

Chapter 1: Introduction

Global climate change has a strong natural cycle of warming and cooling (Humlum *et al.* 2011) but recent assessments have confirmed a significant contributory role of anthropogenic greenhouse gases (GHG) in current global temperature rises (IPCC 2007). Agriculture is a major contributor of GHGs, particularly carbon dioxide (CO₂), methane (CH₄) and nitrous oxide (N₂O), accounting for approximately two-thirds of anthropogenic CH₄ sources (IPCC 2007). Methane with a global warming potential of 25 times CO₂ and a short atmospheric lifetime of approximately 12 years, is a potent GHG (IPCC 2001). Globally, ruminant livestock produce approximately 85 million metric tonnes of CH₄ annually, equivalent to approximately 26% of net anthropogenic emissions (Fitton *et al.* 2011; Sejian *et al.* 2012).

Methane is a by-product of the microbial fermentation process in the rumen referred to as enteric fermentation. Formed primarily when CO₂ acts as the final electron donor, the resultant CH₄ is released into the atmosphere through eructation, respiration, and small amounts (2%) by flatulence (Murray *et al.* 1976; McCaughey *et al.* 1999; Chhabra *et al.* 2009). Methanogens, specific microbes belonging to the Archaea family, principally form CH₄ from CO₂ and hydrogen (H₂) substrates; allowing NADH to donate electrons and regenerate electron carriers such as NAD⁺.

Reducing the quantity of enteric CH₄ emitted from ruminant livestock can be achieved by improving diet quality (Beauchemin *et al.* 2008), dietary supplements (Nolan *et al.* 2010), and utilising plant secondary compounds (Tan *et al.* 2011). Many factors affect CH₄ production; including quantity and quality of feed, animal and microbe genetics, endocrine and neural signals, temperature, even the populations of fermenting microorganisms within the reticulo-rumen. A key physiological mechanism underlying CH₄ production is the length of time digesta is retained in the digestive tract, particularly the reticulo-rumen (RR). This mean retention time (MRT) of dry matter (DM) dictates the length of exposure digesta has to

be subjected to the microbial fermentative processes which lead to CH₄ production. Prolonged anaerobic fermentation due to greater MRT increases the amount of substrate fermented, causing greater quantities of hydrogen ions and carbon dioxide to be released, leading to more methane being synthesised by the methanogens. By reducing the amount of time digesta is retained in the rumen for fermentation, i.e. lowering MRT, it may be possible to lower the quantity of methane emitted by agricultural ruminants.

This thesis investigates the role of digesta MRT in the reticulorumen and whole digestive tract on the production of enteric methane in sheep, and the potential to mitigate methane emissions through reducing digesta MRT. It also investigates sheep physiological factors, in particular the thyroid hormone triiodothyronine, which may have a controlling influence on MRT and the possibility of using these regulators for livestock genetic selection to reduce enteric methane emissions.

Chapter 2: *Literature review*

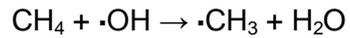
- Methane Production in Agriculture
- Methane Production by Livestock
- Factors Which Impact on Methane Production
- Physiological Regulation of Digesta Kinetics
- Hormonal Regulation of GIT Motility
- Conclusion and Hypothesis

Methane emissions from agriculture

Agriculture in Australia is worth AUS\$41 billion, contributing 2.2% to the nation's gross domestic product, employing 2.9% of the national employed, and utilising around 60% of the nation's land area (ABARE 2010). Associated with Australia's strength in agriculture is the increasing volume of GHG being emitted into the atmosphere by these very same practices. It is estimated that worldwide agriculture emits 5.1 to 6.1 Gt CO₂-eq (CO₂ equivalent) into the atmosphere, predominately through methane (CH₄) and nitrous oxide (IPCC 2007) of which agriculture in Australia contributes 90.1 Mt CO₂-eq (National Greenhouse Gas Inventory 2012). Whilst there are other known sources of methane production, including tundra, wetlands, paddy fields, energy and resource sectors, landfills, biomass burning and fugitive methane emissions from natural gas exploration (Wright *et al.* 2004; Mastepanov *et al.* 2008; Howarth *et al.* 2011; Cubby 2012), agricultural methane emissions currently are a major source (Kumar *et al.* 2009). Methane emissions make up about 11% of Australia's total GHG emissions with ruminant livestock attributing approximately 68% of this component (National Greenhouse Gas Inventory 2012).

2.1 Methane emissions from livestock

Agricultural animal and crop production systems are a major source of methane emissions as well as an important carbon sink. Cattle, sheep and goats lose $6.5 \pm 1.0\%$ of their ingested gross energy as eructed methane but, with only 60-80% of forage gross energy intake (GEI) digested, the actual loss of digested energy as methane exceeds 8% (Blaxter and Czerkawski 1966; Lasseby 2008). The primary methane sink in the environment is its reaction with hydroxyl (OH) radicals in the troposphere (Crutzen 1991; Fung *et al.* 1991). Water vapour in the atmosphere is broken down by oxygen atoms formed from the cleavage of ozone by ultraviolet radiation into OH radicals. These radicals react with the CH₄ to form CH₃ and H₂O (Eq. 1):



Eq. 1

There are three other important, although lesser, sinks of methane. These are the soil (Whalen and Reeburg 1990; Mosier *et al.* 1991), the stratosphere (Crutzen 1991), and the microbes themselves (Morgavi *et al.* 2010). Oxidation of CH₄ into CO₂ and H₂O requires the presence of O₂, something lacking in an anaerobic environment such as the rumen or anoxic marine environments. Instead, a process of microbially mediated anaerobic oxidation of methane (AOM) takes place resulting in a reduction of CH₄ through several possible yet not fully understood biochemical pathways such as reverse methanogenesis, reductive acetogenesis, and methylogenesis (Alperin and Reeburgh 1984; Valentine and Reeburgh 2000; Raghoebarsing *et al.* 2006; Klieve 2009; Wright and Klieve 2011).

It has been demonstrated that methane is produced in both ruminant and non-ruminant animals but the amount produced by non-ruminants is far less than when compared to that produced by ruminants (Klieve 2009). For example, Kempton *et al.* (1976) detected no methane being respired by grey kangaroos and, while some methane was produced in the hind gut, all was lost via the anus with that measured being significantly lower than detected in sheep. Von Engelhardt *et al.* (1978) found that the amount of methane produced in the hind gut of Tammar wallabies (*Macropus eugenii*) and Hyraxes (*Procavia habessinica*) was also far less than that generated by sheep. Studies conducted by Evans *et al.* (2009) and Pope *et al.* (2011) revealed that Tamar wallabies harbour unique gut bacteria and emit only one-fifth the amount of methane as ruminants per unit of digestible energy intake. Ouwerkerk *et al.* (2009) suggested that microbes belonging to the class of hydrogen consuming bacteria called reductive acetogens were common in all kangaroo species. Research into methane production by non-ruminants, while limited, consistently suggests that the amount emitted per animal is far less than that of ruminants (Kempton *et al.* 1976; Klieve 2009; Pope *et al.* 2011). Much of this is due to the absence of the fermentative

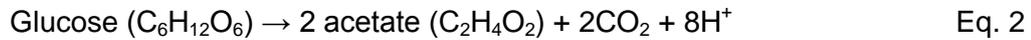
reticulorumen (RR) and the lack of methane producing Archaea, resulting in H₂ accumulation (Evans *et al.* 2009; Pope *et al.* 2011; St-Pierre and Wright 2012).

2.1.1 Hydrogen availability and digestive fermentation

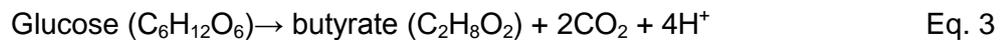
During fermentation, H₂ is released but does not normally accumulate in the rumen because it is immediately used by other microbes, particularly in the production of propionate and by methanogens, which are present in the mixed microbial ecosystem (Wolin and Miller 1989; Kamra *et al.* 2012; Morgavi *et al.* 2012). Accumulation of H₂ has the potential to have adverse effects on the host animal by inhibiting metabolism of rumen microorganisms (Sharp *et al.* 1998). Methanogens act as “electron sinks” by making it energetically feasible for the chemoorganotrophic bacteria present in the rumen to dispose of electrons by forming H₂ in an ongoing manner rather than as other reduced products, for example ethanol (Wolin 1974; Morita *et al.* 2011). As such, in anaerobic ecosystems like the rumen, H₂ produced by the fermenting bacteria is oxidised into CH₄ by methanogens. This collaboration between the fermenting species and H₂-utilisers, such as methanogens, is referred to as “interspecies hydrogen transfer” (Iannotti *et al.* 1973; Stams and Plugge 2009). It is thought that there may even be a physical association between the fermenting species and H₂-utilisers to facilitate interspecies transfer, with methanogens found within or attached to the external pellicle of protozoa (Stumm *et al.* 1982; Krumholz *et al.* 1983; Finlay *et al.* 1994; Sharp *et al.* 1998).

Hydrogen availability for interspecies hydrogen transfer, essential for CH₄ production, often depends on the host's diet and the microbial array. Through glycolysis of digesta, pyruvate is formed and metabolism of pyruvate within the ruminal microorganisms produces the three main volatile fatty acids (VFA) – acetate, butyrate and propionate. Formation of acetate commonly occurs via the pyruvate-formate lyase system which produces formate and acetyl-CoA as intermediate products. The formate released is converted back into CO₂ and H₂, both of which are used to produce CH₄. Acetyl-CoA is converted firstly into acetyl phosphate

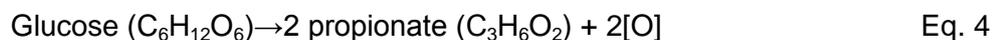
by a phosphotransacetylase reaction then, via an acetate kinase reaction, into acetate (Muller 1988). The stoichiometry of the fermentation pathway can be summarised as follows (Eq. 2):



Acetyl-CoA can alternatively be metabolised through to butyrate. Butyrate is normally formed from two molecules of acetyl-CoA, yielding acetoacetyl-CoA (Duncan *et al.* 2002). With the aid of thiolase, β -hydroxybutyryl-CoA dehydrogenase, crotonase, and crotonyl-CoA reductase, the acetoacetyl-CoA is converted into Butyryl-CoA (Miller and Jenesel 1979). The final step in the conversion process of the fermented glycolytic products into the VFA butyrate usually involves the enzymes phosphate butyryltransferase and butyrate kinase, yielding not only butyrate for absorption through the ruminal wall but also ATP for the microbe (Miller and Jenesel 1979; Yarlett *et al.* 1985) (Eq. 3):



The third main VFA produced by the microscopic organisms within the rumen from digesta is propionate, a major H^+ sink. There are two pathways by which pyruvate can be converted into propionate – via oxalacetate (most common pathway) or via lactate (acrylate pathway) (Bernalier *et al.* 1999) (Eq. 4).



The net result of propionate formation through oxalacetate is two oxygen molecules which, when traded through two molecules of water, are the equivalent to a net disposal of 4H^+ (Van Soest 1994) (Eq. 5):



This indicates that feed type and microbes involved in fermentation determine the quantity of H^+ available in the rumen for CH_4 production. While acetate and butyrate produce excess H^+ , propionate acts as a sink, removing H^+ ions from the rumen. Manipulation of the ruminal environment to produce an increased proportion of propionate relative to acetate and butyrate would result in a lower abundance of reduced cofactors such as NADH.

2.1.2 Biochemistry of methanogenesis

The production of CH_4 in the rumen is a thermodynamically positive process; $\Delta G^\circ = -131\text{kJ/mol}$ (McAllister and Newbold 2008) involving the reduction of CO_2 into formyl-methanofuran (the first stable product of CO_2 fixation) then, through the redox actions of a series of coenzymes plus the addition of H_2 , its ultimate conversion into CH_4 (Rouviere and Wolfe 1988; Thauer *et al.* 1993; Dubey 2005) (Fig 2 - 1). Once reduced, the methanofuran component is cleaved with the formyl group being transferred to the coenzyme tetrahydromethanopterin by formylmethanofuran:tetrahydromethanopterin formyltransferase to form formyl-tetrahydromethanopterin (5-formyl- H_4 MPT) (Donnelly and Wolfe 1986). 5,10-methenyltetrahydromethanopterin cyclohydrolase then converts the formyl group into a methenyl group, methenyl- H_4 MPT (DiMarco *et al.* 1986; Dubey 2005). The methenyl group is further reduced by the reduced deazaflavin coenzyme F_{420} into a methylene group – methylene- H_4 MPT (Hartzell *et al.* 1985). Next, the methylene group is reduced to a methyl group – methyl- H_4 MPT (Dubey 2005). The reduction of methenyl- H_4 MPT is reversible and catalysed by methylenetetrahydromethanopterin dehydrogenase while the reduction of the methylene group, also fully reversible and H_2 dependent, is catalysed by the enzyme methylene-tetrahydromethanopterin:coenzyme F_{420} oxidoreductase (Hartzell *et al.* 1985; Thauer *et al.* 1993; Dubey 2005).

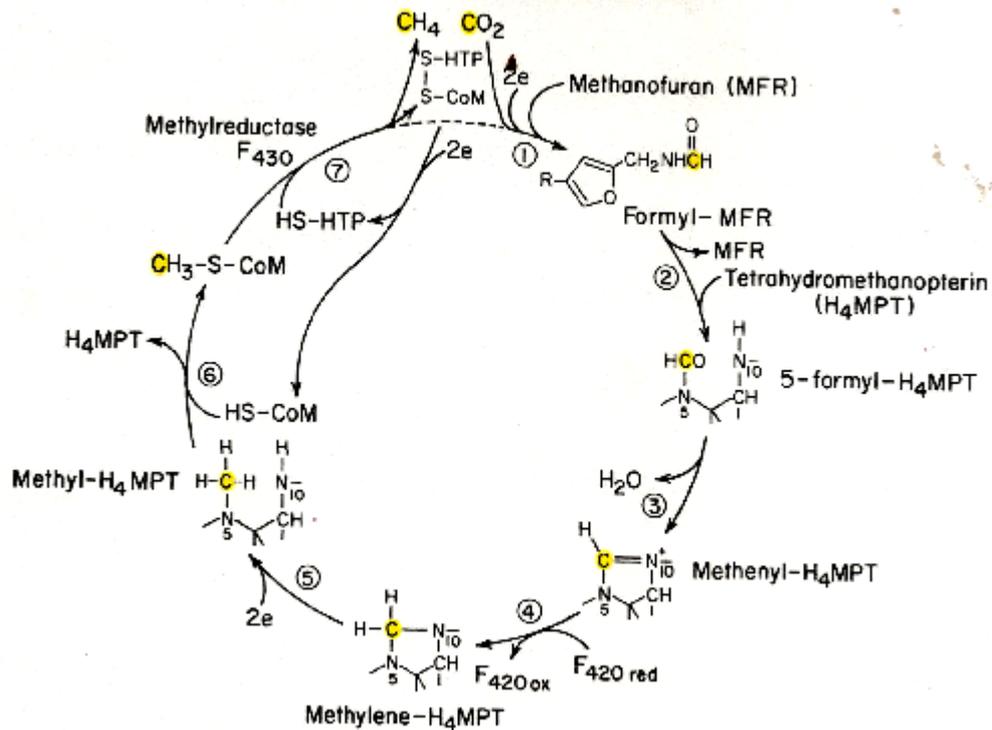


Fig 2 - 1 Diagram of CO₂ reduction to CH₄ (Rouviere and Wolfe 1988)

The methyl group of methyl-H₄MPT is then transferred from N-5-methyl-tetrahydromethanopterin (N-5-methyl- H₄MPT) to coenzyme M (HS-CoM) to form methyl coenzyme M (methyl CoM) (Taylor and Wolfe 1974b). The active form which is involved in the reduction of CO₂ is 2-mercaptoethanesulfonic acid (Taylor and Wolfe 1974b; Dubey 2005). Once methylated with the methyl group from methyl-H₄MPT, it then forms 2-(methylthio)ethanesulfonic acid (CH₃-S-CoM), the substrate for the methyl coenzyme M methylreductase system (Taylor and Wolfe 1974b; Balch and Wolfe 1979). It is this component which undergoes a final reductive demethylation to yield methane (Taylor and Wolfe 1974a).

Fraction B of the methylcoenzyme M methylreductase system, N-7-mercaptoheptanoylthreonine phosphate (HS-HTP), is involved in this last step towards CH₄ production from CO₂ and H₂. HS-HTP has been shown to be the final electron donor (Noll and Wolfe 1987; Ellerman *et al.* 1988; Dubey 2005) for the reduction of CH₃-S-CoM to CH₄

and the resultant production of the heterodisulfide CoM-S-S-HTP from HS-HTP and HS-CoM (non-methylated coenzyme M) (Bobik *et al.* 1987; Ellerman *et al.* 1988) (Eq. 6):



The catalytic component of this methylreductase reaction is the active methylreductase F₄₃₀ – Fraction C (Rouviere and Wolfe 1988; Dubey 2005). CoM-S-S-HTP is later reduced back to HS-CoM and HS-HTP by the enzyme component A1 of Fraction A and H₂ (Rouviere and Wolfe 1988). The reduction of CH₃-S-CoM by H₂ is an exergonic process and is the source of energy for ATP synthesis by the methane producing microorganisms within the rumen (Keltjens and van der Drift 1986).

2.2 Factors which impact on methane production

Although microbial metabolism gives rise to the end-products of rumen fermentation including CO₂ and CH₄, it is recognised that animal and environmental factors also affect the rate and type of fermentation in the rumen. Individual ruminants fed on a common diet could differ significantly in their CH₄ yields (Pinares-Patiño *et al.* 2003; Hammond *et al.* 2009; Garnsworthy *et al.* 2012). Other factors such as mastication, salivation and digesta kinetics affect microbial fermentation (Faichney 1993; Mathison *et al.* 1995; Wilson and Kennedy 1996; Varga and Kolver 1997) and may provide a mechanism for between animal variations. The composition of feed and quality of forage ingested also influence CH₄ production in ruminants (Hegarty 1999; Boadi *et al.* 2004; Eckard *et al.* 2010), even conditions such as environmental temperature (Kennedy *et al.* 1977) and animal genetics have an impact on fermentation (Hegarty *et al.* 2007). Following is a review of factors known to influence methane yield (MY; grams methane produced per kg dry matter intake), including nutrition, particle size and density, water intake and salivation, physical environment, parasites, animal genetics, and digesta kinetics.

2.2.1 Nutrition

Methane production is influenced by the quality and quantity of feedstuff available (Mitsumori and Sun 2008). Dry matter intake (DMI) has been extensively demonstrated to have a significant impact on daily methane production (Blaxter and Clapperton 1965; Shibata *et al.* 1992; Molano and Clark 2008; Muetzel *et al.* 2009). Shibata *et al.* (1992) suggested it was possible to accurately predict daily methane production from ruminants using DMI with the following equation (Shibata *et al.* 1992) (Eq. 7):

$$\text{CH}_4 \text{ (L/day)} = -17.766 + 42.793\text{DMI} - 0.849\text{DMI}^2 \quad (r^2=0.966) \quad \text{Eq. 7}$$

2.2.1.1 Diet composition and ruminal pH

Diet composition has also been shown to affect ruminant methane production. Diet manipulation can decrease organic material fermentation in the rumen and increase post-ruminal digestion, divert H⁺ away from CH₄ production during ruminal fermentation, inhibit methanogenesis, and change digesta residence time and microbial population dynamics in the rumen (Colucci *et al.* 1982; Johnson and Johnson 1995; Benchaar *et al.* 2001; Morgavi *et al.* 2010). The effect of diet on daily methane production and MY of ruminants has been extensively reviewed (Beauchemin *et al.* 2008; Kumar *et al.* 2009; Eckard *et al.* 2010; Martin *et al.* 2010) and will only be addressed briefly in this literature review. The key factors by which diet composition affects enteric methane production are lipid, tannin and saponin content, pH, roughage and starch content.

Wright *et al.* (2004) cultivated methanogens found in Australian sheep and compared those fed on pastures alone with those receiving a lucerne or oaten hay diet. It was discovered that pasture grazed sheep possessed a greater diversity of methanogens than any of the other animals. Within the rumen of the pasture fed sheep were five new species not found before. Nicholson *et al.* (2007) discovered two previously unclustered groups of methanogens within the rumens of pasture grazing sheep in New Zealand while there were also unique

methanogens found within the rumens of Queensland sheep (Wright *et al.* 2006). This demonstrates that methanogens are capable of surviving in animals from different environments.

Diet composition can change the pH in the rumen, impacting on the bacterial populations found within. Optimum pH for methane production is 7.0 to 7.2 but methane production can occur in a range of 6.6 – 7.6 (Kumar *et al.* 2009). Below 6.6 though, the activity of fibre degraders reduce until pH falls below 6.0 where ruminal cellulolysis is totally inhibited (Mould *et al.* 1984; Dijkstra *et al.* 1992). At this point, there is a sharp decrease in feed intake (Ørskov and Fraser 1975). Beharka *et al.* (1998) found that when calves were fed a high grain/low roughage diet which had been finely ground, the pH within the rumen decreased causing a decrease in cellulolytic bacteria numbers with a corresponding increase in amylolytic bacterial abundance. Various ruminal bacterial species have been observed to shift pathways in response to changes in pH while fermenting the same substrate (Dijkstra *et al.* 2012). When pH is falling there is a shift from acetate and butyrate to increased propionate production (Calsamiglia *et al.* 2008) and a reduction in CH₄ production (Poulsen *et al.* 2012). As propionate is the principal alternative H⁺ sink after CH₄ in the rumen, it would be expected that MY would reduce as ruminal pH falls in association with an increased concentrate in the diet (McAllister and Newbold 2008).

Low ruminal pH does have adverse effects on the ruminant. Low pH for prolonged periods each day can reduce voluntary feed intake (VFI), feed digestion and the activity of fibre degraders (Dijkstra *et al.* 1992; Russell and Wilson 1996; Dijkstra *et al.* 2012). With modern ruminant diets possessing high concentrations of starch and protein in an attempt to minimise ruminal degradation of digesta, the benefits may be decreased CH₄ production but the consequences are that rumen pH declines to a point of inefficiency. This leads not only to low ruminal VFA synthesis and microbial protein (MP) production but other complicating issues such as acidosis and even ketosis. Along with this, as the digestibility of fibre

increases a greater daily production of CH₄ occurs, most probably due to increased total fermentation and NADH availability (Pinares-Patiño *et al.* 2003). Therefore, while increasing the concentrate percentage in a diet shifts VFA production towards propionate, decreasing methane emissions, a roughage component within the diet is essential to overcome such complexities as low ruminal pH with its adverse animal health consequences.

2.2.1.2 Dietary fat

Dietary fat is a promising nutritional strategy for suppressing ruminal methanogenesis without affecting other ruminal factors (Martin *et al.* 2010). In almost all studies undertaken, MY was decreased with the addition of dietary fat (Martin *et al.* 2010). Beauchemin *et al.* (2008) reviewed 17 studies on the impact of dietary fat on MY and found that CH₄ was able to be reduced by a mean value of 5.6% per 1% addition to the diet of lipids (DM basis). Martin *et al.* (2010) conducted a similar review of 67 diets containing lipids from 28 studies and found the relationship between level of added fat (% DMI) and CH₄ decrease to be a mean decrease in CH₄ of 3.8% for each 1% additional lipid. This concurs with the meta-analysis conducted by Eckard *et al.* (2010). Moate *et al.* (2011) reported that the 1% addition of dietary lipids to dairy cattle results in approximately 3.5% reduction in MY. Not all studies though reported a reduction in MY due to lipid addition, suggesting the type of fat used could be important (Woodward 2006; Cosgrove *et al.* 2008). Lipid effects on MY are two-fold, lipids are not fermented in the rumen and medium-chain fatty acids are known to affect methanogen numbers, some even having a toxic effect (Nagaraja *et al.* 1997; Machmuller *et al.* 2003; Martin *et al.* 2010). Alternatively, a meta-analysis conducted by Eugene *et al.* (2008) showed that the reduction in MY caused by feeding dietary fat was associated with a decrease in DMI, with a dietary fat content exceeding 6% known to depress DMI (Beauchemin *et al.* 2008). With few known disadvantages, addition of small amounts of dietary fats to ruminant diets for CH₄ abatement could have merit as long as there are no adverse impacts on feed efficiency or performance of the animal.

