women (Aubertin-Leheudre and Adlercreutz, 2009). Although soy stimulated MPS to a similar extent as whey when provided at the same concentration in young males (Tang *et al.*, 2009), therefore proteins with a high rate of digestion may have a greater impact on anabolic potential than protein origin. Thus the AA concentration and a protein with a high rate of digestion to induce a hyperaminoacidemia in the plasma to stimulate MPS.

	Plants								Animal products				Animal		
	Oat[1]	Lupin[1]	Wheat[1]	Soy[1]	Brown rice[1]	Pea[1]	Corn[1]	Potato[1]	Whey[1]	Milk[1]	Casein[1]	Egg[1]	Beef[2]	Chicken[3]	Pork[4]
EAA															
His	0.9	1.2	1.4	1.5	1.5	1.6	1.1	1.4	1.4	1.9	1.7	0.9	3.2	4.5	5.3
Iso	1.3	1.5	2.0	1.9	2.0	2.3	1.7	3.1	3.8	2.9	2.3	1.6	5.2	3.2	5.3
Leu	3.8	3.2	5.0	5.0	5.8	5.7	8.8	6.7	8.6	7.0	5.8	3.6	8.4	6.4	8.7
Lys	1.3	2.1	1.1	3.4	1.9	4.7	1.0	4.8	7.1	5.9	4.6	2.7	9.2	7.9	8.6
Met	0.1	0.2	0.7	0.3	2.0	0.3	1.1	1.3	1.8	2.1	1.6	1.4	3.2	2.6	3.0
Phe	2.7	1.8	3.7	3.2	3.7	3.7	3.4	4.2	2.5	3.5	3.1	2.3	4.3	3.2	4.4
Thr	1.5	1.6	1.8	2.3	2.3	2.5	1.8	4.1	5.4	3.5	2.6	2.0	4.6	3.7	4.8
Val	2.0	1.4	2.3	2.2	2.8	2.7	2.1	3.7	3.5	3.6	3.0	2.0	5.9	3.5	5.9
NEAA															
Ala	2.2	1.7	1.8	2.8	4.3	3.2	4.8	3.3	4.2	2.6	2.0	2.6	5.2	4.7	5.7
Arg	3.1	5.5	2.4	4.8	5.4	5.9	1.7	3.3	1.7	2.6	2.1	2.6	6.9	5.8	7.2
Cys	0.4	0.2	0.7	0.2	0.6	0.2	0.3	0.3	0.8	0.2	0.1	0.4	1.5	1.2	-
Glu	11.0	12.4	26.9	12.4	12.7	12.9	13.1	7.1	15.5	16.7	13.9	5.1	9.6	11.2	15.4
Gly	1.7	2.1	2.4	2.7	3.4	2.8	1.6	3.2	1.5	1.5	1.2	1.4	3.7	3.4	4.3
Pro	2.5	2.0	8.8	3.3	3.4	3.1	5.2	3.3	4.8	7.3	6.5	1.8	3.8	3.2	3.7
Ser	2.2	2.5	3.5	3.4	3.4	3.6	2.9	3.4	4.0	4.0	3.4	3.3	4.3	3.4	3.9
Tyr	1.5	1.9	2.4	2.2	3.5	2.6	2.7	3.8	2.4	3.8	3.4	1.8	3.9	3.7	3.9

Table 1.3: *Amino acid content of raw food materials in g/100g, green and red indicating the respective highest and lowest level for each specific amino acid. [1](Gorissen et al., 2018), [2](Wu et al., 2016), [3](Haščík et al., 2020) and [4]*(B. H. P. Wilkinson *et al.*, 2014).

1.4.7 The effect of dietary amino acids on protein metabolism

As mentioned in (section 1.3.1), AAs are fundamental dietary components to increase protein synthesis and suppress protein breakdown. When AAs are directly increased in the blood at a low, medium, high and very high concentrations (43.5, 87, 162 and 261mg/kgBW/hr), mixed muscle protein synthesis increased by 30, 57, 72 and 88%/hr respectively (Bohé *et al.*, 2001, 2003). The increased AA concentrations have a hyperbolic relationship with MPS, this is in line with the “muscle-full” effect by Atherton *et al,* (2010).

In addition, EAAs also actively stimulate MPS, particularly branch chain AAs and their metabolites (Smith *et al.*, 1998; Katsanos, 2006; Buse and Reid, 1975; Ham *et al.*, 2014; Naseeb and Volpe, 2017), as well as preventing skeletal muscle degradation (leucine metabolite i.e. ketoisocaproate and valine; Mitch and Clark, 1984; Garlick, 2005). MPS is greater when EAA are supplemented at 15g compared to whey protein in elderly people (Paddon-Jones *et al.*, 2006). In addition 15g of EAA stimulated MPS in elderly males, 90 minutes post ingestion, MPS remained increased until 180 minutes post ingestion (Mitchell *et al.*, 2017).

The main EAA for stimulating MPS is leucine (rats; Buse and Reid, 1975; Fulks, Li and Goldberg, 1975 and humans; Smith *et al.*, 1998; Wilkinson *et al.*, 2018). Where 3g, given as an oral or IV bolus (Bennet *et al.*, 1989),

has been shown to stimulate skeletal muscle protein synthesis approximately 2-fold (Atherton, Smith, *et al.*, 2010 oral protein; Wilkinson *et al.*, 2013 oral leucine). Although, as little as 1.5g of leucine stimulated MPS (Wilkinson *et al.*, 2018), with a maximum nutritional stimulation of MPS being achieved by the bolus supplementation of either 3g of leucine or 20g of whey protein containing 2g of leucine in elderly females (Bukhari *et al.*, 2015). However if additional leucine (2.5g) is provided to each meal for 7 days in young men (Backx *et al.*, 2018) and 12 weeks in elderly men (Verhoeven *et al.*, 2009) there is no increase skeletal muscle mass or strength. This could be that to identify noticeable changes requires a greater length of time to impact muscle mass, as in young men myofibrillar FSR increased from 0.04%/hr to 0.08%/hr following leucine supplementation. In addition, the increased rate of muscle protein synthesis lasts for approximately 90 minutes following the ingestion. When leucine (3g) is provided 90 minutes post ingestion, this has no impact on MPS, this means that “muscle-full” effect is reach from 15g of EAA. Although if the EAA are provided in hourly meals for 4 hours at 3.75g rather than a bolus of 15g then the same net increase in MPS occurs except the peak MPS is decrease and the length of time of elevated MPS are increased (Mitchell, Phillips, *et al.*, 2015).

1.1.1.7. The effects of different protein sources on anabolic pathways in the control of protein synthesis

As mentioned above leucine is the main AA for stimulating MPS, this is achieved through direct stimulation of the mTOR signalling pathway

(Rennie *et al.*, 2006; Atherton, Smith, *et al.*, 2010; W. K. Mitchell *et al.*, 2017). This pathway increases MPS through cap-dependent translocation, translational elongation, mRNA biogenesis and ribosome biogenesis (Bodine, 2006). One of the key protein signalling complexes that increases in response to AA stimulation is mammalian target of rapamycin complex 1 (mTORC1; Atherton, Smith, *et al.*, 2010; Mitchell *et al.*, 2015, Beal *et al.,* 2016). The activation of mTORC1 also increases in response to other stimuli such as insulin, glucose and the hormone insulin-like growth factor-1 (Sabatini, 2017). The activation of mTORC1 leads to the activation of P70 S6 kinase, this protein is another prominent signalling marker that is associated with protein synthesis. The activation of P70 leads to the phosphorylation of 4E-BP1 causing a dissociation from eIF4E allowing eIF4G to bind to eIF4E and the subsequent complex can directly increase protein synthesis through cap-dependent translation (Kim *et al.*, 2003). In addition, the activation of P70 S6 kinase blocks eEF2K allowing p-eEF2 to increase protein synthesis through translational elongation. Finally, the activation of P70 S6 kinase allow ribosomal protein S6 to bind to ribosomes biogenesis 5'-top translation (Wang *et al.*, 1998; Bodine, 2006).

1.4.8 The impact of anabolic resistance on MPS in elderly people

As previously stated (section 1.4.vii) amino acid can stimulate muscle protein synthesis, however under certain biological conditions (e.g. aging (Cuthbertson, Smith, Babraj, Leese, Waddell, Atherton, Wackerhage,

Peter M Taylor, *et al.*, 2005a; Katsanos *et al.*, 2005) and obesity) the stimulatory response is blunted. This term is referred to as anabolic resistance. In elderly 10g of EAA increased the rate of MPS to the same level as 5g of dietary EAA in young males. In addition the maximum rate of MPS from dietary EAA is lower in elderly males than young males (Cuthbertson *et al.*, 2005). Under these conditions where anabolic blunting is identified, key MPS signalling proteins (mTOR, 4E-BP1 and p70 S6Kinase) were reduced in both total protein concentration and the level of phosphorylation in elderly males, even though plasma leucine concentration was greater (Cuthbertson *et al.*, 2005). This suggests that anabolic resistance was apparent in elderly males as the plasma leucine concentration was elevated to excess so MPS was maximally stimulated.

1.5 Stable Isotopes as a metabolic tool

Stable isotopes are non-radioactive naturally occurring atoms that have a greater mass than the common element found on the periodic table (Table 1.4), this is due to the nucleus containing an extra neutron but maintaining the same number of protons and electrons to identify it to the common element on the periodic table. This mass difference can be easily measured by mass spectrometric techniques and therefore can be visualised and traced in biological tissues and fluids. As biological systems use common elements (carbon, hydrogen, oxygen, nitrogen and sulphur), the incorporation of stable isotope elements into molecules in the body can be utilised to track dynamic changes in metabolic pathways

and other aspects of metabolism using a range of metabolites ranging from carbohydrates, amino acids and lipids, or labelled water is used as water is found throughout the body. Examples of different stable isotope tracers used are; D_2O (Smith *et al.*, 2007; Gasier, Fluckey and Previs, 2010; D. J. Wilkinson *et al.*, 2014; Brook *et al.*, 2015) and $^{13}C_6$-phenylalanine (D. O Ball and Bayley, 1986; Elango, Ball and Pencharz, 2008).

Element	Abundance of the different masses (%)		
	0	+1	+2
Hydrogen	**99.985, ^{1}H**	**0.015, ^{2}H**	
Carbon	**98.892, ^{12}C**	**1.108, ^{13}C**	
Nitrogen	**99.63, ^{14}N**	**0.37, ^{15}N**	
Oxygen	**99.76, ^{16}O**	**0.037**	**0.204, ^{18}O**

Table 1.4: *The level of natural abundance (%) of the common elemental mass and the respective stable isotope*

AAs labelled with carbon-13, are useful in tracking the flux/movement of the carbon skeleton into protein, AA metabolism and energy metabolism via the TCA cycle. In addition, depending on the AA used as the tracer, the metabolic flux through a specific pathway can also be traced (e.g. leucine to HMB). Stable isotope tracers are extremely versatile and can integrated with a metabolic system without affecting the system. Therefore, stable isotopes can be a powerful tool for measuring rates of metabolism, with appropriate design, timed collection of body fluids i.e. blood, plasma, urine, breath and tissue, the major aspects of amino acid and protein turnover i.e. synthesis, breakdown, oxidation can be determined.

1.5.1 Methods to assess dietary protein and amino acid requirements

Currently more sophisticated methods are available to assess protein and AA requirement levels, including i) the nitrogen balance technique, ii) the 15N labelled AA technique, iii) the 24 hr leucine kinetics technique, iv) the AA oxidation technique and v) the AA balance technique. Each of these methods employs a different approach and set of assumptions and informs upon different aspects of protein metabolism as a way of assessing whole body protein requirements.

1.1.1.8. Balance studies

One of the most common types of techniques for assessing protein requirements are simple net balance studies. These studies compare the amount of a dietary component e.g. protein being ingested - intake with the amount being excreted. Such that when intake exceeds output there is a positive balance (net uptake), and conversely when the output is greater than the intake, the balance is negative (net release), when the two processes are matched then they are balanced (net zero). This can inform on the adequacy, inadequacy or excess of any given nutrient. For example when protein loss exceeds intake this would indicate a net loss of body protein as might occur during starvation, or in disease when body weight/muscle mass is lost. The most commonly and easiest of these techniques to apply is the nitrogen balance technique.

1.5.1.1. Nitrogen balance approach

The nitrogen balance technique is theoretically one of the simplest methods for identifying protein requirements in assessing the balance between protein (converted to an amount of nitrogen) entering (dietary intake) and leaving the body (urinary urea and or ammonia and Nitrogen in faeces). This method was once classified as the gold standard for measuring dietary protein needs, the technique makes several assumptions including:

- Nitrogen entering the body is assumed to be entirely derived from protein and thus AA sources and 16% of protein is assumed to be nitrogen. Therefore, the amount of nitrogen measured in the diet is multiplied by 6.25 (1/0.16; Kopple, 1987) to convert the value into a total crude protein content. However, there are several limitations with this assumption, firstly this excludes the potential of non-protein nitrogen sources in the diet. Secondly depending on the source of the protein, the percentage of nitrogen in protein can vary widely, from 13% to 19% (Jones, 1931). This problem is only associated when the nitrogen content is not assumed or extrapolated from diet diaries.
- The faecal nitrogen is variable, as the total nitrogen leaving the body is comprised of the undigested dietary nitrogen, undigestible dietary nitrogen, and intestine bacterial nitrogen.

- The faecal and urinary nitrogen are assumed to be the sole contributors to nitrogen excretion, however nitrogen is also excreted by other means such as sweat, loss of skin cells and hair.

1.1.1.9. Indicator amino acid oxidation approaches to assess amino acid requirements

Amino Acid Oxidation techniques use a ^{13}C labelled AA to determine the relative oxidation rate of an individual AA from the rate of $^{13}CO_2$ appearance in the breath. The change of this oxidation rate, when similar diets are provided, differing in an individual AA concentration from limiting to excess, can identify the AAs requirement level. There are two approaches commonly used to measure amino acid oxidation to determine AA requirements:

1) **The Direct Amino Acid Oxidation technique (DAAO)**, in which the stable isotope tracer AA is the same as the AA under investigation
2) **The Indicator Amino Acid Oxidation technique (IAAO)**, in which the stable isotope tracer AA is different to the AA under investigation.

Both methods follow a similar principal to Liebig's law of the minimum (Alexander N Gorban *et al.*, 2011). This is that when EAA are below the level of requirement in the body for protein synthesis, protein synthesis is restricted to the lowest AA level with any AA more than this level being assumed to be oxidised. Therefore, in the DAAO the oxidation rate

remains constant and increases when the AA under investigation becomes excess of protein synthesis. Whereas in the IAAO technique, increasing concentrations of the AA under investigation results in an increase in protein synthesis and a decrease in oxidation rates when limiting, the oxidation rate plateaus when the level of protein synthesis has peaked. The point of the shift oxidation rate either to or from a plateau is referred to as the breakpoint. This breakpoint is the AA requirement for the animal under the exact conditions of the study e.g. nutritional status, exercise, age, and reproductive cycle.

Both the DAAO and the IAAO approaches follow the same principles and design of the diets in the study. Each test diet must have similar diets to ensure the energy intake is fixed and that the proportion of CO_2 produced from fat and carbohydrate oxidation in the breath are uniform across the different diets. The protein content is the only component that changes either by varying the amount of protein or varying the amount of an essential AA. Then by measuring the rate of appearance of $^{13}CO_2$ in the breath and comparing the different diets the minimum amount of protein or AA can be identified. The standard methodology requires the diets are provided for 3 days, to establish an equilibrium in protein balance (G. A. Zello *et al.*, 1995).

The technique was first used in pigs (D. 0 Ball and Bayley, 1986) using ^{14}C phenylalanine to assess the tryptophan requirements. Since then this technique has been adapted for application in many monogastric mammals such as horses (Mastellar, Coleman and Urschel, 2016), rats

(Ogawa *et al.*, 2011, 2015; Kato, Nakano, *et al.*, 2016), dogs (Shoveller *et al.*, 2017a) and humans (Lazaris-brunner *et al.*, 1998; Kriengsinyos *et al.*, 2002; Elango, Ball and Pencharz, 2009). One of the major benefits of this technique more recently is that it is non-invasive, requiring only breath collection and therefore utilised to identify nutritional requirements in populations that are often difficult to assess for example, infants, in the first month of life (Hogewind-Schoonenboom *et al.*, 2015), children (Pencharz and Ball, 2004) and elderly (Martin *et al.*, 2019). In addition, it is very quick to measure requirement levels compared to nitrogen balance techniques as is only requires 5 days per diet (3 days on the diet and 2 days wash-out period; Elango, Ball and Pencharz, 2009)

In the IAAO technique, the options for a suitable indicator AA (AA stable isotope) are limited. The criteria for selecting an indicator AA (IAA) for this technique are (G. a Zello *et al.*, 1995):

1) It must be an essential AA, so it cannot be synthesised in the body. This ensures that the label is not diluted through de novo synthesis.

2) It must be irreversibly oxidised, this ensures the oxidation of the labelled carbon to CO_2 can be quantitatively calculated from the breath.

3) It would have a small pool to allow rapid labelling and reduce the costs of providing the labelled amino acid

4) The indicator AA would undergo limited metabolism, except for protein synthesis and oxidation. Additional metabolic pathways connected with the indicator AA must be inhibited/minimised to ensure that any change in the CO_2 production is related only to the utilisation of AA for protein synthesis.

Previous studies have used several different AAs as the indicator including leucine and phenylalanine (Hiroyuki Kato, Suzuki, *et al.*, 2016; Bross *et al.*, 2017; Turki *et al.*, 2017). ^{13}C-Phenylalanine, is the AA of choice in the indicator AA oxidation approach (IAAO). As phenylalanine is an EAA with a small pool size and it can be synthesised into protein, metabolised into tyrosine or oxidised. There are two main phenylalanine tracers that are used $^{13}C_6$ phenylalanine and 1-^{13}C phenylalanine, $^{13}C_6$ phenylalanine reflects a greater extent of oxidation than 1-^{13}C phenylalanine due to the position of the carbon 13 molecules. However, 1-^{13}C Phenylalanine reflects the first step in phenylalanine oxidation due to the carbon 13 molecule being positioned on the carboxyl group rather than the aromatic ring, reflecting oxidation occurring prior to phenylalanine carbon entering the Krebs cycle, when hydroxyphenyl pyruvic acid is converted into homogentisic acid. This reaction is after the phenylalanine molecule has been converted into tyrosine (Teraishi *et al.*, 2012). However, the formation of other tyrosine metabolites (dopamine, melanin and tyramine) also oxidise on the carboxyl carbon, therefore to ensure the oxidation is due to phenylalanine being excess to protein synthesis and not due to a demand of an aromatic metabolite tyrosine

must be provided to a sufficient quantity as to support protein synthesis and provide excess substrate for these alternative pathways. Another AA that is used as an indicator is leucine, however it has been reported that leucine is not suitable as an indicator AA due to leucine stimulatory effect on MPS (Hsu *et al.*, 2006). Although the levels required for leucine as a tracer compared to the level for leucine to stimulate MPS are insignificant. When the breakpoint values are compared using phenylalanine as a tracer there was no difference in the breakpoint levels (Hsu *et al.*, 2006).

1.6 Research ethics in nutrition research

The thirst for increasing scientific knowledge is a constant cycle. This is a cycle of questions, the answering of old questions, that then leads to the discovery of new questions needing answering. This is the core basis of all scientific knowledge, however restraint must be shown as sometimes the easiest way to answer these questions may not be the right way to answer the question. This is referring to the scientific method and how research should be conducted particularly when the research involves any animals as the life of the animal (human or otherwise) must be taken into consideration. It is from this that any research involving animals with vertebrae (plus cephalopods) ethical approval granted. The ethical approval came into being in 1986 (*The Animals (Scientific Procedures) Act 1986 Amendme Regulations 2012*, 2012)

Nutritional research in whole organisms in the category mentioned now requires evaluation as to balance the overall benefits that the research can provide for the scientific community and society against any potential negative implications that the animal could endure throughout the experiment. This cost benefit analyse is carefully measured and depending on the species of the animal depends on the importance of the life that is involve. For example, experimentation on a mouse would be easier to get ethical approval than a monkey for the same experiment, this would be a careful consideration of the worth of the animal. However before ethical approval is granted there are certain steps to protect the unnecessary use of animals in experimentation these are known as the 3 R's (Russell and Burch, 1959) they are;

- **Replacement** – to wherever possible to use alternative methods that do not use animals removing them from experiments.
- **Reduction** – where the animals cannot be replaced by an alternative experiment to use the least amount of animals possible to gain statistical significance. Therefore, power calculations are an important tool for assessing this.
- **Refinement** – When experimentation on animals is unavoidable the welfare both physical and mental must be protected to minimum amount of distress, including but not limited to adequate housing for the animals.

1.6.1 Ethics for human research studies

The premise behind the ethics for human research is to ensure that scientists wanting to carry out the experiments are responsible with the data, the tissue that they are using, and they are not taking advantage of the participants. In the early 1900s there was no effects required for research on humans however following the events of World War 2 the Nuremberg code requires any subject to volunteer for a study. Since then, human ethics has advance from this to assess the minimum number of volunteers but to research being integrated with good clinical practice. Therefore all research involving humans should (Mandal, Acharya and Parija, 2011);

- Be based on work from previous experiments in the lab or animals studies
- Have a protocol that has been independently vetted before the start
- Ensure all volunteers have given informed consent
- Any procedures are performed by an individual qualified to do so
- Undertake the experiment when the benefits are greater than the risks

1.6.2 Ethics for canine research studies

In the UK there were 4227 experiments conducted on dogs, between 2014-2019, with the majority of these experiments being conducted on beagles (4055), representing 0.2% of the total number of experiments that are performed on animals in the UK (Home Office, 2020). The deployment of the 3R's for research in dogs has been used to minimise the use of experiments in dogs (Hasiwa *et al.*, 2011):

- Optimise the experiments e.g. reducing the number control groups in parallel studies, using dogs for multiple studies
- Refinement of dog care e.g. improving staff training, re-homing after experiments
- Replacement of *in vivo* experiments and use *in vitro* and computer modelling.
- Increasing the use of non or minimally invasive experiments

1.7 Overall Thesis aims

The main aims of the thesis was to gain a greater understanding of protein and amino acid metabolism throughout the body, to try and optimise the efficiency of protein utilisation using various stable isotope techniques in three different models, dogs, humans and *in vivo,* whilst maintaining the 3 R's where possible. The thesis will achieve this by:

- Refining an amino acid oxidation technique in dogs to determine methionine requirements.
- Comparing the fractional synthetic rate of muscle and albumin with and without HMB supplementation in males and females
- Comparing two methods for assessing protein synthesis *in vivo,* whilst grown on either hydrolysed plant or animal based protein source
- Designing a refined indicator amino acid oxidation experiment to reduce the study day burden on participants.

2. The assessment of methionine requirement using Indicator Amino Acid Oxidation technique in dogs

2.1 Introduction

2.1.1 Chapter Synopsis

This chapter refines the indicator amino acid oxidation technique, a stable isotope method to assess amino acid requirements, in dogs. This method measured the methionine requirement as there are unique parameters that define the methionine requirement level for dogs, which will be addressed later in this chapter. To ensure the robustness of the method it was repeated on 3 separate occasions, with the results showing minimal variability in both the breakpoint range (the concentration at which the amino acid becomes surplus to the requirement level in the test diet) of 0.32 – 0.39g/1000kcal. The variability didn't alter even when different concentrations of stable isotope priming (7 and 5mg/kgBW) doses where used. The results from this chapter have suggested that:

1) The indicator amino acid oxidation technique can be measured non-invasively in dogs.
2) The methionine requirement level can be reduced.

3) The breakpoint can be detected in a non-steady state provided that the concentration is sufficient to detect the changes in oxidation.

2.1.2 The uniqueness of the minimum methionine requirement in dogs

The minimum recommended requirements for EAA, provided by the NRC (2006) based on the research are presented in Table 1.1. From this table the minimum sulphur amino acid (SAA) recommendations are unique compared to the other EAA recommendations. As the minimum recommendations for the majority of the EAA are based on the optimum growth rate via a dose response method, whereas the methionine minimum requirement level is based upon the prevention of dilated cardiomyopathy (DCM) via taurine deficiency (a SAA metabolite; Sanderson *et al.*, 2001). DCM is the dilatation of the hearts left ventricle resulting in systolic dysfunction (Elliott *et al.*, 2008). The condition is associated with low concentration of plasma and whole blood taurine levels for 1-2 years (<40µmol/L; Sanderson *et al.*, 2001; Delaney *et al.*, 2003; Ko *et al.*, 2007). The main theories for how low blood taurine levels lead to DCM are:

1) An imbalance in the metabolizable energy and sulphur AA intake in larger dog breeds (Tôrres *et al.*, 2003)

2) Reduction in the bioavailability and absorption of taurine into the body (Tôrres *et al.*, 2003).

3) An increased demand of conjugated taurine in bile salts, when a high fat diet is provided (Sanderson *et al.*, 2001).

DCM is most common in large breed dogs (Tôrres *et al.*, 2003; Vollmar *et al.*, 2013) being fed diets containing high levels of lamb and rice (Delaney *et al.*, 2003; Tôrres *et al.*, 2003), pulses or potatoes (FDA, 2019). Fortunately, this condition is reversable, when plasma taurine levels are increased, via the dietary supplementation of either taurine or methionine (Sanderson *et al.*, 2001; Backus *et al.*, 2003; Mansilla, Fortener and Shoveller, 2006). Thus, the minimum methionine requirement level is greater than the level to support protein synthesis as a preventative measure. However recent research reassessing the methionine requirement in Labrador retrievers, a large breed dog more susceptible to DCM, identified a mean breakpoint value for methionine for protein synthesis as 0.55g/1000kcalME (with a 95% CI of 0.41 and 0.71) on a 95 kcal/kgBW$^{0.75}$, using the IAAO technique (Harrison *et al.*, 2020), compared to the minimum requirement of 0.65g/1000kcalME on a 130kcal/kgBW$^{0.75}$. If the different requirement values are converted to the same units (g/kgBW) to compare, the values are 0.017, 0.023 and 0.030 (0.41, 0.55 and 0.71 g/1000kcalME respectively for Harrison *et al* 2020) and 0.037 and 0.047 (the minimum requirement and recommended allowance respectively from the NRC; 2006). These results suggest that the upper 95% CI (0.71 g/1000kcal), would be a suitable concentration for most of the population, however due to the high level of variability from this study the methionine requirement in dogs

requires further investigation. In addition, the study by Harrison *et al* (2020), also compared the chronic effect (32 weeks) for 3 methionine concentrations (the mean - 0.55 g/1000kcalME on a 95kcal/kgBW$^{0.75}$, the upper 95%CI - 0.71 g/1000kcalME on a 95kcal/kgBW$^{0.75}$ and the NRC recommend allowance - 1.37g/1000kcalME on a 95kcal/kgBW$^{0.75}$) there was no reduction in the plasma taurine concentrations in any of the diets, suggesting that the methionine requirement can be lower than suggested. However, the 95% CI range 0.41-0.71 g/1000kcal is highly variable thus requires further investigation to identify an accurate minimum methionine requirement with the IAAO technique in dogs.

2.1.3 The development of the methionine requirement in dogs

One of the first papers to report the importance of methionine in dogs for both sulphur retention and nitrogen retention in adult and growing dogs was by Stekol and colleagues in the 1930's (Stekol, 1935). They investigated the supplementation of methionine in dog diets and then measured the sulphur excreted in the urine and calculated the recovery. The study supplemented methionine to young and adult dogs in a low protein diet and measured the excretion of sulphur and nitrogen, it was shown that that nitrogen retention increased in relation to methionine supplementation. This is due to the increased dietary methionine that would have increased protein synthesis and decreased the excess concentration of AAs being broken down for energy, this results in increased nitrogen retention/decreased nitrogen excretion. The study

only investigated the effect of a bolus doses of methionine rather than optimum requirement for protein synthesis. Rose and Rice (1939) later identified that methionine was an EAA required for growth in young dogs, this was later supported by Milner and colleagues (Milner, 1979) who, through a nitrogen balance technique, identified that a diet without methionine resulted in a negative nitrogen balance (-1.33 g of nitrogen/week) in Beagles. Miller (1944) also identified the supplementation of choline in a low protein diet had a protein-sparing effect, it was suggested that choline can be used to re-synthesize methionine. In addition, it indicated that methionine can be a rate limiting AA in low protein diets. Gessert and Phillips (1956), also identified methionine and lysine are the rate limiting AA in low protein diets for dogs, the growth rate can be recovered when these AA are supplemented in the diet.

Based on this identification of methionine as one of the most limiting AA for growth in dogs, numerous assessments of methionine requirements have since been attempted (Table 2.1). Most of these studies have assessed methionine requirement levels in young dogs who are still growing, which increases the reported requirement level for methionine, as these animals would have a greater level of protein synthesis than older animals (NRC, 2006).