2.2.1.3 Plant secondary metabolites

Plant secondary metabolites (PSM) have been shown to improve animal performance and reduce enteric methane emissions (Goel and Makkar 2012) (Fig 2 - 2). Tannins and saponins constitute the major classes of PSM currently under research for their antimicrobial actions and effect on ruminal fermentation. Both work at reducing enteric methane emissions by suppressing ruminal protozoa, selectively inhibit some bacteria, and reduce hydrogen production by reducing feed degradation (Tavendale *et al.* 2005; Goel and Makkar 2012; Jayanegara *et al.* 2012). The effectiveness of these PSMs as anti-methanogens is uncertain as conflicting results have been reported under various situations (Sliwinski *et al.* 2002; Santoso *et al.* 2004; Waghorn and Woodward 2006; Tiemann *et al.* 2008). Some PSMs can also have a detrimental effect on the fermentation process and can lead to an increase in methane production. For example, catechol, found in many tropical legumes, drastically depresses the fermentation process and impairs ammonia uptake by microbes while mimosine, nicotinic acid, 3,4-dihydroxyphenylalanine (DOPA), and L-canavanine all stimulated fermentation but caused an accumulation of CH₄ in the rumen (Dominguez-Bello *et al.* 1993). This demonstrates careful identification and isolation of PSM is needed to deliver assured mitigation of enteric methane emissions without adversely affecting animal production.

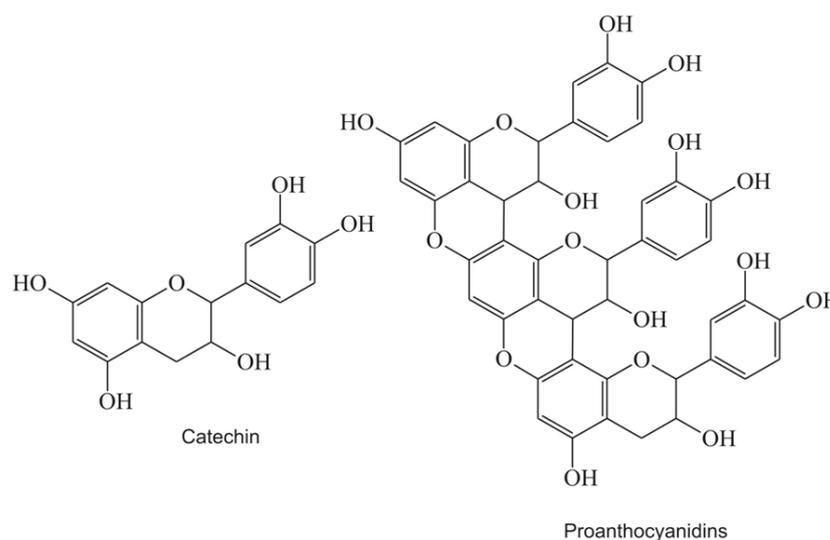


Fig 2 - 2 Basic structures of two condensed tannins, catechin and proanthocyanidins (de Amorim *et al.* 2012)

2.2.1.4 Mean retention time of digesta and voluntary feed intake

Voluntary feed intake as well as feed type and/or supplementation have been shown to impact on the ruminal MRT of digesta. Consuming feed that requires less fermentation time in the rumen causes an increase in VFI, often due to reduced MRT of digesta (Mathers *et al.* 1989). Restricting the rate of flow (ROF) of digesta through the gastrointestinal tract (GIT) may result in distension of one or more segments of the GIT, reducing VFI (Allen 1996). Supplementing poor quality roughage feed with higher quality concentrates has been demonstrated to increase voluntary intake, increase ROF, and reduce MRT (Warren *et al.* 1974). McCollum & Gaylean (1985) demonstrated that the addition of cottonseed meal to prairie hay fed to beef cattle not only increased the total quantity of feed consumed but also particulate ROF through the GIT. This is because retention time of organic matter in the rumen is negatively correlated with volume of daily intake of digestible material; that is increased MRT of digesta leads to decreased VFI (Thornton and Minson 1973; Colucci *et al.* 1982; McCollum and Galyean 1985; McCaughey *et al.* 1999).

2.2.2 Particle size and density

Particle size and density of the feed consumed by the ruminant affects the amount of time digesta is retained in the rumen. By increasing the mean size of the particles consumed it is possible to increase both the amplitude and frequency of the rumen contractions (Colvin Jr and Daniels 1965; Pharr *et al.* 1967) but this does not necessarily lead to an increase in particle ROF through the GIT (Waghorn and Reid 1983). With the rumen contents of grazing livestock usually stratified, a fibre “mat” often develops in the rumen (Hummel *et al.* 2009). This mat acts as a filter, allowing smaller, heavier particles to pass through into the fluid phase below while larger, lighter particles continue to mix within the dorsal portion of the RR (Martz and Belyea 1986; Hummel *et al.* 2009; Clauss *et al.* 2011). As a result of increasing the size of the feed particles ingested, the RR increases its contractions leading to further mechanical breakdown of particles serving to better mix the material with the fermentative microorganisms and their enzymes (Ulyatt 1983). This also facilitates an increase in eructation and mastication as the animal attempts to digest the material consumed (Ulyatt *et*

al. 1986). The end result is that larger food particles are retained for longer periods of time within the RR with an increase in MY (Hummel *et al.* 2009).

2.2.3 Water intake and saliva production

Composition and form of the animal's diet greatly affect the quantity of saliva produced and water consumed. It has been shown that pelleted and high concentrate diets are not conducive to rumination, salivation, good ruminal function, or even high VFI (Grovmum 1988). High roughage diets though encourage rumination to reduce the size of the particles within the rumen by mechanical breakdown which will facilitate forward passage through the ROO, and in the process stimulate saliva production (Carter and Grovmum 1990; Maekawa *et al.* 2002). Addition of salt in the diet increases water intake due to the increase in tonicity within the rumen (Carter and Grovmum 1990; Ishida *et al.* 2012). This additional intake of fluids results in a dilution of the ruminal contents, further assisting the flow of ingested material through the ROO and reducing ruminal MRT (Martinez *et al.* 2009).

Fermentation within the rumen results in the release of H⁺ ions which actively reduce the pH within the ruminal environment. Ruminal mucosa does not secrete either mucus or buffers to stabilise pH and, as such, the buffering capacity of ruminal fluid depends primarily on salivary secretions (Carter and Grovmum 1990). Buffers present within the saliva act to stabilise the pH environment within the RR as many VFA producing microbes are highly sensitive to pH variations (Carter and Grovmum 1990; Calsamiglia *et al.* 2008; Poulsen *et al.* 2012). Phosphates provide the primary buffering capacity in the rumen as well as an important intermediate in microbial synthesis and fermentation (Bravo *et al.* 2003). Saliva is the major source of endogenous phosphate found in the rumen (Dias *et al.* 2009). Furthermore, maintenance of pH is important as excessive acidity is detrimental to rumen motility, resulting in decreased VFI and rumen contractions (Keunen *et al.* 2002). Along with pH, tonicity of the ruminal fluid increases during and after feeding (Warner and Stacy 1965;

Bennick *et al.* 1978). Saliva aids in the control of hypertonicity of the fluid by increasing in quantity to dilute the rumen contents (Carter and Grovum 1990). Rumination of ingested material not only aids in the mechanical breakdown of the feed but also stimulates saliva production, leading to rumen dilution and lowering of rumen tonicity (Carter and Grovum 1990).

The ROF of digesta from the rumen plays a major role in dry matter digestibility (DMD). If fermentation in the rumen is rapid then DMD is high for the diet consumed but should the rate of passage increase, a greater proportion of digesta passes undegraded through to the lower intestines (Nandra *et al.* 1993). The quantity of fluids within the RR and the rate of its influx is a strong determining factor in digesta fermentation. The ruminal fluid's hypertonic state around feeding indirectly stimulates the production of additional saliva in an attempt to restore tonicity (Carter and Grovum 1990; Van Thang *et al.* 2012). As a result the rumen contents become diluted (Harrison *et al.* 1975) with an increase in the RR water pool (Ulyatt *et al.* 1984) and an increase in net flow of material through to the intestines (Harrison *et al.* 1975). It has been suggested that this influx of salivary fluid could improve the efficiency of MP by the ruminal microorganisms and aid in its passage through to the intestines for absorption (Harrison *et al.* 1975). Kelly *et al.* (1991) found that the administration of the parasympathomimetic, slaframine, increased salivation and shifted the site of digestion more towards the post-ruminal regions. Ultimately though, it would be anticipated that the increased rate of passage through the rumen by elevated saliva production would lead to a decrease in CH₄ production due to reduced retention time and, therefore, fermentation of the digesta in the RR.

2.2.4 Physical environment

Ambient temperatures experienced by the ruminant have been demonstrated to have an impact on the amount of fluids consumed. When ruminants experience heat stress their water intake increases to stabilise homeostasis (Hirayama *et al.* 2004). At the same time as

increasing their water intake, ruminants tend to have a lower feed intake with a slower ROF of digesta and methanogens through the RR into the abomasum, resulting in longer ruminal MRT of digesta (Hirayama *et al.* 2004). The result is a greater daily production of enteric methane per unit of DMI (Shibata and Terada 2010). In contrast, cold stress causes a decrease in voluntary water intake as the animal attempts to maintain its core body temperature but an increase in VFI and digesta ROF, resulting in less CH₄ produced (Blaxter *et al.* 1959; Todini 2007). This suggests that environmental factors have an impact on rumen motility and CH₄ production.

During periods of cold weather, animals increase their feed intake due to an increase in heat loss to the environment (Todini 2007; Zhu *et al.* 2011). In contrast, during periods of hotter weather, animals decrease their feed intake but their metabolic rate tends to either not change (Olbrich *et al.* 1972) or increase (Colditz and Kellaway 1972; Hirayama *et al.* 2004). It has been discovered that by increasing the environmental temperature which ruminants are exposed to outside their thermoneutral zone, a decrease in both amplitude and frequency of the RR occurs even if feed is restricted (Attebery and Johnson 1969; Hirayama *et al.* 2004). When the ambient temperature is below the animal's thermoneutral zone, rumen motility and digesta ROF from the rumen increases, causing a decrease in DMD digestibility (Young 1981; Li *et al.* 2000). However, the decrease in DMD due to cold stress was not supported by findings of Kelly *et al.* (1989) and Lourenco *et al.* (2010) who both reported no change to DMD when sheep were exposed to cold ambient temperatures. This indicates that temperature has a direct (not indirectly through a change in feed intake) effect on rumen motility and MRT. Therefore, it may be possible to influence CH₄ production by manipulating rumen kinetics through variations in temperature exposure.

2.2.5 Animal genetics

Phenotypic divergence among ruminant species in CH₄ traits is starting to make selection for high and low MY possible, and appropriate selection tools are being developed. Positive phenotype correlations have been found between the size of the RR, the rumen fill, the amount of organic matter ingested and the quantity of CH₄ emitted (Pinares-Patiño *et al.* 2003). Ulyatt *et al.* (2003) noted that CH₄ emissions increased as the size of the organic matter pool within the rumen increased due to an increase in the amount of organic matter consumed and the size of the rumen. The rumen fill (grams of wet digesta within the rumen) naturally increases as the volume of the rumen increases. This, therefore, suggests that larger feed intakes result from an increased physiological capacity of the rumen, which allows longer MRT and higher DMD (Grovmum 1984) leading to greater CH₄ emissions. Goopy *et al.* (Submitted) found that sheep selected for high MY possessed larger rumens, had longer particle MRT, and consistently a higher proportion of retained rumen contents than sheep selected for low MY. This is just one physiological factor that ties phenotype of the ruminant with its capacity to emit CH₄.

Residual feed intake (RFI), the difference between actual DMI and expected DMI for individuals in a population of similar size and level of production, has shown potential in reducing CH₄ emissions by genetic selection. An efficient animal is one which eats less than expected to achieve the desired production outcome (wool growth, milk production, BW increase, etc.) and, therefore, has a lower RFI than the mean of the population (Hegarty *et al.* 2007; Arthur *et al.* 2010; Hegarty *et al.* 2010; Waghorn and Hegarty 2011). Residual feed intake is a moderately heritable trait making it potentially useful for reducing the emissions intensity (EI) or amount of CH₄ emitted per unit of animal product produced (Waghorn and Hegarty 2011). Little evidence exists though to demonstrate that a lower RFI results in a reduction in MY, suggesting that RFI has the potential to reduce CH₄ EI but may not be useful in reducing the amount of CH₄ yielded per animal.

Improving animal genetics for MY offers opportunity to reduce emissions, but progress is expected to be slow due to its relatively low heritability (Alcock and Hegarty 2011). However, Pinares-Patino *et al.* (2011) found that even though diet affects the magnitude of the sheep's MY rankings there was enough animal variation in CH₄ emissions that selection for low CH₄ could be heritable. With further research, more phenotypic traits correlating with CH₄ are likely to be discovered, making genetic improvement in MY easier.

2.2.6 Internal parasites

Internal parasite infections markedly decrease ruminant feed intake. Symons (1978) discovered that sheep infected with *Trichostrongylus colubriformis* not only decreased their feed intake but also had a significant increase in cholecystokinin (CCK) levels in the plasma. Following treatment with an anthelmintic, both CCK and VFI returned to normal levels. Cholecystokinin is a peptide hormone responsible for suppression of VFI and has been demonstrated to inhibit muscular contractions within the rumen, significantly slowing down rumen motility and rate of passage through the digestive system. Infection of the abomasum with either *Haemonchus contortus* or *Ostertagia circumcincta* induces a reduction in VFI and rumen motility in sheep primarily due to hypergastrinaemia (Anderson *et al.* 1976; Anderson *et al.* 1981; Grovum 1981; Honde *et al.* 1985). Along with this, *H. contortus* infections cause a significant increase in abomasal pH (Nicholls *et al.* 1987), somatostatin (Honde *et al.* 1985) and plasma gastrin concentrations (Honde *et al.* 1985; McLeay and Wong 1989), most probably through the release of hormonal inhibitors in response to the irritating actions of the parasite on the epithelial lining. Somatostatin, a hormone known to reduce contractions in the GIT, increased within 3 h of infection, probably as a result of antigenic reaction (Honde *et al.* 1985). These actions would have an impact on secretive digestion and ultimately gastric emptying as well as slowing ruminal contractions. With this understanding of the impact parasitic infection has on both VFI and rumen motility, gastro intestinal parasite control may have a direct influence on methane emissions from ruminant livestock.

2.2.7 Digesta kinetics

Since Hungate (1966) noted that between-animal variations of digestion, fermentation, and digesta kinetics offered an opportunity to select ruminant livestock for increased productivity, it has been recognised that the period which feed is retained in the rumen significantly affects its fermentation (Hegarty 2004). Pinares-Patiño *et al.* (2003) showed that MY could be significantly reduced by reducing the MRT of digesta in the rumen. Repeatability of the relationship between digesta kinetics and enteric CH₄ emissions has been assessed and found (Pinares-Patiño *et al.* 2011). This repeatability is sufficient to allow genetic selection of animals for MY, subject to a suitable emission measure being used. Other studies have shown that a reduction in digesta MRT is associated with an increase in propionate concentration and proportion relative to acetate and butyrate in the rumen (Janssen 2010), an increase in MP outflow from the rumen (Chen *et al.* 1992), and increased wool growth (Smuts *et al.* 1995). Therefore, manipulation of digesta kinetics has the potential to be used as a tool for CH₄ mitigation. There may be enough between-animal variation in digesta MRT to indirectly select for low MY in ruminant breeding programs.

2.2.7.1 Structure of the ruminant stomach and passage of ingested material within

The stomach of ruminants consist of four compartments; rumen, reticulum, omasum and abomasum (Sellers and Stevens 1966). The rumen and reticulum are the primary sites of fermentative digestion within the GIT, due to the presence of specific microorganisms (Hungate 1966). The contents of the RR are also mechanically broken down as they are continuously mixed together by the rhythmic contractions of the RR wall (Ulyatt 1983; Hummel *et al.* 2009). These contractions not only mix the digesta with saliva and symbiotic micro-organisms but are also responsible for the movement of material from the RR into the omasum along with the evacuation of gaseous by-products (particularly CO₂ and CH₄) into the oesophagus during eructation (Phillipson 1939; Ruckebusch 1989). Therefore, the contractile forces of the RR and the mechanisms controlling their actions play a significant

role in the MRT of digesta and, hence, MY (Okine *et al.* 1989; Okine and Mathison 1991; Bernard *et al.* 2000).

The type and quantity of feed consumed can have an impact on the physical appearance of the rumen. Ruminants which browse or are fed high concentrate diets have a complete absence or very little stratification of digesta in the rumen. In contrast, grazing ruminants and those fed a high roughage diet have a stratified rumen with a gas layer at the top, under which lies a mat of consumed material covering a large layer of fluid (Hofmann 1973; Welch 1982; Tschuor and Clauss 2008; Clauss *et al.* 2010) (Fig 2 - 3). This stratification is considered to be essential to allow the selective retention of particles within the rumen for further processing (Lechner-Doll *et al.* 1991; Tschuor and Clauss 2008).

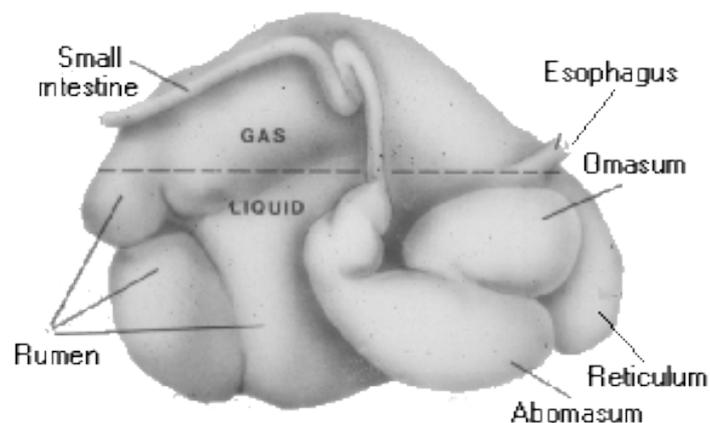


Fig 2 - 3 Structure of the ruminant's forestomach from the right side showing typical gas and liquid stratification (Bowen 2006)

The physical appearance of the epithelial lining of the rumen is dominated by the abundance of papillae (Fig 2 - 4), whose growth is stimulated by the presence of volatile fatty acids (VFA), in particular butyrate and propionate (Warner *et al.* 1956; Sakata and Tamate 1978).

In contrast, there is an absence of these same papillae on the dorsal mucosa where a gaseous layer exists (Hofmann 1973). Hofmann (1973) suggested this is due to the continuous presence of high levels of CO₂ and CH₄, preventing significant concentrations of VFAs developing in this region but the exact mechanism is still unclear. It has been demonstrated that the presence of VFAs, in particular butyrate, promotes proliferation of these epithelial cells of the rumen (Sakata and Tamate 1978), so the absence of VFAs in the region would lead to limited papillae development. The primary role of the papillae upon the epithelial lining of the rumen is to absorb the VFAs produced (Van Soest 1994; Baldwin 1998).

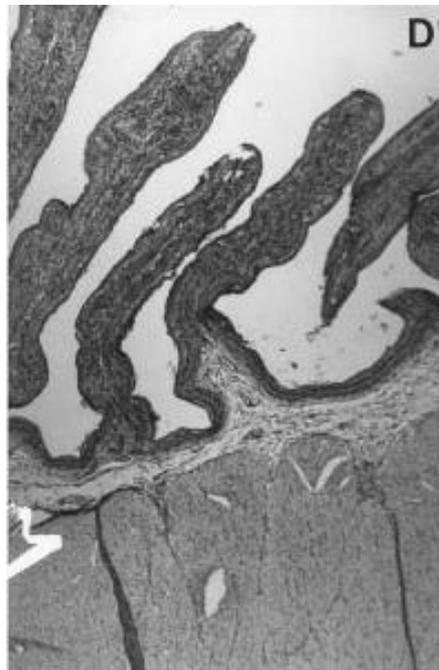


Fig 2 - 4 Sections of stained ruminal papillae from the dorsal blind sac of calves fed unground diets (Beharka *et al.* 1998)

Once rumen digestion of digesta is complete, the material or bolus passes out of the RR into the omasum via the ROO. This passageway is located on the right wall of the reticulum and is directly located with the parallel ridges of the oesophageal groove which, during suckling, forms a direct channel for milk to flow to the abomasum (Schalk and Amadon 1928). The flow of the ingesta into the omasum is mainly dictated by the diameter of the opening of this

reticulo-omasal orifice (ROO) (Bueno 1972). The material passing into the omasum is high in fluid content with little dry matter. This is due to water, predominately saliva, being an important transport vehicle of solid digesta within and from the RR. This water is largely reabsorbed back into the body from the omasum (Ekman and Sperber 1953).

The omasum serves to control the amount of digesta which passes into the abomasum. It was originally suggested that the absorption of the VFA and buffers from the digesta in the omasum would result in the abomasum reducing its secretion of hydrochloric acid needed to lower the digesta's pH (Moir 1984). It was found that the omasum was capable of regulating the in-flow of digesta, as a way of protecting the abomasum, by controlling the amount of material passing through the reticulo-omasal orifice (Afzalzadeh and De B. Hovell 2002). Once digesta has entered the omasum it must be able to continue its journey through the abomasum and into the small intestine (SI). A wave of contraction passes over the omasal body which allows the digesta to pass through the omasal leaves and into the abomasum (Bueno and Ruckebusch 1974). These contractions start at a point on the omasal groove just posterior to the reticulo-omasal orifice and continue slowly over the omasal body (Bueno and Ruckebusch 1974). They are very closely associated with the contractions found in the RR which are designed to continuously mix the ingesta (Bueno and Ruckebusch 1974). These slowly drive the material into the final compartment of the ruminal forestomach, the abomasum. Unlike the rumen, reticulum and omasum, the abomasum is not dependent upon the contractions of the RR for the passage of digesta. Digesta is propelled from the abomasum through to the SI via normal peristaltic movements even after a complete vagotomy, although with greatly reduced capacity (Duncan 1953; Balch 1959).

To enable researchers to understand ruminant digesta kinetics and its role in MY, methodologies are required to enable accurate estimation of kinetic parameters. One such methodology is the use of multicompartmental mathematical models to calculate the flow of particles and solutes through the gut.

2.2.7.2 Estimation of digesta kinetics with mathematical modelling

Models are simplified representations of systems and modelling is the process of developing a model or set of equations to simultaneously represent the structure and behaviour of a system (Wastney *et al.* 1999). Initial attempts to predict digesta flow were limited in fitting the observed data effectively, especially when particle material kinetics were considered. These models only consisted of two (Blaxter *et al.* 1956) or three (Milne *et al.* 1978) compartments and a delay time with only a single marker administered (Blaxter *et al.* 1956; Grovum and Williams 1973). This limited the ability of the early models to provide reliable parameter estimates of digesta kinetics (Aharoni *et al.* 1999).

Grovum & Phillips (1973) noted that there was a poor fit between the observed concentration of marker and the predicted values for a two-compartment, one-marker model. As a result, new improved models were devised, including the multiplicative equation by Dhanoa *et al.* (1985), that is still in use, containing an exponential term and a double-exponential term to describe digesta flow through the gut as a multicompartmental process (Eq. 8); some publications using this model include Huhtanen and Kukkonen (1995), Savoie *et al.* (2001), Hristov and Ropp (2003), Dijkstra *et al.* (2007), and Pérez-Ruchel *et al.* (2012):

$$y = Ae^{-k_1 t} e^{-Be^{-(k_2 - k_1)t}} \quad \text{Eq. 8}$$

where y is faecal marker outflow; A is a scale parameter dependent on k_1 , k_2 , and B ; B is the number of compartments in the model; t denotes time; and k_1 and k_2 represent the rate constants of the two compartments of the GIT having the longest retention times – the rumen and caecum (Dhanoa *et al.* 1985).

As digesta solutes and particles progress along the gastrointestinal tract (GIT) at different rates (Faichney 1975b; Faichney and Griffith 1978; Ellis *et al.* 1982), the use of either a solute or particulate marker alone cannot describe kinetics of whole digesta as well as a

dual-marker system. As a consequence a sequential, irreversible, multicompartmental model was developed with simultaneous fitting of results from more than one marker, allowing a more reliable estimate of the digesta kinetics, including MRT, transit time (TT), and faecal DM output (FDMO) (Dhanao *et al.* 1985; Frances *et al.* 1988; Aharoni *et al.* 1994; Reese *et al.* 1995; Aharoni *et al.* 1999). FDMO could be obtained from these models by including additional variable inputs that included DMD, DMI and marker dose administered.

While this new generation of dual-marker models explained digesta flows and FDMO with more accuracy than earlier single-marker models, there were still discrepancies in their abilities to predict observed recordings of marker concentrations. Faichney *et al.* (1989) noted that particles and solutes moved at different rates through the GIT, particularly within the rumen. In 1994, Aharoni *et al.* devised a sequential model of particulates and solutes with parallel movement of each within each compartment of the digestive tract. Later, Aharoni *et al.* (1999) expanded upon this idea and included the possibility that finer particles moved from the particulate route to the faster solute route. A third model was also proposed that was based on the principles originally suggested by Faichney (1975a; 1975b) and further expanded by Reese *et al.* (1995) where the rate of passage of particles and solids is different in the rumen but is identical for all other pools and delays are identical for all markers.

Kinetic modelling developed further with the introduction of complex mathematical computational programs, such as CONSAM (Boston *et al.* 1982). These programs undertake compartmental analysis by a set of linear and non-linear differential equations designed to simulate a system consisting of several compartments of markers, which are interconnected by flows of those markers (Aharoni *et al.* 1994). The program is used to calculate values of marker concentration at the time of the observed data points based on the model being used, and calculates the differences between the model predicted and observed (actual) data (Berman *et al.* 1962). The program then changes the values of the adjustable

parameters within the model to minimise the sums of squares of these differences by a non-linear, least-squares technique (Berman *et al.* 1962). The least-square method applied to goodness of fit denotes that the best model is the one which minimises the sum of squared residuals (Mumenthaler *et al.* 2000). A lower residual sum of squares (least-squares) represents a more accurate parameter estimate by the model (Mumenthaler *et al.* 2000). A model that yields a better curve fit after successive iterations is identified as producing a smaller sum of squared residuals.

2.3 *Physiological regulation of digesta kinetics*

Reticulorumen and omasal contractions, and the opening and closing activity of the ROO, play major roles in the MRT of digesta in the GIT (Bueno 1972; Bueno and Ruckebusch 1974; Okine *et al.* 1989; Okine and Mathison 1991). To facilitate fermentation, constant mixing of the digesta needs to occur. This allows complete microbial coating of digesta, propulsion of VFA to the dorsal surface for absorption, regurgitation of large material back to the mouth for further mechanical breakdown, and lubrication of the digesta for ease of passage through the ROO (Ruckebusch 1989). This is achieved as a result of specific motility patterns of the RR which are distinct from that witnessed within the GIT of the monogastric (Ruckebusch 1989). Monogastrics experience a series of peristaltic and segmented movements of the intestine to propel digesta through the GIT (Schemann and Ehrlein 1986) while ruminants propel their digesta in the RR through a series of cyclic contractions designed to mix and propel the material either towards the abomasum or forward for mastication and eructation (Phillipson 1939; Ruckebusch 1989). In order to manage digesta MRT to reduce MY, it is important there is a better understanding of the factors regulating RR, omasal, and ROO contractions; and the means of physiological regulation involved, be they neural or hormonal.

2.3.1 Contractile sequences of RR

The contractions within the rumen are best described as a wave impulse designed to propel ingested material in a cyclic manner throughout the RR (Fig 2 - 5). Two regular cyclical sequences of ruminal contractions have been identified. Firstly, one associated with contraction of the reticulum serving to mix rumen contents and progress ingesta from the RR and, secondly, one independent of reticulum contraction and concerned with the removal of gases from the rumen via eructation (Akester and Titchen 1969). The mixing sequence involves a series of coordinated biphasic contractions of the reticulum followed successively by contraction of the cranial dorsal rumen, the caudal dorsal rumen, the caudal ventral rumen and cranial ventral rumen structures (Phillipson 1939; Akester and Titchen 1969; Ruckebusch 1989). The eructation sequence consists of a slightly different series of contractions within the rumen, firstly the caudal blind sac of the rumen, then the more caudal followed by the more cranial regions of the dorsal sac of the rumen contracts, and finally the main ventral sac of the rumen contracts (Akester and Titchen 1969). Eructation occurs when the cranial parts of the dorsal sac are contracting (Akester and Titchen 1969). It is this eructation sequence that expels the rumen headspace gases into the oesophagus before mixing in the airways and being exhaled into the atmosphere (Dougherty and Cook 1962). Cyclic RR contractions in ruminants are primarily regulated by the vagal cholinergic nerves (Ruckebusch 1989) but other mediating factors have a significant influence.

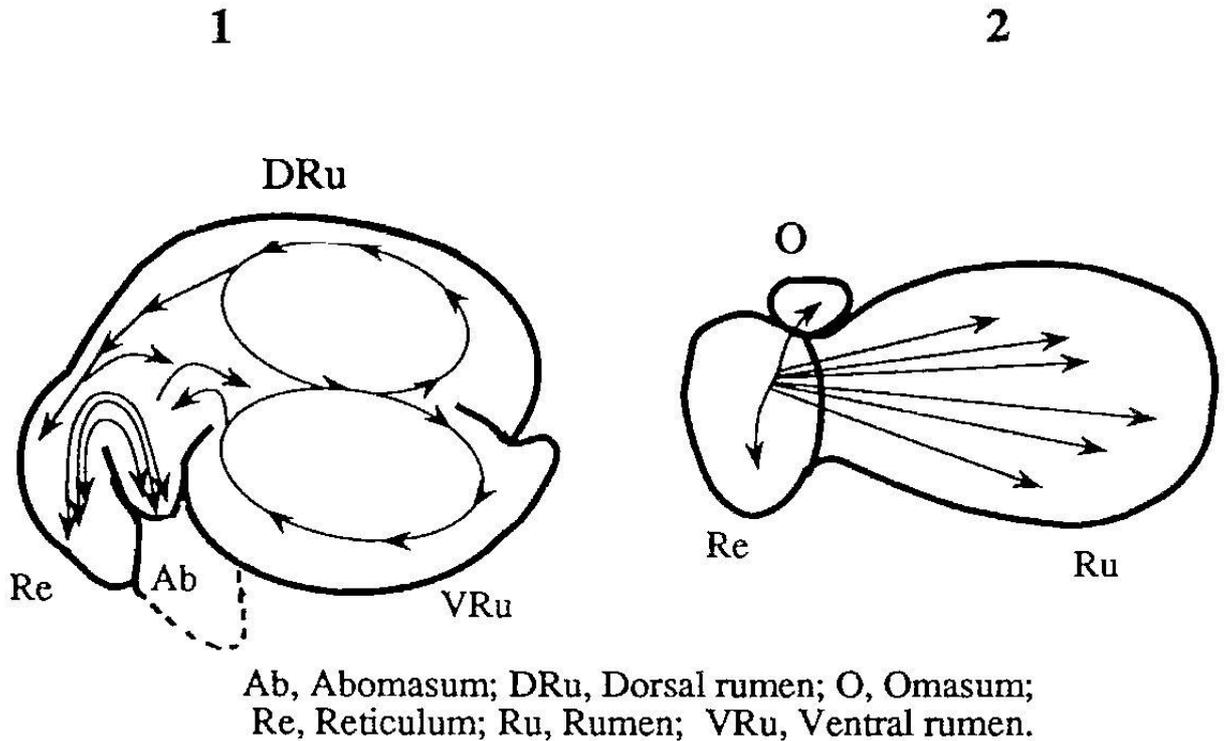


Fig 2 - 5 Drawing summarising the movement of digesta in the reticulo-rumen as seen radiographically in the horizontal (1) and vertical (2) planes. Arrows indicate direction of movement. (Poncet 1991)

Contractile sequences within the RR are not only coordinated and frequent to allow continuous flow of both digesta and gases, but also vary in amplitude to help regulate the quantity of material propelled. Digesta passage from the RR to the abomasum is not so determined by contraction frequency as by duration and amplitude of contractions (Okine and Mathison 1991). Before digesta can pass through the RR into the omasum, certain requirements for passage need to be met. These requirements include the ROO being open and not masked by the lips of the reticular groove, particle size and density allowing passage of digesta down through the stratified filter bed, and there being sufficient force to propel digesta into the omasum (Okine and Mathison 1991; Li *et al.* 2000; Clauss *et al.* 2011). Once these requirements are met, the duration of RR and ROO contractions become the most important attribute associated with passage of digesta from the RR (Okine and Mathison 1991; Onaga *et al.* 2008).

2.3.2 Omasal contractions

As digesta is propelled from the RR, it enters the omasum before continuing on to the gastric stomach, the abomasum. Omasal contractions are synchronised with the RR contractions (Bueno and Ruckebusch 1974). They gradually develop prior to reticular contractions and cease when the reticulum contracts (Bueno and Ruckebusch 1974). Omasal contractions play a key role in the gastric transit in ruminants by restricting digesta flow from the RR to the abomasum where gastric digestion takes place (Hauffe and Von Engelhardt 1975). While synchronisation of omasal contractions to RR contractions occurs, it has been shown that omasal contractions persist after vagal and cholinergic blockades (Bueno and Ruckebusch 1974; Gregory 1982; Onaga *et al.* 2011), indicating that non-cholinergic intrinsic factors are also involved in the omasal contractions (Onaga *et al.* 2008; Onaga *et al.* 2011). Frequency of omasal contractions appear to be regulated by neurogenic intrinsic factors like substance P (Groenewald 1994), while it is also possible that the amplitude of contraction is regulated by humoral factors like gastrin (Onaga *et al.* 2008). In fact, gastrin released by the abomasal antrum binds to its receptors found within the smooth muscle of the omasum, called CCK-1R and CCK-2R receptors, and either raises contractile amplitude or works as a mediator of negative feedback on gastrin secretion (Onaga *et al.* 2008).

2.3.3 Regulation of RR, ROO and omasal contractions

Regulation of the contractile processes within the RR and omasum is vital for the efficient functioning of the ruminal digestive system. Cholecystikinin (CCK) (Fig 2 - 6), a peptide hormone secreted by the duodenal mucosa is recognised as a physiological mediator in fat- and protein-induced feedback inhibition of gastric motility and has been widely accepted as regulating ruminal contractions (Onaga *et al.* 2008). Baile *et al.* (1986) discovered that CCK elicited changes in ruminal contractile amplitude that simulated those experienced in ruminants at differing stages of satiation. CCK has also been shown to inhibit cyclic RR contractions (Grofum 1981; Ruckebusch 1983) as well as mediate VFI by ruminants that consume diets high in fat content (Choi *et al.* 2000). It also acts as an inhibitor of rumen

motility in the presence of intestinal acidification (Bruce and Huber 1973). Onaga *et al.* (2008) demonstrated the existence of a duodeno-omasal feedback regulation via CCK when infusion of sheep with CCK increased omasal electromyographic (EMG) activity or convulsions at much lower plasma levels than those required to inhibit RR contractions.

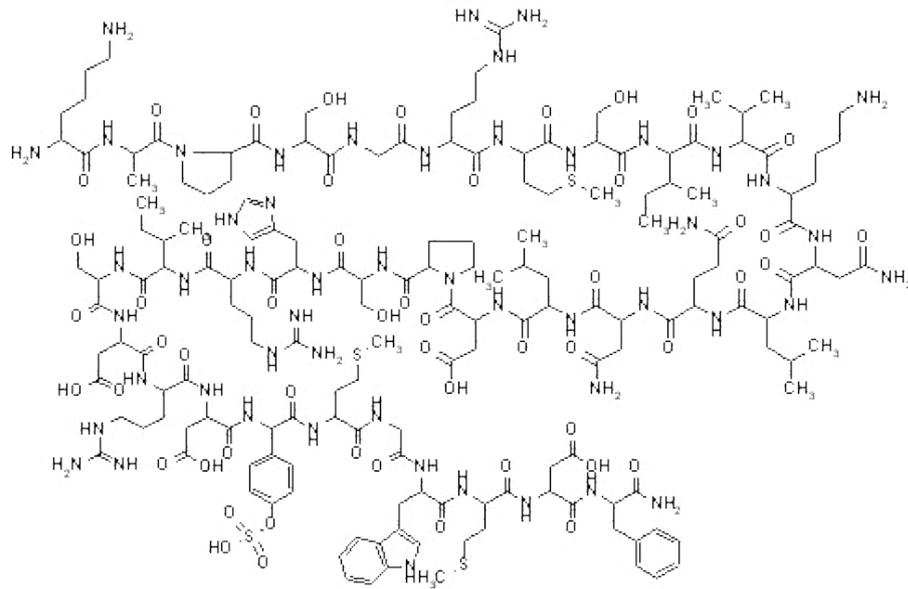


Fig 2 - 6 Chemical structure of cholecystokinin (Gordon 2000a)

Regulation of ROO contractions along with coordinated contraction and relaxation of the RR is important in controlling rate of passage and MRT. Relaxation of the ROO is mediated by neurological and endocrinological systems while being coordinated with other determinants of passage from the RR like contractile amplitude and frequency (Bueno 1972). The muscular surface of the ROO is extensively populated with immunoreactive cells which excrete neurotransmitters, including acetylcholine, noradrenaline and adrenaline (Reid *et al.* 1991). However, quiescence of the ROO still occurs even after blockade of both muscarinic and adrenergic receptors, but not after ganglionic blockade (Reid *et al.* 1991). As a result, it was suggested that the nonadrenergic, noncholinergic polypeptide called vasoactive intestinal polypeptide (VIP) may be involved in the relaxation of the ROO (Reid *et al.* 1988a).

Vasoactive intestinal polypeptide is a member of the glucagon-secretin family of peptides and a neurotransmitter in the central and peripheral nervous system (Fig 2 - 7). VIP is a highly basic octacosapeptide with a broad range of biological effects including vasodilation, relaxation of smooth muscle cells, and influence on the secretory processes in glands (Fahrenkrug 1980). Along with its proposed role in the control of ROO inhibition, VIP has been proposed as a mediator in a variety of physiological processes (Said 1984). VIP induces full quiescence of both the ROO and the abomasum in lambs during suckling to allow direct passage of the milk through to the gastric stomach, bypassing the degrading processes of the rumen (Reid *et al.* 1988b). VIP can also induce quiescence of the ROO's activity in adult sheep fed solid feed at either maintenance and above maintenance levels and lower the amplitude of contractions within the RR (Okine and Mathison 1996; Lalatta-Costerbosa *et al.* 2011). Li *et al.* (2002) found an interaction between VIP and environmental temperature existed in that VIP improved duodenal amino acid passage when sheep were in the thermoneutral zone but not under cold stress conditions. With demonstrable immunoreactivity to VIP in the RR, omasum, abomasum, small and large intestines (Wathuta 1986) and co-habitation of VIP with nitric oxide synthase especially in the omasum (Vittoria *et al.* 2000), VIP seems to play a major role in regulating relaxation of the omasal body, particularly the ROO (Onaga *et al.* 2009; Lalatta-Costerbosa *et al.* 2011).

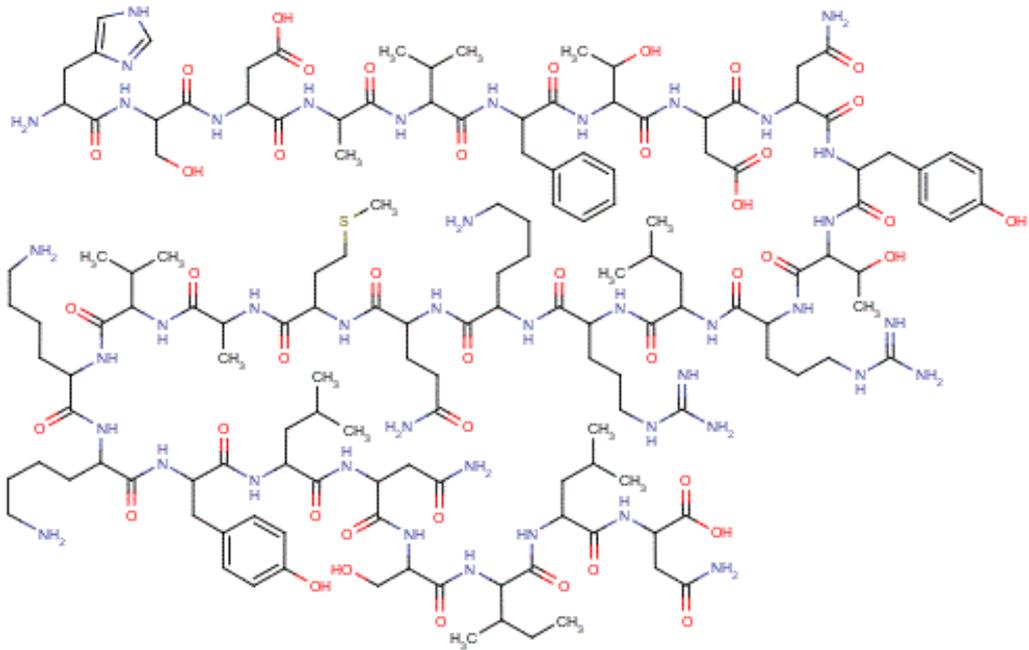


Fig 2 - 7 Chemical structure of vasoactive intestinal polypeptide (VIP) (Gordon 2000b)

Mean retention time has been strongly linked to MY (Pinares-Patiño *et al.* 2003; Pinares-Patiño *et al.* 2007). The retention time of digesta in the RR significantly affects its fermentation which leads to a modification in MY (Hegarty 2004). Understanding the mechanisms controlling digesta MRT within the GIT of ruminants may result in the creation of suitable selection tools for MY and improvement in animal productivity. The preceding review has revealed several physiological regulators of digesta kinetics; such as VIP, CCK, and gastrin; all responsible for modification of RR contractions and quiescence of the ROO. Recent studies have revealed new possible digesta kinetics regulators as well as insights into potential regulators yet to be confirmed (Saras *et al.* 2007; Onaga *et al.* 2008; Nakajima *et al.* 2010; Jin *et al.* 2011; Onaga *et al.* 2011; Oh-ishi *et al.* 2013). The following section briefly looks at the current research in digesta kinetics regulators and the hormones showing potential for selection as digesta kinetics regulators.

2.4 ***Hormonal regulation of GIT motility***

Motility of the ruminant GIT is controlled by intrinsic and extrinsic control mechanisms (Münnich *et al.* 2008). In recent years, research has become focused on the role intrinsic control mechanisms play in ruminal retention time (Okine *et al.* 1998; Vittoria *et al.* 2000; Li *et al.* 2002; Onaga *et al.* 2011). Calingasan *et al.* (1984) undertook an immunocytochemical study of the sheep GIT in an attempt to understand the distribution and frequency of endocrine cells known to influence GIT motility. Endocrine cells are distributed throughout the epithelium and glands of the GIT, and synthesise hormones that play a regulatory role in the physiological functioning of the digestive system (Calingasan *et al.* 1984). Calingasan *et al.* (1984) found somatostatin- (SRIF) and gastrin-immunoreactive cells in the abomasum; SRIF-, gastrin-, CCK-, motilin-, neurotensin (a neuropeptide associated with VFI and digestion)- secretin (a neuropeptide associated with VFI and digestion)-, substance P-immunoreactive cells in the SI; and SRIF- and substance P-immunoreactive cells in the large intestine. Kitamura *et al.* (1985) undertook a similar study on cattle and found a similar distribution except serotonin-immunoreactive cells (5-hydroxytryptamine; 5-HT) were also found to be present in the cattle abomasum, small and large intestines. Mimoda *et al.* (1998) detected 5-HT and substance P specific immunoreactivity in endocrine cells of the SI of cattle, sheep, and goats with immunoreactivity for Met-enkephalin-Arg⁶-Gly⁷-Leu⁸ (MENK8) also detected in duodenum endocrine cells of cattle but not sheep or goat (Mimoda *et al.* 1998). MENK8 is a bio-active member of the endogenous opioid peptides (EOP) known to inhibit intestinal motility (Mitznegg *et al.* 1977; Dobbins *et al.* 1981). Münnich *et al.* (2008) noted the presence of choline-acetyltransferase (ChAT), nitric oxide synthase (NOS), VIP, NPY (a neuropeptide associated with VFI and digestion), substance P, and somatostatin within the rumen of sheep, cattle, goats, and fallow deer. CCK-receptor type 1 (CCK-1R) was present in the rumen (Yonekura *et al.* 2002) while both CCK-1R and 2R are localised in the omasum (Yonekura *et al.* 2002; Onaga *et al.* 2008). Leptin mRNA was also found in the rumen and abomasum of calves but was not present in adult cattle suggesting leptin plays

little role in forestomach contractions (Yonekura *et al.* 2002). This suggests expression of peptides and hormones involved in gastric motility may be species specific within the *Bovidae* family. Other intrinsic factors have also been detected in the ruminant GIT but, as they currently are not associated with GIT motility, they have been excluded from this review.

2.4.1 Wild-type variation

Guilloteau *et al.* (2009) noted an evolutionary variation between domesticated and wild ruminants. European roe deer (*Capreolus capreolus*), a common wild ruminant in Europe and concentrate-selector (Hofmann and Stewart 1972), consumes a diet high in diterpenes and polypholic compounds, such as lignin and tannins (Tixier *et al.* 1997). As a result, roe deer possess a greater concentration of amylolytic than cellulolytic bacteria in the rumen (Fickel *et al.* 1998). They secrete 9 to 14 times more saliva than sheep (Fickel *et al.* 1998), and this saliva contains high amounts of proline-rich proteins (PRP) (Guilloteau *et al.* 2009). In contrast, sheep and cattle possess very low levels of PRP in their saliva (Guilloteau *et al.* 2009). PRP is used in the GIT, in combination with chymotrypsin, to cleave the amino acid bonds in proteins (Guilloteau *et al.* 2009), and the PRP also effectively bind tannins, reducing their deleterious effects on the animal (Verheyden-Tixier and Duncan 2000). Tannins, as mentioned previously, are a current focus of research in methane mitigation and abatement. It may be possible that as a consequence of domestication, where sheep and cattle are removed from a high tannin-rich plant diet, agricultural ruminants may have lost the ability to secrete high amounts of PRP.