Technique	***SAA***	***PRRL***	***Units***	***Breed***	***Age***	***Additional unique features to the study***	***Reference***
N balance	Methionine	0.025	g/g of casein nitrogen	Mongrels	NA	Casein fed at 3g/day/m^2 of body Surface area	(Allison, Anderson and Seeley, 1947)
N Balance	Methionine	0.6	g/1000kcalME	Beagles	8-16 wks.	With cysteine at 0.45g/1000kcalME	(Robert A. Burns and Milner, 1981)
N balance	Methionine	1.17	g/1000kcalME	Beagles	8-16 wks.	Without cysteine	(Robert A. Burns and Milner, 1981)
Weight gain	TSAA	0.45	%	English Pointer	Young	Methionine: cysteine ratio of between 3:1 and 1:1	(Hirakawa and Baker, 1985)
N retention	TSAA	1.55	g/1000kcalME	Labradors and Beagles	6 wks.	soya based diet, so digestibility =80%	(Blaza *et al.*, 1982)
Plasma taurine conc.	Methionine	1.3	g/1000kcalME	Beagles	Adults	High fat and low-fat diet comparison	(Sanderson *et al.*, 2001)
IAAO	Methionine	0.71	g/1000kcalME	Labradors	Adults		(Harrison *et al.*, 2020)
IAAO	Methionine	0.82	g/1000kcalME	Miniature dachshunds	Adults		(W. D. Mansilla *et al.*, 2020)
IAAO	Methionine	1.24	g/1000kcalME	Beagles	Adults		(W. D. Mansilla *et al.*, 2020)
IAAO	Methionine	1.40	g/1000kcalME	Labradors	Adults		(W. D. Mansilla *et al.*, 2020)

Table 2.1: *A compilation of studies assessing methionine requirements in dogs identifying the breeds, age experiments used the compounds measuring and the unique aspects of the studies that could cause variability in the results. PRRL- Potential recommended requirement level N_2 – Nitrogen, TSAA- Total Sulphur Amino Acids*

The first study assessing the methionine requirement in dogs, used nitrogen balance technique to assess the optimum dietary methionine level using casein as the main protein source, at a ratio of 1:40 (methionine to casein; Allison, Anderson and Seeley, 1947). However, no true requirement can be identified from this study as there is a variability of methionine in casein that would increase the methionine requirement. Therefore the methionine requirements provided by the National Research Council (NRC, 2006) are based upon Burn and Milner's work in the 1980's (Burns, Milner and Corbin, 1981; Burns and Milner, 1981; Burns and Milner, 1982; Burns, Garton and Milner, 1984; Milner, Garton and Burns, 1984), the recommendation of 0.6 g/1000kcalME of methionine, provided that cysteine is fed at 0.45 g/1000kcalME, or 1.17 g/1000kcalME of methionine if given alone. These values were derived using nitrogen balance and crystalline AA that are highly digestible, in immature Beagles. The use of crystalline AA may have increased the digestibility of the amino acids thereby not providing a realistic value for nitrogen balance studies, as greater absorption would decrease faecal nitrogen excretion. Following these studies, an alternative methionine requirement was suggested of 1.55g/1000kcalME, the digestibility was assumed to be 80% (Blaza *et al.*, 1982). Therefore, although this level appears greater than the previous studies, when the digestibility factor was 'corrected' for, then the recommended methionine requirement to provide both methionine and cysteine was 1.24g/1000kcalME. This value was only 0.07 g/1000kcalME greater than the requirement level recommended by

Burns and Milner (1981), with this slight difference likely attributable to differences in breed or age (Hawthorne *et al.*, 2004).

A key paper in defining the safe dietary SAA and methionine requirements, measured secondary effects of a sulphur metabolite (taurine) and the association with DCM, rather than the minimum requirement for protein synthesis (Sanderson *et al.*, 2001). It assessed the effect of dietary fat content in a low protein and maintenance level of methionine (1.2-1.3 g/1000kcalME) diet in dogs for 48 months. The diets that were tested were a high fat (24% fat content), low fat (10% fat content) and a high fat with carnitine supplementation. Through changing the fat content of the diet intentionally puts a strain on the SAA pathway and specifically taurine levels, as conjugated taurine is used in bile for fat metabolism in dogs (Wildgrube *et al.*, 1986). The high fat diet with carnitine increased the availability of methionine, by reducing the demand for SAM. However, even with carnitine supplementation, one dog developed DCM and another required taurine supplementation to alleviate symptoms. The paper concluded that low taurine causes DCM in dogs. However, the only diet to show signs of DCM was the carnitine supplemented diet and plasma taurine concentration was unaffected, suggesting that carnitine supplementation was a factor in DCM rather than taurine. This questions the usefulness of the paper to be utilised as a method for defining methionine requirements. Particularly because of a few key reasons:

1) The diets provided were deliberately high in fat to increase the strain on the SAA pathway through increasing a demand for taurine in digestion.
2) The only dogs to develop DCM were dogs that were given carnitine, this was provided to protect the SAA pathway with the aim to increase taurine availability, the results indicated the opposite effect.
3) Dietary taurine was used afterwards to reverse the effect of DCM, rather than providing dietary methionine.

The results from Sanderson *et al* (2001) should only be used to recommend a minimum dietary taurine requirement as a protective measure rather than increasing the minimum methionine requirement to 0.65g/1000kcal from 0.6g/1000kcal (NRC, 2006). Although the increase is not a great increase, it suggests that low levels of methionine are associated with DCM which has not been proven.

More recently, a lower methionine requirement has been determined via the indicator amino acid oxidation (IAAO) technique, using a stable isotope tracer to measure amino acid oxidation relating to amino acids in excess for protein synthesis. The results determined that the predicted mean of methionine requirement was 0.55 g/1000kcalME with an 95% Confidence Interval (CI) of 0.41 and 0.71g/1000kcalME, (lower and upper respectively), with an energy intake of 108kcal/kgBW$^{0.75}$ (Harrison *et al.*, 2020). A long-term study with the mean, upper and excess current methionine requirement levels (0.55, 0.71 and 1.37 g/1000kcalME), it

identified no indications of health problems at any level of dietary methionine. However, there was increased choline oxidation with lower dietary methionine, suggesting that utilisation of betaine and choline was directed into methionine resynthesis to maintain the methionine concentration to support normal bodily functions. As another role for choline is transporting fat from the liver as well as a precursor for acetylcholine (a common neurotransmitter in mammals) (Zeisel and Blusztajn, 1994), this shift in metabolic pathways suggests that the methionine requirement could be lower than the current advised methionine level, provided the choline level is adequate to support this without compromising its other roles.

A separate IAAO study estimated methionine requirement in small, medium and large breed dogs (miniature dachshund (D) , beagle (B) and Labrador (L) respectively) with 4 dogs in each group (W. D. Mansilla *et al.*, 2020). The methionine requirement and 95% confidence interval (CI) were 0.57-0.7 g/1000kcal with 95% CI of 0.822 for D, 0.914g/1000kcal with a 95% CI of 1.238 for B and 0.973g/1000kcal with a 95% CI of 1.397 for L. However, the mean energy intake of the diets differed depending on which breed was being assessed (92.4, 111.6 and 123.78kcal/kgBW$^{0.75}$, for D, B and L respectively). The D breakpoint value is closest to the previous methionine IAAO estimate (Harrison *et al.*, 2020). However, the breakpoint value could be even lower in D because there was only 1 diet before the breakpoint, making it difficult to determine an accurate breakpoint value. The data suggests that the

breakpoint value was between the lowest two methionine levels. This could imply that the breakpoint value was lower than the level reported with more oxidation values when methionine was below the breakpoint level. Also, there was a large amount of variability surrounding the line post breakpoint 0.9-1.9mmol/kg/hr $F^{13}CO_2$ in the D therefore making it difficult to identify an exact breakpoint with this sample size.

From these results in the IAAO technique the predicted mean for the minimum requirement (95% CI) was vast (0.41-0.71g/1000kcalME). Technical and practical issues may limit the robustness and accuracy of this study, therefore we looked to optimise the application and measurement accuracy of the IAAO technique in dogs.

2.1.4 Previous assessment of IAAO in Dogs

In the previous mentioned IAAO work in dogs (Harrison et al 2020), the mean methionine requirement was estimated to be between 0.41-0.71g/1000kcalME on a 108kcal/kgBW$^{0.75}$. This was a large range for the predicted mean with the highest being 42% greater than the lowest value (figure 2.1). This variability around the breakpoint can display an imprecise measurement for the methionine breakpoint (Harrison *et al.*, 2020). It was suggested that this may be due to either natural biological variation and/or a need to optimise the technique. If the biological variation was the cause this would mean that the dogs (Labradors) processed methionine to differing degrees questioning the variability of all other nutrient metabolism within dogs such as fat metabolism (as it is

linked with the SAA pathway) and amino acid metabolism. Therefore, it is more plausible that the method requires optimisation, the main area for improvement was the concentration of stable isotope provided. This is because there are some dogs with phenylalanine oxidation rates less than 0.005µmol/kg/hr in a diet with no methionine (figure 2.1). It would be unlikely that the oxidation rate of phenylalanine would be that low in this situation. Therefore, suggesting that the enrichment was not great enough to detect the oxidation rate. This study had a priming dose of 3mg/kgBW and a top-up dose of 2mg/kgBW (Harrison *et al.*, 2020), usually to achieve a steady state in stable isotope experiments the priming dose is greater than the top-up dose (Rachelle Bross, Ball and Pencharz, 1998). Thus, the priming dose would be the most plausible concentration to increase the overall enrichment of CO_2.

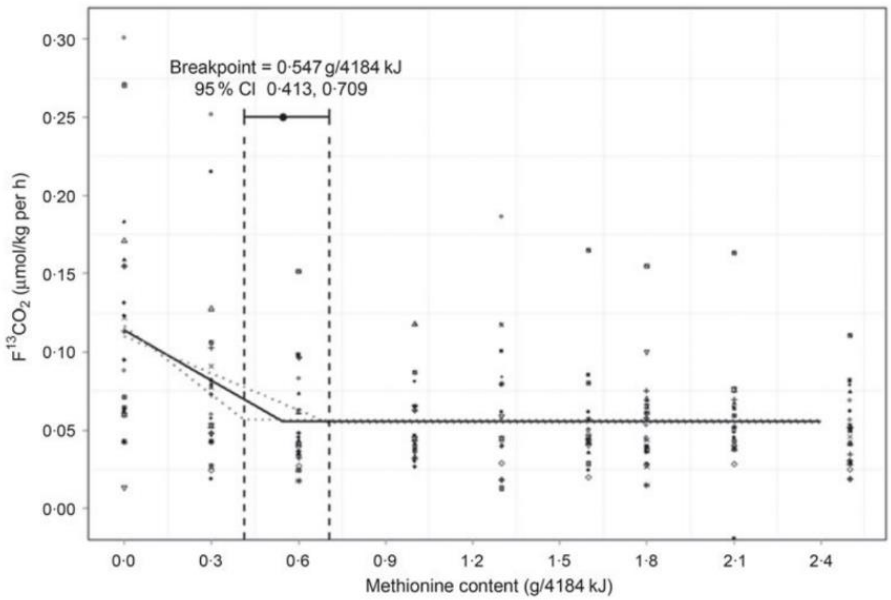


Figure 2.1*: The breakpoint analysis of methionine from Harrison et al (2020)*

Table (2.2) displays the breakpoints for each of these studies and a percentage difference between the upper 95% CI and the mean breakpoint. From this, the methods by Templeman *et al* (2019) and Mansilla *et al* (2020) contain the lowest variability around the breakpoint (<20%) for some of their experiments. However, the method may need some improvement, as some of their data (Mansilla *et al* (2020) was either in excess of 30% or the breakpoints were unable to be determined with the miniature dachshunds. Therefore, to achieve accurate and robust requirement levels with minimal variability the optimisation of the IAAO technique is required.

Breed of dog	No. of dogs analysed	Technique	No. of diets	Amino acid assessing	Breakpoint[b]	Upper 95% CI[b]	Percentage difference[c]	Papers
Mixed breed	5	DAAO	6	Phenylalanine	0.535	0.645	21%	(Shoveller *et al.*, 2017)
Mini. Dachshund	4	DAAO	7	Phenylalanine	0.262	0.358	37%	(W. D. Mansilla *et al.*, 2018)
Beagle	4	DAAO	7	Phenylalanine	0.258	0.365	41%	(W. D. Mansilla *et al.*, 2018)
Labrador ret.	4	DAAO	7	Phenylalanine	0.304	0.463	52%	(W. D. Mansilla *et al.*, 2018)
Mini. Dachshund	4	IAAO	7	Tryptophan	0.154	0.187	21%	(Templeman *et al.*, 2019)
Beagles	4	IAAO	7	Tryptophan	0.218	0.269	23%	(Templeman *et al.*, 2019)
Labrador ret.	5	IAAO	7	Tryptophan	0.157	0.204	30%	(Templeman *et al.*, 2019)
Mini. Dachshund	4	IAAO	7	Threonine	NA	NA	-	(W. Mansilla *et al.*, 2020)
Beagles	4	IAAO	7	Threonine	0.361	0.549	52%	(W. Mansilla *et al.*, 2020)
Labrador ret.	5	IAAO	6	Threonine	0.427	0.518	21%	(W. Mansilla *et al.*, 2020)
Labrador ret.	21	IAAO	9	Methionine	0.547[a]	0.709[a]	30%	(Harrison *et al.*, 2020)
Mini. Dachshund	4	IAAO	7	Methionine	0.635[a]	0.822[a]	29%	(W. D. Mansilla *et al.*, 2020)
Beagles	4	IAAO	7	Methionine	0.914[a]	1.238[a]	35%	(W. D. Mansilla *et al.*, 2020)
Labrador ret.	4	IAAO	7	Methionine	0.973[a]	1.397[a]	44%	(W. D. Mansilla *et al.*, 2020)

Table 2.2. *The different amino acid oxidation techniques breakpoints and 95% confidence interval for both the DAAO (the top 4) and the IAAO (bottom 7) measured on dogs and their breeds measured in. [a]g/1000kcalME [b](percentage of the diet (as-fed basis) [c]percentage difference between the mean breakpoint and the upper 95% confidence interval (CI)*

2.1.5 Objective

The primary objective of this study was to improve the accuracy and validity of the indicator amino acid oxidation (IAAO) technique reported previously (Harrison *et al.*, 2020), and to increase the precision of the IAAO technique to try and better determine the methionine requirement of adult large breed dogs.

2.2 Methods

A total of thirteen Labradors (5:8; male: female ratio with one neutered female) participated in the trial; with an average age of 4.7±1.5 years. Energy intake was optimised to maintain an ideal body condition score (4-5) using the Royal Canin 9-point scale (Freeman *et al.*, 2011). Body condition and bodyweight were assessed weekly, and the caloric intake of the dogs altered accordingly. This technique was adopted to reduce variability in body mass and composition.

2.2.1 Diets

All diets were semi-purified (manufactured by Ssniff Spezialdiaten Gmbh) allowing manipulation of the methionine content whilst other nutrients remained constant. The ‘base’ diet was nutritionally complete in accordance to 1.5x NRC guidelines, adjusted to a daily energy intake of 95kcal/kgBW$^{0.75}$(NRC, 2006). Test diets (A-D) were devoid of phenylalanine, and only differed in methionine content (Table 2.3).

Amino acids (g/1000kcalME)	Base diet	Diet A	Diet B	Diet C	Diet D
Methionine	1.7	0	0.3	0.6	1.5
Phenylalanine	2.39	0	0	0	0

Table 2.3: *The amount of methionine and phenylalanine in each diet*

2.2.2 Study design

The dogs were first acclimatised to the base diet for 14 days (1 meal/day), before being transitioned to 3 days on a test diet. Days 1 and 2 of these 3 days was diet habituation where the dogs were provided 4 meals/day. Day 3 was the day of IAAO sampling (breath samples) in which the dogs were provided 8 meals/day. The nutritional provision on each of the 3 test diet days was identical. After completion of the IAAO sampling day, dogs returned to the base diet for 4 days (1 meal/day) before again being habituated to the next test diet (Figure 2.2). This routine was repeated to ensure all dogs received all diets on three separated occasions (rotation 1, 2 and 3). The order of the diets was randomly allocated, to account for any carryover effect from a previous diet.

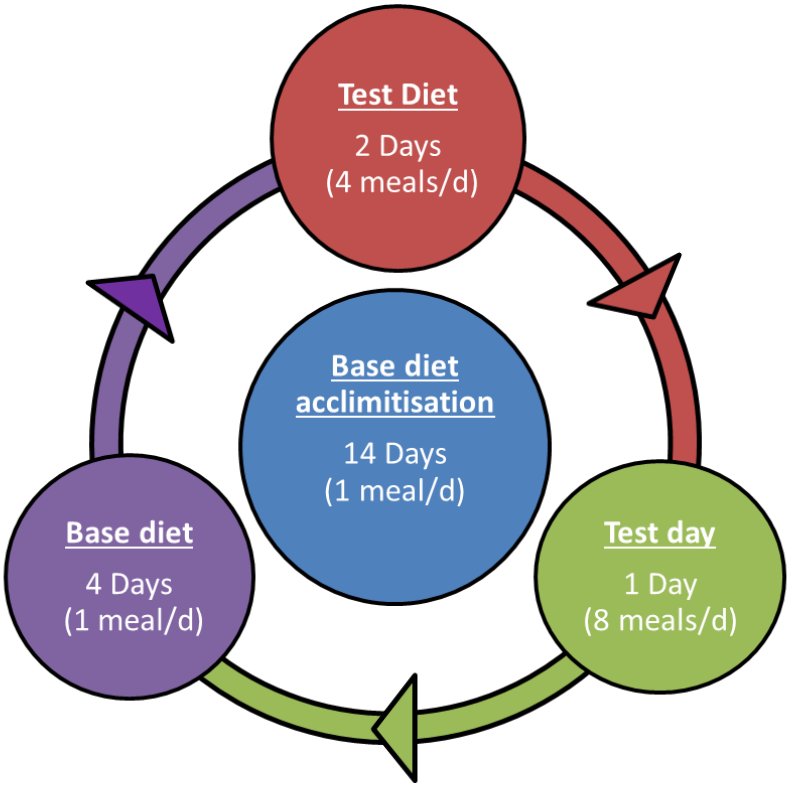


***Figure 2.2**: Outline of the weekly dietary schedule, Base diet in purple, test diet pre acclimatisation period in red and the sample collection day of test diet in green. This was repeated for each test diet*

On the IAAO sample collection days, the daily ration of food (95kcal/kgBW$^{0.75}$) was divided into 8 equally weighed meals fed at hourly intervals (Figure 2.3). Meal 1 contained the complete daily phenylalanine which was added as a powder on top of the food pellets. Meals 2 and 3 did not have any additions. Meal 4 contained the priming dose of 1-^{13}C-phenylalanine; which was 7mg/kgBW for rotation 1 or 5mg/KgBW for rotations 2 and 3. The priming dose for rotation 1 was chosen as the results indicated in the previous IAAO study in dogs (Harrison *et al.*, 2020) suggested that the priming dose used (3mg/kgBW) was under primed, as some of the dogs in the study had no change in oxidation rate

when fed diets with no methionine in their diet. However the priming dose was reduced between rotation 1 and 2 as there was an over priming effect, where the difference between the priming and top up dose was too great, leading to the top up dose being insufficient to replace the oxidised ^{13}C-phenylalanine, resulting in a non-steady state. To correct to a steady state condition either decreasing the priming dose, or increasing the top up dose, is required. The decision was made to reduce the priming dose to ensure that the enrichment of the samples remained at a similar level. This also resulted in 3 priming doses being used to measure the methioinine requirment with the same or similar methods, with rotation 1 and previous IAAO work (Harrison *et al.*, 2020). Meals 5-8 contained a top-up dose of 1-^{13}C-phenylalanine (2mg/kgBW) added on top of the respective meals. The dogs were encouraged to lick the bowls clean once each meal was consumed to ensure the tracer was consumed. A total of 8 breath samples were collected throughout the sampling day; 2 fasted samples (-210 and -180 minutes before the priming dose was supplemented), 2 fed samples between meals 3 and 4 to act as the baseline samples (-60 and 0 minutes i.e.pre-^{13}C-phenylalanine) and then 4 futher samples at 30 minute intervals beginning 25 minutes after meal 7 (210, 240, 270 and 300 minutes), when it is assumed there is a steady state of plasma ^{13}C-phenylalanine labelling (Harrison *et al.*, 2020).

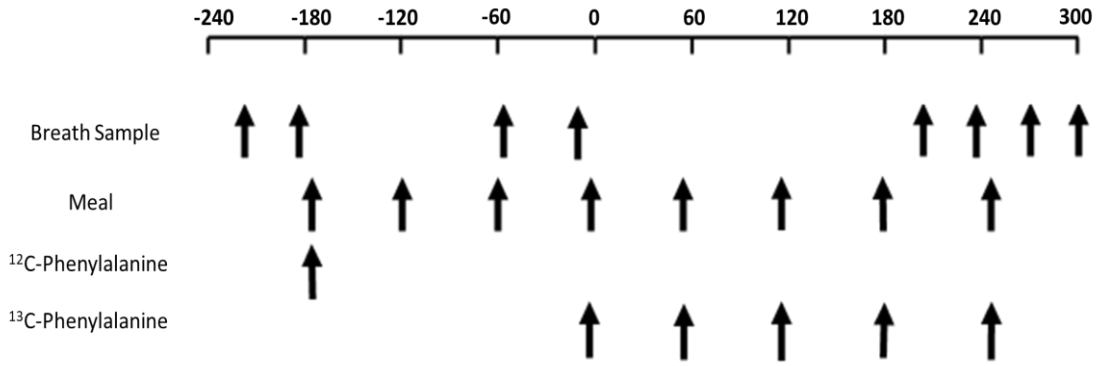


***Figure 2.3**: The protocol on sample collection days*

It should be noted that two changes to the protocol outlined above caused the rotations to differ slightly:

1) Rotation 1 had an N of 10 rather than 12 due to two of the dogs having incorrect tracer dosings provided to them on one of the sample days causing their data to be removed from analysis.

2) Rotation 3 had one of the dogs was swapped out of the study due to palitability issues, and therefore an alternative labrador of similar age was used.

2.2.3 Sample collection and analysis

Breath samples were collected with the use of a canine respiratory mask and breath collection bags (Quintron, QT00830-P). Once a normal breathing rhythm in the mask was established, the collection bags were attached, and the breath samples collected. Approximately 12 ml of breath was removed from the sample bag using a syringe and injected into an evacuated vacutainer. Two samples were collected from each

breath sample. The samples were analysed on a continuous flow isotope ratio mass spectrometer (CF-IRMS; AP2003, Analytical Precision, UK; see figure 2.4 for trace). Instrument quality controls were run throughout each sample batch to ensure the accuracy and reliability of the analyses, including a peak centre (where carbon dioxide reference gas is passed into the source and the optimum voltage set at the centre of the plateau of voltage to CO_2 signal) before the start, at the end and in between each set of breath samples. In addition, a stability test (six 20 second peak centres were obtained over 4 minutes to test instrument reproducibility) was run at the beginning of and at set periods during a batch run. If either of these tests failed the instrument would stop the batch and await operator intervention.

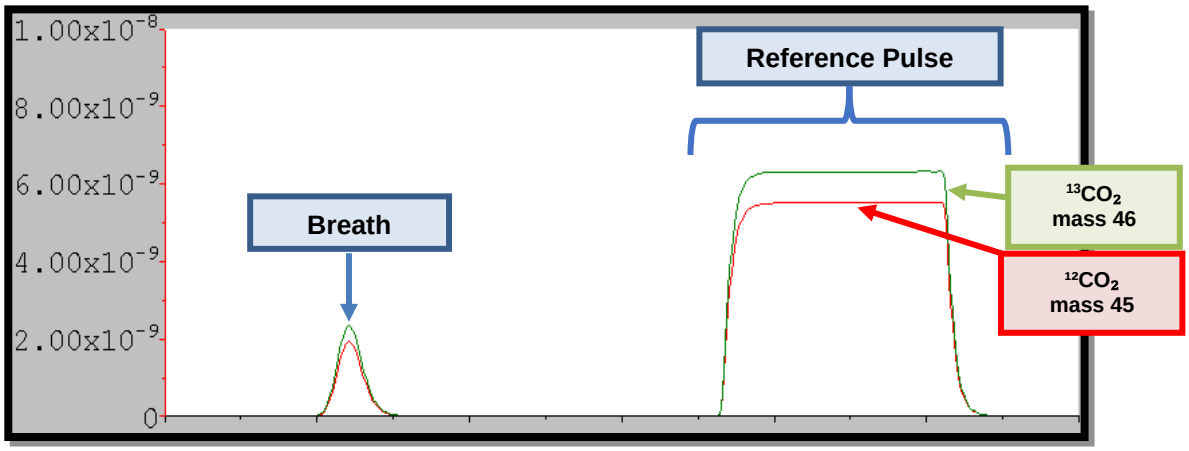


***Figure 2.4**: Trace of the Continuous flow isotope ratio mass spectrometry of $^{13}CO_2$ breath*

2.2.4 Data analysis

The Isotope ratio mass spectrometer results quantify the breath samples in a delta ^{13}C value. This is a the ratio of $^{12}CO_2$ to $^{13}CO_2$ of the breath sample relative to an international standard (pee dee belemnite (PDB; Craig, 1957)). Using the calculation below an Atom percent (AP) (the isotopic enrichment from the baseline sample) can be calculated from the delta ^{13}C value, where R is the ratio of ^{13}C to ^{12}C of the international standard PDB (R=0.0112372) and the change in enrichment from baseline is defined as the atomic percent Excess (APE).

$$\mathbf{AP=100/1/(((delta\ ^{13}C_{sample}/(100+1))*R)+1)}$$

$$\mathbf{APE = AP_{sample} - AP_{Baseline}}$$

The rate of appearance of $^{13}CO_2$ in the breath ($F^{13}CO_2$) (µmol/kg/h) was then calculated with the equation:

$$\mathbf{F^{13}CO_2=(RCO_{2(bicarb.)})*(ECO_2)*44.6*60/BW*RF*100}$$

where the ECO_2 is the $^{13}CO_2$ expired after ^{13}C-Phenylalanine consumed (APE) and BW is the bodyweight in kg. The constants 44.6 and 60 were used to convert FCO_2 into µmol/hr. The RCO_2 for 6 of the dogs was calculated from the breath samples of ^{13}C sodium bicarbonate supplementation in a previous study (Harrison *et al.*, 2020), using the equation below:

$$RCO_2=(D/AUC)RF$$

Where D is the tracer dose administered (mol), AUC is the area under the ^{13}C enrichment-time curve (ppm/min) and the RF is the fractional ^{13}C recovery in breath CO_2 was assumed at 0.82 (Elango *et al.*, 2009; Harrison *et al.*, 2020). The remaining 6 dogs had their RCO_2 substituted with a constant mean of 13.307 mol/ml, (Kerry Grey, Pers Comm from Internal WALTHAM report) measured using CF-IRMS a similar method to Larsson, Junghans and Tauson (2010). From previous work conducted at Waltham Petcare Science Institute where these 6 dogs conducted a bicarbonate RCO_2 oxidation study the asses the bicarbonate retention in dogs like other experiments conducted in humans, horses and rats (Pouteau *et al.*, 2002; Jensen *et al.*, 2015; Junghans *et al.*, 2015).

2.2.5 Statistical analysis

A segmental linear regression in a two-phase linear regression model was used to predict the breakpoint, the AA oxidation threshold for methionine dietary intake levels, and the 95% confidence interval around the breakpoint and the preceding plateau. An analysis of variance (ANOVA) was then used to calculate the difference between breakpoint variables in each of the rotations. A students T-test was used to compare the difference between the breakpoint of the individual RCO_2 and the constant mean RCO_2 of 13.307 mol/ml. All statistical analysis was

performed in GraphPad Prism (version 8.4.2. San Diego), and the level of significance was set at $p<0.05$.

2.3 Results

2.3.1 Methionine delta c values

The mean raw breath samples (table 1.3.1 A-C, rotation 1-3 respectively) indicate that the fasted baseline breath samples (T= -210 and -180) and the fed baseline breath samples (T=-60 and 0) have very little variability around each mean (SD>1.04), whereas some of the enriched breath samples have a greater variability (SD>4.06) particularly in the lower levels of dietary methionine which suggests that the treatment has had an effect. To assess the extent of the effect the individual breath samples were converted into AP values and then the baseline average (-60 and -180 minutes) were then subtracted from one of the enriched breath samples (210, 240, 270 or 300) to calculate the APE (table 2.4 A-C).

A) Rotation 1	***Dietary Methionine Content (g/1000kcal)***			
Time (min)	**0**	**0.3**	**0.6**	**1.5**
-210	-25.24 ±0.81	-25.99 ±0.94	-26.13 ±0.76	-25.81 ±0.86
-180	-25.26 ±0.76	-26.01 ±1.04	-26.34 ±0.93	-25.68 ±0.89
-60	-24.54 ±0.62	-24.66 ±0.88	-25.00 ±0.71	-24.63 ±0.53
0	-23.13 ±0.62	-24.63 ±0.64	-24.96 ±0.55	-24.60 ±0.36
210	-12.78 ±4.06	-20.63 ±2.64	-23.44 ±0.56	-23.57 ±0.67
240	-14.27 ±3.81	-21.55 ±1.83	-23.49 ±0.56	-23.68 ±0.43
270	-14.09 ±3.20	-22.19 ±1.43	-23.56 ±0.57	-23.68 ±0.44
300	-15.13 ±2.28	-22.73 ±1.06	-23.65 ±0.52	-23.72 ±0.40

B) Rotation 2	***Dietary Methionine Content (g/1000kcal)***			
Time (min)	**0.0**	**0.3**	**0.6**	**1.5**
-210	-25.09 ±0.68	-25.72 ±0.84	-25.75 ±0.99	-25.82 ±0.94
-180	-24.92 ±0.60	-25.74 ±0.90	-25.86 ±1.01	-25.85 ±0.96
-60	-23.59 ±0.39	-23.86 ±0.50	-23.88 ±0.60	-24.06 ±0.38
0	-23.54 ±0.50	-23.68 ±0.42	-24.13 ±0.62	-24.11 ±0.33
210	-14.38 ±2.53	-20.94 ±1.54	-22.96 ±0.68	-23.21 ±0.60
240	-14.98 ±.86	-21.55 ±1.49	-23.35 ±0.86	-23.42 ±0.41
270	-15.55 ±1.87	-21.97 ±1.22	-23.26 ±0.38	-23.37 ±0.39
300	-16.87 ±1.27	-22.63 ±1.22	-23.43 ±0.60	-23.52 ±0.41

C) Rotation 3	***Dietary Methionine Content (g/1000kcal)***			
Time (min)	**0.0**	**0.3**	**0.6**	**1.5**
-210	-25.14 ±0.76	-25.41 ±0.80	-25.96 ±1.02	-25.58 ±0.88
-180	-25.24 ±0.79	-25.40 ±0.89	-26.04 ±0.96	-25.50 ±0.81
-60	-23.26 ±0.44	-23.52 ±0.60	-23.73 ±0.54	-23.80 ±0.79
0	-23.32 ±0.52	-23.51 ±0.42	-23.96 ±0.48	-23.88 ±0.60
210	-14.86 ±1.95	-21.80 ±1.05	-23.23 ±0.69	-23.04 ±0.65
240	-15.39 ±2.53	-22.27 ±0.82	-23.32 ±0.43	-23.39 ±0.50
270	-15.58 ±2.28	-22.54 ±0.79	-23.36 ±0.54	-23.45 ±0.60
300	-16.23 ±1.75	-22.77 ±0.66	-23.40 ±0.55	-23.48 ±0.46

Table 2.4: *The average delta c ± SD, at each time point A,B and C Rotations 1, 2 and 3 respectively*

2.3.2 Methionine Breakpoint

From phenylalanine oxidation a breakpoint was reached at 0.35 g/1000kcalME of methionine in the diet (Figure 2.5) with upper and lower 95% confidence limits of 0.39 and 0.32g/1000kcalME, respectively. The breakpoint points for each rotation were 0.37, 0.36 and 0.33 g/1000kcalME.