2.4.2 Enkephalins

Neural encoding for nociception (the neural process for encoding and processing of noxious stimuli, including deleterious changes within the RR) can impact on digesta MRT. Originally discovered by Hughes *et al.* (1975) as a series of EOP in brain extracts of several different animals, enkephalins directly impact on digesta ROF by evoking a blockade of the cyclic contractions of the RR (Ruckebusch *et al.* 1984; Kania and Domański 1996). MENK8

(Fig 2 - 8) has been shown to increase VFI of sheep (Bueno 1987) but diminish amplitude and frequency of ruminal contractions while increasing smooth muscle tone (Ruckebusch *et al.* 1984; Kania 1992; Kania and Domański 1996). Enkephalins inhibit RR contractions by acting as Ca-channel blockers (Dobbins *et al.* 1981), and by causing the localised release of acetylcholine and specific noncholinergic compounds including histamine (Mitznegg *et al.* 1977). Specific δ -opioid receptors participate in the inhibitory actions of enkephalins on the myoelectrical activity of sheep forestomach (Kania and Wielgosz 2009). In some species, enkephalins are capable of reducing metabolism, heart and respiratory rates, and inducing hibernation (Krzymowski and Stefanczyk-Krzyszowska 2012). Enkephalins, along with other neurally activated EOP, demonstrate the hypothalamic influence on rumen motility and MRT, offering further evidence of physiological regulation of gut kinetics within ruminants.

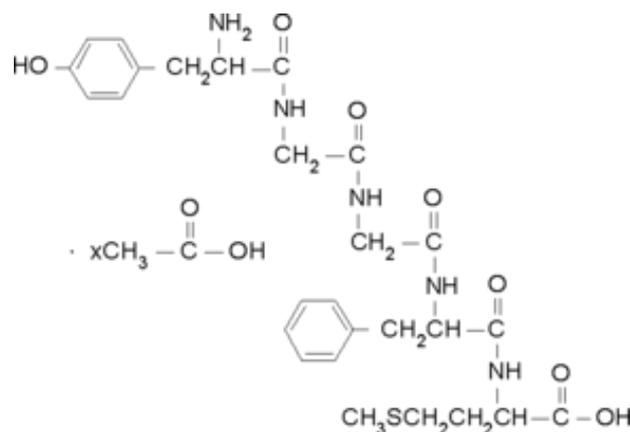


Fig 2 - 8 Chemical structure of Met-enkephalin (Sigma Aldrich 2013)

2.4.3 Gastrin

Another endogenous peptide which exerts significant influence on GIT motility and digesta MRT is gastrin (Fig 2 - 9). Considered a pivotal substance in the digestive system, this gut-regulatory peptide is a chemical messenger mostly implicated in the regulation of secretions, motility, absorption, digestion, and cell proliferation (Guilloteau *et al.* 2006). Plasma concentrations of gastrin dramatically increase late gestation and, due to known trophic effects, are implicated in GIT development of the young ruminant (Guilloteau *et al.* 1998; Guilloteau *et al.* 2006; Guilloteau *et al.* 2009). Gastrin can influence cellular function either in

an endocrine, paracrine, or autocrine fashion. Secreted from peptide-producing cells in the GIT, primarily G-cells, into the interstitial fluid, blood, and GIT lumen; gastrin specifically acts on its target cells and/or organs to regulate the function of the GIT, including the pancreas and gall bladder (Walsh and Dockray 1994; van der Schaar *et al.* 2001; Guilloteau *et al.* 2006). It was originally thought that gastrin's mode of signalling was solely as an endocrine (Fox *et al.* 1987) but it has been discovered that gastrin can act in a paracrine (van der Schaar *et al.* 2001) and autocrine fashion (Guilloteau *et al.* 1997), and even stimulate vagal afferents as a neurotransmitter (Akiyama *et al.* 2012; Katzung 2012).

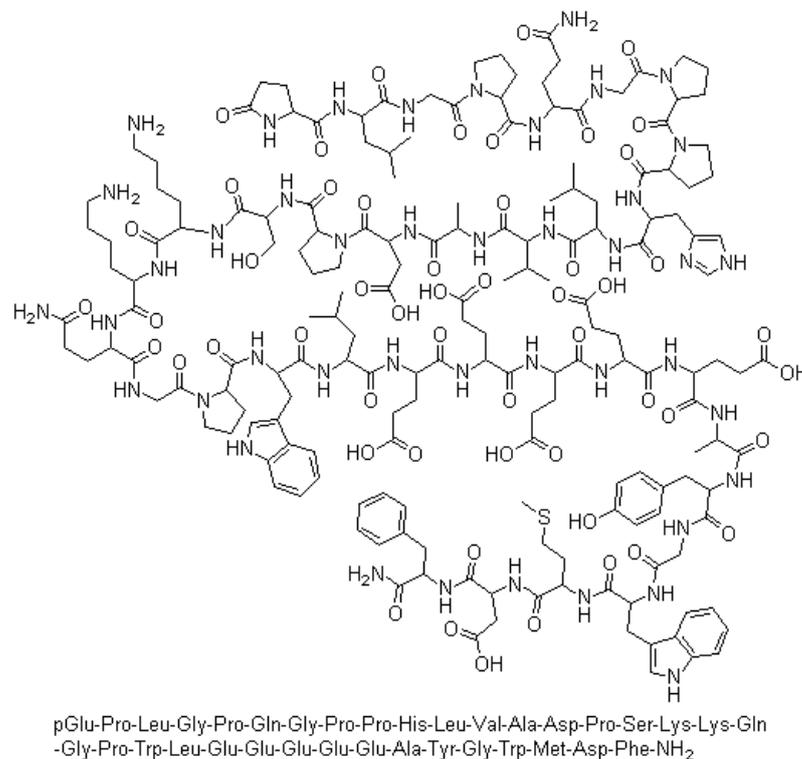


Fig 2 - 9 Chemical structure of gastrin (Lookchem 2013)

Gastrin is capable of inhibiting VFI (Grovm 1981), reducing frequency of reticular contractions (Grovm 1986), inhibiting abomasal emptying (Gregory *et al.* 1985), and seriously affecting the normal migrating myoelectric complex (MMC) pattern of the small intestine (Parkins and Holmes 1989). The effects on GIT motility appear to be more mediated by humoral than by neurogenic intrinsic factors (Onaga *et al.* 2008). Grovm and Chapman (1982) suggested that gastrin's inhibitory effect on RR contractions was through

direct action on the hypothalamus by a negative-feedback response associated with hypergastrinaemia. Antalis *et al.* (2007) also suggested that gastrin released from the abomasal antrum acts as a mediator of negative-feedback regulation but found that gastrin raised omasal tone as an activator of omasal contractions. Gastrin binds to both types of CCK/gastrin receptors in the omasal smooth muscle, CCK-1R and CCK-2R, inducing omasal tonic contractions (Onaga *et al.* 2008).

Elevated plasma levels of gastrin have also been shown to reduce VFI, through inhibition of ghrelin secretion (Lippl *et al.* 2004) and decreased gastric pH (Fox *et al.* 2002). In response to elevated circulating gastrin levels, bacteria within the ruminant abomasal microbiota produce a gastrin inhibitor, resulting in reduced gastrin levels in the abomasum and SI (Simcock *et al.* 2006). Ruminal fluid also displays similar gastrin secretion inhibition, suggesting the same gastrin inhibitors are produced by ruminal micro-organisms which are capable of surviving in the abomasum (Li *et al.* 2011). Therefore, the composition of the microbiota in the rumen may have a significant impact on MRT of digesta within the GIT.

2.4.4 Motilin

The MMC is designed to stimulate motility in the small intestine to allow clearance of digesta in preparation for the next meal, and motilin has been suggested to play an important role in the initiation of the MMC (Nakajima *et al.* 2010). A putative regulatory peptide primarily localised in the wall of the duodenum and proximal jejunum, motilin (Fig 2 - 10) seems to be necessary for the initiation and consolidation of gastric phase III, regular high-amplitude contractions, of the duodenal MMC (Yokohata and Tanaka 2000; Tanaka 2002). Phase III contractions of the MMC start in the duodenum and lower abomasal region, then migrate down the entire length of the SI (Yokohata and Tanaka 2000; Tanaka 2002). For ruminants, the MMC occurs in both a fed and fasted state unlike non-ruminants which only exhibit MMC during fasting (Yokohata and Tanaka 2000; Tanaka 2002). Unlike CCK and gastrin which disrupt MMC cyclic activity (Fioramonti 1988; Parkins and Holmes 1989), motilin has been

suggested in initiating and continuing MMC activity, possibly through cholinergic neural pathways (Yokohata and Tanaka 2000; Englander and Greeley Jr 2006). Motilin's role in MMC activity has been questioned. Plaza *et al.* (1996c), using sheep, found no changes in plasma motilin concentrations during MMC activity but suggested a complex interaction between 5-HT, bombesin, and SRIF was more involved in MMC regulation. Zabielski *et al.* (1998) found that motilin, along with secretin, CCK, and SRIF became elevated in calves during increased MMC activity but it was dependent upon the source of dietary protein consumed. It was suggested that fluctuations of gut regulatory peptides was a result not a cause of MMC (Zabielski *et al.* 1998). Rises in plasma motilin concentrations have been shown to be associated with a rise in plasma SRIF concentrations suggesting their combined role in enterogastric feedback mechanisms (Vantrappen 1982). A better understanding of this peptide and its role in GIT motility is required.

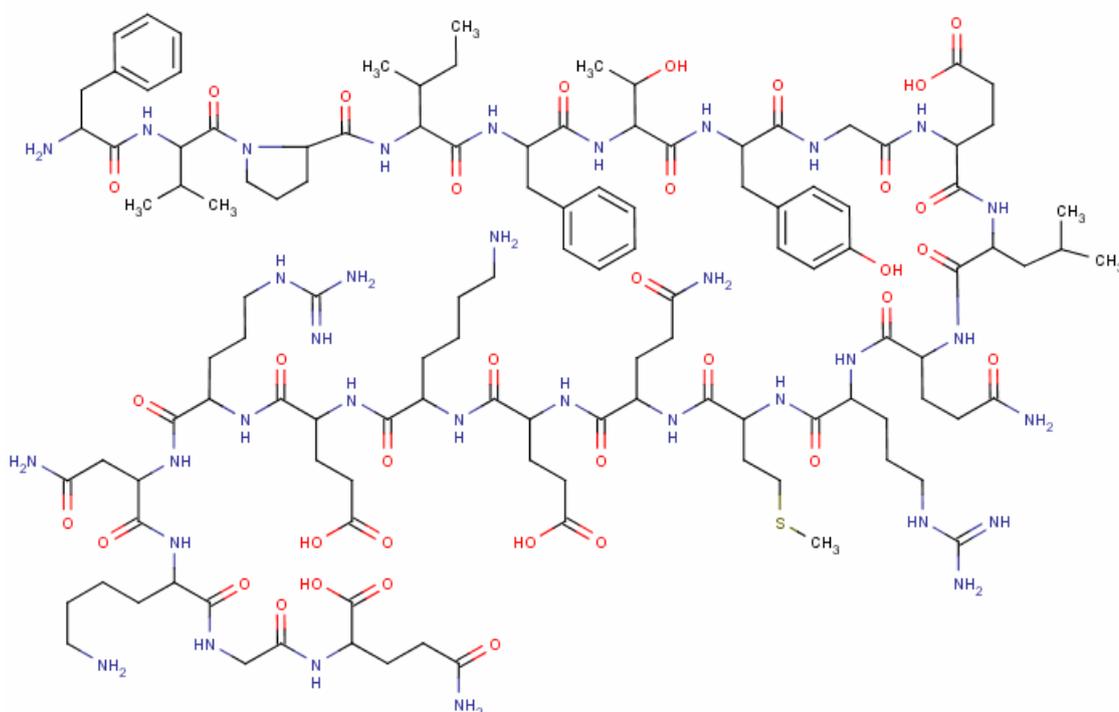


Fig 2 - 10 Chemical structure of motilin (Guidechem 2013)

2.4.5 Antagonists and agonists

Okine *et al* (1989) discovered that it wasn't the frequency or amplitude but the duration of RR contractions that significantly influenced passage rates of ruminal fluid and particulate matter. Antagonists and agonists have an impact on the smooth muscle contractions of the RR, ROO and omasum. Bradykinin and 5-HT have been shown to inhibit the contractile sequences of the smooth muscle surrounding the rumen (Veenendaal *et al.* 1980) while proglumide, a CCK antagonist designed to block both CCK-1R and CCK-2R subtype receptors, have been found to significantly increase ruminal contractions at low doses (Onaga 2007). At high doses, proglumide inhibits cholinergic-induced contraction of the ruminal muscles or acts as an agonist to inhibit contractions in sheep (Onaga 2007). 5-HT agonists induce inhibition of forestomach myoelectric activity through 5-HT₁, 5-HT₂, 5-HT₃ and 5-HT₄ receptors, these actions being mediated by cholinergic neural pathways involving muscarinic and nicotinic receptors (Plaza *et al.* 1996a; Plaza *et al.* 1996b). Dopamine and apomorphine, a dopamine agonist, cause inhibition of extrinsic ruminal contractions, effects which are completely prevented by the antagonist domperidone (Maas *et al.* 2008). These results suggest that apomorphine-induced inhibition of rumen motility is due to dopaminergic receptor activation (Maas *et al.* 2008).

2.4.6 Tachykinins

Tachykinin (TK) receptors have recently been studied in the regulation of gastric motility in ruminants. Semiquantitative reverse transcription (RT)-PCR revealed preprotachykinin and neurokinin (NK) receptors were distributed in the omasal muscle layers (Onaga *et al.* 2011). Application of specific tachykinins; substance P (Fig 2 - 11), neurokinin A (NKA), and neurokinin B (NKB); induced tonic contractions of RR, omasal, and abomasal muscle (Onaga *et al.* 2011; Oh-ishi *et al.* 2013). Administration of NK antagonists did not inhibit substance-P induced contractions but did significantly inhibit NKA contractile effects (Oh-ishi *et al.* 2013). NK receptors are involved in regulation of omasal contractions, and NKA is probably a primary non-cholinergic excitatory neurotransmitter released from motor neurons

in the ruminant omasum (Onaga *et al.* 2011). Substance P also prevents ghrelin's inhibitory action on thyroid hormones, T₃ and T₄ (thyroxine) (Khazali and Mahmoudi 2009) . Onaga *et al.* (2011) also found that cyclic contractions of the omasum are vagal cholinergic nerve-independent further suggesting that localised autocrine/paracrine activity is the main regulatory mechanism of omasal MRT.

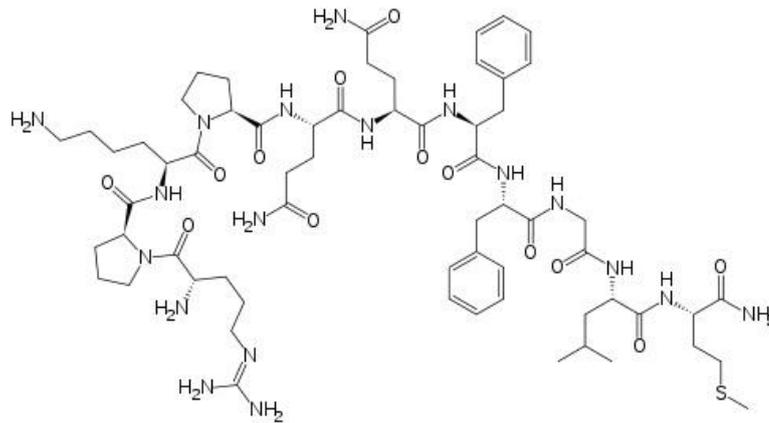


Fig 2 - 11 Chemical structure of tachykinin, substance P (Gordon 2000b)

2.4.7 Triiodothyronine (T₃) and thyroxine (T₄)

Thyroid hormones (T₃, T₄; Fig 2 - 12) are iodinated derivatives of the amino acid tyrosine and have been associated with control of digesta MRT and kinetics through one of their main physiological functions, regulation of metabolism (Todini 2007; Barnett *et al.* 2012). Synthesised in the thyroid gland under the control of the hypothalamus, thyroid hormones induce an increase in basal metabolic activity, increase plasma glucose concentration, and stimulate protein synthesis, lipid metabolism, cholesterol catabolism, while heightening cardiac and neural function (Larsson 1988; Capen and Martin 1989; Todini 2007). Regulated extensively by temperature and season, thyroid hormones peak at times of low temperature and the onset of increasing daylength (Souza *et al.* 2002; Todini 2007). Kennedy *et al.* (1977) induced an elevation in plasma thyroid hormones by exposing sheep to ambient temperatures below their thermo-neutral zone (2°C). The increase in plasma thyroid concentrations corresponded with a major reduction in digesta MRT, particularly in the RR, and a decrease in apparent DMD (Kennedy *et al.* 1977). Similar results were recorded by

Westra and Hudson (1981) in cattle with elevated thyroid hormones being associated with decreased DMD and digesta MRT. Thyroid hormonal influence on digesta MRT in other species has been well documented, occurring predominately in the antrum region of the SI with T₃ receptor mRNA detected in small intestinal mucosa and marked small intestinal villus hyperplasia resulting from T₃ exposure (Hodin *et al.* 1992; Wegener *et al.* 1992; Vassilopoulou-Sellin and Sellin 1995). While it is unclear whether thyroid hormones act directly or indirectly on digesta kinetics and MRT, their influence on digesta kinetics is certain, making them useful hormones in the study of the impact of digesta MRT on MY.

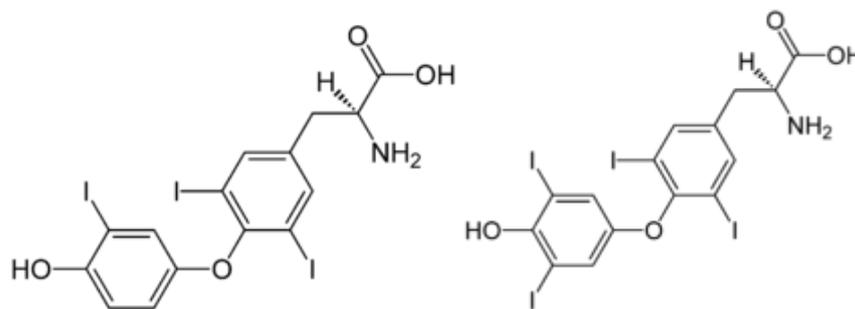


Fig 2 - 12 Chemical structure of triiodothyronine (T₃) and thyroxine (T₄) (Rang *et al.* 1999)

2.4.8 Somatostatin

Somatostatin (SRIF; Fig 2 - 13) is a tetra-deca peptide hormone, first isolated from sheep hypothalamic cells and known to be a potent inhibitor of peptide hormone secretion and smooth muscle contraction in the GIT (Saras *et al.* 2007). Along with reduced gastrointestinal (GI) motility and peptide hormone secretion, SRIF regulates the rate of nutrient absorption from the GIT by inhibiting the secretion of digestive enzymes, reducing gallbladder contraction, and intestinal blood flow rates (Christensen *et al.* 1990; Briard *et al.* 1998; Tulassay 1998; Strowski *et al.* 2000; Mushtaq and Cheema 2009; Jin *et al.* 2011). SRIF is primarily produced in the hypothalamic and GI regions of the ruminant, particularly the basal hypothalamus and periventricular region of the hypothalamus (Leshin *et al.* 1994; Willoughby *et al.* 1995; Iqbal *et al.* 2005) and D-cells of the GI lumen and pancreas (Barry *et al.* 1985; Darvodelsky *et al.* 1988; Simpson 2000). SRIF has two biologically active forms produced by translational cleavage of the preproprotein, prosomatostatin - 14 amino acids

ganglia and nerve fibres of the oesophagus, reticulum wall and groove, rumen pillar and wall, omasum sulcus and abomasum (Vergara-Esteras *et al.* 1990; Groenewald 1994; Pfannkuche *et al.* 2003; Münnich *et al.* 2008). Saras *et al.* (2007) also noted that SRIF induced rapid morphological changes in neuroendocrine cells, causing retraction fibres to form, and cells to round up and contract; breaking cell-cell contacts. It is possible that part of the SRIF inhibitory effect is a result of cellular contractions causing the secretory vesicles to have less contact with plasma membranes (Saras *et al.* 2007).

SRIF is a known inhibitor of gastric secretory peptides and hormones as well as a major regulator of metabolic hormones (Jin *et al.* 2011). SRIF suppresses insulin secretion and plasma concentrations of glucagon, glucose, long chain fatty acids (LCFA) and non-esterified fatty acids (NEFA), although its effect on glucose is a secondary effect of glucagon secretion inhibition (McLeod *et al.* 1995; Mushtaq and Cheema 2009; Chen *et al.* 2011). Mushtaq and Cheema (2009) found that administration of SRIF to goats resulted in a plasma concentration reduction of LCFA and VFA but a rise in glucose. It was proposed the elevation in plasma glucose concentration was due to the goat's utilisation of the VFA in gluconeogenic synthesis of glucose (Mushtaq and Cheema 2009). Thyroid hormones (TSH, T₃, T₄, and thyrotrophin), which are known to have an influence of metabolism and digestive kinetics, are controlled and regulated by SRIF (Christensen *et al.* 1990; Todini 2007; Barnett *et al.* 2012). Elevated SRIF concentrations in plasma and the gastric portal vein also actively suppresses ghrelin, a peptide known to increase VFI (Sugino *et al.* 2004; Iqbal *et al.* 2006), further emphasising the GIT regulatory role of SRIF. With the primary role of SRIF being as an inhibitory peptide, reducing digesta kinetics, digestion, absorption, growth and development; the active suppression of SRIF or genetic selection for reduced secretion of this peptide hormone may prove to be highly beneficial to producers (growth, development, and feed conversion).

2.5 Conclusion and hypothesis

2.5.1 Conclusion

Balch (1952) noted there was simultaneous abolition of motility of the RR and digesta passage from the RR as a result of bilateral vagotomy. He concluded that motility of the RR coordinated with quiescence of the ROO are prerequisites for caudal movement of digesta from the RR (Balch 1952). From this conception, Okine and Mathison (1996) suggested that the regulation of the relaxation of the ROO coordinated with muscular contractions of the RR may be important in controlling digesta passage. However, there has been a long accepted concept that the physical properties of the diet is the main factor determining digesta ROF and, therefore, MRT (Freer and Campling 1963; Weston and Hogan 1967). Diet does impact on digesta ROF from the RR with increased forage content in the diet increasing digesta MRT due to a high proportion of indigestible and resistant fibres (Hungate 1966; Baker and Hobbs 1987) but these physical properties have been shown to be overcome by physiological regulators of the animal (Sections 2.3, 2.4, and 2.5)

Kennedy *et al.* (1977) found that elevation of plasma concentrations of the thyroid hormone triiodothyronine within the physiological range, a hormone found to increase when ambient temperatures decrease (Todini 2007), caused a reduction in digesta MRT and MY even when sheep were fed a high roughage diet. During pregnancy, ruminal outflow rates of sheep steadily increase as a result of increased primary rumen wall contractions and enhanced mixing motility of the RR, regardless of diet (Zaenuri and Godwin 1997). Thus, it may be possible that not just the physical properties of the diet determine the MRT of digesta in the rumen and MY of the animal, but also the physiological and metabolic state of the animal (Okine *et al.* 1998). Should this be true, indirect genetic selection for reduced MY based on physiological traits associated with controlling digesta kinetics may be possible.

2.5.2 Hypothesis

Key elements in methane production are the amount of time that ingested material is subjected to the fermentative processes of ruminant microorganisms and the level of interspecies interactions between the various microbes. Therefore, the hypothesis being tested in this thesis is that changing the retention time of ingested material in the digestive tract (MRT) will change the amount of methane produced by rumen microbiota, and that divergence in the MRT is a mechanism contributing to differences in methane production between-animals of divergent MY genetics.

2.5.3 Aims

The objective of this work was to quantify the role of digesta passage rate in moderating methane production and methane yield. This objective was met by pursuing three specific research aims:

- Investigate thyroidal hormonal regulation of rumen motility and determine whether it results in a modification of MY and MRT
- Determine whether changes in ambient temperature can modify MRT and MY
- Assess whether thyroid hormones are useful indicators of ruminant digesta kinetics and MY

Additional to investigating the role of digesta kinetics on methane production and yield, a new multicompartmental mathematic model of sheep digesta kinetics was developed to allow estimation of digesta retention times.

Chapter 3: *General Materials and Methods*

Materials and methods described in this chapter were common to both experiments undertaken. Specific procedures associated with each experiment are described in the relevant experimental chapter.

3.1 Location, facilities and experimental animals

3.1.1 Location

Experiments I and II were conducted in Animal House facilities at the University of New England (UNE), Armidale between July 2010 and September 2011.

3.1.2 Selection of experimental animals

Fifteen two-tooth Merino wethers were obtained from the University of New England research station “Kirby” at Armidale, NSW and used in experiments I and II. In experiments I & II, animals with the least quantity of feed refusals were used, as feed intake has a major impact on methane measurements. Their entry weights ranged from 36 to 45 kg with each animal possessing a body condition score of between 3 and 3.5 out of 5 (NSW Department of Primary Industries 2008).

3.1.3 Diet and feed analysis

During experiments I and II a blended diet of 50% wheaten and 50% lucerne chaff was supplied to each animal at a rate of either 1.2 times (experiment I) or 1.35 times (experiment II) maintenance requirement. The level of maintenance requirement for each animal was calculated using the formula: $ME_M = 0.46MJ/kg \text{ Liveweight}^{0.75}$ (Dawson and Steen 1998). Samples of the feed were obtained prior to commencement of each experiment and sent for estimation of metabolisable energy (ME) by a commercial laboratory (Wagga Wagga Feed Quality Testing Laboratory, NSW DPI, Wagga Wagga). The laboratory operates under

ISO/IEC 17025 standards and is fully accredited by the National Association of Testing Authorities (NATA).

3.1.4 Pens, metabolism crates and procedures

Sheep were housed individually during experiments I and II in metabolism crates within temperature controlled rooms. A maximum of five animals were kept in each room with all having *ad libitum* access to water. Feed availability was restricted as described in Section 3.1.3 and delivered in portions approximately every 2 h at a rate of 1/12th the total days ration per portion via an overhead automated feeding apparatus (Fig 3 - 1).



Fig 3 - 1 Sheep housed in metabolism crates receiving a fixed portion of diet every 2 h by an overhead automated feeding apparatus

3.2 ***Apparatus and procedures for methane collection and measurement***

3.2.1 ***Open circuit respiration chambers***

Enteric methane production in experiments I and II was measured by open circuit respiration chambers in accordance with Bird *et al.* (2008) (Fig 3 - 2). The animals were placed in the chambers for a period of 22 h while their methane emissions were recorded. Each animal was fed 1/11th its daily ration every two hours during methane recording by sealed automated overhead feeding tubes. Air was drawn through each chamber at a rate of approximately 120 l/min using a household vacuum cleaner (Sony Corp. Japan) connected to 37mm i.d. flexible hosing from outside the chamber room. Air within each chamber was continuously mixed by an oscillating fan mounted in the roof of each chamber. Total airflow through each chamber was measured using an AL800 airflow meter (Elster American Meter Co., Nebraska, USA) with samples of incoming and outgoing air analysed for CH₄ concentrations every 13 mins.



Fig 3 - 2 Sheep respiration chamber used in experiments I and II showing automated feed dispenser on top of chamber

3.2.2 Methane concentration and production determination

Concentrations of methane in air leaving the chamber in experiments I and II were determined using an Innova 1312 photo-acoustic gas analyser (Air Tech Instruments, Denmark), calibrated with a two point calibration (0 and 100 ppm CH₄) and automatic moisture correction. Data was recorded on a personal computer using proprietary software. Temperature and humidity was measured via temperature and humidity probes placed inside the chambers and attached to Easysense advanced data loggers (Data Harvest, UK).

Methane production was calculated as the product of the average net increase in methane concentration (outflow – inflow) and the total airflow through the chamber adjusted to STP conditions. As a second measure of methane production, average methane concentrations were analysed from a Tedlar bag containing sub-samples of air from within each chamber, drawn into it by a peristaltic pump continuously over the 22 h period. Recovery of methane released from a cylinder of pure methane (BOC Ltd – 99% purity) was between 98 and 100%.

3.3 Rumen fluid collection and analysis

3.3.1 Sampling

In experiments I and II, rumen content samples were taken for the purpose of VFA analysis and measurement of protozoal abundance (PA) immediately prior to 6 d total faecal collections. Ruminal fluid was obtained via oesophageal intubation using a length of high-pressure hose (8 mm internal diameter) capped with a blind brass sieve (48 x 1 mm holes). Attached to the external end of the hose was a Terumo 50 mL syringe with a catheter tip (model DS-NO46) which was used to draw up the sample. Upon completion of obtaining the sample, the syringe and tube were thoroughly rinsed and used again to obtain further samples from other animals.

Approximately 15 mL of rumen fluid was placed into an acidified (0.3 mL x 18 M H₂SO₄) narrow necked McCartney bottle and shaken gently to mix. This was immediately placed on ice before being frozen and stored for VFA analysis. A further 4 mL was added to another narrow necked McCartney bottle containing 16 mL of formal saline (4% formaldehyde v/v; 0.9% NaCl w/v in H₂O) and stored at room temperature for protozoal enumeration.

3.3.2 VFA analysis

Rumen fluid samples collected for VFA analysis (Section 3.3.1) were thawed overnight before being centrifuged at 1630 g for 10 min to separate the supernatant from any solids collected. Supernatant (500 µL) from each rumen fluid sample was transferred in duplicate by a 1 mL Gilson pipette into 1.5 mL micro-centrifuge tubes containing 1000 µL of internal standard (1.6% isocaproic acid/ethanol and 12% metaphosphoric acid in deionised water). Along with the VFA samples, two blank samples (500 µL Mili-Q water plus 1000 µL of internal standard) and two calibration standards (500 µL calibration standard plus 1000 µL of internal standard) were included. Each tube was vortexed and then centrifuged for 3 min at 2000 g in a Tomy centrifuge (Tomy Tech USA Inc., California, USA). At least 1.2 mL of the solution was transferred to autosampler vials for use in a gas chromatograph (Varian CP 3800). The gas chromatograph being fitted with an autosampler and flame ionisation detector was used to determine VFA concentrations as reported by Nolan *et al.* (2010). Injector, column and detector temperatures were fixed at 200, 130 and 200°C respectively. Each 1 µL sample was injected into a 1.8 m x 2 mm i.d. packed glass column with 15% neopentyl glycol adipate and 2% phosphoric acid phase on chromosorb W2W (80/100 mesh) with helium used as the carrier gas (27 mL/min). A calibration standard (118.7 mM acetic, 103.3 mM propionic, 9 mM isobutyric, 29.1 mM butyric, 7.6 mM isovaleric, 8.1 mM valeric acid; Supelco/Sigma-Aldrich, Pennsylvania, USA) was used to determine the relative VFA responses for each acid. Peak areas were integrated and concentrations of each VFA calculated via the Varian Star chromatography software (version 6.41).

3.3.3 Protozoa abundance

Rumen ciliate populations were counted for all animals at the end of experiments I and II. After thoroughly shaking to insure suspension of all particulate matter within the formal saline/rumen fluid mixture, an aliquot was drawn by a wide-mouthed plastic pasteur pipette and placed onto a Fuchs Rosenthal ultra plane counting chamber (Weber Scientific International Ltd, England) of 0.2 mm depth with 1.0 mm² grids. Counts of protozoa (nine sub-grids counted for each animal) were made under an Olympus light microscope (model EH) at 100 x magnification. Large and small Holotrichs (*Isotrichia* and *Dasytrichia*) and Entodiniomorphs were identified and counted separately.

3.4 Preparation and analysis of digesta kinetic markers

3.4.1 Chromium-mordanted fibre

Chromium (Cr) was mordanted to fibre in accordance to the method described by Uden *et al.* (1980) and used to estimate rate of passage of the solid portion of digesta. Wheaten chaff was ground then dry sieved through a 1 mm screen with the chaff particles smaller than 1 mm discarded. The remaining chaff was washed, boiled, treated with sodium lauryl sulphate, acetone, sodium dichromate, ascorbic acid, and oven dried to leave Cr mordanted neutral detergent fibre (NDF) as a non-digestible digesta kinetics marker.

3.4.2 Cobalt-EDTA

Co-EDTA was prepared in accordance to the method of Uden *et al.* (1980) and used as a liquid tracer to estimate rate of passage of liquids through the GIT. Co (II) acetate-4H₂O, EDTA, and lithium hydroxide monohydrate (LiHO-H₂O) were mixed with Milli-Q water, 30%

hydrogen peroxide, and ethanol before being filtered and dried (100°C) to form Co EDTA crystals.

3.4.3 Sample treatment

Each feed and faecal sample collected was put into an individual aluminium foil containers, weighed and placed into a drying oven at 80°C. Each day the samples were reweighed until no further weight loss was detected. After drying was complete, the samples were cooled in a desiccator and final weights recorded to determine DM content. The dried samples were ground in a hammer mill with a 1 mm screen. Each ground sample was then thoroughly mixed and a sub-sample collected and stored in 70 mL screw-top plastic containers to await digestion and analysis of markers.

All sample organic matter was removed prior to marker analysis by a modified sealed chamber digestion (Anderson and Henderson 1986). Aliquots of each ground and dried sample (0.2 ± 0.02 g) were placed in 100 mL Schott bottles that had been previously weighed (minus cap). Two mL of freshly prepared perchloric acid (HClO_4) and 30% hydrogen peroxide (H_2O_2) mixture (7:3 v/v) was placed into each bottle using a gravimetrically calibrated Gilson pipette. With the caps placed loosely on top, the bottles were allowed to stand overnight in an operating fume cupboard. Next day, 1 mL of 30% H_2O_2 was added to each bottle which was then tightly sealed and placed into an oven at 80°C for 30 min. The bottles were then removed, allowed to cool down (approximately 20 min) and another 1 mL of 30% H_2O_2 was added. Again, the bottles were tightly sealed and returned to the oven but this time for 1 h. After allowing cooling once more, each digested sample was brought to a standard known weight (25 g) by the addition of Milli-Q water. Samples were thoroughly shaken and then filtered (Whatman No.1, England) to remove silica precipitates, eluting into 30 mL plastic tubes with screw top lids and stored at 2°C until analysis.

3.4.4 Marker analysis

Cobalt and Cr concentrations in the digested feed and faecal matter were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian Vista Radial MPX). A stock solution containing $100 \mu\text{g mL}^{-1}$ of both Co and Cr in Milli-Q water was made from concentrated solutions containing $1000 \mu\text{g mL}^{-1}$ Co and Cr (Australian Chemical Reagents, Australia). This was used to prepare working standards consisting of 0, 0.01, 0.05, 0.1, 0.5, 1, 2, 10 and $20 \mu\text{g mL}^{-1}$ of each element within 5.6% (v/v) HClO_4 and Milli-Q water. The digested faecal samples were removed from cool storage and allowed to return to room temperature before being decanted into 15 mL glass ICP test tubes and processed under standard conditions with marker concentrations being derived by reference to the regression curve created from the working standards.

3.5 Faeces & urine collection

3.5.1 Faeces collection

For a period of six consecutive days immediately following administration of the digesta markers, a total faecal collection was conducted to allow determination of digestibility and digesta retention times of liquid and solid phase digesta for each animal based on appearance of marker in the faeces.

Plastic bags were attached to the bottom of the metabolism crates in order to collect any faeces passed by each animal. The first bag was replaced with a new one 8hrs after administration of the markers, with subsequent bag changes occurring at set intervals over the following six days – every 2 h for the first 24 h, every 4 h for the next 48 h, every 8 h for the next 24 h and every 12 h for the final 48 h. A total of 28 samples were collected from each animal. Only faeces excreted in the final 2 h of each collection period were used for analysis of Co and Cr concentrations. Twice a day, samples were weighed, thoroughly

mixed and sub-sampled. Each sample bag was recorded with the animal's identification, sample number and date of collection. All faecal output over the 6 days was collected and weighed to determine DMD.

3.5.2 Urine collection

Total urine output was also collected over six days from each animal. The urine was collected into 10L plastic buckets placed underneath the metabolism crates. Each bucket contained approximately 100 ml of 10% (v/v) H₂SO₄ to prevent bacterial degradation of purine derivatives (PD). Once a day, each animal's urine was decanted into a pre-tared bucket and weighed. The collected urine was then diluted to 4L with water and thoroughly mixed to prevent precipitation of PD. Subsamples were taken and the pH adjusted to <3 by the addition of 18 M H₂SO₄ before being frozen at -20°C until required for analysis.

3.6 Apparent dry matter digestibility (DMD)

After drying each feed and faecal sample at 80°C in a fan-forced oven to constant weight, the DM content was determined by using the following formula:

$$\text{DM content (\%)} = 100 \times (\text{Dry weight of sample} / \text{Original sample weight})$$

Once the DM content of the faeces was determined, apparent DMD was able to be calculated by using the following formula:

$$\text{DMD (\%)} = 100 \times (\text{Feed DM Intake} - \text{DM content of faeces}) / \text{Feed DM Intake}$$

3.7 Estimation of microbial protein outflow from the rumen

Microbial biomass provides the ruminant with a variety of nutrients, in particular the majority of the amino acids which become available for absorption by the host. Estimation of the

amount of MP produced was determined by measuring the excretion rate of the purine derivative (PD) allantoin in the urine in accordance to the colorimetric method originally devised by Young and Conway (1942) and later modified by Chen and Gomez (1992). Allantoin excreted in sheep urine represents between 60 and 80% of total PD present (Chen and Gomes 1992).

Allantoin in the urine samples was firstly hydrolysed under weak alkaline conditions at 100°C to allantoic acid, then hydrolysed to urea and glyoxylic acid in dilute hydrochloric acid. The glyoxylic acid reacts with phenylhydrazine hydrochloride to produce a phenylhydrazine derivative. Oxidation of this derivative with potassium ferricyanide resulted in colour formation with the absorbance measured at 522 nm on a spectrophotometer. Plotting the resulting absorbance against a standard curve of known allantoin concentrations provided a linear regression used for the calculation of allantoin concentration, which was then adjusted for dilution and multiplied by stored urine volume to obtain daily allantoin and PD excretion (Section 3.7.3).

3.7.1 Preparation of samples, standards and reagents

An allantoin stock solution (100 mg/L) was prepared by dissolving 50 mg of allantoin (Sigma Pharmaceuticals, USA) in 100 mL 0.01 M NaOH then made up to 500 mL with Milli-Q water. Working standards (5, 10, 20, 30, 40 and 50 µg/mL) were prepared from the stock solution. Phenylhydrazine-HCl and potassium ferricyanide reagents were also prepared by weighing out and dissolving 0.0665 g of phenylhydrazine-HCl and 0.334 g of potassium ferricyanide into separate beakers and making up to 20 mL with Milli-Q water. Both reagents were made fresh and kept refrigerated until used. Urine samples were left to thaw overnight then placed in an ultrasonic water bath for 5 min to resuspend solids. Urine samples were then diluted 1 in 40 with Milli-Q water (0.1 mL urine + 3.9 mL water) before analysis.

3.7.2 Spectrophotometric determination of allantoin

To 0.25 mL of each sample (in duplicate), 1.25 mL of Milli-Q water was added then 0.25 mL of 0.5 M NaOH. A 0.25 mL water blank and 0.25 mL of each working standard (all in duplicate) were prepared at the same time as the samples. All tubes were then vortexed and placed in a boiling water bath for 7 min. The tubes were cooled in ice and 0.3 mL of 0.5 M cold HCl added. Phenylhydrazine-HCl reagent (0.25 mL) was added to each tube, vortexed and returned to the boiling water bath for another 7 min. The tubes were removed from the bath and cooled in ice for several minutes before adding 0.75 mL of cold concentrated HCl then vortexing. Potassium ferricyanide reagent (0.25 mL) was finally added to each tube at 12 sec intervals and vortexed.

At 18 min after addition of potassium ferricyanide, all tubes (samples, water blanks and standards) were again vortexed to ensure mixing. Each tube had its absorbance read on a UV-1201 Spectrophotometer (Shimadzu, Japan) set at 522 nm using 10x10x45 mm acrylic cuvettes (Sarstedt, Germany) 20 mins after the potassium ferricyanide reagent was added. The water blank and allantoin working standards were used to produce an allantoin standard curve with the allantoin content of the urine sample determined from this curve.

3.7.3 Estimation of microbial protein

Purine derivative excretion and microbial N supply were estimated from allantoin concentrations in the urine samples in accordance to calculations established by IAEA (2003). In brief, allantoin excretion was determined as the product of daily urine volume and allantoin concentration in the sample. As allantoin concentrations are estimated to represent between 60 and 80% of PD excreted (IAEA 2003), it was assumed that the concentrations in the urine samples accounted for 70% of total PD excretion and adjusted accordingly. Purine absorption was calculated by means of the Newton-Raphson iteration process with absorbed purines being equal to microbial N supply divided by 0.727 (IAEA 2003).

3.8 ***Terminology of digesta kinetics mathematical modelling***

To determine MRT of digesta within the sheep GIT, a mathematically based, multicompartmental double-marker digestive tract model was developed (refer Chapter 4) and used throughout this thesis. The multicompartmental simulation program, WinSAAM, was used to calculate variables rates and defined parameters within the model boundaries. Initial estimates were assigned to models as adjustable variables then, using a nonlinear, least-square technique, the program adjusted the variable parameters to enable the model to better fit the observed faecal marker data. To allow these calculations, notations were assigned to specific variables within the model:

Compartment is a homogenous entity into which material flows and departs at a steady-state rate of passage. When combined with other compartments, in a linear fashion, it is designed to represent a body in whole. These are not physically visible compartments but kinetic compartments.

Component is the address for a solution of either calculated or observed values.

Steady state is when numerous properties of a system are unchanging in time and that the recently observed behaviour of the system is constant within the experimental period.

L(i,j) is defined as the fractional rate of flow (ROF) from compartment j to compartment i and represents the fraction of the donor compartment leaving in unit time (e.g. per h or per day). The reciprocal of this flow rate is the mean retention time (MRT) of the substance within compartment j.

F(i) is the quantity of material estimated to be present in compartment i at time t.

F'(i) is the net rate of change in size of compartment i due to flow to or from compartment j. $F'(i) = U(i,j) - R(j,i)$ being the difference between rate of input of material from

compartment j into compartment i and rate of output from compartment i into compartment j at any given time point.

U(i,j) is the rate of input into compartment i from compartment j per unit of time (e.g. grams per hour). It represents the mass increase of a compartment due to the fractional outflow from a preceding compartment.

R(i,j) is the rate of output from compartment i into compartment j per unit of time. It represents the mass decrease of a compartment due to the fractional outflow from the associated compartment.

P(i) is an unassociated, nonlinearly estimated parameter to which the user assigns model significance. It can be incorporated into an equation or assigned as an input but are neither component nor compartment associated.

DT(i) is the length of time material resides in the delay compartment i. It establishes a delay chain associated with transit of a particle between two compartments.

DN(i) is the delay number. It specifies the resolution or number of pseudo-compartments which make up the delay (DT).

M(i) is the mass or volume of marker material in compartment i.

IC(i) is the initial conditions designed to initialise compartments prior to solving. $F(j,0)=IC(j)$, where $F(j,0)$ is the value of the solution to the j^{th} component of the system of differential equations at time=0.

k₁ and k₂ are the rate constants of the two sections of the ruminant GIT possessing the longest retention times – rumen and caecum.

These notations are utilised throughout this thesis.

Particle route (P) and solute route (S)

Due to WinSAMM programming language restrictions, different series of numerals were required to represent particle and solute flow. Compartments 1 to 6 in the model were used to represent particle flow through the animal while compartments 11 to 16 represented

solute passage. For ease of understanding within this thesis, compartments representing particle flow were labelled P1 to P6 (e.g. L(2,1) referred to as L(P2,P1)) and compartments representing solute flow were labelled S1 to S6 (e.g. L(12,11) referred to as L(S2, S1)).

With the establishment of these specific variables, it was possible to calculate estimates of specific parameters required to describe digesta kinetics:

Rumen MRT – the average time digesta components reside in the RR. This was calculated as the reciprocal of $L(i,j)$, the ROF of digesta, between compartments representing the RR in the model developed for this thesis (refer Chapter 4); $1/L(i,j)$. The model contained two compartments representing the RR – Particles L(P2,P1) and L(P3,P2); Solutes L(S2,S1) and L(S3,S2). Calculation of Rumen MRT was the addition of the each compartment's reciprocal ROF – Particle $1/L(P2,P1) + 1/L(P3,P2)$; Solutes $1/L(S2,S1) + 1/L(S3,S2)$.

Hindgut MRT – the average time digesta components reside in all compartments post-ruminally (omasum, abomasum, intestines and caecum/proximal colon). This comprised the time digesta resided in the delay compartment (DT) of the model and the reciprocal ROF between compartments in the model post-delay, representing the caecum/proximal colon; Particles $DT(P3) + 1/L(P4,P3) + 1/L(P5,P4)$; Solutes $DT(S3) + 1/L(S4,S3) + 1/L(S5,S4)$.

Total MRT – the average time that digesta components reside within the entire animal's GIT. Calculation of this parameter was the combination of rumen MRT and hindgut MRT.

Rumen non-digestible DM mass – an estimation of the mean quantity (mass) of NDF present in the rumen. It was calculated by combining the mass, $M(i)$, of marked material in the compartments representing the RR; Particles $M(P1) + M(P2)$.

Faecal Dry Matter Output (FDMO) – The estimation of amount of dry matter present in the faeces. Can be estimated using the formula $FDMO=DMI*(1-DMD)$, where DMI is the daily feed DM intake, and DMD is the DM digestibility of the feed.

Chapter 4: Development and Comparison of Mathematical Models for Estimating Digesta Kinetics and Faecal Output in Sheep Using Faecal Concentrations of Dosed Particulate and Solute Markers

4.1 Introduction

Methane yield has been positively correlated with digesta MRT in ruminants (Pinares-Patiño *et al.* 2003; Nolan *et al.* 2010) and studies have shown that reductions in digesta retention times can lead to reductions in MY (Nolan *et al.* (2010), 23%; Okine *et al.* (1989), 29%). Therefore, MRT may provide an indicator of ruminant MY, and a possible proxy measurement of methane production for use in genetic selection. Therefore, it was necessary to develop a system capable of accurately estimating the digesta kinetics of non-rumen cannulated animals. One such system is mathematical-based models utilising recovery data of non-digestible markers in faeces. These models are designed to partition the animal's GIT into a series of continuous compartments, all linked together, estimating the flow of digesta under steady state conditions.

Compartmental models describing the gut have been used to fit digesta marker concentrations in faeces after an animal was given a single oral or intraruminal dose of non-absorbable markers. The MRT of digesta in the whole gut, within the rumen, and other compartments of the GIT can be estimated by the change in concentration of these non-digestible, non-absorbable markers in faeces post-dosing. Various mathematical models have been developed (Blaxter *et al.* 1956; Grovum and Williams 1973; Dhanoa *et al.* 1985; Pond *et al.* 1988; Reese *et al.* 1995; Aharoni *et al.* 1999) for both sheep and cattle to

analyse the changes in faecal concentrations and fit them into two faecal marker curves representing the rumen and caecum-proximal colon.

To develop a system for estimating sheep digesta kinetics using compartmental mathematical models, existing peer-reviewed cattle and sheep models were critically analysed and compared for accuracy (goodness of fit), output and usability. After evaluation to find an appropriate platform, revision of this model was undertaken to remove any unnecessary design parameters and include any additional functions to develop a new mathematically-based compartmental model appropriate for sheep MRT calculation.