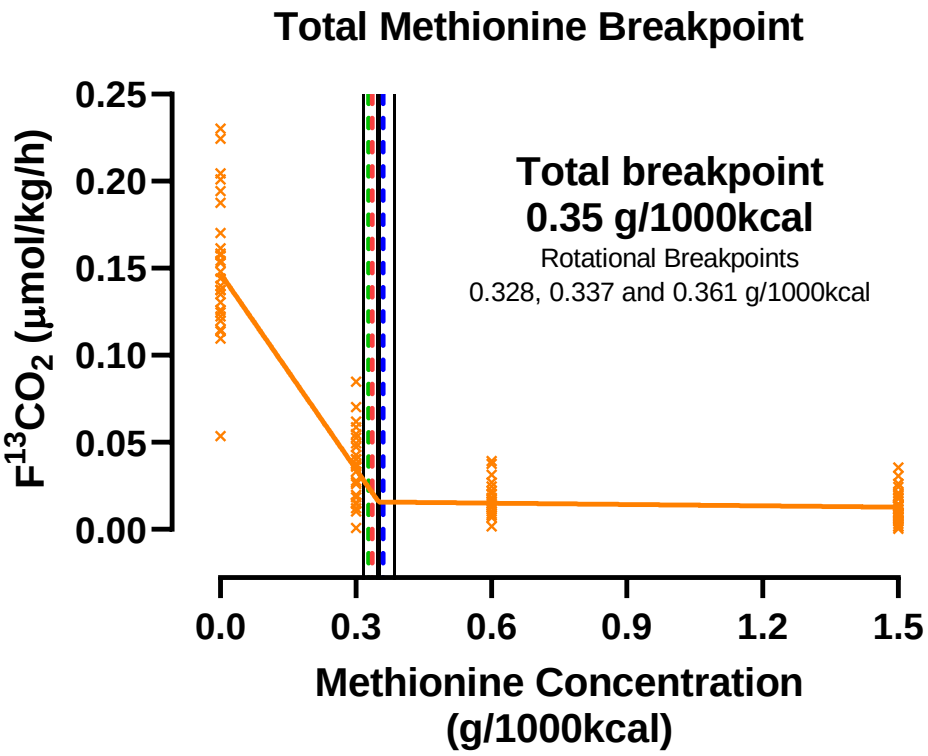


Figure 2.5. *The effect of methionine intake on the production of $^{13}CO_2$ from 1-^{13}C phenylalanine oxidation ($F^{13}CO_2$) by IAAO in adult Labradors in 3 different rotations. Two phase linear regression analysis of $F^{13}CO_2$ data (orange line) identified a breakpoint of 0.35g/1000kcal. Breakpoint and the 95%CI represent by solid black lines. The rotational breakpoints being red = rotation 1, blue = rotation 2 and green = rotation 3.*

The methionine breakpoint for each of the rotations lies at 0.366, 0.361 and 0.328g/1000kcalME for each respective rotation (figure 2.6). There is a significantly lower ($P \leq 0.01$) between the breakpoint of rotation 1 and 2 and rotation 3. This analysis also showed that there was no significant difference of the methionine breakpoint value between the different priming doses (rotations 1 and 2).

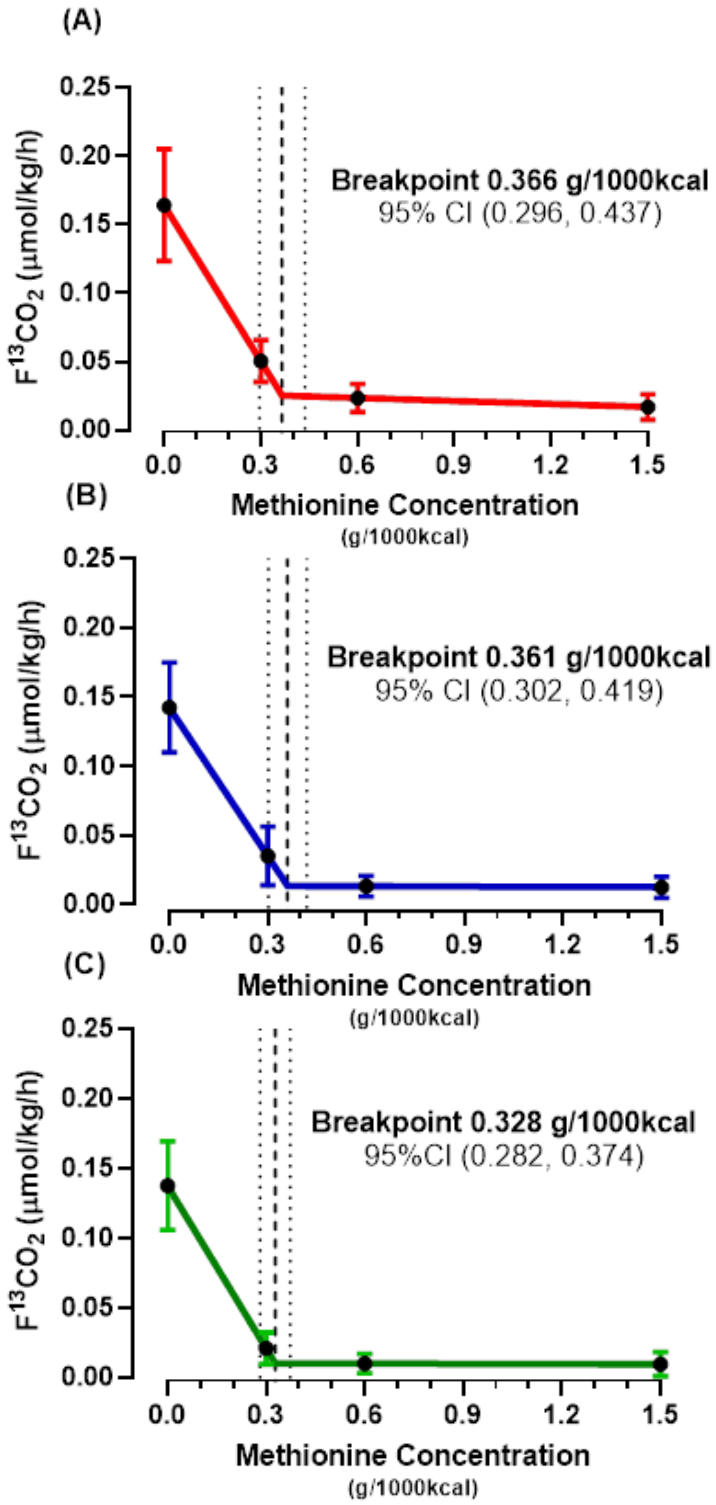
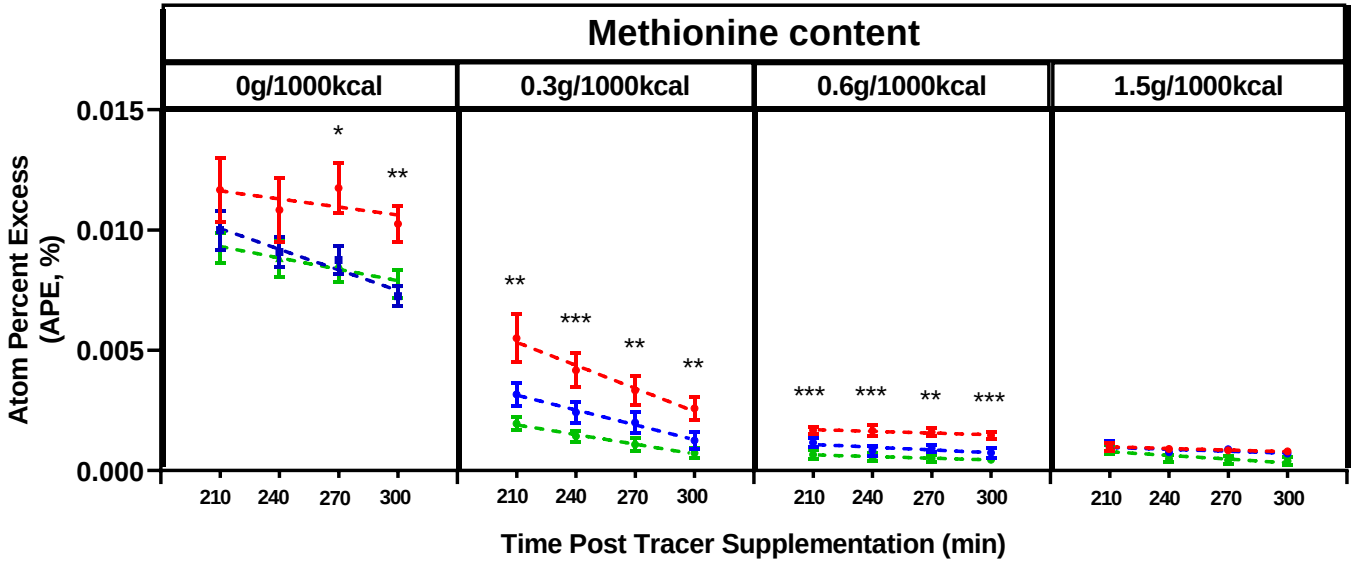


Figure 2.6. *Individual rotation figures breakpoints for the methionine content; A- rotation 1, B- rotation 2 and C- rotation 3. The dashed vertical line represents the mean predicted breakpoint value and the dotted line the 95% Confidence Interval. The error bars on each of the figures represent the standard deviation of each of the individual dogs at the different methionine concentration.*

2.3.3 Priming dose comparison

Although there was no significant difference between the breakpoints of the dogs in rotations 1 and 2, there was, as expected, a significant difference between the breath enrichments when the priming dose was reduced from 7mg/kgBW (rotation 1) to 5 mg/kgBW (rotation 2 and 3; Figure 2.7); between rotation 1 and 2 in diet A, T= 270 and 300 minutes ($P \leq 0.05$ and $P \leq 0.01$ respectively) and diet C, T=240 minutes ($P \leq 0.05$), between rotation 1 and 3 in diet B in all time points ($P \leq 0.01$ or greater) and diet C at all time points ($P \leq 0.01$ or greater). Unexpectedly, there was a significant difference ($P \leq 0.05$) between rotation 2 and 3 at 210 minutes diet B. There was also no significant difference between any of the breath enrichments in diet D.



Time post Tracer Supplementation (min)	Diet A	Diet B	Diet C	Diet D
210	NSD	*R1-R3	**R1-R3	NSD
240	NSD	**R1-R3	*R1-R2, **R1-R3	NSD
270	* R1-R2	*R1-R3	*R1-R2, ***R1-R3	NSD
300	**R1-R2	**R1-R3	*R1-R2, ***R1-R3	NSD

Figure 2.7. *(A) Line graphs comparing the APE of the breath samples on each diet. The dashed lines relate to the trendline of each data set and the error bars signify the standard error of the mean (SEM); Red - rotation 1, Blue – rotation 2 and Green - rotation 3. (B) A table of significant differences between the different rotations on each diet; *** P≤0.001, ** P≤0.01 and * P≤0.05*

2.3.4 RCO_2 comparison

The RCO_2 measurement is an important measurement for identifying the breakpoint as it is used as an alternative to VCO_2, to measure the rate of carbon dioxide production. The RCO_2 measure is an additional study day for the IAAO experiment that is required in the calculation for determining the breakpoint value. The breakpoint values for methionine were compared with either the individuals RCO_2 or a constant RCO_2, it was shown that there was no significant difference between the breakpoint values (Table 2.5). This was probably due to the dogs in this study being adults of the same breed with a similar activity level and body condition score (4-5). As this measure requires an additional sample collection day, therefore by using a constant value for the RCO_2 would reduce the number of study days.

Rotation	***Individual RCO_2***	***Constant RCO_2***	***P value***
1	0.380 ±0.115	0.368 ±0.126	NSD
2	0.343 ±0.253	0.342 ±0.052	NSD
3	0.334 ±0.275	0.333 ±0.043	NSD

Table 2.5: *The comparison of the methionine breakpoint value in dogs calculated with either an individual's RCO_2 value and a constant RCO_2 of 13.307 mol/ml for all three rotations.*

2.4 Discussion

From the results the IAAO method predicts the breakpoint value for methionine in Labradors on a highly digestible diet, to lie between 0.317-0.386 g/1000kcalME with a mean breakpoint of 0.35 g/1000kcalME, when cysteine is fed at 3.345g/1000kcalME. This minimal variability in 95% CI (0.317-0.386 g/1000kcalME) suggests the primary objective was met in optimising the IAAO technique in dogs. In addition, there was no significant difference between the breakpoints of rotations 1 and 2, even though the apparent $^{13}CO_2$ oxidation rates were significantly different, due to the different priming doses, 7mg/kgBW and 5mg/kgBW. This could mean AA oxidation rates could be measured in non-steady state conditions, provided the tracer is in excess. There was also no significant difference between breakpoints when using a RCO_2 that is unique to the individual or an average RCO_2 of 13.307mol/ml. This suggests that provided the parameters such as breed, body weight and body condition score are kept constant the RCO_2 may not be necessary, and a constant could be used as an alternative.

The breakpoint value of 0.317-0.386g/1000kcal supports the conclusion of by Harrison *et al* (2020), that the methionine requirement could be less than currently suggested by the NRC (1.3g/1000kcalME; NRC, 2006) cysteine is provided at a sufficient level. Although further research into the wider impact of reducing the methionine requirement on cysteine and diets with other protein and fat compositions to safely confirm a

reduction. This group also found that in the diets with low methionine levels there was an increase in choline and betaine utilisation towards the production of methionine (Allaway *et al.*, 2021).

2.4.1 The optimisation of the breakpoint variation in amino acid oxidation techniques in dogs

The breakpoint showed a reduction in the percentage difference of the mean and upper 95% CI from 30% to 10%, (0.547 - 0.709 g/1000kcalME and 0.35 – 0.39 g/1000kcalME), when compared to the previous work by Harrison *et al, (*2020). This meets the primary objective of this study to improve the accuracy of the breakpoint in the IAAO technique in dogs.

In the current study, there was a significant difference between rotation 2 and 3, however it is not physiologically important as the 95% CI overlap (0.302-0.419 and 0.282-0.374 g/1000kcalME, upper and lower 95% CI for rotation 2 and 3 respectively). The main difference between the two rotations is that one of the dogs was replaced from the study due to palatability issues with the diet. When the data sets were compared without the two dogs there was no significant difference between the breakpoints.

However, there was no significant difference in the 95% CI when the priming doses were reduced, between rotations 1 and 2 (7 to 5mg/kgBW). In both rotations the oxidation rate of phenylalanine was not in a steady state during breath sample collection, indicating that steady

state labelling of the ^{13}C Phenylalanine was not essential to determine the breakpoint providing the breath CO_2 enrichment can be measured robustly. However, the enrichment of ^{13}C phenylalanine must be sufficient to detect changes in oxidation rate, this could have been the reason for the increased variability (0.41 - 0.71g/1000kcal) in the 95% CI of the breakpoint in the work by Harrison *et al* (2020). It is clear when no methionine was provided in the diet some of the dogs had no increase in the oxidation rates, as would be normally expected. The main difference between this study and Harrison *et al* (2020) was the increased priming dose (3 vs 7 and 5 mg/kgBW, priming doses of Harrison *et al* (2020) and this study respectively), this could have led to the decreased variability. However, the decreased 95%CI in the breakpoints could also be due to a reduction in the number of diets, as this experiments breakpoint value was determined by only 2 diets either side of the breakpoint, whereas Harrison *et al* (2020) had 9 diets. A breakpoint is detected using a two-phase linear regression model, therefore a minimum of 2 data points either side of the line is required. This would mean that in the current study the R^2 value for both lines would always be 1 for each dog, however with Harrison *et al* (2020) it will be less than 1, this would increase the variability of the breakpoint value.

An alternative IAAO method in dogs detected a methionine breakpoint value greater than that of the current study in 3 different dog breeds (0.57-0.822, 0.914-1.238 and 0.973-1.397 g/1000kcal in dachshunds, beagles and Labradors respectively; Mansilla *et al.*, 2020). The method

provided 13 meals, the first 3 every 10 minutes and all subsequent meals every 25 minutes, with a priming dose of 9.4mg/kgBW and a top-up of 2.4mg/kgBW, the same as previously published IAAO techniques (Mansilla *et al.*, 2018; Templeman *et al.*, 2019). This study provided a greater amount of stable isotope tracer and a greater number of meals (13 vs 8), thus making this more time invasive for the dogs increasing the likelihood of stressing the dogs. In addition, the diet was provided at a higher level of energy intake 1.5-1.8 times greater than the dogs resting energy expenditure (130kcal/kgBW$^{0.75}$), less 5% of their normal energy intake. This high level of energy intake could have altered protein metabolism in the dogs leading to a reduction in the proportion of protein being oxidised in the diets resulting in an increase in the breakpoint value due to a greater demand for amino acid and their metabolites in other areas of metabolism.

As previously mentioned above the first 3 meals in Mansilla *et al.*, (2018), Templeman *et al.*, (2019), Mansilla *et al.*, (2020) and Shoveller *et al.*, (2017) were given in quick succession to prime the AA pool to get to an AA steady state quicker and shorten the study day length. Also, additional AA (i.e., ^{13}C phenylalanine) supplemented into the diets were given in liquid form rather than in powdered form in the method used in this chapter. The use of dissolved AA would reduce the uncertainty of the intake of the AA than powder added on top of each meal. The compliance of the uptake of the AA's is a challenge that is unique to animal experimentation as the method needs to ensure complete uptake of the

additional AA. Of these two methods a liquid is the better method for ensuring the uptake however this requires the animal to willingly drink immediately after eating to ensure the tracer uptake time points remain consistent, as forcing the stable isotope would go against ethics and provide additional stresses that could affect results. Both of these studies can detect an AA breakpoint value with the IAAO technique, even though the methods are different. However, the method used in this chapter was able to identify a breakpoint using less stable isotope tracer and less meals allowing for a free-living experience, refining amino acid oxidation technique in dogs. Although the method used by Mansilla *et al.*, (2018), Templeman *et al.*, (2019) and Shoveller *et al.*, (2017) did use a more efficient method for providing the tracer and primed the meals to reduce the study day. Therefore, the IAAO method used in this chapter could still be further improved by utilising those parts of their method.

2.4.2 The importance of the RCO_2 in determining a breakpoint value for the IAAO technique

The two different methods for detecting the rate of CO_2 production either the RCO_2 or the VCO_2. The method for assessing RCO_2 requires the dog to complete an additional study day of a similar design to the IAAO method that was used, by providing the diets over 8 hourly meals then at meal 4 providing ^{13}C sodium bicarbonate followed by 10-minute breath samples until the end of the study to measure the total CO_2 production indicated by the area under the $^{13}CO_2$ oxidation curve. The other measure uses the VCO_2 and requires no stable isotope tracer or extra

study day, however it does require simultaneous collection of VCO2 over representative periods during feeding, usually in a respiratory chamber that is both expensive and restrictive. For some dogs the restrictiveness of the respiratory chamber may cause them increased stress and impact VCO2 determination. This would not make it representational of a relaxed free-living situation. Both methods have pros and cons however the results from this study have indicated that it might not be necessary to use an individual RCO_2. As there was no significant difference between using a mean value from unpublished data of 13.307mol/min (Kerry Grey, Pers Comm from Internal WALTHAM report) for the RCO_2 verses an RCO_2 value unique to the individual dog. Providing the dogs in the study are similar (i.e., age-adult 3-8yrs, body condition- 4-5 on the 9-point body condition score and the same breed), a constant value can be used to estimate the CO_2 recovery. This also means that an $F^{13}CO_2$ would not be required to display the results and the raw values from the mass spectrometer could be used, to compare the differences from a baseline and an enriched sample. However, if the animals vary too greatly in morphology, age and breed then this would increase the variability of a breakpoint value.

2.4.3 Limitation of the IAAO method

The IAAO technique is a relatively quick (4-8h), minimally invasive method suitable to predict the minimum concentration of an essential amino acid for protein synthesis in dogs. The breakpoint value provides

a minimum threshold for methionine and should not be used as a requirement level because:

1) **The length of time consuming each test diet.** Although 3 days is long enough to detect a minimum requirement level for protein synthesis, it is unable to detect potential chronic effects at any new requirement level detected. Therefore, to validate any requirement detected from the IAAO technique a chronic study needs to be used to ensure there are no shifts in metabolisms that could cause harm over time. This is particularly necessary if the new requirement level is lower than the previous requirement level.

2) **Breakpoint specific to the exact parameters of the experiment.** The breakpoint values in these experiments are specific to the breed and age of the dogs and therefore to gain a complete understanding of the requirements in dogs every amino acid must be analysed for most if not all breeds of dogs at a variety of different ages.

2.4.4 Limitations of the method applied in this chapter

Although this method has reduced the variability of the study, some of the adaptations may limit the robustness of this method. The method still requires further optimisation to perform a comprehensive analysis of amino acid requirements in dogs across all life stages and different

breeds. These include the optimum number of diets, defining the phenylalanine to tyrosine ratio across each meal, the administration of the stable isotope tracer (dosing levels and the method of oral entry) and the energy intake of the diet.

1) As previously mentioned in section 2.4.1, **the number of different diets used to determine the breakpoint value**.
2) **The rigidity of the concentration of the AA in the diets**. Rather than providing the test AA in the same units as the breakpoint value being detected (g/1000kcal in this chapter), causing figures lying at 4 distinct concentrations the diets could be provided similar to research conducted by Kato *et al* (2016). In this study they provided the diet in fixed grams of protein and then the breakpoint was determined in g/kgBW allowing the data points to be scattered across the predicted 2-phase linear regression line. This would allow for an increase in the accuracy of the breakpoint value as it would be showing multiple different AA concentrations.
3) **The ratio between the phenylalanine and tyrosine throughout the study day were not constant.** In traditional IAAO studies phenylalanine and tyrosine are fed evenly in each meal (Shiman and Gray, 1998). However, in this study 100% of the ^{12}C phenylalanine was provided in the first meal and tyrosine was evenly distributed throughout the 8 meals. This would mean that the phenylalanine would spike following the consumption of meal 1 and tyrosine and the rest of the AA would all be at 12.5% of their

requirement level. This should then follow Lebig's law of the minimum (Alexander N Gorban *et al.*, 2011) and cause more phenylalanine to be converted to tyrosine and oxidised initially. As such, the tyrosine level in the body increased from two ways in the diet; from tyrosine directly and excess phenylalanine, until the level balanced out, therefore the phenylalanine was lower than the dietary requirement level as a greater proportion of phenylalanine has been convert into tyrosine. This means that when ^{13}C phenylalanine was added in the diet a greater proportion of the enrichment phenylalanine would remain as phenylalanine to compensate for the previous loss. Which potentially means that the enrichment of the breath samples would be lower, and the efficiency of the experiment was decreased. The easiest solution to correct for this is to ensure that ^{12}C phenylalanine is equally distributed throughout the meals on the study day.

4) **The method of providing the phenylalanine to the meals.** The phenylalanine in this study was added in powdered form directly on top of the meals. This potentially impacted the amount of phenylalanine consumed by the dogs, it was therefore unclear if all the powder had been completely consumed. This then had the potential to alter the level of enrichment, as it could have caused an unequal concentration of the stable isotope to be consumed, that would then result in fluctuations in the enrichment level of the breath samples. Also, as the ^{12}C phenylalanine was provided as a powder, there was the potential for a reduced intake of

phenylalanine and could have caused a false positive breakpoint value, with the breakpoint detected being the phenylalanine level rather than the methionine level. Therefore rather than providing the phenylalanine as a powder, the ^{12}C phenylalanine could be incorporated in the kibble pellet and the ^{13}C phenylalanine could either be dissolved in water (Templeman *et al.*, 2019) or encapsulated to increase the accuracy of the stable isotope uptake.

2.4.5 The methionine breakpoint value as a requirement in dogs

The results from this study support the findings from Harrison *et al,* (2020) that the mean minimum methionine requirement is less than the current recommended level in dogs (1.3 g/1000kcalME with an energy intake of 95kcal/kgBW$^{0.75}$; NRC, 2006). An exact value cannot be exactly determined as both studies have differing methionine breakpoint values (0.34 and 0.71 g/1000kcalME with an energy intake of 95kcal/kgBW$^{0.75}$). However, the diet that was used in both studies was a highly digestible diet and therefore perhaps not representative of a “normal” dog diet where the digestibility would likely be lower i.e., 80-90%. In addition, this newly identified breakpoint value discovered in this chapter would require a long-term evaluation to determine the safety of this lower level. However, the lower methionine requirement level suggests that methionine dietary supplementation into diets could be reduced and

protein sources that are high in protein but lower in SAA could be used as predominate protein sources in diets i.e., plant base.

2.4.6 Scope for future work in IAAO technique in dogs

The aim of the study was to further optimise the IAAO approach in dogs and I believe this has been achieved, however there is the potential for further improvements in the application of the IAAO to potentially reduce the burden on the animal by reducing duration of the sample collection day and the number of samples collected and analysed. As the IAAO technique measures excess amino oxidation beyond that required for protein synthesis, and measures the rate of oxidation when it is switched on, or no oxidation when it is off, the tracer does not necessarily have to be in a steady state (as is obvious in Figure 2.7) this is shown when there was no significant differences between rotation 1 or 2 and 3, when we hadn't achieved a true steady state. Therefore, a faster, non-steady state IAAO approach could potentially be used to identify amino acid requirements in the future.

Another area within this technique that could be developed in future studies is the identification of the optimum number of diets needed to provide a robust breakpoint measure that reflects biological variation. In addition, further work needs to be assessed on whether individual RCO_2 values are necessary in calculating the breakpoint or a RCO_2 constant for the diet provided the participants are within a constant threshold.

In addition, further testing is required to identify if this minimum dietary requirement for methionine (0.34 g/1000kcalME) is the minimum value for sustain protein synthesis in addition to the other metabolism demands on methionine. A long-term study would be useful to support the validity of this value, without compromising the health and welfare of the animal. This study would determine if the identified lower level of methionine could be provided without causing a reduction in plasma methionine or any early indicators of health problems, such as reduced taurine status. Further studies with the sulphur amino acids could identify the impact of altering cysteine using the breakpoint level of methionine to help establish the critical threshold of total sulphur amino acid requirement diet.

2.4.7 Conclusion

The results from this study have shown that the mean minimum methionine requirement for protein synthesis using the IAAO technique in dogs to be between 0.39-0.32 g/1000kcalME, when cysteine is provided at 3.345g/1000kcalME with an energy intake of 100kcal/kgBW$^{0.75}$. This detected level is lower than the current minimum recommended level of 1g/1000kcalME for the same energy (NRC, 2006). The IAAO was reproduced 3 times with minimal variability 9% difference between the breakpoint at the upper and lower 95% CI. However, the breakpoints were detected in a non-steady state, which is uncommon, therefore further experiments need to be conducted to assess if the breakpoint in a non-steady state is an accurate

representation of a minimum requirement before the breakpoint from this experiment can be concluded as an accurate minimum requirement value. With the overall aim to utilise the IAAO technique to assess the minimum amino acid requirements across the different breed sizes in all the EAA.

3. The impact of acute HMB supplementation to protein feeding on muscle and albumin protein synthesis

3.1 Introduction

3.1.1 Chapter synopsis

This study looked at protein synthetic responses to whey protein with and without β-hydroxyl β-methyl butyrate (HMB) on mixed muscle and albumin protein synthesis in older adults. Measuring the fractional synthetic rate (FSR) of albumin and mixed muscle as indicators for protein synthesis, with the aim to assess whether HMB supplementation in addition to a whey protein meal could augment the effect of whey protein on protein synthesis. Given separately, both whey protein (Bukhari *et al.*, 2015) and HMB (Wilkinson *et al.*, 2013) have been identified to stimulate muscle protein synthesis.

3.1.2 Anabolic resistance: A contributing factor to sarcopenia

As previously stated (section chapter 1.4.8), anabolic resistance occurs in aging individuals and contributors to sarcopenia, due to the reduced MPS response to anabolic stimuli (e.g. ingestion of leucine or whey protein). The link was first identified in 2000 where there was a depleted response to muscle FSR in elderly, compared to young despite both

groups being in an hyperaminoacidemia state (Volpi *et al.*, 2000). This blunted response to MPS was due to a uniformed decrease in both the concentration and phosphorylation of proteins in the mTOR signalling pathway (mTOR, 4E-BP1 and P70-S6kinase; (Cuthbertson, *et al.*, 2005). It is currently unclear if anabolic resistance only influences MPS through the mTOR signalling pathway.

3.1.3 The impact of HMB on protein metabolism

β-hydroxyl-β-methyl butyrate (HMB) is a metabolite of leucine that stimulates MPS to the same level as leucine when provided at the same concentrations (Wilkinson *et al.*, 2013). Approximately 5% of dietary leucine will be synthesised into HMB, this reaction occurs mostly in the liver (Matthews *et al.*, 1980), with an intermediary step of α-ketoisocaproate acid (KIC, Van Koevering and Nissen, 1992) with the respective enzymes being branched-chain alpha-ketoacid dehydrogenase and 4-hydroxyphenylpyruvate Dioxygenase (Van Koevering and Nissen, 1992). Although HMB synthesis occurs in the liver, the enzyme 4-hydroxyphenylpyruvate dioxygenase is present in the kidney and skeletal muscle (Wilkinson *et al.*, 2013) which suggests that this step can be performed within these tissues.

The natural plasma HMB levels, differ between gender and age with lower concentrations in females than males and an inverse relationship with age (Deutz *et al.*, 2013, Kuriyan *et al.*, 2016). Further, plasma HMB concentration is positively correlated with appendicular lean mass in

adults, there is a stronger affiliation in young adults (30-45 yrs) than older adults (65-75 yrs; Deutz *et al.*, 2013; Kuriyan *et al.*, 2016), either the increased lean mass is due to the increased plasma HMB level or the opposite is true, the increased lean mass leads to an increased capacity for HMB synthesis and thus increased plasma HMB. Also in rats, the enzyme 4-Hydroxphenylpyruvate Dioxygenase (that converts KIC to HMB) is reduced with age (Shreeram *et al.*, 2016), this could be similar in humans as the plasma concentration of HMB reduces with age it correlates with muscle mass (Kuriyan *et al.*, 2016). Also the supplementation of HMB increased whole body strength (Nissen *et al.*, 1996) and increased protein turnover and lean body mass in elderly men and women when supplemented for a year (Baier *et al.*, 2009). In addition whole body protein synthesis increased by 20% in elderly women when supplemented for 12 weeks in combination with arginine and lysine (Flakoll *et al.*, 2004).