The purpose of this study was to produce a multicompartmental double-marker digestive tract model capable of fitting data collected from the first experiment (Chapter 5), and give estimates of digestive kinetic parameters such as rumen and hindgut MRT, FDMO and marker recovery for sheep. These values could then be used in testing the relationship between digesta MRT and enteric methane production. The model developed for estimating digesta kinetics in sheep and used throughout this thesis is based on models published by Aharoni *et al.* (1994; 1999) that were devised to determine digesta kinetics in cattle. The model demonstrating best goodness of fit as identified by the least-square method was then utilised throughout this thesis.

4.2 *Materials and Methods*

4.2.1 *Selection of modelling method to use*

Analysis of digesta marker concentrations in faeces of ruminants enables estimation of MRT in gut segments with no surgical intervention. Faecal marker curves can usually be resolved into two exponential components being the rumen and caecum-proximal colon (Warner 1981; Faichney and Boston 1983). Faichney and Boston (1983) though suggested that the

digestive tract of ruminants has at least three mixing compartments (rumen, abomasum and caecum-proximal colon) and the rumen MRT is not always longer than the caecum-proximal colon MRT. They also suggested that particle and solute phases move at the same rate in the caecum-proximal colon. Thus, allocation of the rate constants for rumen and caecal compartments can be ascribed with certainty provided that they are obtained for both a solute- and particle-phase marker (Faichney and Boston 1983). Early models (Blaxter *et al.* 1956; Grovum and Williams 1973) lacked this multi-phase dual marker system, which may have resulted in underestimation of MRT and errors in compartment identification.

Matis (1972) suggested using a different approach to fitting faecal marker data by expanding on the Blaxter *et al.* (1956) two-compartment model for passage of particles through the ruminant GIT. He proposed the exit probabilities of each particle was dependent on its time of arrival in the RR and introduced a gamma distribution of lifetimes of particles in the first compartment of his model – the time-dependent theory (Matis 1972). This made passage from the compartments time-dependent and improved the calculation efficiency of compartmental outflow rate constants. Ellis *et al.* (1979) also suggested that the inclusion of a gamma distribution time dependency in the fast compartment of a two-compartment model would appropriately describe the slow initial increase in faecal marker concentrations. While the initial application of this theory by Ellis *et al.* (1979) improved the accuracy of digesta kinetics predictions, it was found to be very sensitive to initial data points and often poor in fitting predicted to observed values in the ascending phase of the excretion curve (Dhanao *et al.* 1985). Much difficulty also exists when attempting to fit gamma function models using software such as ConSAAM and WinSAAM and, therefore, these models were not considered for development of the sheep digesta kinetics model.

Dhanao *et al.* (1985) suggested the use of a multicompartmental model, assuming first order kinetics, whereby a continual series of compartments connected by fluxes could better describe digesta flow along the ruminant GIT. Describing the flow of digesta along the

tubular section of the gut as longitudinal diffusion, they suggested that the inclusion of an unspecified number of sequential compartments, instead of using increasing orders of gamma distribution or a tubular component, could account for the delay preceding the appearance of markers in faeces (Dhanao *et al.* 1985). As a very large number of sequential compartments behave in a similar manner to a tubular component, Dhanao *et al.* (1985) were able to refer to all of the sequential compartments in their model after the second compartment as the 'tubular section' of the system. This system of digesta kinetics model was found to be unreliable as a predictor, with de Vega *et al.* (1998) noting that the model "did not provide reliable digesta kinetics estimates, and discrepancies between outflow rates estimated from slaughter or faecal marker excretion curves were large for both rumen and large intestine". This was primarily because the model was developed on the premise that $k_1 > k_2$, whereby k_1 being slow and k_2 fast rates of particle- and liquid-phase passage through the GIT. This meant the RR was assumed to have the longest MRT, something proven not to always be the case (Faichney and Boston 1983; Cruickshank *et al.* 1989).

Aharoni *et al.* (1994) expanded upon the idea that the ruminant digestive tract is a series of sequential compartments in which digesta are mixed followed by a delay portion, simulating the SI, where digesta advances without further mixing (Appendix 1). Aharoni *et al.* (1994) argued that this was based upon the assumption that fluxes of undigested material in all compartments along the whole GIT are equal, i.e. that the flux from compartment 1 to compartment 2 equals that of the flux from compartment 2 to compartment 3 and so on, when this possibly was not the case. Aharoni *et al.* (1994) suggested the possibility that bypass fluxes existed within the ruminant digestive tract. It was proposed that particulate matter in the rumen moved directly to the omasum/abomasum instead of through the slower fine particulate matter compartment in the rumen (Aharoni *et al.* 1994). They also proposed that digesta moved directly from the SI to the colon instead of through the caecum which is sometimes considered a mixing organ (Aharoni *et al.* 1994). This idea contradicted the previous assumption that all fluxes from compartment_(i) to compartment_(j) are equal and

introduced the concept of bypass compartments. Aharoni *et al.* (1999) expanded on this idea by introducing bypass fluxes of very fine particles to pass from the slower particle to the faster solute route (Appendix 2). This additional passage was based on the idea that very small particles and microbial matter move along the digestive tract at rates similar to solutes (Ellis *et al.* 1982; Owens and Goetsch 1986; Faichney and White 1988; Faichney *et al.* 1989; Cherney *et al.* 1991). It is this approach to ruminant digestive kinetics that was selected as the basis of the model design in this thesis.

The kinetic models tested, based upon previously published, peer reviewed models (Aharoni *et al.* 1999) intended to fit marker excretion data from cattle, were modified to represent digesta kinetics in sheep through the use of measured faecal chromium and cobalt concentrations following a single intra-ruminal dose of Cr-mordanted non-digestible fibre and CoEDTA (Experiment I – Chapter 5).

4.2.2 Animals and feed

In experiment 1 (Refer Chapter 5), 10 mature Merino wethers were injected with T₃ (300µg; IM injection) on two different protocols (daily; n=5 and every second day; n=5) or saline (control; n=10). Each animal received a diet of 50% lucerne/50% wheaten chaff to provide 1.2 x maintenance ME requirement. The feed was delivered by an overhead belt mechanism that released approximately 1/12th of the daily ration every 2 h during the 6 day period of total collection of faecal samples. Each animal received via intubation direct into the rumen a single dose of two non-digestible markers, prepared in accordance with Uden *et al.* (1980); 1.1g of Cr as Cr-mordanted NDF (Section 3.4.1) and 0.6g Co as CoEDTA (Section 3.4.2). The resulting time-course of faecal concentrations of Cr and Co were used to modify Aharoni *et al.* (1999) models from cattle to sheep, develop new models, and assess their goodness of fit.

4.2.3 Faecal sampling

Faeces passed by each animal were collected in 28 successive time periods over the six days of total collection (Section 3.5.1). Following dosing with markers, the faecal Cr and Co concentrations were used for estimation of digesta kinetics.

4.2.4 Co & Cr marker analysis

The DM content of faecal samples was determined by drying to constant mass at 80°C in a fan forced oven. The Cr and Co concentrations in faecal, feed, background faecal samples and Cr-mordanted fibre samples were determined via inductively coupled plasma optical emission spectroscopy (Section 3.4.4) after sample preparation by modified sealed chamber digestion according to Anderson and Henderson (1986) (Section 3.4.3).

4.2.5 Digesta kinetics computational analysis

Model development and analysis of sheep digesta kinetics was undertaken using the non-linear curve fitting algorithms of WinSAAM (Stefanovski *et al.* 2003).

4.2.6 Aharoni's digestive tract kinetics models M1, M2 & M3

Mean faecal concentrations of Cr and Co measured for both treated and control animals were fitted to each model in Aharoni *et al.* (1999) to determine particle and solute total MRT, rumen MRT and hindgut MRT as well as estimated rumen indigestible DM mass, DM digestibility, and estimated faecal output. Fractional rates of exchange between compartments within the digestive tract for each of Aharoni *et al.* (1999) models were adjusted in accordance with the physiological differences in compartments between sheep and cattle. This was to address the differences between species of compartmental size, mass and fluxes through the GIT.

Flow charts of all three models, M1 (Aharoni *et al.* 1994), M2 & M3 (Aharoni *et al.* 1999) are shown in Fig 4 - 1

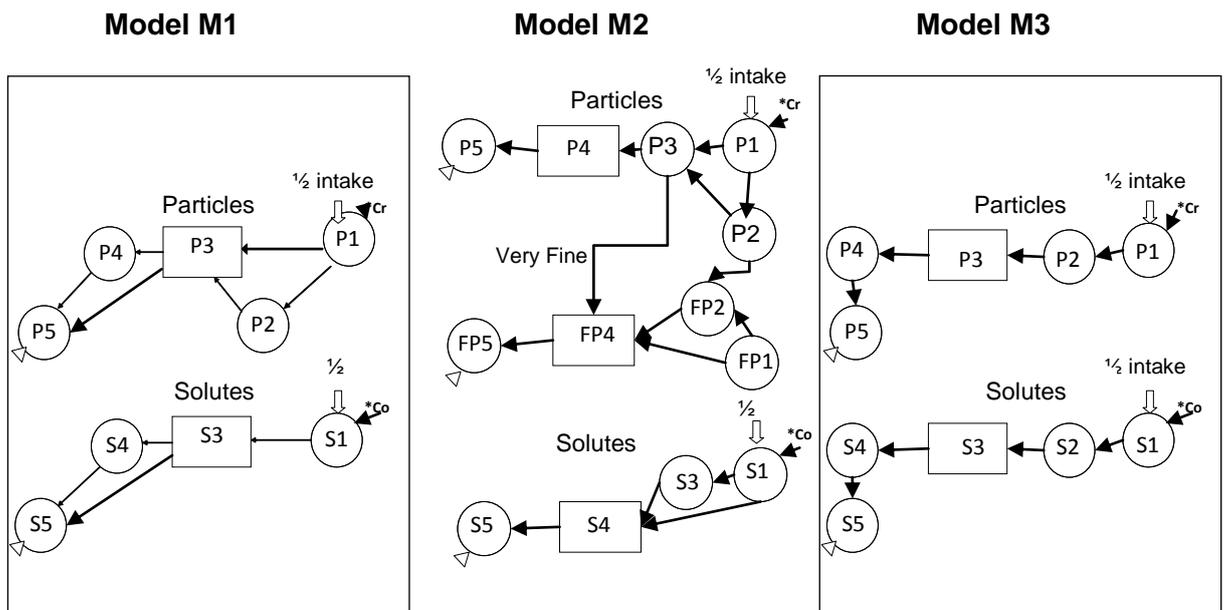


Fig 4 - 1 Flow charts of rumen kinetics models M1, M2 & M3 altered from Aharoni *et al.*(1999) showing particle route (P1 to 5) labelled by Cr-mordanted feed (Cr), and solute route (S1 to 5) labelled by Co-EDTA (Co) in all models; and a proposed fine particle route (FP1 to 5) in model M2. Feed intake was set arbitrarily to the two routes in equal amounts

Model M1 is comprised of two separate routes for digesta flow through the GIT. Route P1 to P5 represents particulate flow through two compartments within the rumen (P1 and P2), a delay representing the non-mixing component of the lower gut (P3), a further mixing compartment within the lower gut (P4), and the rectum (P5) from which the faeces is expelled. Route S1 to S5 represents the movement of solutes through the digestive tract with one compartment in the rumen (S1), a delay in the lower gut similar to the particulates (S3), another compartment in the lower gut (S4) and the rectum (S5). Included in the model were a series of small by-pass fluxes (P1 to P3 and P3 to P5 for particulates and S3 to S5 for solutes) to represent rapid by-pass movement of minute particles and fast free-flowing liquids which avoid mixing. Compartments P4 and S4 are assumed to represent any further mixing which may occur within the lower digestive tract. Aharoni *et al.* (1994) stated that it was not possible to determine whether mixing within the lower gut occurred at the beginning, middle or end of the delay compartment as samples of digesta are only obtained at the end of the path. They therefore concluded that delay and compartments below the rumen represent all the organs of the lower gut together.

A second model, M2, (Aharoni *et al.* 1999) was later devised, which not only repeated the assumption of separate particulate and solute routes within the digestive tract but also allowed for a separate route for very fine particles (FP) to move along at the faster rate of the solutes rather than at the slower rate of larger particles (route FP1 to FP5); Fig 4.1 – M2. This third phase was based upon the principle that very small particles and microbial matter move along the digestive tract at a rate similar to water and solutes. These very small particles are assumed in the model to leave the larger sized particle route at compartments P2 and P3 and travel along the digestive tract at a rate similar to the solutes. Particles which leave compartment P2 enter the faster route at compartment FP3 (identical to S3 in the solute route) while those which leave from compartment P3 go into the faster route at delay FP4 which is identical to solute delay S4. In addition to this, the compartments in the lower gut for model M1 (compartments P4 and S4) were removed and included in the forestomach region as compartments P3 and S3. This was based on the assumption that no further mixing occurs once the digesta reach the lower gut (Faichney and Boston 1983).

Model M3 was developed by Aharoni *et al.* (1999) after reviewing the model published by Reese *et al.* (1995) which expanded on the idea that particles and solutes travel at different rates within and from the rumen but travel together post-ruminally (Faichney 1975a; Faichney 1975b). The model consists of two separate routes for particles and solutes with two compartments in the rumen representing either particles (P1 & P2) or solutes (S1 & S2), delay compartments (P3 for particles & S3 for solutes) representing the lower gut region with an additional compartment (P4 for particles & S4 for solutes) before digesta exit the GIT. When Aharoni *et al.* (1999) tested this proposal they found data for the Co marker representing the solute phase did not fit the model because the first appearance of Co in the faeces substantially preceded that of Cr (particulate marker). They therefore varied the model to allow different delay times in the lower gut region (P3 & S3) as well as within the rumen (compartment P1 to P2 for particles & compartment S1 to S2 for solutes), with all other rates of passage being the same for both markers.

4.2.7 Model modifications

As the original models by Aharoni *et al.* (1999) were developed to explain digesta kinetics in cattle and the data in this thesis was associated with sheep, modifications to the models were necessary to account for physiological differences between the species. Such differences include compartment size (mass), differences in flow of particles and solutes through the GIT, and volume of initial intake. All models modified and developed were compared for best fit.

To reflect the different rates of digesta passage between sheep and cattle, an adjustment to the rate constants for digesta flow from compartment j to compartment i , $L(i,j)$, was made. This was necessary because the published models were developed for cattle rather than sheep.

A modification made to both M1 and M2 models was the arbitrary value assigned to the delay number. The delay number (DN) was set by Aharoni *et al.* (1994; 1999) to 5. This value was changed to 6 instead due to a programming recommendation within the WinSAAM program to set DN values to an even number. By setting the value to 6 this allowed a tighter curve fit for the digesta departing from the delay and entering the hindgut. This was applied to both the particle and solute phase compartments – Model M1: Particles DN(P3) and Solutes DN(S3); Model M2: Particles DN(P4) and Solutes DN(S4); respectively.

Modifications were made to model M1 to enable it to better fit the sheep faecal marker results. Flow rates from the delays to the main compartments in the hindgut for both particles $L(P4,P3)$ and solutes $L(S4,S3)$ were modified as they were found to be too slow when set to the original non-adjustable rate of $1.00E+00$ but too fast to be physiologically achievable when allowed to be adjustable. It was therefore decided that a nominal set value of $2.40E+01$ or 24 h be assigned as the flow rate to allow the model to discount the parameter

when calculating the fit of the other kinetic parameters but too small to significantly affect the retention times of digesta estimated by the model. The same was applied to the passage rate from compartments P5 and S5 to 0 (out) – L(0,P5) and L(0,S5).

In model M1, a bypass was originally included for particles and solutes which precluded any further mixing in the hindgut. When applied to sheep digesta kinetics, the amount taking this route, estimated by the model, was too small to be calculated. Accordingly, the bypass routes of L(P5,P3) for particles and L(S5,S3) for solutes was removed from the model.

A major development of model M2 was the inclusion of a separate flow path for very small particles which move with solutes but need to be accounted for as particles (compartments FP1 to FP5). We discovered an error in the published WinSAAM working file (coding error in Aharoni *et al.* 1999) resulting in compartment FP1 being redundant as there was no connection made to other compartments. When fitting the model to data from sheep, the rate of passage of particles from compartment P2 to compartment FP3, L(FP3,P2), and compartment P3 to compartment FP4, L(FP4,P3), was deemed to be so rapid that the model was unable to account for it. This caused distortion of the output and, therefore, the parallel flow path for very fine particles was removed from the model.

Aharoni *et al.* (1999) included a bypass from the rumen directly to the post-ruminal portion of the digestive tract for very small particles, L(P3,P1), and fast flowing solutes, L(S4,S1). When the sheep faecal marker concentration data was fitted to model M2, the digesta marker calculated by the model to be utilising these bypasses was so small that the inclusion of the bypasses distorted the mathematical output. As a result, both bypasses [particles L(P3,P1); solutes L(S4,S1)], were removed from the model. It should be noted that these bypasses may be beneficial inclusions in the model under different conditions such as a high concentrate diet with low roughage. As with model M1, the rate of passage for particles and

solutes out of the animal was too slow in Aharoni *et al.* (1999) so a much faster nominal rate of $2.40E+01$ or 24 h was applied to particles [L(0,P5)] and solutes [L(0,S5)] respectively.

With no computational coding for model M3 included in the published document, it was not possible to determine what modifications may have been required to convert the cattle based model to a sheep model. Accordingly, coding for model M3 in WinSAAM for this comparison was based on the details included in the Materials and Methods and the diagram published by Aharoni *et al.* (1999). All compartments and conditions associated with passage rates as detailed in the publication were adhered to in producing this model.

Flow charts of the three modified models; M1, M2 and M3; are shown in Fig 4- 2.

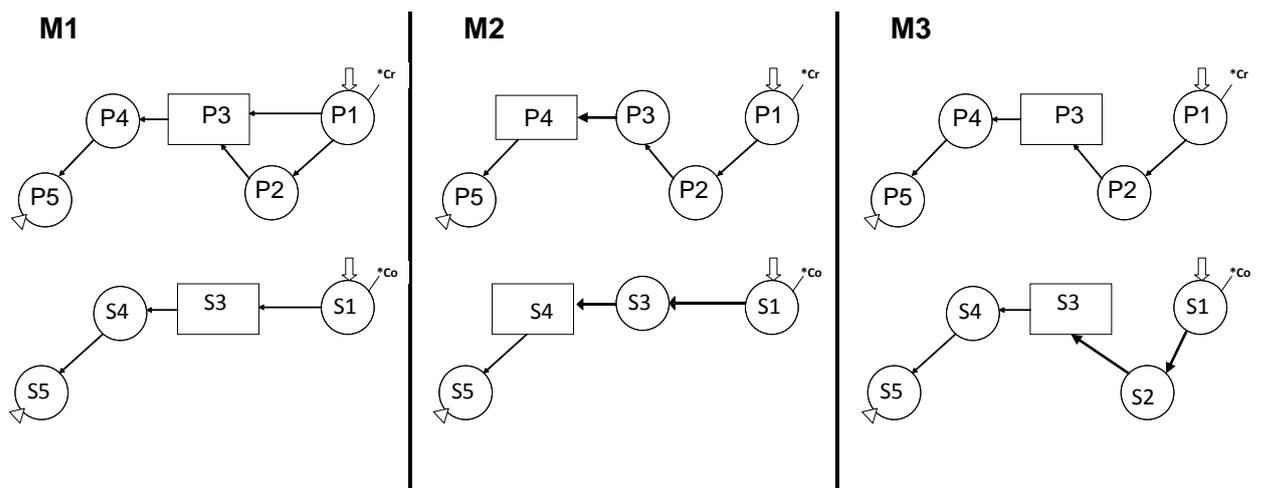


Fig 4- 2 Flow charts of digesta kinetics models M1, M2 and M3 modified to allow calculation of sheep digesta kinetics

4.2.8 Development of new models

Aharoni *et al.* (1999) stated that, if the purpose of the models is to estimate total retention time and faecal output, the most appropriate model will be the one that yields the best fit to the observed data. They compared the fit of the concentration-time curves by means of fractional standard deviation (FSD), i.e. the difference between the calculated and observed marker concentration for each data point, divided by the observed value; ratio of the

deviation from the true value. Aharoni *et al.* (1999) used the sum of squares of the fractional standard deviation (SSFSD) to compare models for the Cr (particles) and Co (solutes) curves, and the sum of the two curves to determine which model produced the best goodness of fit. SSFSD is the sum of all ratio of deviations between calculated and observed values from the model, with a higher value indicating greater error by the model in predicting observed values (Mumenthaler *et al.* 2000). Using this method, model M2 performed best when fitting cattle data, particularly concerning particle SSFSD. With this in mind, it was decided for this thesis that any development of new models to calculate sheep digesta kinetics would be based on the M2 model.

Correlation is the degree of relationship between two or more variables, and is an indicator of how independent one variable is of the other. It is possible to summarise the correlations that exist between these variables using a correlation matrix. Values in cells of a correlation matrix range from -1 to 1 whereby -1 is negatively correlated (variables counter each other) and 1 is positively correlated (variables fully dependent on each other). A correlation matrix score close to 0 indicates high independence of the two variables, i.e. one variable potentially adds value to the other variable. After modifications of model M2, the correlation matrix revealed a perfectly negative correlation (scale -1) or relationship between rumen compartments 2 and 3 (Table 4 - 1). Therefore, a simpler model was developed, referred to as model MB1 throughout this thesis, without the second rumen compartment. This created a model with two compartments in the rumen (P1 and P3 for particulates; S1 and S3 for solutes), a delay in the hindgut (P4 for particulates; S4 for solutes) and a collection compartment at the distal end of the GIT (P5 for particulates; S5 for solutes). A flow diagram of this new model, MB1, is shown in Fig 4 - 3.

Table 4 - 1 Correlation matrix between compartments for modified model M2

Correlation Matrix							
Column	L(P2,P1)	L(P3,P2)	L(P4,P3)	DT(P4)	L(S3,S1)	L(S4,S3)	DT(S4)
L(P2,P1)	1.00	0.43	-0.46	0.44	0.46	-0.46	-0.15
L(P3,P2)	0.43	1.00	-1.00	0.08	1.00	-1.00	-0.32
L(P4,P3)	-0.46	-1.00	1.00	-0.09	-1.00	1.00	0.33
DT(P4)	0.44	0.08	-0.09	1.00	0.09	-0.09	0.00
L(S3,S1)	0.46	1.00	-1.00	0.09	1.00	-1.00	-0.30
L(S4,S3)	-0.46	-1.00	1.00	-0.09	-1.00	1.00	0.34
DT(S4)	-0.15	-0.32	0.33	0.00	-0.30	0.34	1.00

The correlation matrix for the revised model, MB1, also revealed a perfect negative correlation between compartments P1 and P3 (Table 4 - 2). This suggests that a single compartment rumen model would best account for the foregut digesta movement within sheep. Physically though, sheep possess more than one compartment in their foregut, rumen and reticulum, therefore, a single compartment model would not be capable of mimicking true digesta kinetics in sheep.

Table 4 - 2 Correlation matrix between different compartments for new model MB1

Correlation Matrix						
Column	L(P3,P1)	L(P4,P3)	DT(P4)	L(S3,S1)	L(S4,S3)	DT(S4)
L(P3,P1)	1.00	-1.00	-0.34	0.99	-0.99	-0.32
L(P4,P3)	-1.00	1.00	0.39	-0.99	0.99	0.33
DT(P4)	-0.34	0.39	1.00	-0.37	0.38	0.17
L(S3,S1)	0.99	-0.99	-0.37	1.00	-0.99	-0.28
L(S4,S3)	-0.99	0.33	0.38	-0.99	1.00	0.35
DT(S4)	-0.32	0.31	0.17	-0.28	0.35	1.00

In an attempt to develop a rumen model with a single compartment, neither the goodness of fit (SSFSD = 24.674) or alignment of the fitted curve to actual data points were sufficient to accept the model for assessment. Therefore, a model with two rumen compartments was adopted. With this in mind, another model like model MB1 but with a hindgut compartment as in models M1 and M3, comprising two rumen compartments was developed. This new model, MB2 (Fig 4 - 3), incorporated the concept suggested by Aharoni *et al.* (1994) that

both particles and solutes experience further mixing in the post-ruminal region of the GIT, particularly in the caecum and colon.

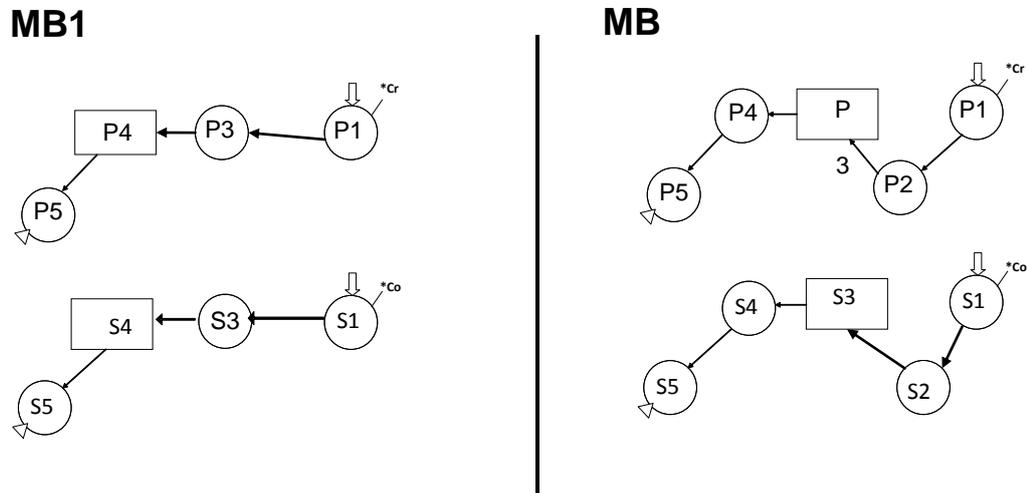


Fig 4 - 3 Flow diagram of a two-rumen compartment model (MB1) and a two-rumen compartment plus hindgut compartment model MB2

4.2.9 Assumptions and analysis

The fit of the concentration-time curves was compared by Aharoni *et al.* (1999) between models by means of the FSD and the SSFSD as mentioned previously. This same method of comparison of goodness of fit has been applied extensively by other authors (Pond *et al.* 1989; Aharoni *et al.* 1991; Moore *et al.* 1992) when determining the effectiveness of a model describing ruminant digesta kinetics. As such, the same goodness of fit test was also applied when deciding which model was the best to use for this thesis.

Some debate still exists as to the correct allocation of rate constants to specific compartments of the digestive tract. Grovum and Williams (1973) argued that the slow rate constant represented ruminal outflow and, therefore, suggested that the faster rate constant probably represented the digesta retention time post-ruminally. Contrastingly, Faichney and Boston (1983) found that sometimes fractional rates of outflow from the caecum are slower

than fractional rates of outflow from the rumen and an assumption that the rumen has the longer digesta MRT can lead to errors. However, they suggested that the correct identification of the two components can be achieved by the simultaneous use of solute- and a particle-phase markers because, in ruminants, such markers do not behave independently in the caecum-proximal colon (Faichney and Boston 1983). In general, with simultaneous application of both particulate and solute markers, the slower rate constant can be assumed to represent ruminal outflow with the faster rate constant being the passage of material post-ruminally (Dhanao *et al.* 1985). This general assumption was applied in this thesis when comparing models for goodness of fit.

Each model consisted of compartments leading to a delay then passing either to another compartment and to faeces (M1, M3 and MB2) or directly to faeces (M2 & MB1). Each model also possessed separate, parallel routes for particulates and solutes. All compartments prior to the delay are assumed to represent the RR portion of the digestive tract while the delay and any additional compartments post delay constitute parts of the digestive tract post-ruminal (hindgut). Rumen MRT of particulates was considered to be the sum of retention times in compartments P1 and P2 for models M1, M3 and MB2, sum of retention times in compartments P1 and P3 in model MB1, and sum of retention times in compartments P1, P2 and P3 in model M2. Retention time in the particulate route delay compartment (DT) plus the retention time in any particulate compartment post delay compartment (M1, M3 and MB2) were deemed to represent particulate hindgut MRT.

A model representing physiological parameters is designed to mimic the normal functions within an animal. A physiological function included in each model was to estimate the marker associated (indigestible) DM mass in the rumen. As the marker added and measured was Cr mordanted NDF, it was therefore assumed that the masses calculated by the model are associated with the concentrations of NDF DM material in each compartment instead of an estimation of DM present in each compartment as traditionally thought. Further investigation

of this theory is required. Adding the masses of NDF DM in the RR compartments represented the total rumen indigestible DM mass. These values were then compared to actual NDF masses measured in sheep of similar size. Estimates in the literature of rumen NDF DM content range from 11 to 14% of rumen volume (Hungate *et al.* 1971; Kennedy *et al.* 1992; Pinares-Patiño *et al.* 2003; Park *et al.* 2011). It was therefore assumed in the models that 12.5% of the rumen volume represented NDF DM content. This assumption also requires further investigation. The variation of the predicted mass from the expected mass was included in the measure of SSFSD.

A model of good design should be able to provide desired outputs for the user. The models compared in this thesis were all designed to enable calculation of commonly required digesta kinetics measures – DMD, FDMO, dose recovery and DMI. By knowing two of these parameters, it is possible to calculate the others. For example, FDMO could be estimated using the equation $FDMO = DMI * (1 - DMD)$, where DMI is the daily feed DM intake, and DMD is the DM digestibility of the feed. Alternatively, DMD can be determined from mean feed DMI and mean FDMO when these have been determined during balanced trials. As DMI was restricted to 1.2 x maintenance, this parameter was fixed within the models. To test the accuracy of each model, DMD was made an adjustable parameter, allowing the prediction of each animal's DMD. This predicted digestibility was compared to actual measures of DMD devised from total collection of faecal samples collected over 6 d and the variance included in the measure of SSFSD.

4.2.10 Statistical method

Analysis of variance (ANOVA) using R statistical software (R Development Core Team 2008) was used for statistical analysis. SSFSD for each model tested were compared for model effect using the ANOVA. The comparisons of MRT and FDMO were made within the experiment (Chapter 5) with the ANOVA assessing model effects with model and treatment

effects and all their interactions assessed by multifactorial design. Effects were reported as significant when $P < 0.05$ and as trends when $P < 0.1$.

4.3 **Results**

Goodness of fit for each model (SSFSD) along with particle and solute MRT, estimated rumen DM mass, and faecal DM output using faecal Co and Cr concentrations of six randomly selected control animals are presented in Table 4 - 3. A complete data set of faecal Cr and Co concentrations for the six animals is included in Appendix 3. Aharoni *et al.* (1999) proposed that model M2 possessed a much better goodness of fit than models M1 and M3 when fitting data from cattle. Our study reinforces this suggestion, using data from six sheep, with the SSFSD for models M1, M2 and M3 being 5.92, 3.31 and 4.62 respectively. Comparison of goodness of fit for the two new models developed (MB1 and MB2) showed much similarity between models M2 and MB1 (SSFSD 3.31 and 3.79 respectively) but an improved overall fit for model MB2 compared to all other models (SSFSD 2.73).

The five different models produced significant differences in MRT estimations (Table 4 - 3). For particles, model M1 estimated shorter retention time in the rumen ($P < 0.01$) and longer retention time in the hindgut ($P < 0.001$) than all the other models. Model M2 gave the longest retention time in the rumen and shortest retention time in the hindgut. For all models, total MRT of particles were almost identical with no significant differences found ($P = 0.86$) and MRT of solutes showed similar trends to particles. Rumen MRT estimation was longest in M2 and MB1 and shorter in M1 ($P < 0.01$) while hindgut MRT was longest in M1 and shortest in M2 and MB1 ($P < 0.001$). Total MRT of solutes, like particles, were not different.

While not significant, there was a tendency for models to produce different estimates of rumen indigestible DM ($P < 0.10$) with model M1 calculating 373 g or 81.3% of the expected DM mass within the rumen (assuming 12.5% of rumen contents is NDF DM - refer 4.2.9.

Assumptions and Analysis) while M2 estimated the greatest mass of 470 g or 103.0% of expected rumen DM mass. MB2 gave the estimate most closely matching the expected mass of rumen NDF, to within 0.1% of expected values. There were no significant differences in ruminal DM mass estimations by M2, MB1 and MB2.

Table 4 - 3 Mean sum of squares of fractional standard deviation (SSFSD), mean retention times (MRT) of both rumen and hindgut, and total MRT of particles and solutes, predicted mass of indigestible material within the rumen (Rumen Indigestible DM) and expressed as a percentage of mass expected to be in the rumen (Expected DM Mass*), predicted faecal DM output and expressed as a percentage of actual faecal DM output (estimated FDMO) as estimated with models M1, M2, M3, MB1 and MB2 in 6 control sheep.

Parameter	M1	M2	M3	MB1	MB2	SEM
SSFSD	5.92 ^c	3.31 ^a	4.62 ^b	3.79 ^a	2.73 ^a	0.65
Particle MRT (h)						
Rumen	22.3 ^b	30.2 ^a	28.9 ^a	30.1 ^a	29.1 ^a	2.3
Hindgut	23.7 ^b	15.4 ^a	17.2 ^a	15.8 ^a	16.2 ^a	1.3
Total	46.0	45.5	46.1	45.9	45.4	2.5
Solute MRT (h)						
Rumen	16.7 ^b	21.6 ^a	20.5 ^a	21.6 ^a	21.0 ^a	1.4
Hindgut	18.1 ^b	12.6 ^a	14.3 ^a	12.6 ^a	13.2 ^a	1.3
Total	34.8	34.2	34.8	34.2	34.2	1.7
DM Digestibility (%)	59.5	59.0	59.7	59.1	58.9	2.6
Rumen Indigestible DM (g)	373 [#]	470	439	468	456	48
▲ Expected DM Mass (%)	81.3 ^b	103.0 ^a	96.1 ^a	102.4 ^a	100.1 ^a	4.7
Faecal DM Output (g)	305	307	302	307	308	15
Estimated FDMO (%)	98.6	99.3	97.8	99.3	99.7	1.4

^{a,b,c} Means with different subscripts were significantly different.

[#] Trend in difference between means ($0.05 \leq P < 0.10$).

▲ Expected Mass: Calculated rumen DM mass based on the assumption that 12.5% of total rumen volume is NDF DM (refer Section 4.2.9. Assumptions and Analysis)

Faecal DM output, like DM digestibility, did not differ significantly across models or when compared with actual faecal output values obtained during the 6 day total collection period (refer chapter 5). All models under-estimated the measured amount of FDMO from the animals within 1.4%.

Mean masses of indigestible (NDF) material estimated in each compartment by the models are given in Table 4 - 4. Compartment P3 for models M1, M3 and MB2 is representative of the post-ruminal portion of the digestive tract (hindgut) and, therefore, is not included within the rumen for particles while compartment S3 represents solute passage through the hindgut for models M1, M3 and MB2. Compartment P2 in model MB2 and Compartment S2 in models M1, M2 and MB1 do not exist. Due to the different designs of each model, the estimated compartmental masses vary significantly between each model. Exceptions to this are compartments P3 in the particles and S3 in the solutes when included within the RR portion of the digestive tract. When both compartments are associated with the hindgut, significant variations between models occur ($P < 0.01$ particles; $P = 0.04$ solutes). There was no difference in the mass of indigestible particles or the total mass of indigestible DM (particles) estimated by each model to be present within the digestive tract but the estimates of total solutes in the digestive tract differed significantly between models ($P = 0.02$), although the absolute differences were very small ($SEM < 3$ g).

Table 4 - 4 Mean mass of indigestible material (particles and solutes) from 6 control sheep estimated to be present within each compartment by models M1, M2, M3, MB1 and MB2.

Compartment mass	M1	M2	M3	MB1	MB2	SEM
Particles (g DM)						
Within the rumen						
M(P1)	304	9 ^a	185 ^b	274 ^c	286 ^c	36.9
M(P2)	28 ^a	183 ^b	250 ^{bc}	-	163 ^b	36.9
M(P3)	-	273	-	188	-	92.4
Within the hindgut						
M(P3)	229 ^b	-	213 ^a	-	209 ^a	0.9
M(P4)	136 ^b	231 ^c	41 ^a	236 ^c	37 ^a	8.9
M(P5)	12 ^b	12 ^b	0.7 ^a	12 ^b	12 ^b	0.4
Total Particles	711	708	690	711	708	7.3
Solute (g)						
Within the rumen						
M(S1)	237 ^b	133 ^a	67 ^a	194 ^b	213 ^b	35.3
M(S2)	-	-	250 ^b	-	105 ^a	12.9
M(S3)	-	197	-	135	-	66.6
Within the hindgut						
M(S3)	175 ^b	-	170 ^b	-	155 ^a	3.2
M(S4)	103 ^b	176 ^c	41 ^a	176 ^c	28 ^a	5.9
M(S5)	12 ^b	12 ^b	0.7 ^a	12 ^b	12 ^b	0.4
Total Solutes	528 ^b	518 ^a	529 ^b	517 ^a	514 ^a	2.8
Total Mass	1239	1226	1219	1228	1221	9.5

^{a,b,c} Means with different subscripts were significantly different.

The curve fit of mean values of marker in the faecal DM by model MB2 of the 6 randomly selected control sheep after introducing single doses of Cr-mordanted non-digestible fibre and Co-EDTA into the rumen are given in Fig 4 - 4. A comparison of fitted marker curves for all models to mean observed faecal Co and Cr concentrations is shown in Appendix 4. Observation of the curve fit shows that all the models gave a good fit to the observed data.

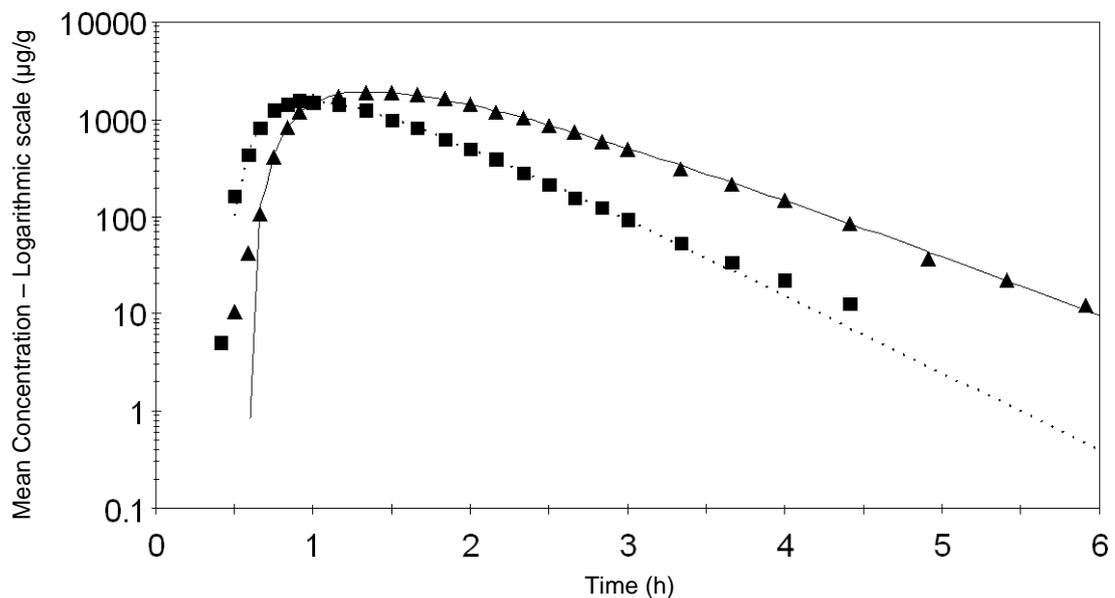


Fig 4 - 4 Curve fit of faecal concentrations of Co (■, broken line) and Cr (▲, solid line) by model MB2 of the mean values from the 6 randomly selected control sheep (all faecal concentrations are less measured background concentrations)

The flow between compartments, $L(i,j)$ (Table 4 - 5), is characterised by the fractional rate of flow or the portion of digesta in the donor compartment which flows to another compartment per unit of time. Similar to the masses mentioned earlier, not all compartments exist in each model due to each model's different design and, therefore, not all fractional rates of flow exist in each model.

Table 4 - 5 Calculated fractional rate of flow (g/h) of digesta between compartments [L(i,j) and delay post-ruminal (DT)] for both particles and solutes in each model

Fractional Rate of flow (g/h)	M1	M2	M3	MB1	MB2
Particles					
- Rumen					
L(P2,P1)	5.11E-01	2.42E+01	1.97E+00	-	1.49E+00
L(P3,P1)	8.61E-01	-	-	1.55E+00	-
L(P3,P2)	2.62E+00	1.65E+00	1.44E+00	-	1.75E+00
L(P4,P3)	-	1.60E+00	-	1.69E+00	-
- Post Ruminal					
DT	6.62E-01	6.31E-01	5.68E-01	6.94E-01	5.96E-01
L(P5,P4)	2.51E+00	-	6.31E+00	-	1.07E+01
Solutes					
- Rumen					
L(S2,S1)	-	-	6.51E+00	-	1.83E+00
L(S3,S1)	1.61E+00	1.94E+00	-	2.15E+00	-
L(S3,S2)	-	-	-	-	3.03E+00
L(S4,S3)	-	2.51E+00	-	2.36E+00	-
- Post Ruminal					
DT	5.04E-01	4.91E-01	4.54E-01	4.94E-01	4.45E-01
L(S5,S4)	3.31E+00	-	-	-	1.44E+01

4.4 Discussion

Aharoni *et al.* (1994) proposed an alternative model structure to the previous solely-sequential approach of estimating ruminant digesta kinetics. The new approach was to allow some parallel flow of particles to occur in subunits of the sequential system. Model M1 allowed some parallel movement in both the particle and solute routes by inclusion of two bypass fluxes in the particle route, L(P3,P1) and L(P5,P3), and one in the solute route, L(S5,S3). A new model proposed by Aharoni *et al.* (1999), M2, took this idea further and allowed particles to transfer from the slower particle route to the faster solute route by means of an additional pathway commencing in the RR, L(FP1 to FP5). Our study found that when estimating the digestive kinetics of sheep, remembering both Aharoni's models were based on cattle; the amount calculated to bypass the main sequential system was so insignificant that inclusion of the bypass routes distorted results and reduced the goodness of fit (SSFSD). Therefore, this additional component of the original M2 model was removed. A

possible explanation of this could be filter-bed effect, described below, which occurs with small particles within the RR.

The filter bed effect is associated with the stratification of the rumen. Ingesta on entering the rumen is propelled and mixed by contractions of both the rumen and reticulum, causing a stratified fibre mat to develop on top of a fluid layer and below a gaseous layer (Hofmann 1973; Welch 1982; Sutherland 1988). This fibre mat is considered essential for the selective retention of certain particles within the rumen and is a characteristic of many grazing ruminants such as sheep and cattle (Lechner-Doll *et al.* 1991; Clauss and Lechner-Doll 2001; Clauss *et al.* 2006). Originally it was thought that rate of flow from the rumen was limited by a critical particle size (<1mm in sheep and <2mm in cattle), whereby smaller particles had a faster rate of passage than larger ones (Poppi and Norton 1980; Poppi *et al.* 1985; Mathison *et al.* 1995). Mathison *et al.* (1995) noted though that a large number of particles present in the rumen were smaller than the critical size. It was suggested that the ruminal fibre mat served as a filter for small particles, trapping finer particles and allowing larger particles to continue to mix within the RR (Mathison *et al.* 1995). A particle's functional specific gravity (FSG), the ratio of mass of a sample to mass of gas-free fluid that takes up an equal space of the sample, may aid the filter bed effect in trapping finer particles (Bhatti and Firkins 1995). Smaller particles possess a larger FSG than larger particles which, in theory, would increase the likelihood these smaller particles would progress to the ROO (Sutherland 1988; Kaske *et al.* 1992; Mathison *et al.* 1995; Clauss *et al.* 2011). Hristov *et al.* (2003) found that particles with a FSG of greater than 1.02 were more likely depleted of substrate available for microbial fermentation, smaller in size, and had shorter MRT in the rumen than lighter particles. The filter bed appears to trap many of the lighter particles, preventing their escape from the RR by binding them together to effectively produce a larger particle, and increasing their MRT to something similar to larger particles and fibres (Mathison *et al.* 1995).

The particle-phase marker chosen for use in this study was Cr mordanted NDF. Mordanted chromium remains tightly attached to NDF (Udén *et al.* 1980), making it a popular marker in digesta kinetics. There are constraints when using Cr-mordanted NDF though, as the concentration of Cr on the feed affects the density of the marked feedstuff (FSG), affecting its passage characteristics (Ehle *et al.* 1984; Ramanzin *et al.* 1991). Moore *et al.* (1992) noted an 11% greater ruminal and total retention time in hay mordanted with Cr compared to hay marked with rare earths whereas Mader *et al.* (1984) found a 31% increased retention using Cr mordanted hay as opposed to rare earths. Even so, mordanted markers were developed to overcome the issue of marker migration associated with rare earths. Depending on the method of marker application, as much as 20% of the rare earth marker could migrate intraruminally to small particles (Erdman and Smith 1985; Siddons *et al.* 1985; Owens and Hanson 1992) or even phosphates (Van Soest *et al.* 1988), overestimating digesta passage rates. Of the different markers investigated, chromium mordanted feedstuff appeared to be the best representative of actual passage of digesta, even with these constraints. Burns *et al.* (1997) reported that faecal output from Cr-mordanted ingesta kinetics was well correlated with actual outputs, but faecal output using Ytterbium (Yb) was poorly correlated ($r^2=0.08$). Luginbuhl *et al.* (1994) also reported significant underestimations of faecal output when rare earth labels were used as markers in comparison to Cr mordanted feed.

Processing of all modified models by the non-linear curve fitting algorithms in WinSAAM allowed a number of alternative parameters to be determined to meet the objectives of the study. This is to provide accurate estimates of kinetic parameters to compare to enteric methane production. Important analytical data can be obtained from the models, such as FDMO, DMD, mass of indigestible (NDF) material in the rumen, marker dose administered and recovered, along with estimations of digesta kinetics, when certain key components are known. For example, FDMO and apparent DMD can be determined if dose of marker and DM intake are known. Estimations such as these rely on the accuracy with which the

predicted faecal marker concentration curve fits the observed data and the biological opportunities of the model's design. All five models tested in the study were found to be quite accurate in their estimations of FDMO, based on the recorded apparent digestibility results obtained from a balanced 6 d total collection, with all models underestimating FDMO by between 2.2% (model M3) and 0.3% (model MB2). These results were more accurate than those achieved by Aharoni *et al.* (1999) when applying cattle data, where all models overestimated digestibility (10% - model M2; 13% - model M3), suggesting that the modifications made to Aharoni's M2 cattle model produced a more accurate fit to data from sheep. Considering the size of the underestimation of FDMO in comparison to other models developed (Moore *et al.* (1992) – 23%; Aharoni *et al.* (1999) – 10%), these underestimations could be considered small.