The optimum concentration of daily HMB supplementation to maximise myofibrillar MPS is 2.42g (0.043 to 0.073%/hr), a similar response to 3g of leucine (0.042 to 0.088 %/hr; Wilkinson *et al.*, 2013). Any dietary HMB greater than 2.42g is excreted into urine or converted into either acetyl CoA, to enter the TCA cycle or directed towards cholesterol synthesis. In addition, HMB supplementation in young men can limit MPB and increase fat free mass when combined with whole body resistance exercise for 3 weeks (Nissen *et al.*, 1996). Further, 2.42g of HMB suppressed MPB by 57% in young men (Wilkinson *et al.*, 2013) therefore

2.42g of HMB impacts both MPB and MPS this compounds the effect on muscle protein turnover.

3.1.4 Objective

The aim of this experiment to determine the effect of 40g of dietary whey protein with and without 3 g of HMB on mixed muscle and albumin protein metabolism in older adults and to determine if there is an effect of gender.

3.2 Methods

3.2.1 Subjects

Healthy, elderly males and females, (14:12 with an average age of 69.7 ± 2.8: 67.2 ± 2.9 years respectively) were recruited to this study (demographics shown in Table 3.1), based on the following exclusion criteria:

- Underlying metabolic, musculoskeletal, cerebrovascular or cardiovascular disease
- Recent active malignancy (<5 years since remission)
- Uncontrolled hypertension, a systolic blood pressure > 140mmHg or an average diastolic blood pressure of 90 mmHg
- Body mass index (BMI) >30kg/m^2
- Taking medication known to impact protein metabolism or vascular tone (e.g. Metformin; Oliveira and Gomes-Marcondes, 2016)

The eligibility was identified using the exclusion criteria by an initial health screening session lasting ~1h, comprising a medical history, anthropometric measurements (height and weight), an ECG and blood pressure assessment and an assessment of strength and physical function. Throughout the study participants were requested not to consume any nutritional supplements (e.g., fish oils, omega-3 supplements (>0.5 g/day), including high doses of vitamin D (>400IU/d).

Parameter	***Male (n=14)***	***Female (n=12)***
Age (y)	69.7 ± 2.8	67.2 ± 2.9
Height (m)	1.74 ± 0.05	1.64 ± 0.08
Weight (kg)	80.5 ± 4.3	67.7 ± 8.3
BMI (kg/m^2)	26.7 ± 1.8	25.1 ± 1.9
Total Lean Leg Mass (kg)	18.3 ± 1.7	12.9 ± 1.9
Appendicular Skeletal Mass (kg)	25.2 ± 2.1	16.9 ± 2.4
Skeletal muscle index (kg/m^2)	8.3 ± 0.5	6.3 ± 0.6
Fasting plasma glucose (mM)	5.2 ± 0.5	4.9 ± 0.4

Table 3.1. *Participant demographics*

3.2.2 Study design

The crossover study design invited participants to attend the research facility on two occasions (after their initial health screening session) 28±7 days apart. Before each study day participants fasted overnight (from 2200h to 0900h) with water *ad libitum*. Each study day lasted ~8 hours,

including study set-up, a 6-hour tracer infusion period and post-infusion monitoring (Figure 3.1). For the duration of both study days participants lay semi-supine on a bed, with dual energy x-ray absorptiometry (DXA) performed at the start of the first study day only, to determine appendicular lean mass.

A cannula was inserted into forearm for the infusion of $^{13}C_6$ phenylalanine (Cambridge Isotope laboratories, MA USA). The $^{13}C_6$ phenylalanine tracer was given as a priming dose of 0.4mg/kg and then a constant infusion of 0.6mg/kg/hr for the duration of the study day (Figure 3.1). In the middle of the study day, 40g whey protein either with or without 3g of HMB was given to the participants in a double-blind design. The order of studies was randomised via www.sealedenvelope.com. Blood samples were collected throughout the day with the first being prior to the start of the tracer infusion. Five muscle biopsies were collected across the study day at: i) 0 minutes (after achieving an isotopic steady state of phenylalanine), ii) 120 minutes (immediately before ingestion of the supplement to measure postabsorptive MPS), and iii) at each hour for three hours following the supplement to measure the temporal effect on MPS.

The muscle biopsies were collected using conchotome forceps (Dorph, Nennesmo and Lundberg, 2001), the samples were dissected of all

visible fat and connective tissue, frozen rapidly in liquid nitrogen and then stored in a -80°C freezer until analysis.

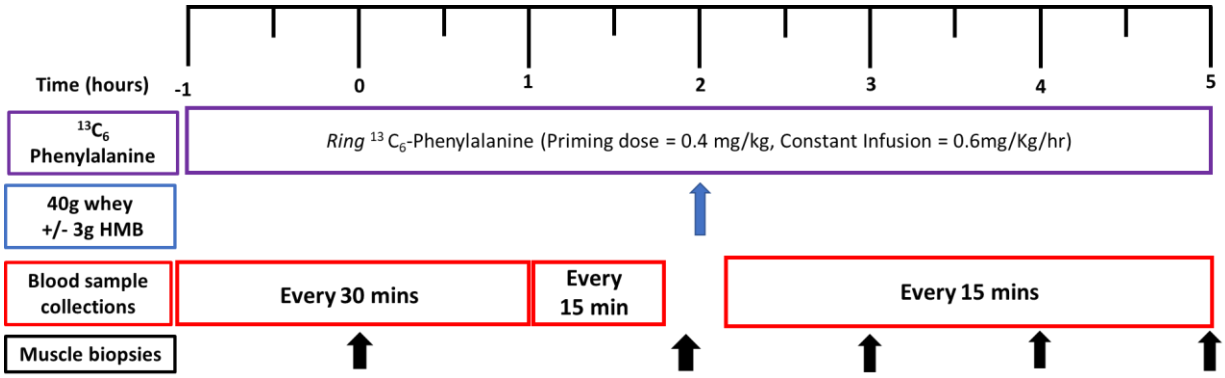


Figure 3.1: *Schematic representation of the study day protocol*

3.2.3 Analysis methods

3.1.1.1. Isolation of albumin from plasma

Ice cold trichloroacetic acid (1ml at 10%) was added to plasma (50µl) and vortex mixed. Samples were then centrifuged (4°C, 10,000 rpm for 20minutes) before the supernatant was removed and discarded. Ice cold ethanol (1ml) was vortex mixed with the remaining sample to completely resuspend and placed on ice for 20 minutes. The samples were then centrifuged (4°C, 10,000 rpm for 20 minutes) before the supernatant was removed and dried down (N_2 gas at 90°C), resuspended in 0.3M NaOH (200µl) and vortex mixed and then solubilised (37°C for 60 minutes) and precipitated out using 1M perchloric acid (400µl) and vortex mixed and centrifuge (4°C, 3000g for 20 minutes). The supernatant was then discarded, the remaining pellet washed with 0.2M perchloric acid (2ml) and centrifuged (4°C, 3000g for 20 minutes) with the supernatant discarded. The remaining pellet, representing the plasma proteins was

resuspended with 0.1M hydrochloric acid (1ml) and Dowex H^+ resin slurry (1ml; see Dowex H^+ resin preparation method in section 1.2.3.3) to hydrolyse at 110^{o}C overnight to be run through 5ml pipette tip columns for the assessment of the protein bound ^{13}C phenylalanine.

3.1.1.2. Isolation of muscle protein bound phenylalanine

Mixed muscle (~25mg) was minced with fine scissors in 0.2M perchloric acid, homogenised and centrifuged (11,000G, at 4^{o}C for 15 minutes) into a pellet of total protein. The supernatant was extracted to measure the intramuscular phenylalanine enrichment. Similar to the albumin isolation, the pellet was solubilised in 0.3M NaOH for 20min at 37^{o}C then transferred to a boiling tube and precipitated with perchloric acid, centrifuged into a pellet and washed twice with 70% ethanol before being hydrolysed overnight with 0.1M HCl as preparation for analysis via gas chromatography- combustion -isotope ratio mass spectrometer (GC-C-IRMS).

3.1.1.3. Preparation of Dowex H+ resin

Approximately 500ml of Dowex H^+ resin was washed with doubly distilled water until clear. Dowex H^+ resin is transferred into a Buchner funnel and 1M hydrochloric acid (1L) is added to the Dowex H+ resin and filtered through. This changes the Dowex H+ resin into the acidic state. The Dowex H+ resin is then neutralised with ddH_2O and 2M ammonium hydroxide (1L) is added, changing it to the alkaline state. This was then

neutralised with ddH_2O followed by a further 1M hydrochloric acid (1L) and neutralised with ddH_2O to be stored for use later.

3.1.1.4. Amino acid isolation with Dowex H+ resin using columns

A column is made by cutting the tip off a 5ml pipette tip and wedging a filter in the lower third of the inside of the pipette tip. This column has prepared Dowex H+ resin (2ml) added (see section 1.2.3.3), is washed with 2M ammonia hydroxide neutralised with ddH_2O and then 1M hydrochloric acid. Following this, the hydrolysed samples from albumin (section 1.2.3.1) and muscle (section 1.2.3.2) were added on top of the Dowex H+ resin. Once added, 1M hydrochloric acid (1ml) is added to the columns to elute through the column, this step is repeated. The column is then neutralised with ddH_2O (5ml). 2M Ammonium hydroxide (1ml) is then added, as the solution eluting from the Dowex H+ resin changes from neutral to base, the remaining eluate is collected. An additional 2M Ammonium hydroxide (3ml) is eluted through the column and collected. The samples were dried down to the approximate volume of 1 ml, on a Techne block™ at 90^oC with a continuous flow of nitrogen gas. Following this the samples were transferred to a 2ml autosampler vial for the remainder to be dried down on a Turbovap LV™ sample concentrator, for subsequent derivatisation and analysis by GC-C-IRMS.

3.1.1.5. N acetyl, n-propyl ester derivatisation of amino acids

Following from section 1.2.3.4, propyl acetate (200µl) and BF3:propanol (14%;100 µl) were added to each sample. Samples were then capped, vortex mixed and heated at 110^{o}C for 30 minutes. Once cooled the samples were dried on a turbo vap with nitrogen at 70^{o}C. Acetonitrile (50µl) and 1, 4 dioxane (5µl) was then added to the dried sample and vortex mixed. Triethylamine (37.5µl) was next added, the sample vortex mixed, and acetic anhydride (22.5µl) added. The sample was vortex mixed again and then heated at 55^{o}C for 15 minutes. After heating Chloroform (50µl) and 0.001M sodium bicarbonate (150µl) were added before the upper aqueous layer was removed. A molecular sieve added to each sample tube for 2 minutes to remove any remaining aqueous layer. The samples were transferred into vials suitable for mass spectrometry analysis. The samples were run on a gas chromatography combustion delta Isotope Ratio Mass spectrometer (GC-C-IRMS, Delta-plus XP, Thermo, Hemel Hampstead, UK) with separation achieved on a 25m/ 0.25mm/ 1.0µ-film DB 1701 capillary column (Agilent Technologies, West Lothian, United Kingdom).

3.1.1.6. Plasma and intracellular Phenylalanine enrichment

Plasma was thawed and then centrifuged to bed the fibrin clots. Urease solution was added at room temperature for 20 minutes to remove urea interference. Samples were then de-proteinised using ice-cold ethanol, before centrifugation (4^{o}C, 13,000*g* for 20 minutes). The supernatant

containing plasma free amino acids were decanted and evaporated on a Techni block at 90°C under N_2. Dried amino acids were solubilised in 0.5 M HCl and the lipids extracted with Ethyl Acetate, before being evaporated to dryness. Amino acids were derivatised by adding acetonitrile (70µl) and N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) and heating at 90°C for 60 min, cooled then transferred to autosampler vials for quantification, The enrichment [atom percent excess (APE)] of arterialized and venous $^{13}C_6$ phenylalanine was determined using Gas Chromatography-Mass Spectrometry (GC-MS), from the ratio of labelled to unlabelled phenylalanine (m/z 240 to 234) with reference to an enrichment calibration curve (0-10 APE; Thompson *et al.*, 1989).

3.1.1.7. Fractional synthetic rate calculation of mixed muscle and albumin

Fractional synthetic rate (FSR) was calculated via measurement of the increase in L-[ring-$^{13}C_6$]-phenylalanine enrichment into myofibrillar protein between two consecutive biopsies (mixed muscle), or plasma (albumin) samples using:

FSR [expressed as %.h^{-1}] = ($\Delta E_m/E_p \times 1/t$) × 100 (Wilkinson *et al.*, 2015)

where ΔE_m is the change in labelling of muscle-bound L-[ring-$^{13}C_6$]-phenylalanine between 2 biopsy samples, E_p is the average L-[ring-$^{13}C_6$] free phenylalanine precursor enrichment in the intramuscular pool, and t

is the time in hours between biopsies. A similar approach was used to calculate the rate of albumin protein synthesis, using changes between bound phenylalanine in plasma albumin over time, relative to the average venous plasma $^{13}C_6$ phenylalanine (Aizhong, Sreekumaean Nair and Strong, 1998).

3.2.4 Statistical analysis

In complete data sets (albumin FSR) a two-way ANOVA for time x supplement was used with Tukey's multiple comparison post-hoc analysis to determine differences between groups. Where full data sets were not available (muscle FSR) due to missing biopsy samples, a mixed effects analysis was performed. All analyses were performed on Prism 9.0.1 (GraphPad, San Diego, CA), with data presented as mean ±SD.

3.3 Results

3.3.1 The postprandial rate of albumin and mixed muscle protein synthesis in response to whey protein with and without HMB

There was no significant difference between the post absorptive rates of muscle or albumin FSR between the two study days. Similarly, there were no differences in rates of post absorptive muscle or albumin FSR between males and females during either study day (Table 3.2).

Protein FSR	***With HMB***	***Without HMB***	***Diet P value (+HMB, -HMB)***	***Treatment p value***
Albumin FSR				
Male				
No. of participants	12	13		
Fasted	0.217 ±0.093	0.220 ±0.076		>0.9999
postprandial 1-2 hr	0.398 ±0.128	0.376 ±0.131	0.0010, 0.0035	0.9967
postprandial 2-3 hr	0.409 ±0.166	0.365 ±0.109	0.0005, 0.0066	0.9379
Female				
No. of participants	9	9		
Fasted	0.206 ±0.07	0.234 ±0.049		0.9923
postprandial 1-2 hr	0.319 ±0.109	0.350 ±0.096	0.2735, 0.2525	0.9884
postprandial 2-3 hr	0.363 ±0.188	0.315 ±0.128	0.0614, 0.6141	0.9242
Mixed Muscle FSR				
Male				
No. of participants	13	14		
Fasted	0.034 ±0.015	0.032 ±0.015		0.9959
postprandial 1-2 hr	0.081 ±0.042	0.080 ±0.041	0.0044, 0.0043	>0.9999
postprandial 2-3 hr	0.052 ±0.033	0.062 ±0.029	0.8708, 0.1871	0.7665
Female				
No. of participants	12	12		
Fasted	0.039 ±0.016	0.039 ±0.011		>0.9999
postprandial 1-2 hr	0.085 ±0.032	0.058 ±0.027	0.0047, 0.5624	0.2567
postprandial 2-3 hr	0.084 ±0.042	0.058 ±0.031	0.0052, 0.5624	0.2567

Table 3.2: *Mean fractional synthetic rate for fasted, 1-2hr post ingestion and 2-3 hr post ingestion with the respective P-values for both diet and HMB treatment*.

3.3.2 The effect of HMB on postprandial muscle protein synthesis rates in males and females

When assessing MPS across the study period 0-3hr, the muscle FSR increased to the same extent in response to 40g whey protein when given without or with 3g HMB in both males (-HMB: 0.032±0.015 vs. 0.064 ± 0.021 %/h^{-1}, p=0.0002; +HMB: 0.034 ± 0.015 vs. 0.058 ± 0.015 %/h^{-1}, p=0.0015) and females (-HMB: 0.039 ± 0.011 vs. 0.059 ± 0.017 %/h^{-1}, p=0.0172 ; +HMB: 0.039 ± 0.016 vs. 0.078 ± 0.01 %/h^{-1}, p<0.0001). In males, postprandial rates of muscle FSR were not different after whey protein supplementation with or without HMB (0.064±0.021 vs. 0.058±0.015%/h^{-1}, p=0.6447). In females, postprandial rates of muscle FSR were higher when HMB was provided in addition to whey protein (0.059 ± 0.015 vs. 0.078 ± 0.01 %/h^{-1}, p=0.0173). In addition, postprandial rates of muscle FSR in response to the combination of whey and HMB were significantly higher in females than in males (0.058±0.015 vs. 0.078±0.01%/h^{-1}, p=0.0443; Figure 3.2).

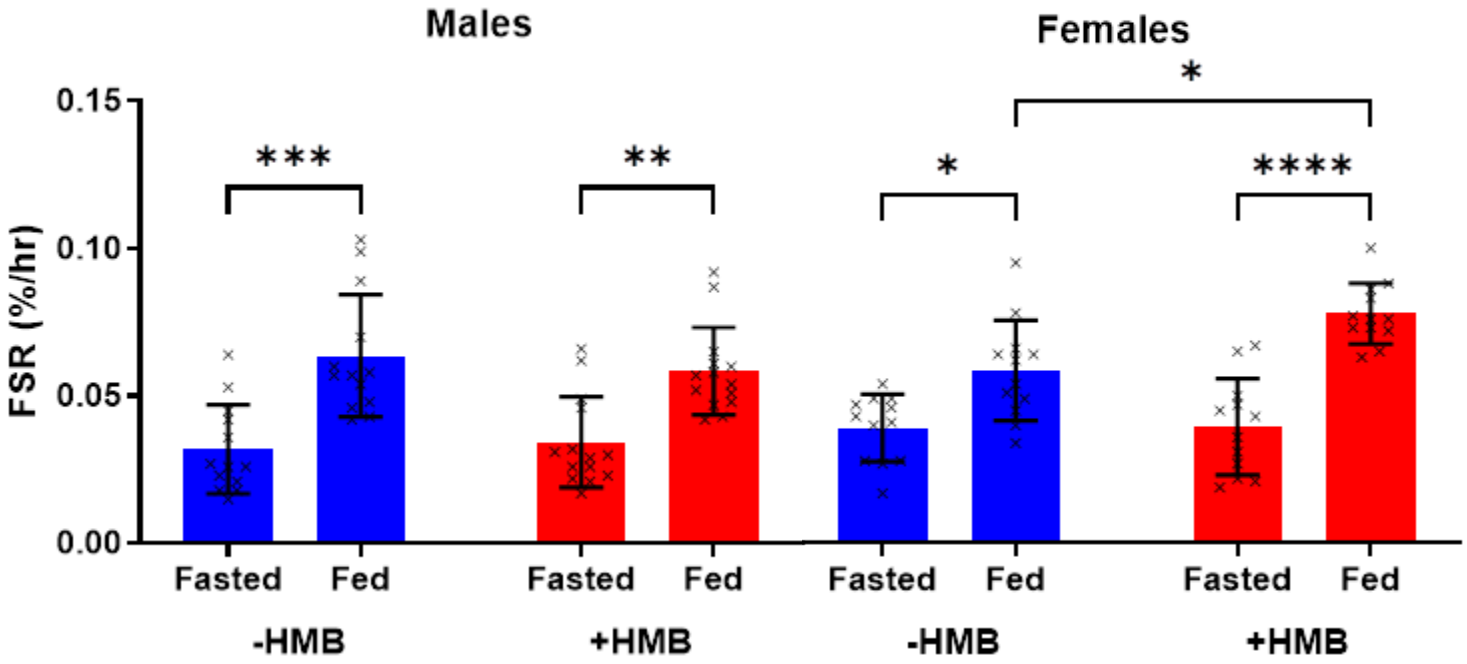


Figure 3.2*: muscle FSR before and after 40g whey protein supplementation both with (red) and without (blue) HMB over a 3 hour period in males and females. With the black crosses indicating individual data points and the black error bars indicating the standard deviation. *P<0.05 ** P<0.01, *** P<0.001, **** P<0.0001*

3.3.3 The effect of HMB on postprandial albumin protein synthesis rates in males and females

As with the muscle FSR, the albumin FSR increase to the same extent in response to 40g whey protein when given 3g HMB in males (-HMB: 0.22 ± 0.076 vs. 0.342 ± 0.074%/h^{-1}, p=0.0012; +HMB: 0.221 ± 0.09 vs. 0.368 ± 0.101 %/h^{-1}, p= 0.0002). However, in females there was only an increase in albumin FSR when 3g HMB was provided with 40g whey (-HMB: 0.234 ±0.049 vs. 0.292 ±0.0626 %/h^{-1}, p=0.2973; +HMB: 0.207 ±0.069 vs. 0.313 ± 0.102 %/h^{-1}, p= 0.032). In both males and females, the postprandial rates of albumin FSR following whey protein supplementation with or without HMB (males: 0.342 ± 0.074 vs. 0.368 ± 0.101 %/h^{-1}, p=0.694; females: 0.292 ± 0.0626 vs. 0.313 ± 0.102 %/h^{-1}, p=0.8955). Also, there was no significant difference between male and female postprandial rates of albumin FSR in response to the combination of whey and HMB (0.368 ± 0.101 vs. 0.313 ± 0.102 %/h^{-1}, p= 0.7727; Figure 3.3).

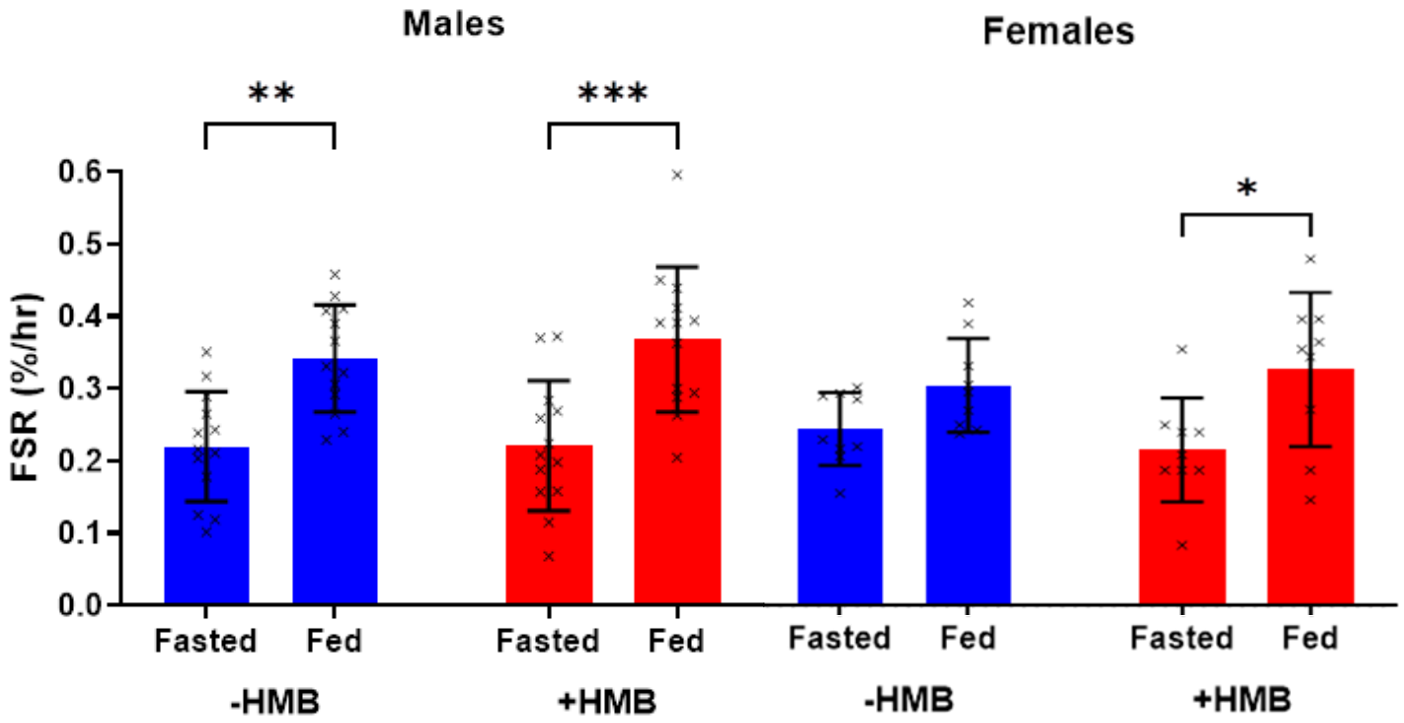


Figure 3.3: *Albumin FSR before and after 40g whey protein supplementation both with (red) and without (blue) HMB over a 3-hour period in males and females. With the black crosses indicating individual data points and the black error bars indicating the standard deviation. *P<0.05 ** P<0.01, *** P<0.001*

3.4 Discussion

From the results it was shown that the combined supplementation of HMB and whey protein increased MPS in elderly women, however the same effect was not replicated in elderly males or in the rates of albumin synthesis in both genders. In addition the supplementation of whey protein, both with and without the addition of HMB, increased MPS in elderly women, but neither strategy was able to elicit an increase in elderly men. Conversely, albumin protein synthesis significantly increased in response to 40g whey protein both without and with HMB in older men, while in older women the addition of HMB was needed to elicit a significant increase in albumin protein synthesis.

3.4.1 The effect of HMB on muscle protein synthesis in response to whey protein in older men and women

In both elderly males and females the ingestion of whey protein significant increase muscle FSR, complimenting the literature (Koopman *et al.*, 2008; Daniel R. Moore *et al.*, 2009; Smith *et al.*, 2009; Pennings *et al.*, 2011; Burd *et al.*, 2012; Wilkinson *et al.*, 2018). However, the additional supplementation of HMB (3g) increased muscle FSR in elderly females only. This increase was greater than the muscle FSR in elderly males, indicates a sexual dimorphism. Sexual dimorphism with muscle protein metabolism has previously been reported in elderly people as women have a greater capacity for muscle protein synthesis (Smith *et al.*, 2008) and the basal rate of muscle protein synthesis (Smith *et al.*,

2012) although the results in this chapter did not indicate any difference in basal MPS rates between males and females. Also men lose muscle mass at a greater rate than women (Gallagher *et al.*, 2000; Zamboni *et al.*, 2003). Due to these differences, it indicates that methods for protecting muscle mass could require gender specific considerations.

The enhanced muscle FSR response in elderly women from HMB supplementation may represent an increase in anabolic signalling proteins making a potential supplementation to reduce the effect of anabolic resistance. As EAA (3g) supplementation increased phosphorylation of anabolic signalling proteins (i.e., p70s6Kinase) greater than whey protein (20g) in a comparable cohort of older women (Bukhari *et al.*, 2015). This is likely due to the leucine content in the EAA feed (Bukhari *et al.*, 2015), as leucine stimulates the mammalian target of rapamycin (mTOR) pathway (Atherton, Etheridge, *et al.*, 2010). Further, as HMB is a metabolite of leucine, it stimulates the mTOR signalling pathway as well as having an anticatabolic effect this could have had an additional impact of MPS (Wilkinson *et al.*, 2013). The whey protein supplemented in the current study is half the concentration of work by Bukhari and colleagues (2015; 40g vs. 20g of whey protein). As such, the “muscle full” phenomenon (Atherton, Etheridge, *et al.*, 2010) maybe occurring here, with the HMB being able to stimulate MPS on its own (Wilkinson *et al.*, 2013), but when supplemented with whey protein a blunted response occurs rather than further stimulating MPS. Further, the work supporting the muscle full phenomenon was conducted only in

men, therefore there could potentially be gender differences in this intrinsic regulation that allow additional stimulation of MPS only women e.g. a greater stimulation of an anabolic stimuli to trigger the muscle full effect. No significant difference in the FSR of older men when HMB was provided in line with previous work, where leucine adjuvant to whey protein was not able to further stimulate MPS (Koopman *et al.*, 2008) further indicating a “muscle full” effect.

3.4.2 The effect of HMB on albumin protein synthesis in response to whey protein in older men and women

The supplementation of HMB with whey protein has no impact on albumin FSR in both elderly males and females. The sexual dimorphism exhibited in the muscle FSR data was not replicated in the albumin FSR data (Figure 3.2 and 3.3). Contrary to the literature where previous work has identified males have a greater albumin FSR in response to mixed meals (Aizhong, Sreekumaean Nair and Strong, 1998) and dietary protein (Thalacker-Mercer *et al.*, 2007) than females. Although with regard to the dietary protein study (Thalacker-Mercer *et al.*, 2007), the protein was fed hourly and first postprandial blood samples were collected at 180 minutes, therefore due to the study design the initial response to the supplementation of albumin FSR may have been missed.

The difference between the fed and fasted state in albumin FSR in response to whey protein and HMB was expected and occurred in most

of the groups (females; +HMB and males; +/-HMB). These results are in line with the literature of increased albumin turnover in response to dietary AA in children (James and Hay, 1968), young (Caso et al., 2007; Moore et al., 2009) and older adults (Caso *et al.*, 2007). Older adults also have a greater increase in rate of albumin synthesis in response to nutrient ingestion (Caso *et al.*, 2007) compared to younger individuals. Due to the increased AA availability either in response to dietary protein increasing the plasma AA or surplus pool from the skeletal muscle (De Feo, Horber and Haymond, 1992). It was more likely to be due to the increased dietary protein causing the increase in albumin FSR aiding in transport of its various nutrients throughout the body (e.g., AA and Fatty acids).

3.4.3 Future areas to expand this field of research

The results of this study have highlighted subtle differences in the muscle protein synthetic responses to whey protein with and without HMB between older males and females. Therefore, further research is needed to better understand:

1. **Differences in protein metabolism in elderly people-** From the research shown elderly males and females respond differently to the combined supplementation of HMB and whey protein, therefore further investigation is required to assess other external stimuli that impact protein metabolism to identify if potential sexual dimorphisms, particularly nutritional supplementation. This may help future

treatments of mitigate age-associated declines in skeletal muscle 'health'.

2. **The impact of HMB with and without whey protein on other tissues -** Based on our findings of improved albumin synthetic responses with the combined supplementation of whey protein and HMB in older females, future research should explore the impact of HMB on other tissues. The impact of HMB and whey protein in the elderly needs to be assessed in other pools of protein to identify if there was other changes in the protein metabolism for example the impact of HMB on the changes in the rate of liver protein metabolism, due to HMB and albumin being synthesised in the liver.
3. **The impact of HMB on protein metabolism in other mammalian species -** This research could be expanded into other mammalian species such as companion animals, to explore the impact of HMB on aspects of health and sufficient protein intake.

3.4.4 Conclusion

From this study it suggests that there is a sexual dimorphism in the stimulation of muscle protein synthesis with the combined supplementation of HMB (3g) and whey protein (40g) in elderly people. With elderly women having an additional positive response to muscle protein synthesis with this supplementation, suggesting that muscle protein synthesis in elderly women was affected to differing degrees to nutritional stimuli than elderly males. This same dimorphism was not apparent in the rate of albumin synthesis, under the same conditions,

suggesting that this response was specific to certain protein pools. Therefore, the treatment for preventing/ delaying the effects of sarcopenia may require gender specific methods, particularly with regards to nutritional supplements.

4. The comparison of animal and plant hydrolysates on muscle protein synthesis in C2C12 cells

4.1 Introduction

4.1.1 Chapter synopsis

This chapter compares two methods (SUnSET technique and ^{13}C proline) for assessing MPS in a skeletal muscle cell line (C2C12) model using hydrolysates from specific protein sources (plant and animal). Following on from the comparison of the two methods, the anabolic signalling responses were compared when the cells were provided with different concentrations of media and the different hydrolysed protein sources. The first study compared the two methods where it was hypothesised that both methods would increase as the AA concentration increased. Although only the ^{13}C proline method showed this relationship. The second experiment, used information from the first, investigated the effect of different protein sources. It was hypothesised that animal-based protein sources would have a more beneficial impact on MPS due to the greater concentration of all EAA. However, there was no significant difference between the FSR when the cells were provided with either plant or animal protein source.

4.1.2 In vitro studies in the field of nutrition

In many scientific settings *in vitro* studies are a crucial tool that enables experiments to be performed without utilising the whole organism. For example, *in vitro* approaches allow compounds that could unintentionally harm whole organisms to be tested with minimal risk and can also facilitate a better understanding of mechanisms underlying cellular physiological changes in response to experimental paradigms. For example, in relation to nutrition and skeletal muscle, *in vitro* studies showed that leucine can stimulate MPS through anabolic signalling to a greater degree than other AA (Atherton, Smith, *et al.*, 2010). There are multiple benefits to *in vitro* studies in that are they:

1) are often quicker to conduct that whole-organism experiments;

2) reduce the use of animals;

3) can enable a large sample size;

4) are often cheaper than whole-organism experiments;

5) facilitate toxicity experiments (e.g., using puromycin to assess protein synthesis at high concentration can have a negative effect on protein synthesis, and it is unclear if the effect is permanent (Yarmolinsky and Haba, 1959).

However, there are also multiple considerations when conducting *in vitro* studies, primarily related to extrapolating findings to whole organisms, as multiple organ system models may respond differently to the same stimulus/treatment/compound (Polli, 2008). For example leucine

supplementation in rats increases liver, muscle and adipose protein synthesis rates but has no effect on kidneys and heart protein synthesis rates (Lynch *et al.*, 2002). Therefore *in vitro* studies are often best complemented with *in vivo* studies to achieve a more complete understanding (Polli, 2008).

4.1.3 Methods to assess protein synthesis in cells

There are different ways that the rate of protein synthesis can be determined. In *in vitro* these methods revolve around the inclusion of a labelling molecule to fix to proteins during synthesis. The labelled molecules can either be incorporated into a protein with a labelled amino acid or attached to the protein during synthesis. Both methods will be assessed in this chapter, the surface sensing of translation (SUnSET) blotting method measuring protein synthesis via attachment during protein synthesis and a stable isotope labelled amino acid to measure protein synthesis via amino acid incorporation using ^{13}C proline.

4.1.3.1. Surface sensing of translation method to assess protein synthesis

The SUnSET blotting method is a nonradioactive fluorescence-activated cell sorting-based assay (Schmidt *et al.*, 2009) which measures protein synthesis within a cell with puromycin an aminonucleoside antibiotic, produced by *Strepomyces alboniger.* Puromycin is a structural analogue of aminoacyl tRNAs that gets incorporated into polypeptide chains preventing peptide elongation (Goodman and Hornberger, 2014).

However, elongation only shuts down at high concentrations of puromycin to inhibit protein synthesis (Yarmolinsky and Haba, 1959). Low concentrations have been shown not to inhibit translation but can reflect overall rates of protein synthesis (Schmidt *et al.*, 2009). The first use of this technique to determine rates of protein synthesis was to assess whole tissue rates with ^{3}H-puromycin in rats (Nakano and Hara, 1979). Since then, a relatively large body of work has used puromycin to assess the rates of protein synthesis in different fibre types in both *in vitro* and *ex vivo* experiments (Goodman *et al.*, 2011). When provided *in vivo* it has been shown that the rate of puromycin binding over a 24 hr period is fastest between 2 and 6 hours and between this time the rate is constant (Crossland *et al.*, 2017). Therefore, puromycin provided for ~4 hours is the most sensitive and robust time-period to assess differences between treatments. This method has also been used to assess nutritional interventions on MPS in mice (e.g. fasted vs. leucine vs. leucine fortified whey protein, with the leucine fortified whey increasing MPS compared to fasting but not leucine sole supplementation; Dijk *et al.*, 2018). Therefore, as this method has been proven to measure MPS *in vivo* and from nutritional intervention it was taken forward for the experiments reported in this chapter (section 1.17.2 on for more detail).

4.1.3.2. ^{13}C proline to assess the rate of protein synthesis

Addition of the stable isotope ^{13}C proline to cell culture can be used to measure the rate of protein synthesis by measuring the incorporation of labelled proline into protein (Atherton *et al.*, 2009). This approach works

by providing a large bolus of 50% enriched ^{13}C proline to the cell culture media. This flooding dose equilibrates with the proline in the media, flooding all the amino acid pools (i.e., extracellular, intracellular and amino-acyl tRNA) to the same level of enrichment. The labelling of proline taken up into protein can then simply be measured in the media i.e., the precursor pool for protein synthesis. In addition, the amount of ^{13}C proline incorporated into the cell protein is also measured (the product) over time and the rate of protein synthesis can be calculated from the precursor-product relationship. This approach has been validated in cells (Atherton *et al.*, 2009) and theoretically avoids uncertainties about the choice of precursor pool from which to estimate protein synthesis. An added advantage of using a proline flood approach is that it does not affect the pathway it is being used to measure, opposed to using a labelled EAA such as leucine or phenylalanine. Indeed, previous work in young males has shown that there is no significant difference between the myofibrillar FSR using a primed constant infusion of ^{13}C leucine (the more common tracer used for assessing protein synthesis) and ^{13}C proline (Babraj *et al.*, 2005). This suggests that FSR measured by ^{13}C proline is accurate, can overcome uncertainties around precursor labelling and does not impact protein synthesis directly.

4.1.4 Objective

The primary aim for this chapter is to compare the sensitivity of the SUnSET blotting method and ^{13}C proline method to determine the level rate of protein synthesis in C2C12s, on a media with increasing

concentrations of hydrolysed animal protein dictated by a fixed leucine concentration (2mM to 0.016mM leucine) over a 4 hour period. The secondary aims are to:

- assess the changes in the anabolic signalling protein phosphorylation and concentrations levels when the media protein source is altered uniformly according to the leucine concentration.
- Compare the rates of protein synthesis of C2C12 when grown on a plant or animal protein source matched by the concentration of leucine and how the AA concentrations from the different protein sources impact the anabolic signalling proteins.

The hypothesis for this chapter is:

- The data from the ^{13}C proline and SUnSET techniques used to detect muscle protein synthesis will correlate when animal hydrolysate media concentrations increase;
- The anabolic signals regulating protein synthesis will increase in response to increasing AA concentration in the media.
- Cells grown on animal hydrolysate will have the highest rate of protein synthesis and cells grown on plant hydrolysate will having the lowest rate.

4.2 Methods

4.2.1 Cell culture methods

Murine C2C12 myoblasts (ECACC, Salisbury, UK) at passage 6-8 were cultured at 37ºC and 5% CO_2 in Dulbecco's modified Eagle media, high glucose, pyruvate, no glutamine (DMEM, Cat no. 21969035, Thermo Fisher Scientific, UK) supplemented with 10% (V/V) foetal bovine serum (FBS), 1% (V/V) of 200mM L-Glutamine (Cat no. G3126, Merck, UK) and 1% (V/V) antibiotic-antimycotic (Sigma-Aldrich, UK). The cells were seeded in a T75 culture flask (Thermo Fisher Scientific, UK) with 12ml of DMEM per flask with fresh media every 2 days. The cells were split into 6 well plates (Nunclon™ Delta; Thermo Fisher Scientific, UK) at between 80-90% confluency. To split the cells, the media was removed, and the cells were washed twice with Hanks Balance Salt Solution (HBSS, Sigma Aldrich, UK) for 30 seconds each time. Trypsin at 1ml and the flasks were incubated (37ºC with 5% CO_2) for 3-5 minutes until the cells detached. The trypsinised cells were resuspended in fresh DMEM media solution (15ml), and a 10ml strippette was used to gently disperse any clumps of cells. Once the cells were separated into single cells the 6 well plates were incubated (37ºC with 5% CO_2) for 4 hours, to allow the cells to adhere to the surface of the plate. Once at approximately 90% confluency, differentiation was induced by substituting the DMEM with 10% (V/V) FBS media with DMEM with 2% (V/V) horse serum. The

experiments were performed on day 5 following the addition of DMEM with 2% horse serum media (see figure 4.1).

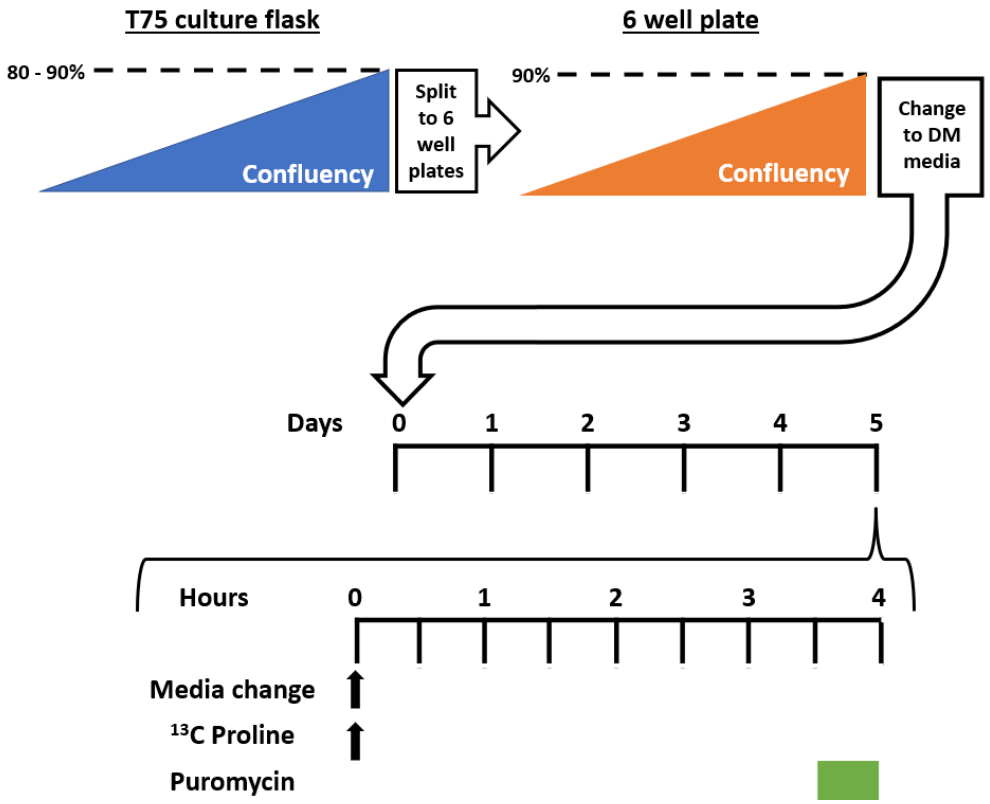


Figure 4.1: *Schematic overview of the experimental protocol for the cell culture aspect of both experiments*

4.2.2 Experimental media preparation

For both experiment 1 and 2, peptone from animal proteins (cat no. 77180-100G, Sigma Aldrich, UK) was used, this is a mixed animal protein source derived from pig. In experiment 2, the peptone special (vegetable) (cat no. 92976-500G-F, Sigma Aldrich, UK) was also used, this is a mixed vegetable source with the original vegetables used unknown. As the proteins are hydrolysates this means that the proteins are partially

broken down into various peptide chain lengths as well as some free AAs (Manninen, 2009). This meant that it was easier to hydrolyse the samples further to increase the concentration of free AAs provided in the media for the cells.

In experiment 1, 757 mg of the animal protein was equally aliquoted in 15 boiling tubes and hydrolysed in Dowex with 1M HCl at 110^{o}C overnight. Following hydrolysis, the samples were purified though cation exchange columns (see experimental chapter 2). The samples were then dried and resuspended in amino acid free media (28ml; 20mM hepes buffering solution, 140mM NaCl, 2.5mM $MgSO_4$, 5mM KCl and 1mM $CaCl_2$) to produce media with approximately 2mM leucine concentration (27mg of hydrolysate/ml in media). The media was then diluted with amino acid free media and serially diluted to give 1, 0.5, 0.25, 0.125, 0.0625 and 0.03mM leucine.

In experiment 2, a media of either only plant (PM) or animal (AM) protein was prepared as in experiment 1. However, 676 mg of each protein source was mixed with 25ml of the amino acid free media (27 mg/ml of hydrolysate in media). The highest media concentrations were based on a 2mM leucine concentration in animal protein. This highest concentration was serially diluted to 1, 0.2, 0.1, 0.02 and 0.01mM of leucine in the media. Similar concentrations of a 50:50 blend (BM) of the plant and animal media were made.

4.2.3 Experimental design

In experiment 1, 3 wells were allocated per media concentration (2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03mM leucine), with an additional 6 wells for the positive controls of DMEM with 2% horse serum (3 for the enriched positive control with ^{13}C proline and 3 for the baseline enrichment positive control with ^{12}C proline). In experiment 2, 6 wells were allocated per concentration of each protein source (2, 1, 0.2, 0.1, 0.02 and 0.01 mM leucine) and as with experiment 1 an additional 6 wells contain the positive control.

On the morning of the experiment, the DMEM with 2% horse serum was removed from the cells and the cells were washed twice with HBSS (Sigma Aldrich, UK). Following this, 2ml of the protein free media was added with 5µl of puromycin at 200mg/ml. The 6 well plates were incubated at 37^{o}C and 5% CO_2 for 3 hours 30 minutes, at which point 24µl of a 50% enrich ^{13}C proline at 8mg/ml were added to all wells bar 3 in each experiment allocated for the baseline enrichment positive control wells where 24µl of 100% ^{12}C proline at 8mg/ml was added. The samples were then incubated (37^{o}C and 5% CO_2) for a further 30 minutes. Following this, the samples were removed from the incubator and the media collected and the cells scraped and collected.

4.2.4 Sample Collection

At the end of the experiment, 1ml of the media was collected and frozen (-20^{o}C). The 6 well plates were washed in ice-cold phospho- buffered saline (2ml; PBS) and collected with ice-cold Homogenization buffer (200µl; 50mmol/L Tris-HCL, pH 7.5, 1mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β-glycerophosphate, 50mmol/L NaF and a complete protease inhibitor cocktail tablet; Roche, West Sussex UK) and lysed by repeatedly passing through gel-loading pipette tips. The samples were then centrifuged (12000rpm for 10mins at 4^{o}C) and the supernatant was removed, with 50µl for western blotting (section 4.2.6) and the remaining for measuring intracellular proline enrichment for the FSR calculation (section 4.2.5). The pellet contained proteins and peptides to measure the ^{13}C proline incorporation for the FSR calculation (section 4.2.7).

4.2.5 Amino acid concentration and enrichment

The media collected from the cell experiments was used to measure the AA concentration and ^{13}C proline enrichment (used in determining the FSR of the cells). Both measures utilised method for isolation, derivatisation, and analysis, the only differences were the internal standards used and the specific masses detected.

4.2.5.1. Isolation of amino acids in media and derivatisation

To measure the concentration of AA in the media, 10µl of an amino acid internal standard was added for quantitation with reference to a standard

curve for each AA to fix the ratio of each amino acid to the internal standard. The internal standard was not added during ^{13}C proline enrichment analysis as the internal standard contained the same ^{13}C proline and would interfere with the enrichment measure. The samples were then run through cation exchange columns (see section 3.2.3.4 for method)**,** however the volumes of the solutions were reduced pro rata, since only 1 ml of dowex resin was needed, therefore the volumes of HCl, NH_4OH and ddH_2O were reduced by a third. Once the samples were eluted from the columns the samples were dried at 90^oC with a constant flow of N_2, using a Dri-block heater© (Dri-Block heater, Techne, Staffordshire UK). The samples were derivatised with equal volumes of N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA) and Acetonitrile vortex mixed and heated to 90^oC for 1 hour. The samples were then transferred to an autosampler vial ready to be analysed on the mass spectrometer.

4.2.5.2. Mass spectrometer conditions

To measure the amino acid concentration and the enrichment of ^{13}C proline the samples were analysed on an ISQ Series Single Quadrupole GC-MS Systems (Thermo Fisher Scientific, UK). They were injected 1µl with a 1:20 split into an injector temperature of 250^oC and an initial oven temperature of 100^oC on a 30m RXI -17 Restek GC column (0.25mm and 0.25um, column dimensions and film thickness respectively, Restek Pure Chromatography, US). The oven temperature was held for 1 min at 100^oC and then ramped to 300^oC, at a rate of 12^oC/min. The AA retention

time and masses shown in the table below (Table 4.1; software Thermo Xcalibur).

Amino Acid	Retention Time (min)	Masses (m/z)
Alanine	7.29	260, 261
Glycine	7.51	246, 248
Valine	8.4	288, 289
Leucine	8.83	302, 304
Isoleucine	9.13	302
Proline	9.46	286, 287
Methionine	11.19	320, 321
Serine	11.38	390, 391
Threonine	11.64	404, 405
Phenylalanine	12.18	234, 235
Glutamate	13.54	432, 433
Lysine	14.30	431, 432
Glutamine	14.60	431, 432
Tyrosine	16.05	466, 467
Tryptophan	17.67	244, 249

Table 4.1: Amino acid derivative masses and retention times from the derivative MTBSTFA when analysed on the ISQ Series Single Quadrupole GC-MS Systems (Thermo Fisher Scientific, UK)

4.2.6 Measurements to assess cell signalling and SUNSET technique

Both the SUnSET technique and method for measuring cell signalling have similar protocols as both measures involve western blot analysis. The western blot analysis uses electrophoresis to separate protein solution via weight before transferring the proteins onto a blotting paper, followed by targeting the desired proteins with antibodies and measuring level of adherence to the target protein. To accurately assess the desired output, (either the relative levels of bound puromycin/target protein or relative the level of phosphorylation/activation of a target protein) the

protein concentration of the samples must be measured and matched. This ensures that any difference identified between samples are not false positives.

4.2.6.1. Protein quantification

Protein concentration was quantified using a NanoDrop™ 2000 (Thermo Fisher Scientific, UK; Desjardins, Hansen and Allen, 2010). Once quantified, the lowest protein concentration was used as the reference to match the other samples. Therefore, the samples were diluted to 0.16µg/µl and 0.55µg/µl for experiment 1 and 2, respectively. Once diluted to the same concentration and volume of 33.33µl and 16.67µl, 3x Laemmli Loading dye (24% 1M tris-HCL, pH6.8, 30% of 20% Sodium dodecyl sulphate, 30% glycerol, 16% 2-Mercaptoethanol and 0.6% Bromophenol blue) was added. The samples were then heated at 95^{o}C for 5 minutes prior to loading.

4.2.6.2. Western blot

The protein (10µl at the protein concentrations stated above) from each sample was loaded onto 12% Criterion XT Bis-Tris gels (Bio-Rad Laboratories, Hemel Hempstead, UK) and separated via weight through electrophoresis at 200V for 55 minutes in Bio-rad criterion western tank with 5% Bio-rad XT mops running buffer. The gels were transferred to polyvinylidene difluoride membrane at 100V for 45 minutes, then blocked with 2.5% milk in Tris Buffered saline with tween-20 (TBST, pH 7.6, 0.8% sodium chloride, 0.242% Tris base and 0.1% Tween-20) for 1 hour at

room temperature. Following the blocking the membrane was washed with TBST and then incubated with the primary antibody overnight at 4^{o}C.

- **For muscle protein synthesis**, the surface sensing of translation technique (SUNSET) (Schmidt *et al.*, 2009), using puromycin to label the peptides in experiment 1- a monoclonal puromycin antibody at 1:5000 (12D10; EMD Millipore, Watford, UK)
- **For cell signalling in experiment 1**- mTOR s2481 (Cell Signalling Technology. Danvers, USA), ribosomal protein S6 kinase 1 (p70 S6K1, Cell Signalling Technology. Danvers, USA), eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1, Cell Signalling Technology. Danvers, USA), Elongation factor 2 (EEF2, Cell Signalling Technology. Danvers, USA).
- **For cell signalling in experiment 2** – the same as experiment 1 however an additional phosphorylated protein Kinase B (PKB or AKT, Cell Signalling Technology. Danvers, USA).

Following the overnight incubation with the primary antibody the membranes were washed with TBST and the membranes were washed with an anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody for the SUnSET technique and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody for the cell signalling proteins. The secondary antibodies were incubated at room temperature for an hour. The membranes were then washed with

TBST before the bands were detected using Chemiluminescent HRP substrate (EMD Millipore, Watford, UK) with a Chemidoc XPS imaging system (Bio-Rad Laboratories). To normalise the detected signal response the samples were normalised using Coomassie Brilliant Blue staining of the membrane. The bands were quantified by densitometric analysis using Image Lab software (version 5 build 18; Bio-Rad Laboratories).

4.2.7 Proline enrichment analysis

From the sample preparation in section 4.2.4, equal volumes of 1M perchloric acid were added to the sample to resuspend the pellet. The samples were then centrifuged (12000 rpm at 4^{o}C for 10 minutes), the supernatant removed and then washed with 1ml 70% ethanol before being centrifuged (12000 rpm at 4^{o}C for 10 minutes). The supernatant was removed and the protein taken up in 1ml of 0.1M HCl, combine with 1 ml dowex slurry and incubated at 110^{o}C overnight. The hydrolysed samples were run through columns to purify and isolate the amino acid fraction (see section 3.2.3.4 for method), these were dried down under nitrogen at 90^{o}C.

4.2.7.1. Preparation of protein derived free ^{13}C Proline for enrichment analyses

To the dried down sample, 60µl of doubly distilled water, methanol (32µl) and pyridine (10µl) were added. Methylchloroformate (8µl; MCF) was pipetted directly into the aqueous mix and immediately vortex mixed for

30 seconds, then left at room temperature for 5 minutes. Chloroform (100µl) and 0.001M $NaHCO_3$ (100µl) were added and vortex mixed, allowed to equilibrate, and the upper aqueous layer removed. A molecular sieve was then used to remove any remaining water, after mixing for 30 seconds, the chloroform layer was transferred to a mass spectrometry vial ready to be injected into a gas chromatography combustion Isotope Ratio Mass spectrometry.

4.2.7.2. Gas Chromatography combustion Isotope Ratio Mass spectrometry conditions

MCF derivatisation to analysis ^{13}C proline Isotope Ratio Gas Chromatography To measure the amino acid enrichment and Proline ^{13}C enrichment was determined by gas chromatography combustion Isotope Ratio Mass spectrometry (GC-C-IRMS, Delta-plus XP, Thermo, Hemel Hampstead, UK). A 2.5µl splitless injection was used, the injector temperature was 250^oC, with an initial oven temperature of 100^oC and separation achieved with a 30m RXI -17 Restek GC column (0.25mm and 0.25um, column dimensions and film thickness respectively, Restek Pure Chromatography, US). The oven temperature was held for 1 min at 100^oC and then ramped to 160^oC, at a rate of 15^oC/min followed by a ramp of 2^oC/min to 170^oC and then a final ramp of 20^oC/min to 300^oC where it was held for 3 minutes. Reference peak of CO_2 gas was supplied into the detect between 480 to 495 seconds and 760 to 775 seconds (Figure 4.2 for chromatography form GC-C-MS).

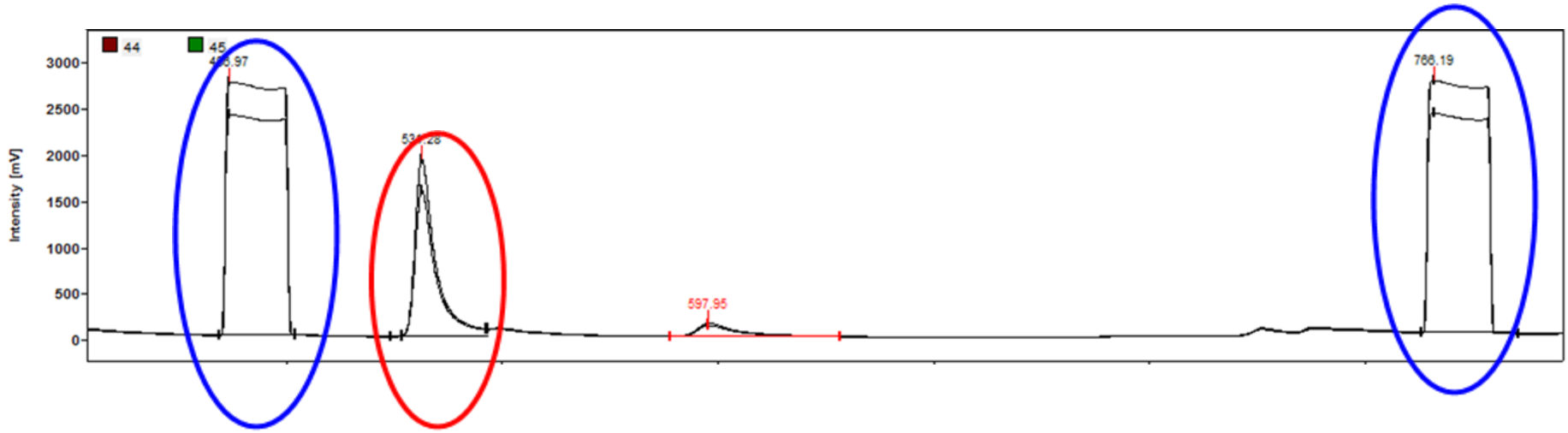


Figure 4.2: *A screenshot of the chromatograph from gas chromatography combustion Isotope Ratio Mass spectrometry, with two reference peaks highlighted by blue circles and the CO_2 peak derived from proline in a red circle*.

4.2.7.3. Proline FSR calculation

Muscle FSR is calculated using the precursor-product approach, the precursor labelling was calculated from analysis of the free ^{13}C proline enrichment in the media and/or the intracellular ^{13}C proline enrichment (by GC-MS) in APE and the enrichment of ^{13}C proline incorporated into protein in APE, following conversion from Delta notation (by GC-C-IRMS). The following equations were used:

The isotope ration R is calculated from the delta value (D) obtained by GC-C-IRMS.

$$\mathbf{R= [(D/1000)+1] \times 0.011}$$

$$\mathbf{\text{Protein proline enrichment (APE, Ep)} =[(R_s-R_{bl})x\ 9.73]/\{1+[(R_s-R_{bl})\ x\ 9.73]\}\ x\ 100}$$

Where R_s is the delta of the sample and R_{bl} is the baseline value, to measure the background enrichment level of the sample the constant factor of 9.73 is used as the dilution factor of the stable isotope of carbon-13 from ^{13}C proline to $^{13}CO_2$.

$$\mathbf{FSR=[(E_{inc}/E_{fp})\ x\ T^{-1}]\ x\ 100}$$

Where E_{inc} is the proline enrichment in the protein, E_{fp} is the free proline enrichment in either the intracellular or media AA and T is time in minutes.

4.2.8 Statistical analysis

All statistical analysis was performed on GraphPad Prism version 9.1.1.225. In experiment 1, results from both protein synthesis tests were analysed against the leucine concentration in the media using regression models. To ensure the correct model was applied to the data a Normality and Lognormality test was performed on both data sets this indicated that the results from the ^{13}C proline FSR and the SUnSET technique had different distributions, the ^{13}C proline FSR data most likely having a Lognormal distribution, so a nonlinear regression analysis was used and the SUnSET techniques data being normally distribution, so a linear distribution was used. In addition, the one-way analysis of variance (ANOVA) was used to compare the rate of protein synthesis of each method verses the concentration of leucine in the media. Also, a correlation analysis was also used to identify a relationship between the two techniques. The anabolic signalling results from this experiment were analysed using a one-way ANOVA to determine increases in either phosphorylated signal or total signal.

In experiment 2 all experiments (media concentrations, ^{13}C proline enrichments and cell signalling) were analysed using a two-way ANOVA or a mixed effect analysis dependent upon if the data contained an equal or unequal number of replicates, respectively. In all experiments that required post-hoc analysis to compare differences between individual data sets a Tukey multiple comparison was used.

4.3 Results

4.3.1 Experiment 1 – Effect of animal hydrolysate concentration on protein synthesis in C2C12 cells

4.3.1.1. The concentration of animal hydrolysate in amino acid free media

The highest concentration of the hydrolysed peptide used an estimated target of 2mM leucine as previously reported to be the maximum nutritional stimulation of MPS in cells (Atherton, Smith, *et al.*, 2010). Subsequent media concentrations were diluted by 50% to ensure a broad range of media concentrations. The average percentage difference between the expected leucine concentration and the actual leucine concentration measured in the media was 16% (Table 4.2). This protein source had approximately 10.6% leucine and 19.8% branch chain amino acids of the total amino acid content measured; cysteine was not measured due to the

complexity of the dimerization of cysteine into cystine preventing accurate measure of the concentration.

Expected concentration (mM)	*Calculated Concentration (mM)*					
Leucine	Leucine	TAA	EAA	BCAA	Aromatic	Aliphatic
2	1.83	16.27	7.10	3.58	0.74	7.42
1	1.07	8.83	3.88	1.92	0.51	4.12
0.5	0.61	5.19	2.17	1.08	0.34	2.39
0.25	0.33	2.60	1.16	0.56	0.22	1.18
0.125	0.16	1.40	0.61	0.28	0.13	0.61
0.063	0.06	0.71	0.30	0.13	0.06	0.28
0.031	0.04	0.55	0.22	0.09	0.05	0.21
0.016	0.03	0.32	0.14	0.06	0.03	0.12

Table 4.2 *Amino acid concentration of the media at the start of the experiment (mM). TAA, total amino acids; EAA, essential amino acids; BCAA, branched chain amino acids.*

4.3.1.2. ^{13}C proline enrichment in media, intracellular water and protein and the impact on FSR

The concentration of ^{13}C proline added to each well was fixed at 8mg/ml, although the concentration of the rest of the amino acids in the media concentration was halved (Figure 4.3), therefore there was not a uniformed flooding dose of ^{13}C proline provided to each of the wells. The ^{13}C proline

enrichment in the media ranged from an APE of 5.7±0.31% to 42.6±0.06% and the ^{13}C proline enrichment in the protein ranged from 0.053±0.013% to 1.96 ±0.029%.

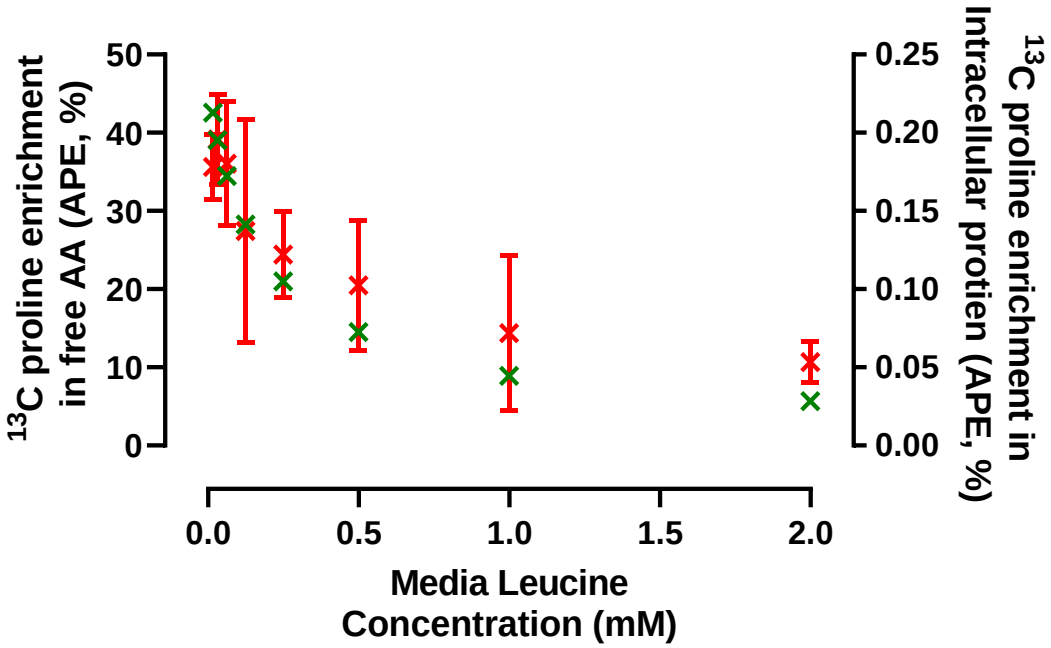


***Figure 4.3**. The ^{13}C proline media (green) and protein enrichment (red) of C2C12 at various concentrations of animal hydrolysates with the error bars representing the standard deviation.*

The FSR calculation using the ^{13}C proline enrichment in the protein and media showed that there was a significant difference ($P<0.05$) between the FSR at the highest media AA concentration (2mM leucine: FSR, 1.88±0.44%/h) and the lowest 0.016mM leucine: 0.84±0.1%/h (Table 4.3). However, it should be noted that variability within measures was high.

Expected media Leucine Concentration (mM)	***Fractional Synthetic Rate (%/hr)***	***Puromycin as a percentage of the positive control (%)***
2	1.88 ±0.44	76.9
1	1.60 ±1.07	66.6
0.5	1.71 ±0.58	77.9
0.25	1.16 ±0.25	58.4
0.125	0.97 ±0.50	69.0
0.063	1.05 ±0.22	58.4
0.031	1.00 ±0.15	70.9
0.016	0.84 ±0.10[a]	69.0

Table 4.3: *Fractional synthetic rate (%/hr) and puromycin as a percentage of the positive control (%) from experiment 1.* [a] *significant difference from the FSR at 2mM leucine concentration P<0.05.*

Figure 4.4 shows an FSR increasing as the predicted media leucine concentration increases, presenting a non-linear relationship with a predicted plateau FSR of 1.8%/hr. However, the data had a high level of variability and therefore the predicted FSR plateau is an inaccurate, although the data suggest it could lie between a media concentration of 1-2mM leucine. This high level of variability was greatest at the higher concentrations this was most likely due to the decrease in the enrichment.

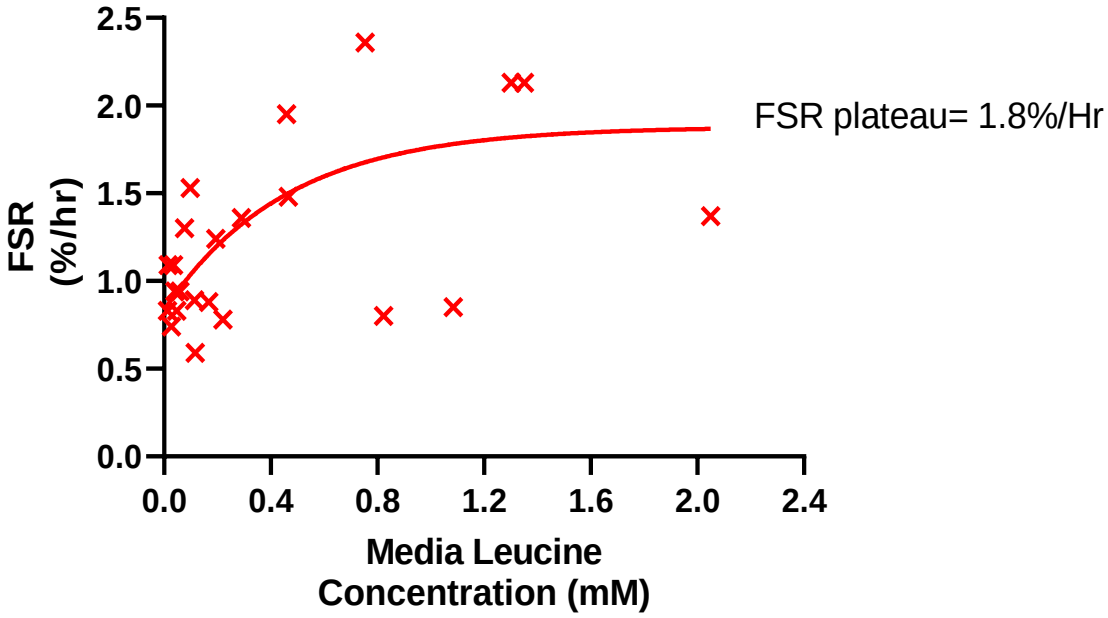


Figure 4.4: *The non-linear relationship of fractional synthetic rate (FSR) of C2C12 cells being provided animal hydrolysate verses the final media leucine concentration. The solid line representing a One-phase association exponential analysis of the data*

4.3.1.3. SUnSET technique measuring protein synthesis in C2C12

The use of the SUnSET technique to assess protein synthesis while providing a range of media concentrations was unable to identify any significant differences between the different concentrations (Figure 4.5). This suggests that the SUnSET technique may not be sensitive enough to identify changes in protein synthesis across varying media concentrations. However, given that this method has previously been used to assess changes in protein breakdown in C2C12 from various treatments (Crossland *et al.*, 2017) and protein synthesis in muscle cells (Goodman

and Hornberger, 2014), this may be due to errors in the experimental design (e.g., the concentration of the puromycin added to the cell) or analysis (e.g., the length of time the primary antibody washed to blotting paper).

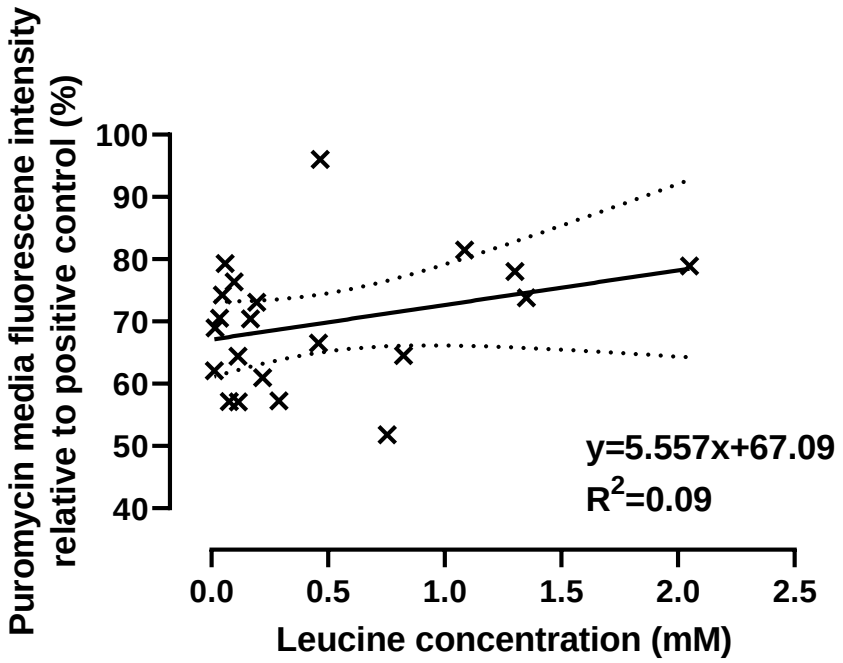


Figure 4.5: *Linear regression of puromycin as a percentage of the positive control versus different concentrations of amino acids (AA) in media using animal hydrolysates in C2C12 cells. The solid line represents the line of best fit and the dotted line the 95% confidence intervals.*

4.3.1.4. Comparison of Proline FSR and SUnSET technique as measures for protein synthesis in C2C12

The SUnSET and the FSR techniques for assessing protein synthesis are not directly comparable without data transformation. FSR measures provide absolute values of the rate of protein synthesis. Puromycin provides relative changes in fluorescence to reflect protein synthesis via the incorporation of puromycin into elongating peptide chains (Goodman and Hornberger, 2014), and is therefore not an absolute rate of protein synthesis. The SUnSET method results were normalised relative to the positive control (puromycin added on C2C12 cells grown on differentiation media) to account for the gel-to-gel variability. After this normalisation, there was no correlation between the FSR and SUnSET technique ($P=0.461$, $R^2=0.0262$; Figure 4.6).

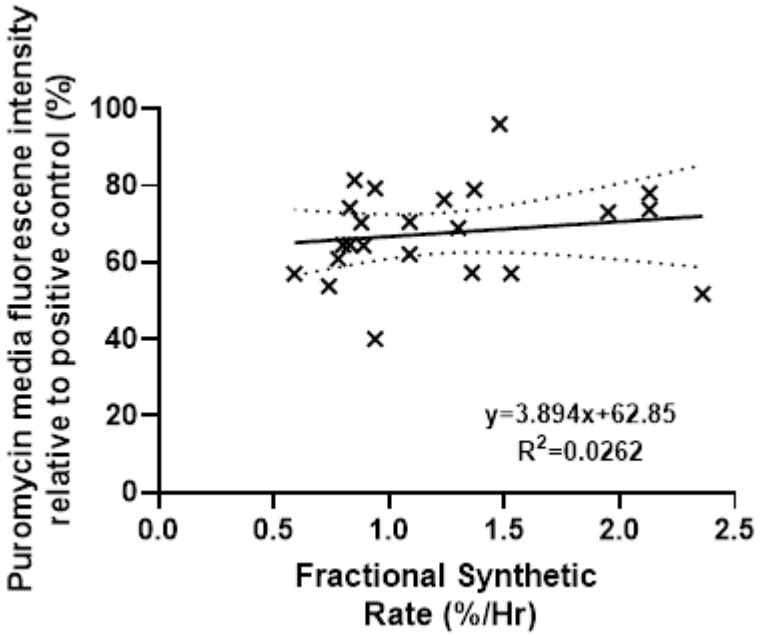


Figure 4.6: *Relationship between two techniques measuring protein synthesis in C2C12 cells: i) SUnSET; using puromycin concentrations relative to a positive control versus ii) FSR (%/Hr) using a stable isotope tracer*. *The solid line represents the line of best fit and the dotted line the 95% confidence intervals.*

Based on this data suggesting that only the FSR technique was able to detect differences in protein synthesis across differing leucine concentrations, only this method of assessment was taken forward to experiment 2.

4.3.1.5. The anabolic signalling response to hydrolysed protein at different concentrations

There was no significant difference in the phosphorylation of any of the signalling proteins assessed (mTOR (A), P70 (B), eEF2 (C) and 4E-BP1 (D)) in response to different media amino acid concentrations (Figure 4.7). All samples were normalised to a positive control to reduce inter-gel variability.

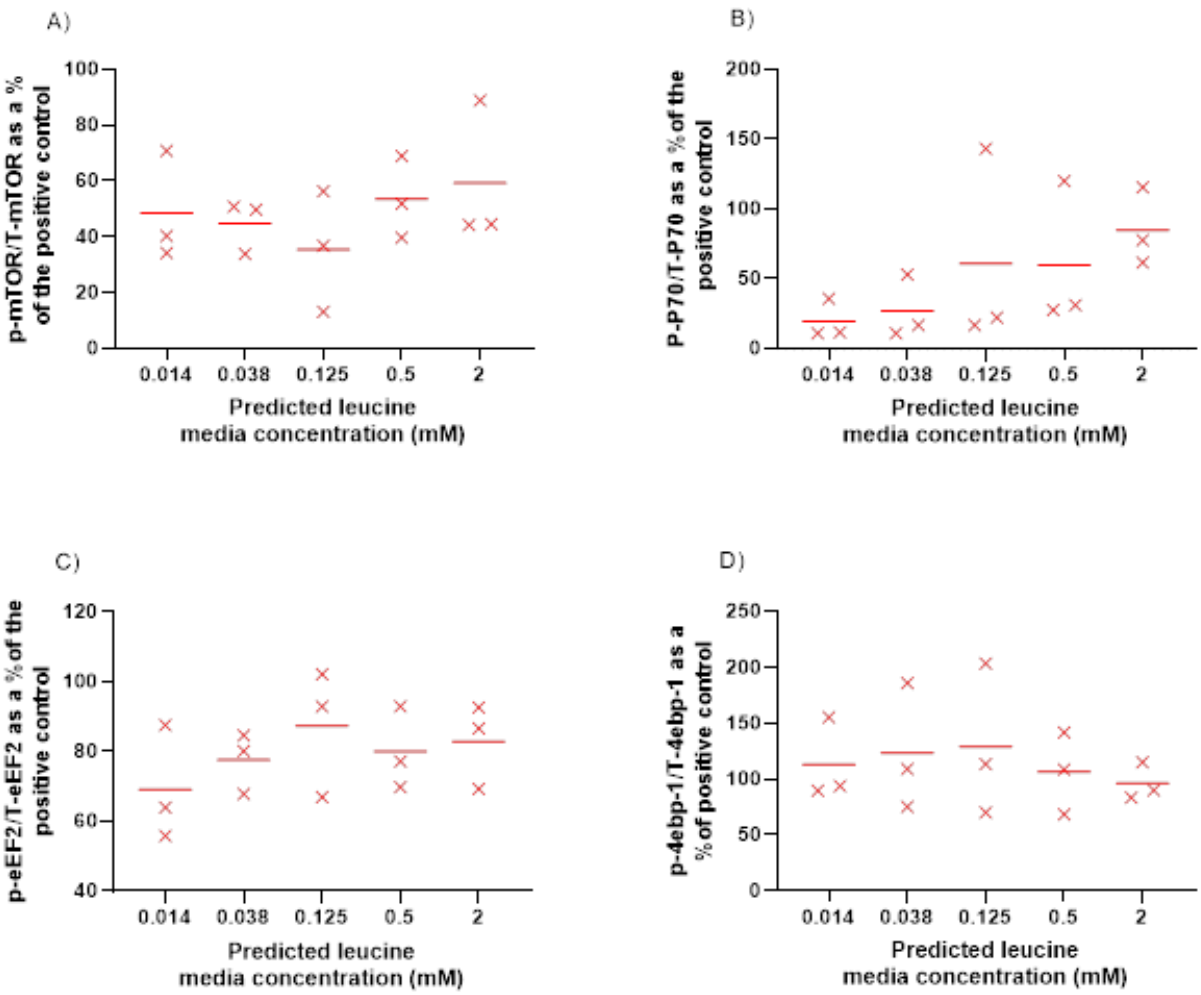


***Figure 4.7**: Anabolic signalling responses of C2C12 provided with different concentrations of animal peptide based on the leucine concentration relative to the positive control. A) p-mTOR, B) p-P70, C) p-eEF2 and D) p-4E-BP1. With the red line representing the mean of each data set*

4.3.2 Experiment 2 – comparison of plant, animal and a 50:50 blend on C2C12

4.3.2.1. The media concentration of plant, animal and 50:50 blend of hydrolysates

At the same apparent leucine concentration there was significant difference ($P<0.05$) in the TAA concentrations (Table 4.4), although Tukey post hoc analysis was unable to determine significant differences between protein sources. Considering individual AA, only alanine, valine, isoleucine, aspartic acid and threonine were significantly greater ($P<0.05$ for alanine, valine and isoleucine and $P<0.01$ for aspartic acid and threonine) in PM than AM. Comparing BM and AM, valine, threonine and aspartic acid significantly greater in BM than AM ($P<0.05$). There was no significant difference between the AM concentrations of glycine, methionine, serine, phenylalanine, glutamic acid, lysine, histidine, tyrosine and as expected leucine and PM or BM. Also there was no significant differences between the AA concentrations in PM and BM.

Amino acid concentrations (mM)	**Animal**	**Blend**	**Plant**	**P value** (Blend vs. animal, Plant vs. animal
TAA	7.52 ±0.89	10.52 ±1.00	10.64 ±1.80	§
EAA	4.11 ±0.35	5.00 ±0.47	5.02 ±0.94	NSD
BCAA	1.96 ±0.24	2.59 ±0.14	2.79 ±0.53	NSD
Alanine	0.74±0.12	1.17 ±0.06	1.20±0.26*	0.034
Glycine	1.22±0.09	1.24±0.03	1.05±0.21[a]	NSD
Valine	0.48±0.06	0.77±0.05*	0.87±0.18*	0.0429, 0.0115
Leucine	1.06±0.09	1.17±0.06	1.15±0.20	NSD
Isoleucine	0.42±0.10	0.65±0.05	0.77±0.17*	0.0227
Methionine	0.24±0.04	0.31±0.02	0.30±0.06	NSD
Serine	0.17±0.01	0.343±0.10	0.40±0.17	NSD
Threonine	0.14±0.01	0.34±0.01*	0.44±0.12**	0.0302, 0.005
Phenylalanine	0.18±0.06	0.15±0.04	0.15±0.04	NSD
Aspartic Acid	0.25±0.03	0.64±0.12*	0.79±0.20**	0.023, 0.0054
Glutamic Acid	0.29±0.04	1.07±0.61	0.83±0.23	NSD
Lysine	1.30±0.08	1.30±0.28	1.03±0.23	NSD
Histidine	0.80±0.13	1.42±0.33	1.60±0.78	NSD
Tyrosine	0.29±0.04	0.31±0.03	0.31±.0.07	NSD

Table 4.4: *The AA concentration (mM) of the animal, plant and 50:50 blend media at the highest concentration used in the experiments (2mM expected leucine). * P<0.05 and P<0.01 denote significant differences in relation to animal media source, with the exact P values provided in the end column. When two values are in the end column the first value relates to Blend media vs. animal media and second value plant media vs. animal media.* § denotes a significant difference P<0.05 however tukey analysis is unable to determine which values are significantly different to others.

4.3.2.2. The ^{13}C proline enrichment in intracellular water and incorporated into protein with each protein hydrolysate – the effect of amount of hydrolysate.

The ^{13}C proline enrichment was measured in media, free intracellular proline and intracellular protein, as in experiment 1. However, in experiment 1 only the ^{13}C proline enrichment in the media was used to calculate the FSR and this resulted in large variability in enrichment between the highest and the lowest concentrations. Therefore, in experiment 2, to minimise the potential impact of this on FSR calculations the ^{13}C proline enrichment from the intracellular water was used. The intracellular enrichment was still inversely proportional to the concentration of the AA in the media but to a lesser extent (Figure 4.8 A-C). However, there was a significant difference ($P<0.05$) in the intracellular enrichment levels across the media concentrations.

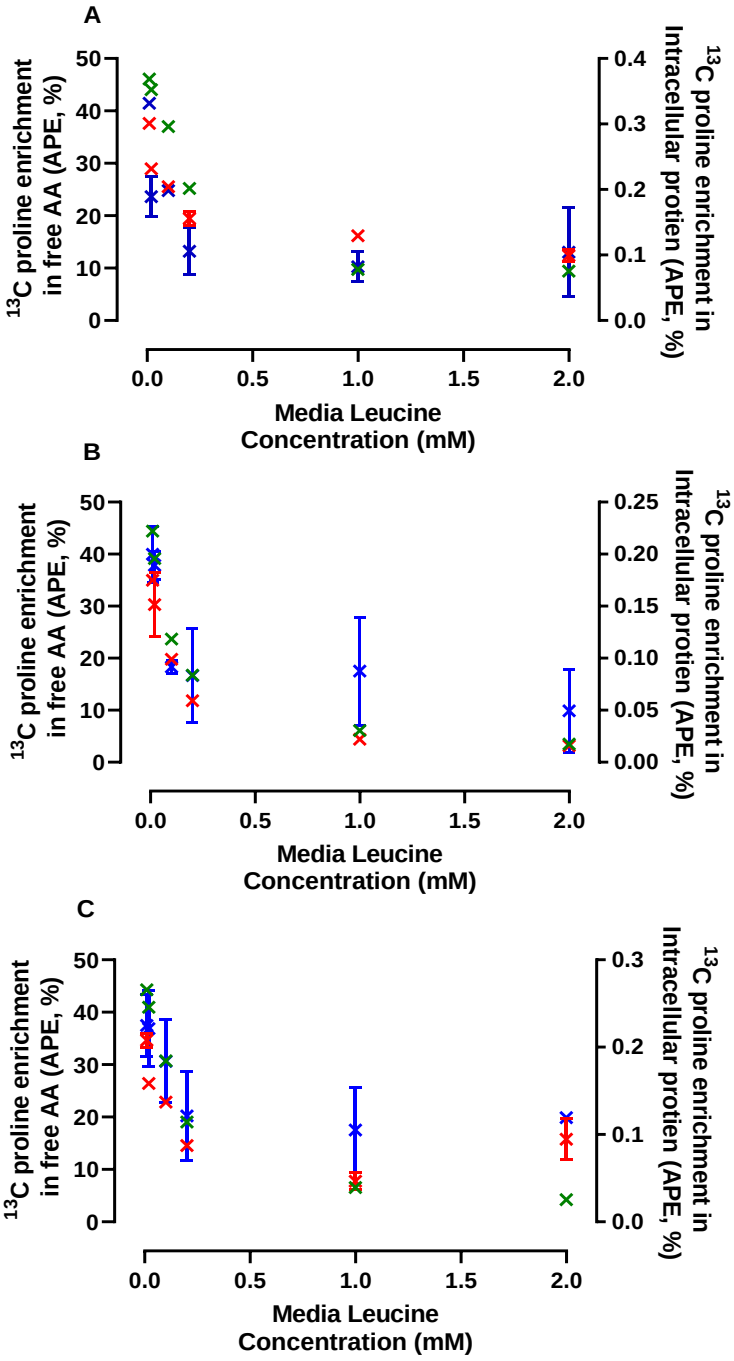


Figure 4.8: *The ^{13}C proline enrichment in the media (green), intracellular water (blue) and intracellular protein (red) of C2C12 at various concentrations of* ***A*** *- animal hydrolysates,* ***B*** *– plant hydrolysate and* ***C*** *– 50:50 blend of plant and animal hydrolysate.*

Even though there was a significant difference in the enrichment levels of intracellular AA and protein, there was no significant difference in FSR across the different concentrations in the media. FSR was significantly greater for AM than BM (1.67±0.04% versus 1.03±0.13%, p=0.0164) only at the lowest leucine concentration. Although there was no significant between FSR in PM and either of the other two media at the lowest leucine concentration despite the mean FSR for PM being lower (PM: 0.93%, AM: 1.67% and BM: 1.03%). There were no significant differences in the rate of protein synthesis within protein sources across differing leucine concentrations (Table 4.7).

Expected Leucine concentration (mM)	**FSR using ^{13}C proline Intracellular enrichment (%/hr)**		
	AM	**BM**	**PM**
2	1.28 ±1.91	0.98 ±1.42	1.30 ±0.81
1	1.00 ±0.49	4.39 ±5.78	3.94 ±2.53
0.2	1.03 ±0.48	1.21 ±0.39	1.03 ±0.61
0.1	1.52 ±0.18	1.26 ±0.24	0.95 ±0.08
0.02	1.29 ±0.32	1.35 ±0.27	1.18 ±0.65
0.01	1.67 ±0.04	1.03 ±0.13[a]	0.93 ±0.31

Table 4.7: *Fractional synthetic rate (FSR) across differing protein sources (AM, animal; BM, blend of 50% animal and 50% plant hydrolysate; and PM, plant) at 6 different concentrations of leucine (2, 1, 0.2, 0.1, 0.02 and 0.01mM). [a] Significant difference (P<0.05) compared to FSR in cells grown in AM.*

4.3.2.3. Cell signalling in response to different protein sources

Even though there was no significant difference between the rates of muscle protein synthesis in C2C12 cells being grown on different protein

hydrolysates matched by the concentration of leucine in the media, anabolic signalling was assessed to determine if this was different when differing hydrolysates were being provided as growth medium.

The mTOR phosphorylation was significantly greater with AM than PM or BM and 2mM leucine concentration (83.4 ±21.5% vs. 57.1±20.4% and 58.0±8.0%, AM vs. PM and BM respectively, $p<0.05$), with no difference at the lower (0.2mM and 0.02mM) leucine concentrations (Figure 4.9).

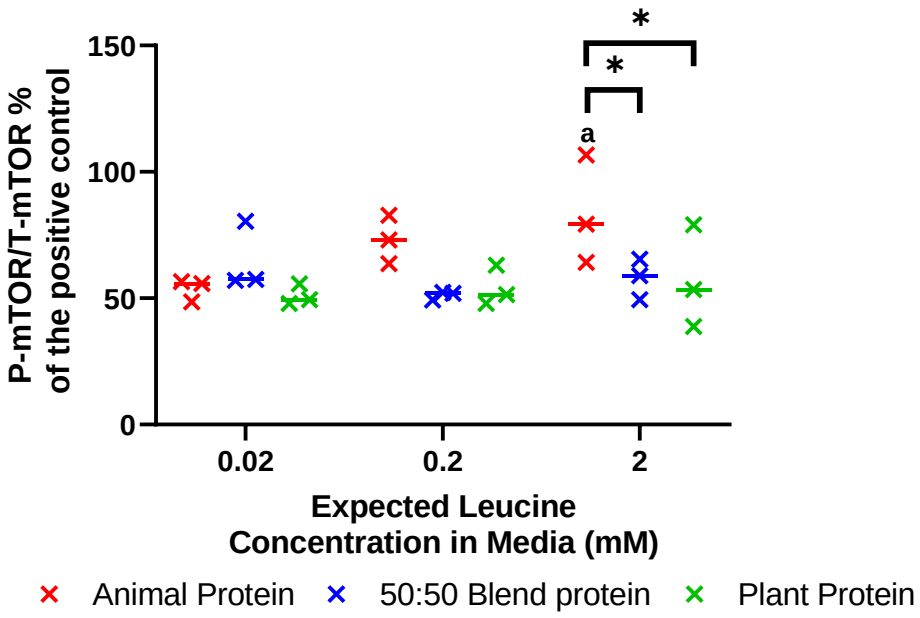


Figure 4.9: *A comparison of mTOR activation in C2C12s in media grown on animal hydrolysate (red), plant hydrolysate (green) and 50:50 blend of plant and animal hydrolysate (blue) The mean represented by the solid line. Significant differences indicated by * P<0.05 between protein sources at the same concentrations and [a]P<0.05 between concentrations of the same protein source*

The P70S6K1 phosphorylation was significantly greater with BM than PM (43.0±2.0% vs. 9.8±3.7%, BM vs. PM respectively) at the lowest concentration of leucine (0.02mM) only, with no significant difference between PM and AM, and no significant difference between BM and AM, despite a marked numerically lower value in AM. Within protein source, P70S6K1 phosphorylation was significantly lower with BM at the highest (2mM) compared to the lowest protein concentration (6.4±2.0% vs. 43.0±2.0% for 2mM and 0.02mM leucine of BM media respectively; Figure 4.10).

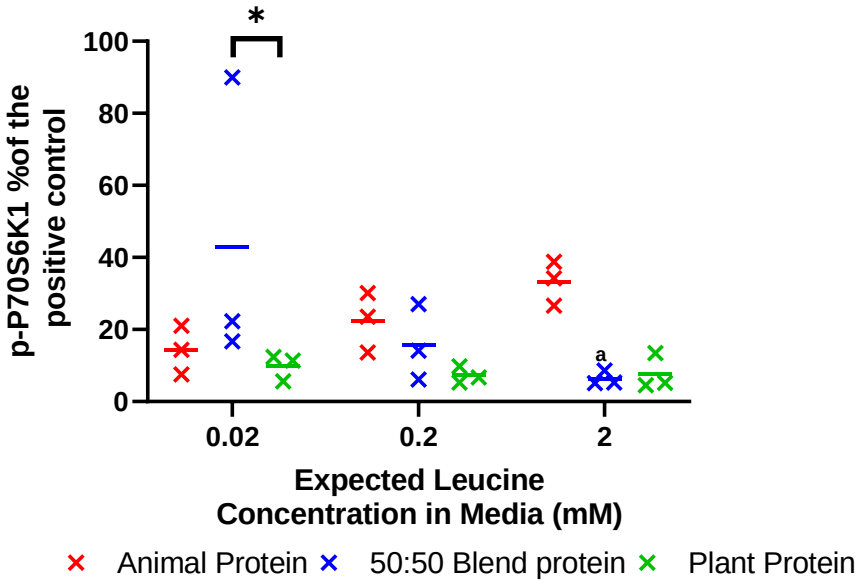


Figure 4.10: *A comparison of phosphorylated P70S6K in C2C12s in media grown on animal hydrolysate (red), plant hydrolysate (green) and 50:50 blend of plant and animal hydrolysate (blue) The mean represented by the solid line. Significant differences indicated by * P<0.05 between protein sources at the same concentrations and [a]P<0.05 between concentrations of the same protein source*

There was no significant difference across media protein source or leucine concentration for phosphorylation of 4EBP-1 (figure 4.11A) or activation of eEF2 (Figure 4.11B).

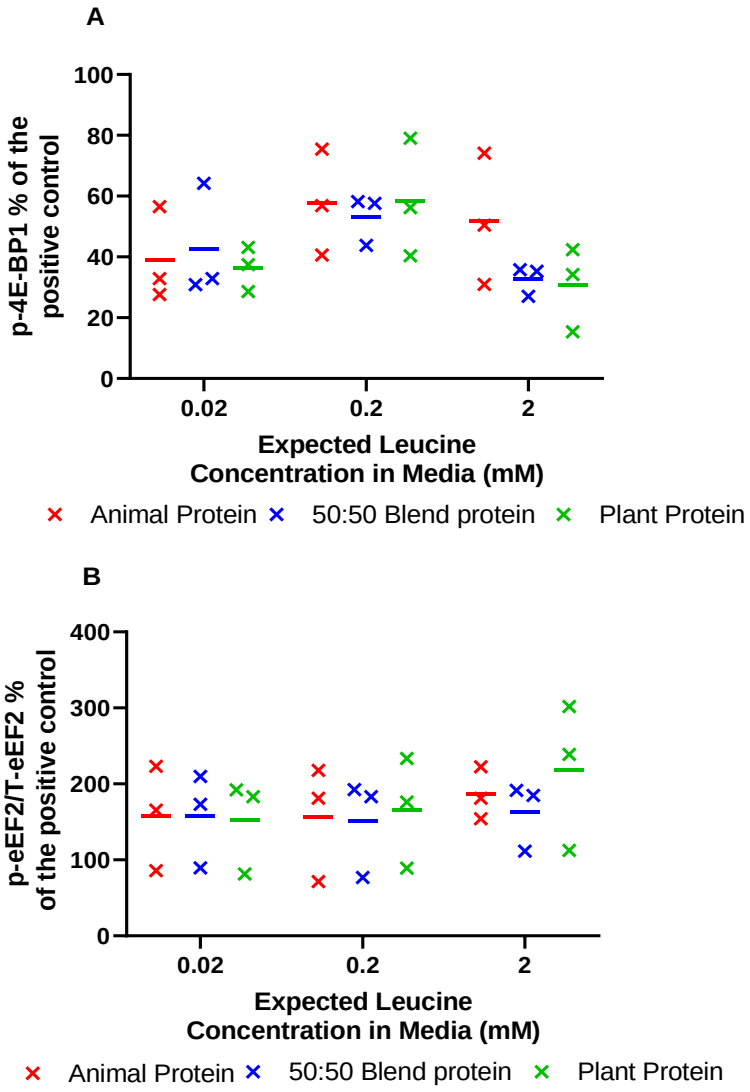


Figure 4.11: *A comparison of cell signals (**A** -phosphorylated-4E-BP1, **B** - eEF2 activation) in C2C12s in media grown on animal hydrolysate (red), plant hydrolysate (green) and 50:50 blend of plant and animal hydrolysate (blue). The mean of each data set represented by the solid line.*

Phosphorylation of AKT (Figure 4.12) was significantly lower with PM compared to both BM and AM (24.4±6.8% vs. 71.5±23.7% and 61.7±14.9%, PM vs.BM and AM respectively) at the lowest (0.02mM) leucine concentration, with no significant difference between BM and AM. Conversely at the highest leucine concentration (2mM) AKT phosphorylation was significantly greater with PM than AM (54.3± 18.2% vs. 23.1±15.1%, PM vs. AM respectively). Across the leucine concentrations, AKT phosphorylation was lower at 2mM media leucine concentration compared to 0.02mM for both AM (23.1±15.1% vs. 61.7±15.0%, leucine concentration of 2mM and 0.02mM respectively) and BM (33.5±6.4% vs. 71.5±23.7%, leucine concentration of 2mM and 0.02mM respectively), yet conversely was higher for PM (54.3±18.2% vs. 24.4±6.8%, leucine concentrations of 2mM and 0.02mM respectively). Finally, at 0.2mM leucine concentration, AKT phosphorylation was lower than 0.02mM leucine concentration in BM (30.8±17.6% vs. 71.5±23.7%, leucine concentration of 0.2mM and 0.02mM respectively).

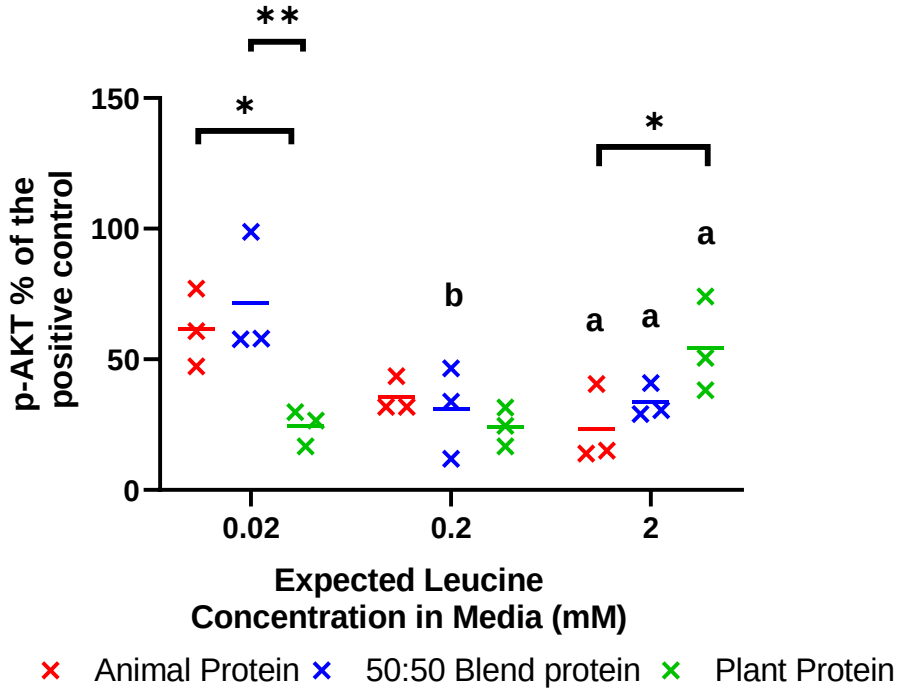


Figure 4.12: *A comparison of AKT phosphorylation of in C2C12s in media grown on animal hydrolysate (red), plant hydrolysate (green) and 50:50 blend of plant and animal hydrolysate (blue) The mean represented by the solid line. Significant differences indicated by * P<0.05 and **P<0.01 between protein sources at the same concentrations and [a]P<0.05 and [b]P<0.01 between concentrations of the same protein source*

4.4 Discussion

The results from this chapter suggest that the ^{13}C proline method had a greater sensitivity for measuring protein synthesis across an AA concentration gradient than the SUnSET blotting method. Further, there was no difference in the rate of protein synthesis when the leucine concentrations were the same in the different media sources using the ^{13}C proline method. Although, the activation of mTOR was greater in AM than

BM or PM at the highest media concentration and phosphorylation of P70S6K1 was greater in BM than PM at the lowest media concentration. In addition, phosphorylation of AKT was greater in AM and BM than PM at the lowest media concentrations and conversely greater with PM than AM at the highest media concentration. Unfortunately, despite these observations, the raw data showed a high level of variability, which when coupled with a low number of replicates, precludes any definitive conclusions from being drawn. To move this work forward, the experiments presented in this chapter need to be repeated, with greater validation (e.g., more method development in hydrolysation of the proteins for the media) and a greater number of replicates (e.g., 8-10 wells).

4.4.1 The Comparison of two methods to assess protein synthesis

There was no correlation between the two methods of assessing protein synthesis (SUnSET and FSR using ^{13}C proline) *in vivo* is unexpected. As in theory both these experiments measured changes in protein synthesis with slightly different approaches (incorporation either directly into the protein for ^{13}C proline or indirectly via puromycin binding during elongation). The expected result for both of these methods would be as the AA concentration in the media increased the rate of protein synthesis should increase, this was due to the increased availability of AA and the

increase in leucine concentration (Cuthbertson, Smith, Babraj, Leese, Waddell, Atherton, Wackerhage, Peter M Taylor, *et al.*, 2005b), as 2mM of leucine stimulates MPS in C2C12 in the same cell culture medium used in this experiment (Atherton, Smith, *et al.*, 2010). However, when the methods were compared against the concentration curve, only the stable isotope (^{13}C proline) method demonstrated increases in MPS when AA concentrations increased. However the SUnSET method could only identify a significant difference between the highest and lowest media concentrations. This could be due the SUnSET method having a lower sensitivity to small changes in the protein synthesis than GCMS analysis (Bass *et al.*, 2017). In addition, lack of apparent sensitivity of the SUnSET method could also be due to an error in analysis (e.g, area selected to measure was determined by eye using a prominent band as a guide, therefore allowing for increased human error regardless of how stringent). Although human error is a potential reason for the lack of sensitivity it is unlikely as there was minimal “bleeding” effect (large signal size that leads to the protein bands overlapping) and a clear detection of individual protein bands on analysis.

Conversely, the assessment of FSR using stable isotopes via IRMS does have the capability of detecting small changes in enrichment (Wilkinson, 2018) and so suggests that could be the reason for the difference between

the two methods. Also, an AA stable isotope is able to integrate with the AA pool and incorporate into proteins, this would allow for more enriched AAs to incorporate into a single protein, whereas puromycin binds to the ends of polypeptide molecules (Goodman and Hornberger, 2014). This means for every protein synthesised, one purmycin would bind but several prolines are incorporated, with 40-50% being enriched (with the standard method (Atherton *et al.*, 2009)) resulting in a greater level of detection. Therefore, for the investigation of small changes in rates of protein synthesis in muscle cells, ^{13}C proline appears to be a preferable method.

4.4.2 The impact of different protein hydrolysates in media on muscle protein synthesis

There was no impact on the rate of muscle protein synthesis when supplemented with protein sources from either animal, plant or a combination of the two *in vivo,* suggesting that the AA composition and/or concentrations of protein sources used were adequate to stimulate MPS sufficiently at the concentrations used. Further, the lack of difference between the rates of FSR may be due to:

1) The AA concentrations in the medias were provided at sufficient quantities for MPS following Liebigs law of the minimum (Alexander N. Gorban *et al.*, 2011; see section 1.3.1).

2) The balanced leucine concentrations of the different medias, rather than balanced nitrogen or total protein content, as has been used previously (Sawano *et al.*, 2020). This standardised the additional stimulatory effect leucine has on MPS (Rennie *et al.*, 2006; Norton *et al.*, 2009) from each protein source and each concentration, thus preventing a protein source with the greatest leucine concentration per gram of protein or nitrogen content having a greater MPS due to leucine stimulation.

3) High variability in the FSR at the highest media concentration, this reduced the clarity of the FSR results at these concentrations. This variability was due to the equal concentration of ^{13}C proline added to all media concentrations. This resulted in a dilution effect with the enrichment leading to the low ^{13}C proline enrichment in the media and intracellular protein. This low enrichment level caused a greater error in detection and interpretation on the mass spectrometer, with the detected peaks being increasingly difficult to distinguish from background enrichment levels, increased the chance of human errors in data collection where small shifts in peak size interpretation having substantial impacts on delta ^{13}C values.

Therefore, even though there was no difference in FSR in response to different protein sources, no firm conclusions can be drawn due to the acquired variability in the data.

4.4.3 The impact of different hydrolysates in media on intracellular signalling

The increase in the activation of mTOR signalling in cells provided with AM instead of PM and BM at the highest media concentrations, suggest that difference in the AA composition between the sources of the difference in activation. From the AA comparison, only threonine, aspartic acid and valine had different AA concentrations in both PM and BM to AM, but were greater than AM. Previously, increased mTOR signalling were associated with an increased in EAA rather than decrease although high increases in mTOR activation have previously been associated with increased EAA (Mitchell *et al.*, 2015) rather than decreased. This further suggests that PM and BM caused a hypertonic state leading to a decrease in protein synthesis (Kruppa and Clemens, 1984). Alternatively, as excess leucine has previously been shown to increase mTOR signalling (Atherton, Smith, *et al.*, 2010), this could also suggest the increase, as although the leucine concentration was kept constant AM had a greater leucine concentration relative to TAA concentration (AM: 14.1%, PM: 10.8% and BM 11.1% of leucine in the media).

There were also significant differences in the level of phosphorylated AKT and P70S6K1 between the protein sources, with BM having greater p-P70S6K1 than PM at the lowest media concentrations and PM having greater levels of p-AKT at the lowest media AA concentrations and AM and BM having the greater levels at the highest media AA concentrations. This suggests that the AA compositions in AM are more suited to promoting signalling pathways as AKT phosphorylation has been shown to increase *in vivo* when deprived of glutamine, arginine, methionine or lysine (Jin *et al.*, 2021); with methionine and lysine being low in plant base protein sources (Gorissen *et al.*, 2018). However, the assessment of the media AA content for the experiments presented in this chapter suggests that there was no significant difference in those AAs in the media.

In experiment 2, three signalling proteins (P70S6K1, AKT and 4E-BP1) were measured for the level of phosphorylation rather than the ratio between the phosphorylation vs. the total expressed. Thus, the results could indicate that 4E-BP1 activation remained unaltered through the change in amino acid concentrations, P70S6K1 had a greater activation at the lowest concentrations of the blended protein and AKT's activation decreases as both the animal and blended media concentrations increase and vice versa for the plant protein media. Although, any result where phosphorylation was only measured could represent a different biological

story to what was detected. As phosphorylation of signalling proteins could either represent a high proportion of activated signalling proteins but a low total concentration of the proteins (Yung, Charnock-Jones and Burton, 2011) or a low proportion of activated signalling proteins with a high concentration of phosphorylated signalling proteins (Tremblay *et al.*, 2007). Therefore, as the total expressed proteins were not measured it is impossible to tell if the phosphorylation changes in these proteins is significant or insignificant. Although only the impact of the different treatments should be the main factor, as if this experiment was conducted on muscle cells from different people rather than C2C12s then the genetic variability would decrease the reliability of the results, as different people have different levels of signalling proteins.

4.4.4 The effect of media amino acid concentration on C2C12 intracellular signalling

From the results in experiment 1, it is indicated that the change in AM concentration has no impact on mTOR, P70S6K, 4E-BP1 and eEF2. However for mTOR, P70S6K and 4E-BP1 this is contrary to the literature as these signalling pathways are commonly associated with increased activation with the addition of free AAs or protein supplementation both *in vivo (mTOR and P70S6K1;* Beals *et al.*, 2016*, P70S6K1 and 4E-BP1;* Mitchell *et al.*, 2016 *and P70S6K1;* Mitchell, *et al.*, 2015)*)* and *in vitro*

*(*mTOR; Atherton, Smith, *et al.*, 2010; Sawano *et al.*, 2020, 4E-BP1; Atherton, Etheridge, *et al.*, 2010*).* Although others have not been able to identify increases in mTOR, P70S6K1 and 4EBP1 in elderly people fed chicken or soya as protein sources, but MPS did increase in response to dietary intervention (Kouw *et al.*, 2021). This could be due to the western blot method used, as the smaller signalling protein signals (4E-BP1) have a very large and clear signal whereas the larger signal proteins (mTOR or P70S6K1) have a very small and faint signal. In most studies that measure signalling proteins in response to AA stimulation, they identify differences in the larger signalling proteins (Mitchell, *et al.*, 2015; Beals *et al.*, 2016; Sawano *et al.*, 2020), however as no method is stated for the western blot in Kouw *et al,* (2021) it is impossible to state this as the exact cause of the difference.

In the cells grown on BM, the significant difference in p-P70S6K1 at 0.02mM and 2mM expected leucine concentration may be a false positive as one of the data points at 0.02 mM expected leucine concentration is 4 times greater than the other two data points or due to the ratio being phosphorylated or total (explained in section 4.4.3). Also combined with the no change in the rate of MPS further supports this result being a false positive, as p-P70S6K1 increases are associated with increases with MPS (Atherton *et al.*, 2009; Mitchell, *et al.*, 2015).

4.4.5 Limitation of the study

The work presented in this chapter is subject to a number of experimental limitations limiting the conclusions that can be drawn from this work:

1) **High level of variability in FSR at higher AA concentrations-** The high level of the FSR is due to the increasing AA concentration in the media causing the ^{13}C proline enrichment in the media and intracellular to be reduced. Previous, ^{13}C proline enrichment is fixed at an APE of 45% (Atherton *et al.*, 2009). Although the variable enrichment levels have indicated it is possible to measure FSR at an enrichment of less than 45% APE. However, the dilution of the tracer at the highest concentration resulted in a media enrichment of less than 10 APE resulting in an increased variability, impacting in the accuracy of the FSR values. Therefore, a possible correction to the experimental design to reduce variability is to ensure that the proline enrichment in the media is constant at 45%.
2) **The hydrolysation of the media-** The current 'gold standard' method of hydrolysing proteins uses 6M HCl and neutralized with a strong base. However, when performed in the lab, following neutralisation the samples were unable to be dried down completely due to the high levels of salts. This made the method unusable as the liquid need to be dried down to dissolve the powdered

hydrolysate into the media solution. Therefore, 1M HCl was used and filtered through dowex as a compromise, maximising hydrolysis whilst reducing the salt concentration. This method of hydrolysis had inconsistencies between the two experiments, as the actual leucine concentration at the highest concentrations of AA in the media were different (1.83mM and 1.29mM for experiment 1 and 2 respectively) even though both used the same initial protein concentration (27mg animal protein/ ml of media). In addition, both hydrolysations were also lower than the predicted leucine concentration (2mM) for the highest concentration, this could be due to:

a) The initial measure of the leucine concentration to calculate the leucine concentration

b) The hydrolysation of the protein not being 100% effective.

c) The sample preparation and derivatisation to measure the media.

4.4.6 Future work to this experiment

Firstly, repeating the experiments presented in this chapter after refining the approaches via fixing the ^{13}C proline enrichment in each of the wells to an APE of 45% (Atherton *et al.*, 2009) to increase the accuracy of the FSR results. In addition, a comparison of various hydrolysation methods and strengths of acids, to utilise a hydrolysation method that can be

optimised to maximise the hydrolysation of the protein source with minimal salt residue. This may ensure that the expected leucine concentration can be achieved consistently and would further improved the accuracy of the analysis.

Once the method is refined, a similar experimental approach could then be transferred into human and dog primary muscle cells to assess potential species-specific protein-source preferences to elicit the greatest increases in MPS. Using these models, a greater number of ratios of plant to animal hydrolysates could be tested to determine the ideal hydrolysate ratio to achieve greatest level of MPS from the lowest concentration of total protein. The results from these experiments could then be the basis for *in vitro* experiments, testing the acute and chronic impact of combined dietary protein sources on rates of MPS. *In vitro* experiments could also be used to identify if preferential protein-source ratios are impacted by age or gender. Clinically, chronic studies in elderly people could assess an optimum ratio of specific protein-sources to have the greatest impact in minimising sarcopenia.

4.4.7 Conclusion

The results from this study suggest that using ^{13}C proline to measure protein FSR in C2C12, is a better method for identifying acute changes in

the rates of protein synthesis than the SUnSET technique. In addition, there was no difference in rate of protein synthesis when cells were provided with protein hydrolysates from animals, plants or a 50:50 blended source, when matched by the leucine concentration, and little impact on intracellular signalling. However, given a number of experimental limitations to the work presented in this study, firm conclusions cannot be made without refined and repeated experimental work.

5 The experimental design for the optimisation of a Non Steady State Indicator Amino Acid Oxidation technique in healthy adults

5.1 Introduction

This chapter describes a study that leads directly from the work presented in earlier chapters of this thesis, specifically Chapter 2. Based on the exploration of the IAAO technique to determine the protein requirement of healthy young human volunteers, this study protocol was developed and approved by the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (FMHS REC; approval number: 422-1911). However, due to the Covid-19 pandemic all human volunteer research at our institution was suspended beyond the timeframe of my PhD studies. This chapter will include the background and rationale for this study, the objectives, and the proposed methodology

5.1.1 The current time intense assessment of protein requirements

As previously outlined in detail in Chapter 2, protein is one of the main macro nutrients required for a balanced diet, and this is true for both

humans and companion animals. The current protein requirement for humans is currently 0.8g/kgBW in the UK, based on Dietary Reference Intake (DRI; WHO/FAO/UNU Expert Consultation, 2007). Since this recommendation it has been suggested that healthy elderly people should have a greater protein requirement level (1-1.2g/kgBW/day) (Elango *et al.*, 2010). In addition, elderly people with acute or chronic diseases have been suggested to have a greater requirement level still (1.2-1.5g/kgBW), excluding elderly people with severe kidney conditions not on dialysis (glomerular filtration rate of <30ml/min/1.73m^2). The current methods for identifying protein or amino acid requirements are either via nitrogen balance (NB) or a steady-state Indicator Amino Acid Oxidation (IAAO) approach. The NB method currently requires participants to be on each diet tested for 5-7 days as this method measures nitrogen intake in the diet and nitrogen excreted in the urine (i.e., net balance); which requires a minimum of 5 days for the urea pool to equilibrate to a new level of protein in the diet. As such this method is time-consuming and not readily applicable. In addition, the measurements for this method are also relatively imprecise, relying on estimates of dietary intake and 24h urine collections to estimate total nitrogen excretion- often looking for small differences in the presence of a very-high background.

In comparison, the steady-state IAAO technique only requires 2-3 days of dietary intervention on each diet, with a stable isotope labelled AA (either ^{13}C-phenylalanine or ^{13}C-leucine) given on day 3, along with a test meal divided over 8-hours (D. 0 Ball and Bayley, 1986). More detail on the steady-state IAAO is provided in Chapter 2, with the results from this chapter questioning the necessity of achieving an isotopic steady state in this IAAO technique. As a breakpoint value was able to detect changes in oxidation rates with increasing dietary methionine requirements in dogs. If a method of a non-steady state IAAO technique could be used, then this could reduce overall cost of the study by reducing the amount of tracer required to perform the IAAO experiment. There is a further aim that in removing the necessity of the steady state to determine a breakpoint value in the IAAO technique combined potentially reducing the length of the diet provided then it could be utilised to reduce the overall length of the study.

5.1.2 The possibility of non-steady state indicator amino acid oxidation

As previously stated in chapter 2, the results imply that the IAAO can be analysed in isotopic non-steady state conditions. As the percentage difference between the methionine breakpoint in dogs and the upper 95% CI value being 11%, whereas the other papers have a percentage difference of 38% (Wilson *et al.*, 2000; Riazi *et al.*, 2004). This data

suggests that although the IAAO technique most commonly assesses a breakpoint value in an isotopic steady state (Moehn *et al.*, 2004), it is possible to achieve this read-out under non steady-state conditions.

The role of the stable isotope in the IAAO technique is required to be at a sufficient enrichment level to detect the oxidation rate at different concentration of dietary AA. Stable isotopes are traditionally provided in a steady state in the IAAO technique, imply that any changes in the oxidation of the indicator AA are the direct representation for the change in the dietary AA concentration. However, a non-steady state should still be capable of detecting changes in AA oxidation, as the stable isotope enrichment, caloric intake and the activity level will remain consistent between the different diets for all participants. Therefore, any changes in the oxidation rate between the diets will still be directly associated with the amino acid breakpoint value. The only difference between an IAAO technique in an isotopic steady state and a non-steady state will be the length of time for the detection of the amino acid oxidation on a study day.

5.1.3 Isotopic steady state and variabilities that can affect the isotopic steady state

An isotopic steady state is when the tracer leaving the metabolic pool is equal to the tracer entering the metabolic pool (Katz and Rognstad, 1967),

this is represented in IAAO technique by a plateau in the $^{13}CO_2$ enrichment in the breath and the indicator AA in the plasma (^{13}C phenylalanine, ^{13}C lysine or ^{13}C leucine; Bross et al., 1998). By having a steady state this ensures that any changes in the enrichment are more likely relating to the changes from the external stimuli of the experiment. Unlike intravenous infusions of stable isotopes which are commonly used for the study of metabolic processes (e.g., muscle protein synthesis with D3-ketoisocaproate infusions (Bohé *et al.*, 2001), oral tracer provision is not provided as a continuous flow, so the enrichment levels have greater fluctuations. Therefore, in oral stable isotope experiments the “top-up” dose provided after the initial tracer provision must be sufficient to replenish the tracer lost from the pool to maintain a steady state (Figure 5.1). This “top-up” could be hourly, daily, or even weekly dependent on the turnover rates of the isotope used in the desired biological pool being measured. One of the main constraints with an isotopic steady state approach is the length of time required to initially achieve a steady state. This time is lessened by priming the enrichment pool with a bolus dose of the isotope being used or an intermediate metabolite. The intermediate metabolite would be a metabolite that is between the stable isotope being provided and the measured endpoint enrichment. For example,^{13}C sodium bicarbonate can be provided as the intermediate for ^{13}C phenylalanine, to

enrich the blood plasma pool reducing the time take for $^{13}CO_2$ to appear in the breath.

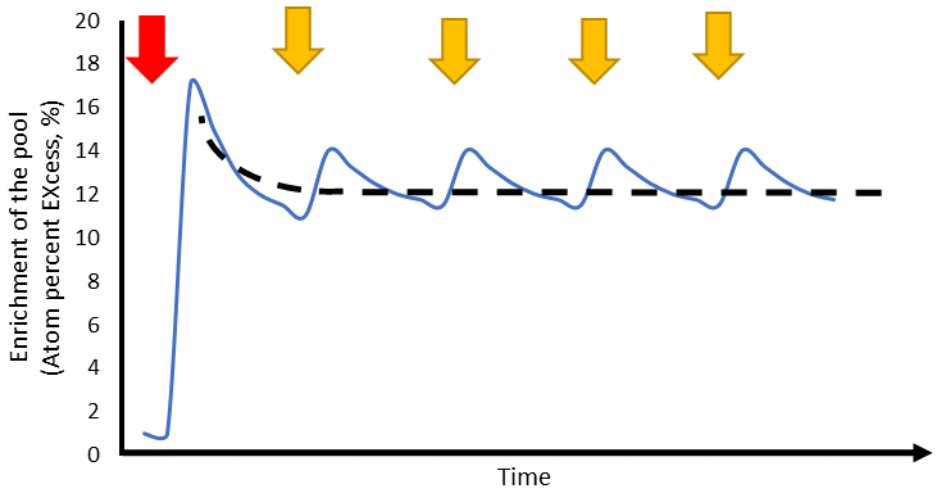


Figure 5.1 *A graphic representation of an oral steady state over time; the blue line - the change in the measured pool enrichment, the dotted black line- the mean enrichment when in steady state, the red arrow- the point of the priming dose of an oral stable and the orange arrow – the point of the top up doses of an oral stable isotope*

However, an isotope steady state requires the "top up" dose to be proportional to the priming dose and enough to compensate for the loss of the enrichment via excretion or transmogrification, as if not it can result in a non-steady state. This can occur when the "top up" dose is either:

- disproportionally **greater** than the priming dose, resulting in the enrichment of the pool increasing over time (Figure 5.2), or
- disproportionally **less** than the priming dose, resulting in the enrichment of the pool decreasing over time (Figure 5.3).

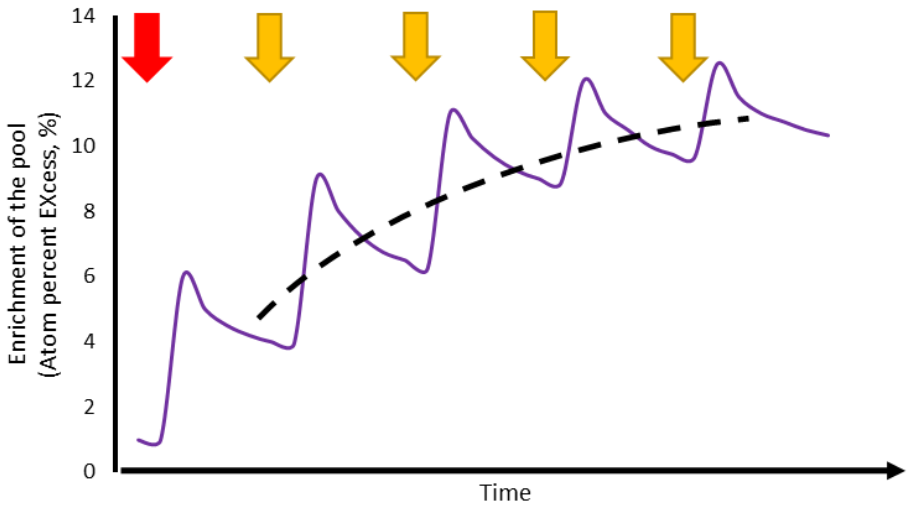


Figure 5.2 *A graphic representation of a non-steady state when the top-up is proportionally greater than the priming dose; the purple line- the change in the enrichment pool, the dotted line- the trend of the enrichment following the priming dose, the red arrow – the priming dose and the orange line the top-up dose*

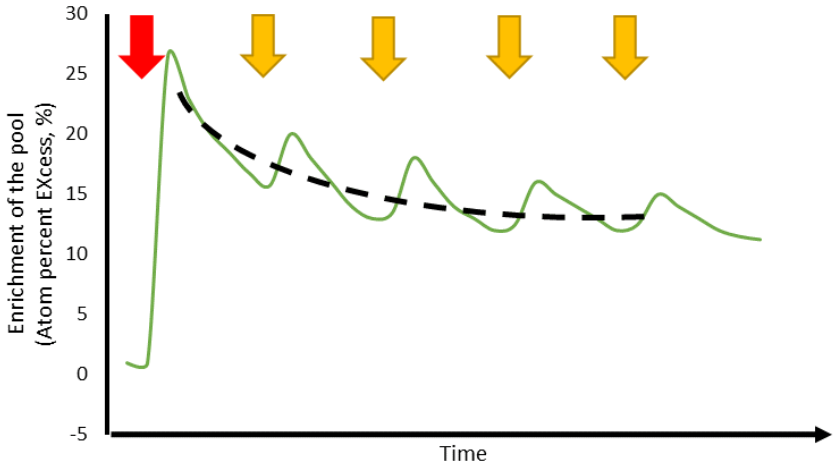


Figure 5.3 *A graphic representation of a non-steady state when the top-up is proportionally less than the priming dose; the green line- the change in the enrichment pool, the dotted line- the trend of the enrichment following the priming dose, the red arrow – the priming dose and the orange line the top-up dose*

The enrichments in either of these non-steady state situations, (Figures 5.2 and 5.3) will eventually reach a steady state, from an equilibrium between the stable isotopes entering and leaving the body. This is provided that the top up doses are continuously given at the same rate. The main impact of the non-steady state infusions is the time required to achieve a steady state (Zamboni *et al.*, 2009). Irrespective of the "top up" doses being too high or too low, the need to achieve a steady state for the determination of protein requirements via IAAO is a significant barrier to the application of this method in larger populations, in situations of financial and/or time constraints and in hard to reach populations (e.g. clinical groups), where the information to be gained from these methods is arguably the most important.

5.1.4 Study aims

The primary aim of this study was to assess the feasibility of using a non-steady state IAAO technique by comparing a non-steady state and steady state section in a steady state IAAO technique, to determine the minimum protein requirement in young males and females. The secondary aim of this study was to assess the suitability of non-purified diets for use in IAAO technique.

5.2 Methods

5.2.1 5.2.1 Participants

To keep within group variability low, but also to try and determine any differences in protein requirements across the life-course using a non-steady state IAAO technique, the recruitment plan was to recruit in to two separate arms:

- Group 1: 12 young males and females targeting a 50:50 split (BMI: 18-35kg/m^2, age: 18-35 years).
- Group 2: 12 older males and females targeting a 50:50 split (BMI: 18-35 kg/m^2, age: 65-80 years).

For participant safety and well-being and to try and minimise within group variability, potential participants would be excluded if they presented with:

- A BMI <18 kg.m^2 or >35 kg.m^2
- Active cardiovascular disease
- Respiratory disease (excluding well-controlled asthma)
- Metabolic disease
- Active inflammatory bowl or renal disease
- Active malignancy

- Recent steroid treatment (within 6 months) or hormone replacement therapy
- Clotting dysfunction
- Musculoskeletal or neurological disorders
- Food allergies to any ingredient included in the diets
- Engagement in any formal exercise training regime

Each potential participant will be screened prior to study enrolment against the inclusion and exclusion criteria listed above. This screening session will have also included the collection of informed consent. To ensure a participant's eligibility for this study the screening session will include an assessment of height, weight, and body composition via bioelectrical impedance analysis. A resting ECG will be performed including measures of heart rate and blood pressure, and a full medical history will be collected. A small blood sample will be collected via venepuncture for standard clinical chemistry analysis, to include U&Es, LFTs, TFTs, insulin, glucose, lipids, and clotting function. Participants will be asked to attend this session fasted overnight (from 10pm) and to have consumed 500ml of water before attending the screening session and then a further 500ml at the screening session. All screening sessions will be conducted between 8 and 10am.

5.2.2 Study design

The study was designed to be run over 6-weeks, with participants being supplied 5 different allocated diets in a random order, with randomisation generated by sealedenvelope.com. Each diet contains different levels of protein (0.6, 0.8, 1.0, 1.2 and 1.4 g/kgBW/day) and is consumed for 3 days of each week (H Kato, Suzuki, *et al.*, 2016), relative to each participants recommended caloric intake, with a 4-day "wash-out" period between diets. The caloric intake of each participant would be based on their basal metabolic rate x1.2 (as recommended for 'minimal activity' (Alfonzo-González *et al.*, 2004)**.** Each diet was made up of commercially available pre-packaged meals to provide the lowest protein requirement (0.6 g/kgBW/day), with additional protein supplementation (in powder form) given to meet the requirements for each specific week.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Week 1	NM	NM	TD	NM	NM	NM	NM
Week 2	DA	DA	TD	NM	NM	NM	NM
Week 3	DA	DA	TD	NM	NM	NM	NM
Week 4	DA	DA	TD	NM	NM	NM	NM
Week 5	DA	DA	TD	NM	NM	NM	NM
Week 6	DA	DA	TD	NM	NM	NM	NM

Figure 5.4: *The weekly program for each participant undertaking the study. NM- normal meal (green), TD- test diet (red) and DA- diet adjustment period (yellow).*

From weeks 2-6 there is a 2-day ‘lead-in’ phase for the diets to allow the body to become adjusted to the protein level in the diet (Figure 5.4). Participants would also be supplied with a diet diary to record all dietary intake for the 4-day “wash-out” period between each study diet. In addition, when participants are on the 2nd day of the ‘lead-in’ diets ^{15}N-L-glycine (20mg) to be taken at 9am, following a void of the bladder. With participants asked to collect a small amount of the urine prior to taking this tracer). Urine will then be collected for 12 hour intervals for 36 hours to measure protein breakdown (Rennie *et al.*, 1981). The urine collection will cease at the end of the study day on day 3 of each week.

On day 3 of each weeks study days participants would attend following an overnight fast (from 8pm the night before, with water *ad lib*). The diet for these study days will be the same as that consumed in the previous 2 days but split over 8 portions provided hourly. The study day diets will also be supplemented with phenylalanine and tyrosine (30.5 and 40.0 mg/kgBW/day, respectively) to be evenly distributed amongst the 8 meal portions. The supplementation of phenylalanine and tyrosine is given to ensure that ^{13}C phenylalanine can only be either synthesised into protein or oxidised. Breath and blood samples will be collected prior to meals 1 and 5 to provide fed and fasted baseline values for each study day. In the 5th meal a priming dose of ^{13}C-sodium bicarbonate and 1-^{13}C

phenylalanine at 0.176 and 1.86mg/kgBW respectively. The 6th, 7^{th,} and 8th meals a top up dose of 1.2mg/kgBW 1-^{13}C phenylalanine. Following the priming dose, 10-minute breath samples for 5 hours and 30 minute blood samples for 2 hours will be collected (Figure 5.5). For the first study week, there are three distinct changes to the other study weeks:

1) There is no 1-^{13}C phenylalanine provided, the only ^{13}C sodium bicarbonate is provided on the 5th meal to measure CO_2 recovery values.

2) Between meals 3 and 4, 5 and 6 and 7 and 8 a resting VCO_2 data will be collected for 10 minutes. This is to measure VCO_2 as an alternative measure for the CO_2 recovery.

3) As the first study day is only used to measure the CO_2 recovery it is not necessary to provide diets for the two days prior to the study day.

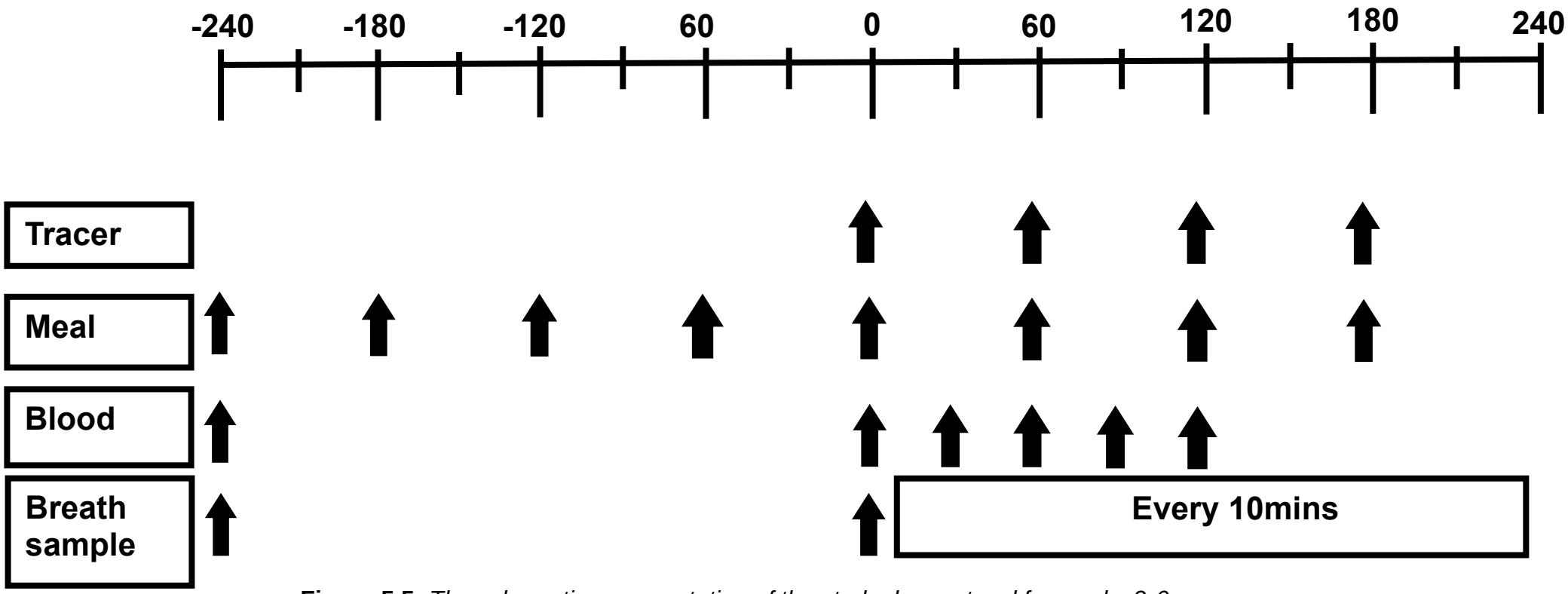


Figure 5.5: *The schematic representation of the study day protocol for weeks 2-6*

5.2.3 Study diet

The diet for this study was designed to try and replicate a normal daily meal plan as closely as possible both in the 'lead-in' periods and on the study days. As such the diet on the study days would be spit as follows:

- Breakfast ingredients split for meal portions 1 and 2
- Lunch and snack ingredients split for meal portions 3, 4 and 5
- Dinner and snack ingredients split for meal portions 6, 7 and 8

The aim of the diet was that it was low in protein (to meet the lowest intake to be tested) yet easy to cook and still able to meet maintenance energy requirements. As the diets were going to be prepared and consumed at home for 2 days (out of 3) for 6 weeks, ease of preparation and palatability were key factors in the designing the diet to try and enhance compliance. As such, the microwavable meals (that were also suitable for oven cooking) were chosen as the main components lunch and dinner. To produce a study protocol that was easy to replicate on a larger scale or in a non-research setting, it was decided that single point of purchase for all meals would be beneficial. Comparing the vegetarian 'ready-meal' options from four of the UK leading supermarkets (ASDA, Tesco, Sainsbury's and Marks and Spencer; Table 5.1). ASDA was shown to have the lowest protein content in their comparable microwave meals.

Product name	***Supplier***	***Serving Size***	***Energy content (kcal/100g)***	***Protein Content (g/100g)***	***Fat Content (g/100g)***	***Carbohydrate Content (g/100g)***	***Method for cooking***
Tesco slim cook butternut and vegetable risotto 500g	Tesco	500	71	**2.1**	0.2	14.4	oven/microwave
Asda Bombay potatoes	Asda	300	106	**2.3**	4.5	13	oven/microwave
Asda Gobi saag Aloo	Asda	300	75	**2.5**	3.4	7.7	oven/microwave
Asda Vegetable Balti	Asda	400	78	**2.5**	3.8	6.4	oven/ microwave
Asda Vegetable Chow Mein	Asda	400	87	**2.5**	2.5	13	microwave/shallow fry
Asda Vegetarian Mushroom stroganoff and rice	Asda	350	120	**3.1**	3.5	18	microwave
Asda Plant based veggie Gnocchi	Asda	380	137	**3.4**	3.3	22	oven/ microwave
Asda Extra special creamy garlic mushrooms	Asda	300	70	**3.4**	4.4	2.7	microwave
Plant Kitchen Thai Green Curry with rice	Marks & Spencer	800	149	**3.6**	6.2	18.8	microwave/ oven
Asda Extra Special Asparagus, Tender stem Broccoli and Kale Medley	Asda	170	78	**3.7**	5	3.5	microwave
Tesco Slim Cook Butternut Dhansak 500g	Tesco	500	75	**3.8**	0.8	10.5	oven/microwave
Asda Plant Based Three bean chilli and rice	Asda	380	118	**3.9**	1.1	22	microwave

Product name	***Supplier***	***Serving Size***	***Energy content (Kcal/100g)***	***Protein Content (g/100g)***	***Fat Content (g/100g)***	***Carbohydrate content (g/100g)***	***Method for cooking***
Deliciously Ella Cauliflower and lentil Dahl 275g	Tesco	275	156	**4**	10.3	10.7	oven
Hearty food Co Mac 'N' Cheese 400g	Tesco	400	121	**4.4**	2.7	19.3	oven/microwave
Asda cheese and tomato pasta bake	Asda	400	126	**4.4**	2.5	21	oven/ microwave
Amy's kitchen Gluten free Vegan Mexican Burrito Bean and rice 156g	Sainsbury's	156	148	**4.5**	3.8	24	oven/ microwave
Asda Extra Special Wyke farm cheddar Mash	Asda	400	127	**4.5**	6.6	11	microwave
Hearty food Co Cheese and Tomato Pasta 400g	Tesco	400	137	**4.6**	2.1	23.7	oven/microwave
Chargrilled vegetable pasta bake	Marks & Spencer	800	142	**4.9**	4.9	18.9	microwave/oven
Asda Macaroni Cheese	Asda	400	155	**5**	6.2	19	oven/ microwave
Amy's kitchen Gluten Free Vegetable Lasagne 255g	Tesco, Sainsbury's	255	136	**5.5**	5.5	16	oven/microwave
Asda little kids spaghetti Carbonara 4+yrs	Asda	300	108	**5.5**	1.8	16	microwave

Product name	***Supplier***	***Serving Size***	***Energy content (Kcal/100g)***	***Protein Content (g/100g)***	***Fat Content (g/100g)***	***Carbohydrate content (g/100g)***	***Method for cooking***
Amy's kitchen 'gluten free Broccoli and Cheddar Bake 270g	Sainsbury's	270	154	**5.9**	7.4	16	oven/ microwave
Amy's kitchen Gluten Free Rice Mac and cheese 255g	Tesco, Sainsbury's	255	157	**6.3**	6.3	18	oven/microwave
Made without wheat Macaroni Cheese	Marks & Spencer	400	174	**6.4**	7.4	20.2	microwave/oven
lazy vegan tikka masala ready meal 350g	Sainsbury's	350	115	**7.4**	4.6	10	shallow fry /microwave
Asda Italian Macaroni cheese	Asda	800	177	**7.4**	8.9	16	microwave/oven
lazy Vegan ready Provençale meal 350g	Sainsbury's	350	124	**7.7**	4.3	12	shallow fry /microwave
lazy vegan ready meal Mexican veggies and chunky pulled peas 350g	Sainsbury's	350	128	**8**	3.6	14	shallow fry /microwave
Amy's Kitchen Breakfast scramble veg scramble and hash browns 255g	Sainsbury's	255	135	**8.6**	7.5	7.5	oven/ microwave
Aunt Bessie's vegetarian toad in the hole 190g	Tesco	171	178	**9.4**	7.6	17	oven
Hearty food Co. 2 cheese Omelettes 200g	Tesco	190	226	**13.2**	17.3	4.1	oven

Table 5.1: A *list of 4 retailers microwaved meals organised by the lowest to highest protein content per 100g*

5.2.3.1. Diet formulation

Based on the daily diet plan outlined above (Table 5.2), formulation of each individual's diet (including the protein supplementation where required) was calculated on a Microsoft Excel sheet, specifically designed for this study.

Diet Ingredients	***Calorie content kcal/100g***	***Protein Content (g/100g)***	***Fat Content (g/100g)***	***Carbohydrate Content (g/100g)***
Breakfast				
brown toast (33g/slice)	228	12	2.9	37
strawberry jam	261	0.5	0.5	62
ASDA spreadable with real butter	730	0.6	78	0.6
Lunch				
Asda vegetable Balti	72	2.5	3.8	6.4
Asda Basmati micro rice	154	2.4	1.4	32
Dinner				
Asda vegetable chow Mein	87	2.5	2.5	13
Asda rice noodles	138	2.6	2.2	26
Snacks				
dried apricots	277	3.4	0.5	63
pear	64	0.4	0.1	15
quavers	337	2.5	30.8	9.9
apple	60	0.3	0.2	14
galaxy	558	6.7	32.4	56
Whey protein		100	0	0

Table 5.2: *A list of the components of the diets with their calorie (kcal/100g), protein, fat, and carbohydrate content (g/100g)*

As for the meal design and the single point of purchase requirement, this Excel sheet was purposefully designed to be easy to use to allow for further roll-out of this study, if indeed the non-steady state technique is proven. Only the participants:

- gender,
- height,
- weight,
- date of birth and
- study start date

are required to be inputted for calculation of each individual's diet, tracer doses, the additive phenylalanine and tyrosine doses and the additional whey protein needed.

5.2.3.2. Initial energy requirement

Using date of birth and study start date an exact age at commencement of the study was calculated for all the participants using the formula:

A =DATEDIF (B, S, "y")

where A=age, B=date of birth, S=start date of the study and "y" to give the results in years.

In the 'gender' cell, a number key is given for distinguishing between genders (1 for male and 2 for female). In order to use an "IF statement"

to automatically select the correct Basal Metabolic Rate (BMR) calculation (Harris and Benedict, 1918):

For male participants,

$$\mathbf{BMR = 66+(13.7 \times BW) +(5 \times H) -(6.8 \times A)}$$

or for female participants,

$$\mathbf{BMR = 665+(9.6 \times BW) +(1.8 \times H) -(4.7 \times A)}$$

where BW=body weight (kg), H=height (cm) and A=age (using the cell equation above).

5.2.3.3. Total energy requirement

BMR was then multiplied by 1.2 as the experiment was going to be conducted under free living situations and so to ensure that the participants were provided sufficient energy (Westerterp, 1998). Participation in a formal exercise regime was an exclusion criterion for this study, and all individuals were asked to maintain their (low level) of habitual physical activity for the duration of the study.

5.2.3.4. The proportioning of the nutrients in and between the diets

The diet was initially calculated to meet the requirements of the diet with the lowest protein concentration (0.6g/kgBW/day). As the main source of protein in the other diets is from the additional whey protein this means that as the protein content in the diet increases the energy content from the main 'whole-food' ingredients decreases. Many of the ingredients in the diet decreased by 2, 4, 7 and 9% as the protein intake increased (to 0.8, 1.0, 1.2 and 1.4 g/kgBW/day) due to whey supplementation). However, in order keep the energy intake consistent between the diets the apples, pears and Quavers were reduced by 4, 8, 9 and 12%, respectively. In addition, with the changing proportion of energy in the diet coming from protein, relative intake from the two macronutrients (fat and carbohydrate) fluctuates. Therefore, to try to reduce the variability in macronutrient contributions between the diets, the 'whole-food' alterations as explained above, allowed the carbohydrate to fat ratio to be maintained at ~2:1 across all diets. Outlined below is the daily intake for a 70kg reference male on each of the 5 different diets (Table 5.3).

Ingredients	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Breakfast					
Brown Toast (33g/slice)	33	33	32	31	30
Strawberry Jam	50	49	48	46	45
ASDA spreadable with real butter	12	11	11	11	11
Lunch					
Asda vegetable Balti	249	244	239	232	227
Asda Basmati micro rice	158	155	152	147	144
Dinner					
Asda vegetable chow Mein	249	244	239	232	227
Asda rice noodles	158	155	152	147	144
Snacks					
Dried Apricots	30	29	29	28	27
Pear	249	239	229	227	219
Quavers	87	83	80	79	76
Apple	241	231	222	219	212
Galaxy	25	24	24	23	23
Whey protein	10.8	25.5	40.2	55	69.7

Table 5.3: *The diet formulation of a 24yr 70kg old male, 0.6, 0.8, 1, 1.2 and 1.4g/kgBW protein contents of diets 1, 2, 3, 4 and 5 respectively*

5.2.4 Analysis methods

The results from this experiment will use a 2-phase linear regression with the oxidation rate of ^{13}C phenylalanine for the average oxidation rate across the time point to determine the breakpoint of the protein requirement. In addition, a 2-phase linear regression to determine the breakpoint from the oxidation rate at each hour post tracer supplementation. From these hourly breakpoint values an ANOVA will be used to compare the protein breakpoint values to compare the effect on the different protein levels. The average breakpoint value would be used to compared against the protein level from the results in the ^{15}N glycine level of the protein. The change in the plasma amino acid concentration will also be compared using an ANOVA analysis.

5.3 Discussion

5.3.1 Chapter summary

This theoretical study is designed to compare the stable isotope supplementation of a steady state vs. non steady state with the IAAO technique. It is achieved by comparing the breakpoint value of oxidation rates of $^{13}CO_2$ in hourly intervals prior to a steady state with oxidation rates in steady state in the same participant. The diet used in this study

is designed to be a more realistic diet, as there are various food ingredients to the diet and not a uniform diet for the 8 meals. The diet was also designed with simplicity to the participant as the main components for lunch and dinner are microwave meals. With this study, the aim is to begin developing a protocol to measure protein and amino acid requirements with a reduced study day. However further experiments would be required to reduce the length of time of the study day through the reduction of the number of diets or the time between the number of diets.

5.3.2 Study limitations

Although it is anticipated that the outcome from this experiment will be that the non-steady state IAAO will be able to detect a breakpoint value in protein requirement in both young and older and males and females, there is a possible significant limitation with the study design potentially interfering with the comparison of the steady state verse non-steady state. This limitation is based on each of the 'meals' within a study day not being identical, as opposed to the traditional method where they would be (R. O. Ball and Bayley, 1986). However, when designing the diet, due to an interest in taking this work forward to determine the true protein requirements of clinical and hard to reach populations (e.g., care home residents), the idea was to mimic a normal daily diet as far as

possible, as unusual foods at certain times of day or a whole day of supplementation feeding may not be possible in these environments. In addition, it is unclear whether there is a necessity for the diet to be completely identical for each meal for the IAAO technique, provided the protein content of the diet remains evenly split over the 8 meals.

5.4 Conclusion

This study is designed to test if the IAAO technique can be used to determine a breakpoint value, and therefore determine a protein requirement with a lesser time and sample collection burden that traditionally used (Kim, McMillan and Bayley, 1983). If this is proven possible and the study day for IAAO can be shortened and less invasive, this opens the possibility of using this technique to determine the true protein requirement of different groups in clinical settings, including those in whom low protein intake has been shown to be associated with poor clinical outcomes (Deutz *et al.*, 2014).

6 General Discussion

6.1 Thesis summary

The overall aim of this thesis was to assess the capabilities of stable isotope techniques for the measurement of protein and amino acid metabolism in three different models (dogs in chapter 2, humans in chapter 3 and cell culture in chapter 4), with a view to better understand amino acid and protein requirements and/or the impact of optimal protein-based nutritional strategies on protein turnover, across these different model systems.

The overall aim of this thesis was achieved through 3 distinct experimental models:

1) a set of experiments to refine the IAAO technique in dogs while assessing methionine requirements, and subsequently designing and gaining regulatory approvals for an IAAO-based clinical study to test the capabilities of the refined technique in humans, with a focus on reducing participant burden and wider applicability in difficult to study populations (i.e., older adults in supported living);

2) an experiment comparing postprandial muscle FSR and albumin FSR in older males and females, in response to a large protein

load, both with and without adjuvant HMB supplementation – with the aim of extending the anabolic window;

3) a set of cell-culture experiments to comparing two methods for measure the rates of protein synthesis when protein hydrolysates are added used as the sole protein source in the media.

4) the effect of plant and animal-derived protein hydrolysates on C2C12 muscle cell protein synthesis and associated anabolic signalling *in vivo* with the plan to assess the impact in human and dog primary muscle cells.

6.2 The indicator Amino acid oxidation technique in dogs

The IAAO method used in chapter 2 was the first recorded method for assessing the minimum AA requirements non-invasively using stable isotopes in dogs. This method was able to detect the minimum methionine requirement for adult Labradors with the lowest variability surrounding the breakpoint value. The experiment was repeated on 3 separate occasions and the variability of the breakpoint value remained fairly constant. The result from this exceeded the expectations of the hypothesis as the aim was to use an experiment to confirm that the lower methionine requirement for dogs detected by (Harrison et al 2020).

The IAAO experiment in dogs is a fairly new technique to be implemented in dogs, so currently there it is a limited field of research, however from the papers that have been published the results in this study have the most accurate level for a minimum AA requirement in dogs. Other studies (Mansilla, Fortener and Shoveller, 2006; Shoveller, 2016; Mansilla *et al.*, 2018), have a higher level of variability surrounding the breakpoint value, although this could be due to the differences in the methods. As the enrichment level in the other method provides a greater enrichment level both priming dose and top up dose (priming dose: 9.40 mg/kg BW vs. 7/5mg/kgBW and top up does: 2.4mg/kgBW vs. 2mg/kgBW for the priming and top up dose, Mansilla et al (2006) vs the enrichment in chapter 2).

There are several areas that future research could be focused on, either in dog research or the further refine the method of the IAAO technique these are:

1) In dogs this IAAO method could be used to determine the EAA requirements for dogs in all lifestages and breeds.
2) The IAAO methods in this chapter has identified potential areas in the method that could be refined such as assessing whether the IAAO could be calculated in a non steady state or to assess if the

RCO_2 value is necessary for calculating the IAAO is the animals body conditions and composition have minimal variability.

6.3 The use of HmB as a nutrient supplement to aid in the prevention of sarcopenia

It was shown in chapter 3 that the supplementation of 3g of HMB with whey 40g protein could circumvent anabolic resistance as MPS increased in elderly Women, even though 40g of whey protein is sufficient to increase MPS to the maximum. The same effect is not seen in elderly men which suggests that a sexual dimorphism occurs in regard to the stimulation of HMB. The hypothesis in regard to this chapter was partially correct as there was no difference in the change of albumin FSR when whey protein and HMB are supplemented together in elderly people.

The supplementation of whey protein at 40g was sufficient to stimulate MPS this is in line with other research in this field (Atherton, Etheridge, *et al.*, 2010; Churchward-Venne *et al.*, 2012; Wilkinson *et al.*, 2018) The sexual dimorphism on the stimulation of MPS has previously been identify in the older women (Smith *et al.*, 2008, 2012). Although the difference between genders in the basal level of MPS identified by Smith *et al* (2012) was not identified in this thesis, the sexual dimorphic effect

was apparent when the anabolic stimuli of HMB and whey protein were supplemented together. Future research in for this chapter would be expanded in two separate pathways:

1) In humans, the assessment of chronic HMB supplementation on muscle mass, function and metabolism in frail older adults, particularly females;

2) In dogs, the assessment of acute and chronic HMB supplementation in dogs to determine if MPS, and/or subsequent muscle mass/ function is affected;

6.4 The use of ^{13}C proline as a method for assessing the rate of protein synthesis *in vivo*

It was shown in chapter 4 that the ^{13}C proline method could detect a changes in MPS *in vitro* to greater sensitivity than the SUnSET technique when the cells are provided an animal hydrolysate for 4 hours at varying concentrations. It was hypothesised that there would be no difference between the two methods, although the benefit of utilising IR-GCMS for detecting small changes in ^{13}C proline enrichment were the key differences in identify the slight changes in MPS compared to a immunoblotting method in the SUnSET method. There were also additional benefits to utilising a stable isotope method rather than

puromycin, the stable isotope incorporates into the protein during protein synthesis multiple times (Atherton *et al.*, 2009) whereas puromycin only binds on during elongation to a lesser degree (Goodman *et al.*, 2011). Therefore, the stable isotope methods enrichment can be several folds greater for a slight increase in the rate protein synthesis, coupled with the increased detection sensitivity, could be the reasons for the difference between in correlation in the rate of protein synthesis in the two methods. This study was a pilot study and there were many areas that could have been improved on which are discussed in detail in the chapter (section 4.4.5). Therefore, the next step in this area of research is to repeat the experiment to confirm these results. However, following on from this the field of research could be expanded to:

1) Compare the various ratios of plant and animal hydrolysates on C2C12s to find an optimum dose for the maximum rate of MPS.
2) To advance the *in vitro* technique by repeating the experiment in chapter 4, as well as testing plant and animal protein sources on human and dog primary cells to gain an understanding on the potential optimum protein ratio of plant to animal protein sources in these species;
3) To determine the effects of different protein hydrolysates on MPS, with a focus on those that may be utilised by the pet food industry

in the future e.g., insect and plant proteins (Bosch, Vervoort and Hendriks, 2016).

6.5 Conclusion

In conclusion, this work covered a broad area of research and therefore the findings of each of these are unique to each chapter. However the key findings from this thesis are

i) a reduced and robust minimum methionine requirement in dogs;

ii) an optimised in the IAAO technique in dogs for identifying requirements;

iii) the additional stimulation of MPS in elderly women from the dual supplementation of HMB and whey protein suggesting an alternative anabolic pathway for hmb that can reduce the effect of anabolic blunting

iv) A proof of concept experiment comparing ^{13}C proline and SUnSET method for measure protein synthesis *in vivo,* with early evidence suggesting that using ^{13}C proline is a better method for measuring acute differences in MPS *in vivo.*

This thesis, focused on the development and utilisation of non- or minimally invasive methods for measuring protein synthesis using stable isotopes, with the findings contributing to each respective field of research. These findings help to increase understanding of protein metabolism in response to nutritional intervention. They can be utilised to further aid research in a number of areas particularly in “hard to reach” clinical populations in humans (e.g. care homes) and a greater fundamental understanding of protein metabolism in dogs.

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