Goodness of fit (SSFSD), when used as a measure for comparison between models, showed that model MB2 was the best tested model to predict concentrations of both particle- and solute-phase markers in comparison to the observed concentrations in the faeces (SSFSD=2.727). Aharoni *et al.* (1999) noted that model M2 provided the best fit of actual to predicted data points and prediction of faecal output with cattle data. The main variation between models M2 and MB2 is the inclusion of an additional compartment in the post-tubular portion of the model (particles – P4, solutes – S4). This improvement in curve fitting due to addition of a compartment post-ruminally suggests there may be a differential passage of liquid and solid digesta in the intestines (Grovmum and Williams 1977; Colucci *et al.* 1989; Huhtanen and Kukkonen 1995), in contrast to suggestions by Faichney and Boston (1983) that both phases travel together post-ruminally. A small portion of the particles may be undergoing further fermentation within the proximal-caecal region as sheep are known to ferment substrates and produce VFA in the caecum (Faichney 1968). This suggests that digesta movement beyond the abomasum is not homogeneous with solutes as previously thought (Faichney 1975b; Faichney and Boston 1983; Huhtanen and Kukkonen 1995) but is subjected to further processing within the intestinal tract.

An iterative physiological model is designed to provide the user with optimised outcomes, based on the initial chosen estimates of biological variables that are expected from animals under such conditions. While goodness of fit (SSFSD) indicates the model's ability to simulate faecal marker values at certain times, comparing outcomes to recorded, published and peer accepted values is possibly the best indicator of a model's soundness. FOR, the proportion of a digesta constituent that leaves a compartment per unit time (Faichney 1980), of particulates in the rumen for sheep have been measured extensively under similar circumstances and diets to this study, and a range of 3 to 4% h⁻¹ is the consensus (Aitchison *et al.* 1986; Faichney and White 1987; Isac *et al.* 1994; Alcaide *et al.* 2000; Pinares-Patiño *et al.* 2003). The mean retention time of liquids and particles in the rumen are calculated as the reciprocal of their respective FOR (Faichney 1980). Using 3 to 4% h⁻¹, this equates to a ruminal MRT of between 25 and 33h (mean 29h). All 5 models, except M1 (22.3h), gave predictions within this ruminal MRT range, with both models M3 and MB2 predicting a mean value of approximately 29 h.

Aharoni *et al.* (1999) stated that total MRT and FDMO estimations are similar among different models, and depend on the quality of the curve fit, but the retention times among compartments vary greatly between models. Poore *et al.* (1991) found that total MRT estimations in all the models they tested were similar, whereas other kinetic variables were sensitive to the design of the model. In this study, total MRT for particles and solutes were less sensitive to the design of the model than the retention times of different compartments describing the gut. Estimates of total MRT of particles from all five models did not differ significantly but rumen and hindgut MRT estimations varied amongst models. Similar results occurred for solutes, although total MRT was found to differ between models. Differences also occurred with compartmental masses for each model yet estimated total mass within the digestive tract was similar. Further development of DM content mass predictions by the models needs to be investigated by comparing masses predicted by the model to actual masses within the animal's digestive tract. Retention time estimates for both particles and

solutes in the rumen and hindgut of model M1 varied significantly relative to all other models, suggesting any rapid bypass of small particles through the RR to the abomasum may be comparatively small in quantity. All other models estimated similar MRT for both the rumen and hindgut.

4.5 ***Conclusions***

The analysis of digesta marker concentrations in faeces in the period after intraruminal marker administration enables estimates to be made of MRT in the whole gut and in gut segments of animals without the need for surgical intervention. Models that fit these faecal marker concentration v time data enable researchers to assess digestive physiological impacts of dietary and environmental factors imposed on animals under certain circumstances. The problem with each of the models tested here is the difficulty of linking certain compartments of the model to the gut anatomy of the animal. For example, it is difficult to know whether the abomasum is represented within the RR section or the tubular portion of the model. Total MRT and faecal output estimations were similar for all five models tested but partitioning retention time of the two main regions, rumen and hindgut, varied considerably between models. The differences in estimates of partitioning could be due to difficulties a model may have in fitting the observed data to the predicted curve. Therefore, the model possessing the best goodness of fit (SSFSD), as long it also retains acceptable physiological limitations, should provide the best and most reliable estimates of digestive kinetics. Since model MB2 possessed the lowest SSFSD it is considered the best of the models tested in this study. This model will therefore be used as the means of studying digesta kinetics throughout this thesis as associations between digesta kinetics and methane production are evaluated (Refer to Appendix 5 for a full WinSAAM computational working file of model MB2).

Chapter 5: Experiment I Effect of exogenous Administration of the Thyroid Hormone Triiodothyronine on Digesta Mean Retention Time and Methane Yield in Sheep

5.1 Introduction

Contractile forces of the RR and omasum propel ingested material through to the abomasum with the flow of digesta into the omasum mainly dictated by the diameter of the ROO (Bueno 1972). These contractile forces and the relaxing of the ROO are major determinants on the amount of time material is retained in the rumen for fermentation (Vittoria *et al.* 2000; Afzalzadeh and De B. Hovell 2002). This retention time period of digesta or mean retention time (MRT) has been positively linked to methane production (Pinares-Patiño *et al.* 2003). The recent discovery of specific chemoreceptors present on the epithelial lining of the RR and omasum as well as upon the ROO have given new understanding of the controlling forces for these RR contractions and ROO relaxation (Reid *et al.* 1988b; Okine *et al.* 1995; Vittoria *et al.* 2000; Onaga *et al.* 2011). These receptors respond to noncholinergic, nonadrenergic intrinsic factors such as substance P, cholecystokinin, gastrin, met- and leu-enkephalin, and vasoactive intestinal polypeptide (Sections 2.3, 2.4 and 2.5). This suggests that hormonal factors may play a major role in ruminal contractions.

One group of hormones with a known regulatory influence on digestive function are the thyroid-produced hormones. Thyroid hormones are involved in an array of different physiological processes, including growth, reproductive function, body temperature and pelage growth (Draper *et al.* 1968; Wilson 1975; Rhind and McMillen 1995). There are three types of thyroid hormones, Thyroxine (T_4) – the inactive reservoir form of the hormone;

Triiodothyronine (T_3) – the deiodised, metabolically active form; and Reverse Triiodothyronine (rT_3) – an inactive isomer of T_3 which blocks T_3 receptor sites. The active T_3 acts upon multiple target tissues throughout the body, stimulating oxygen utilisation and heat production within all cell types in the body (Todini 2007). One important process that thyroid hormones extensively regulate is metabolism. Their direct and indirect actions result in an increase in the basal metabolic rate, increase in plasma glucose concentrations, lipid metabolism, anabolic effect on protein metabolism in physiological concentration and catabolic effect at high concentration of hormones, decreased vascular smooth muscle cell resistance, and cholesterol catabolism, while also heightening cardiac and neural functions (Larsson 1988; Capen and Martin 1989; Davis and Davis 1996; Todini *et al.* 2007).

To assess the association between MRT and MY identified by Pinares-Patiño (2003), sheep were treated to create divergent T_3 levels to induce divergence in MRT that was not confounded by different DMI or substrate availability. By increasing a ruminant's metabolic activity through the administration of T_3 , it is thought that the animal's digesta kinetics could be modified, resulting in a reduction in MRT. The purpose of this study was to assess whether mimicking a state of hyperthyroidism could reduce digesta MRT within the rumen through increased catabolism, resulting in less methane being yielded.

Results from this chapter have been reported at the 2012 NZASAP conference in Lincoln, New Zealand (Barnett *et al.* 2012) – Appendix 6.

5.2 *Materials and methods*

5.2.1 *Animals, facilities and feeding*

Ten mature (two-tooth) Merino wethers were selected from the UNE experimental flock with an entry weight of 40 ± 5 kg, as described in Chapter 3, and fed a lucerne/wheat chaff diet

at a rate of 1.2 times maintenance (853±49g DM/40±5kg BW; 9.40MJ DE/kg DM, 15.0 % CP, 88.3 % DM). After being acclimatised to surroundings and fed for two weeks in individual pens at the UNE Animal House, they were relocated to controlled temperature rooms maintained at 21 ± 2°C and placed into individual metabolism crates, five animals to a room. Sheep were allocated by stratified randomisation into two equal sized groups with one group receiving injections of T₃ during the treatment phase (n=5) while the other group served as a control group (n=5). Another period of two weeks was used to accustom the sheep to surroundings and temperature. This period allowed the animals to adjust to receiving their feed via automatic feeding apparatuses. Once fully adjusted to their environment and feeding regime (consuming at least 90% of their daily ration), each animal was fed 1.2 times maintenance over a 24h period provided in equal portions at regular 2h feeding intervals until completion of the experiment. Any refusals were collected, weighed and recorded. After the adaption period, methane production rates were measured (2 x 2 days) then rumen fluid sampled immediately followed by a 6 day collection of faecal and urine output, followed by another 2 days of methane measurements. This is referred to as Period 1. After measurements were completed, treatment and control groups were reversed with sheep being adapted as for period one and the same parameters measured again (Period 2).

5.2.2 T₃ Injections, blood sampling and analysis

Each treatment animal received, via intramuscular injection, 300µg of sodium 3,3',5-triiodo-L-thyronine (Sigma-Aldrich, St Louis MO). Fresh batches of injections were produced regularly by dissolving 10mg T₃ at a rate of 1:1 (w/v) in a solution of 1N NaOH (200µL) and made up to 10mL with 7.4pH PBS, aliquoted into 5 separate vials then stored at -20°C until required. Each injection was administered at 1100h over a period of 24 days either every second day (period 1) or daily (period 2). Adjustment of the concentration of the T₃ injections to this value was found to be necessary after a preliminary investigation showed that an

injection concentration of 1.2mg or approximately 25µg/kg BW, similar to that administered by Saleh *et al.* (1998), was found to be deleterious to the animal's health. This resulted in the acquisition of replacement sheep which were used for this experiment (Section 3.1.2). In Period 1, injections were made every second day with treatment animals (Group 1) receiving a 300µL T₃ injection while control animals (Group 2) received a 300µL intramuscular injection of 0.9% saline. In Period 2 the injections were administered daily instead of every 2nd day with treatment group (Group 2) receiving a T₃ injection (300µg/d) and control animals (Group 1) receiving saline daily (Table 5 - 1).

Table 5 - 1 Injection protocol of cross-over experiment with 5 sheep per group receiving an intramuscular injection of triiodothyronine (300µg/d) either every second day (period 1) or daily (period 2).

Group	Period 1	Period 2
1	Inject every 2 nd day	Control (Saline)
2	Control (Saline)	Inject daily

Blood samples (10mL) were taken from all sheep twice a week between 1000 and 1100h using sodium heparin vacutainers (BD Vacutainer, USA) and centrifuged at 1400 g for 15min (Beckman TJ-6 tabletop centrifuge) to separate plasma. Plasma from each animal was aliquoted into two 5mL screw top plastic vials and stored at -20°C while awaiting analysis.

Free T₃ concentrations present in the plasma were originally analysed by an Immulite automated, quantitative immunoassay analyser (Siemens, New York, USA) using a Free T₃ competitive, analog-based immunoassay kit (Siemens Medical Solutions Diagnostics, Los Angeles CA). Values for plasma T₃ concentrations in control groups were frequently below assay sensitivity and were therefore reanalysed using Free T₃ (Human) competitive ELISA kits (Abnova Corporation, Taiwan; Ref No. KA0199, Lot No. RN-44291) at an absorbance of 450nm with a sensitivity of 0.05pg/mL. Results from the competitive ELISA kits are reported in this chapter.

5.2.3 Methane and rumen fluid collection

5.2.3.1 Methane and CO₂ emission measurement

Each animal was placed into an individual respiratory chamber and had its enteric CH₄ emissions and CO₂ production rates measured over a 22h period using open circuit respiration chambers (Section 3.2.1). Methane emissions were determined by an Innova 1312 photo acoustic gas analyser (Air Tech Instruments, Denmark) with analysis of sub-samples of air within each chamber collected continuously over the 22h into Tedlar bags (Section 3.2.2). This procedure was repeated four times during the experiment – twice under control conditions and twice during treatment for each group. These collections occurred immediately prior to, and after, the six day total collection required for measurement of digesta kinetics.

5.2.3.2 Rumen fluid sampling

A sample of rumen fluid was collected from each animal via oesophageal intubation immediately prior to administration of rumen markers (Section 3.3.1) and used for determination of VFA concentrations (Section 3.3.2).

5.2.4 Measurement of digesta kinetics

To enable estimation of digesta kinetics within the animals, the model MB2 devised and selected in the previous chapter (Chapter 4) was applied to the concentrations of the administered digesta markers found in the faeces.

5.2.4.1 Administration of markers

Immediately after collecting a rumen sample, each sheep was administered a measured dose of both Co and Cr markers via oesophageal intubation. Each animal received 7.2g of Cr mordanted NDF and 4.5g of CoEDTA in 90mL of Milli-Q water via intubation directly into the rumen. To aid in the introduction of the markers, approximately 20mL Milli-Q water was used to help flush the markers into the rumen after dosing.

5.2.4.2 Faeces collection

Faeces passed by each animal was collected over the six days of total collection (Section 3.5.1) and analysed for DM and digesta marker concentrations.

5.2.4.3 Faeces sample treatment and marker analysis

After collection of faeces, samples were prepared for marker analysis as mentioned in Section 3.4.3. Samples were weighed, dried, ground, digested and analysed for concentrations of Co and Cr markers using ICP-OES as described in Section 3.4.4.

5.2.4.4 Estimation of digesta passage rates

On completion of marker analysis, digesta kinetics for each animal were determined by simultaneously fitting concentrations of Co and Cr in the faeces against sampling time using the double-marker multicompartmental sheep digestive tract mathematical model MB2 developed in Chapter 4. Fitting was conducted using the non-linear curve fitting algorithms in WinSAAM (Stefanovski *et al.* 2003) in order to obtain rate constants of the markers in the reticulo-rumen and hindgut in the digestive tract. Reciprocals of the reticulo-rumen and hindgut rate constants estimated the MRT of digesta in the reticulo-rumen (Rumen MRT) and hindgut MRT respectively. Total MRT was calculated as the sum of Rumen MRT and Hindgut MRT.

5.2.5 Estimation of microbial protein outflow from the rumen

Microbial protein production was calculated from the mean daily excretion of the purine derivative allantoin (Section 3.7) in the urine collected over the 6-d periods (Section 3.5).

5.2.6 Apparent dry matter digestibility (DMD)

Apparent DMD of digesta for each animal was determined as described in Section 3.6.

5.2.7 Statistical methods

A linear mixed effect (LME) model using R statistical software (R Development Core Team 2008) was developed to account for the unbalanced data due to the effect of using different T₃ treatments in Period 1 and Period 2. A time span of two weeks between periods was utilised in anticipation it would be sufficient to eliminate any carry-over effects from treatments (Kennedy *et al.* 1977). Comparison of the data for the 5 control sheep in Period 1 with the 5 control sheep in Period 2 proved there was no period effect on any attribute measured. Consequently, since there was no period effect, it was valid to compare the same sheep across periods, when they were treated and when they were controls. The model also allowed comparison of the control and treated group of sheep within a period. Results were reported as significant when P≤0.05 and as trends when P≤0.1.

5.3 Results

Comparison of data for all parameters measured on the 5 animals as controls in Period 1 and on the 5 sheep used as controls in Period 2 revealed no differences (Table 5 - 2). No significant differences between treated and control animals were found except for methane yield, whether a treatment group was compared with itself as control in the other period, or with the group that was contemporaneously on the control diet in the same period. For this reason, the contrast between treatments is described in relation to control animals in the same period.

Period 1

Sheep injected with T₃ every second day had a higher concentration of T₃ in plasma (54.3 v 16.0 pg/dL; P <0.001) than sheep given saline in Period 1 (Table 5 - 2). When sheep were injected with T₃ every second day, the concentrations of acetate, butyrate and propionate

within the ruminal fluid were significantly reduced ($P < 0.01$). Water intake and urine output both increased ($P < 0.05$), while microbial protein outflow and CO_2 output were unaffected by this T_3 regime.

Digesta kinetics were significantly modified by T_3 injections administered every second day. Total MRT was reduced by 4.0h ($P < 0.001$), primarily as a result of a 3.6h decrease in the MRT of digesta in the hindgut ($P < 0.05$). Rumen MRT and rumen indigestible DM mass were not affected ($P > 0.1$) by T_3 administration every second day.

Table 5 - 2 The effect of intramuscular injections of triiodothyronine (300 μg) every second day (period 1) and daily (period 2) on DM digestibility (DMD), methane yield, microbial protein outflow from the rumen, ruminal VFA concentrations, water intake, urine output, CO_2 and digesta kinetic parameters of sheep fed every two hours (means \pm SE). There were no statistical period effects found.

Parameter	Period 1		Period 2	
	Control (n=5)	T_3 injection Every 2 nd d Treatment (n=5)	Control (n=5)	T_3 Injection Daily Treatment (n=5)
T_3 concentration (pg/dL)	16.0 \pm 8.0 ^a	54.3 \pm 5.7 ^b	12.8 \pm 5.7 ^a	293.4 \pm 9.8 ^c
Methane Yield (g/kg DM)	20.5 \pm 1.2 ^{ab} #	18.9 \pm 0.8 ^b	22.0 \pm 0.8 ^a	22.0 \pm 0.8 ^a
DMI (g/day)	854 \pm 37	816 \pm 22	826 \pm 20	856 \pm 21
DMD (%)	61.2 \pm 1.6	60.2 \pm 1.1	58.9 \pm 1.1	60.3 \pm 2.3
Microbial Protein Outflow (g N/day)	6.9 \pm 1.0	5.0 \pm 1.7	6.4 \pm 0.7	7.1 \pm 1.7
VFA concentration (mM)				
Acetate	51.1 \pm 2.3 ^a	41.7 \pm 1.8 ^b	49.1 \pm 2.6 ^a	50.8 \pm 1.8 ^a
Propionate	18.8 \pm 1.5 ^a	15.0 \pm 0.8 ^b	17.3 \pm 1.1 ^a	18.1 \pm 0.8 ^a
Butyrate	9.7 \pm 0.7 ^a	6.4 \pm 0.3 ^b	9.3 \pm 0.5 ^a	9.8 \pm 0.3 ^a
Acetate:Propionate (Acetate+1/2 Butyrate):Propionate	2.9 \pm 1.0	2.8 \pm 1.1	2.8 \pm 0.9	2.9 \pm 1.1
Water intake (L)	4.1 \pm 0.6 ^a	6.3 \pm 0.7 ^b	5.0 \pm 0.7 ^a	4.8 \pm 0.7 ^a
Urine output (L)	1.5 \pm 0.5 ^a	2.7 \pm 0.5 ^b	1.4 \pm 0.6 ^a	2.0 \pm 0.5 ^a
CO_2 produced (L d ⁻¹ kgDM ⁻¹)	443 \pm 13 ^a	427 \pm 14 ^a	417 \pm 14 ^a	471 \pm 14 ^b
Rumen MRT (h)	25.2 \pm 1.4	24.8 \pm 1.3	26.4 \pm 1.4	27.2 \pm 1.3
Hindgut MRT (h)	19.9 \pm 2.0 ^a	16.3 \pm 1.3 ^b	20.7 \pm 1.5 ^a	18.6 \pm 1.3 ^a
Total MRT (h)	45.1 \pm 2.1 ^a	41.1 \pm 1.7 ^b	47.1 \pm 2.2 ^a	45.8 \pm 1.7 ^a
Rumen Indigestible DM mass (g)	368 \pm 37	400 \pm 23	418 \pm 26	376 \pm 23

^{a,b,c} Means with different subscripts were significantly different within or between period

Trend in difference from control ($0.05 \leq P < 0.10$)

T₃ injections every second day tended to reduce MY (P=0.06) when compared to contemporaneous animals (1.6 g methane/kg DM intake or 8%). Significant reductions in methane yield also occurred relative to when the animals were used as controls (3.1g methane/kg DM intake; a 14% reduction).

When T₃ was administered every second day, MY was correlated (P<0.05) with both rumen MRT and total MRT ($y=0.621x+5.354$; $r^2= 0.43$ and $y=0.357x+5.358$; $r^2=0.53$, respectively) (Fig 5 - 1a) but not with hindgut MRT. There was also a strong linear association between MY and plasma T₃ concentrations in period 1 (Fig 5 - 2a; $y = -0.093x + 24.01$, $r^2= 0.70$; P<0.01).

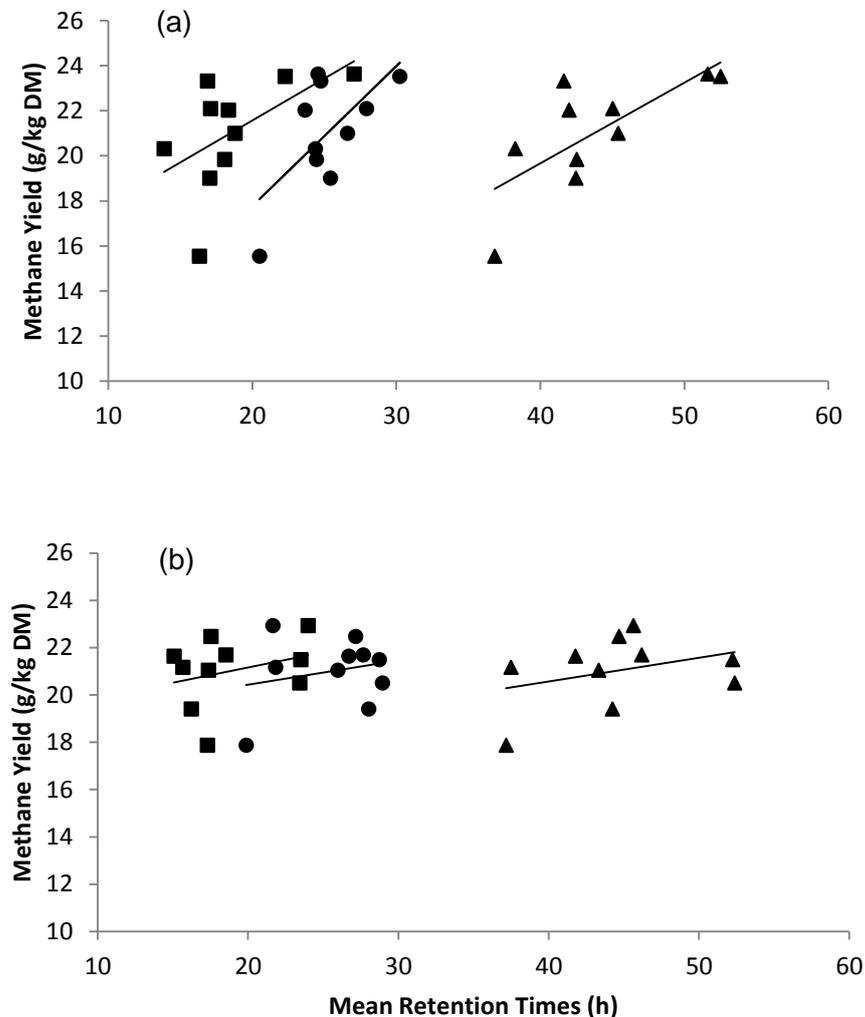


Fig 5 - 1 Relationships between methane yield (g/kg DMI) and mean retention times in the rumen (●), hindgut (■) and total tract (▲) for sheep injected with T₃(n=5) or saline (n=5) every second day (period 1 – Fig 5 - 1a) and sheep injected daily with T₃(n=5) or saline (n=5; period 2 – Fig 5 - 1b)

Period 2

Period 2 showed that injection of T₃ on a daily basis increased plasma T₃ concentrations well in excess of physiological levels (P<0.001; Table 5 - 2) and elevated CO₂ output (P<0.05) but no other parameters showed significant effects of daily administration of T₃. No correlations between MY and any of the digesta kinetics parameters (Fig 5 - 1b) or plasma T₃ concentrations were found when T₃ was injected daily (Fig 5 - 2b).

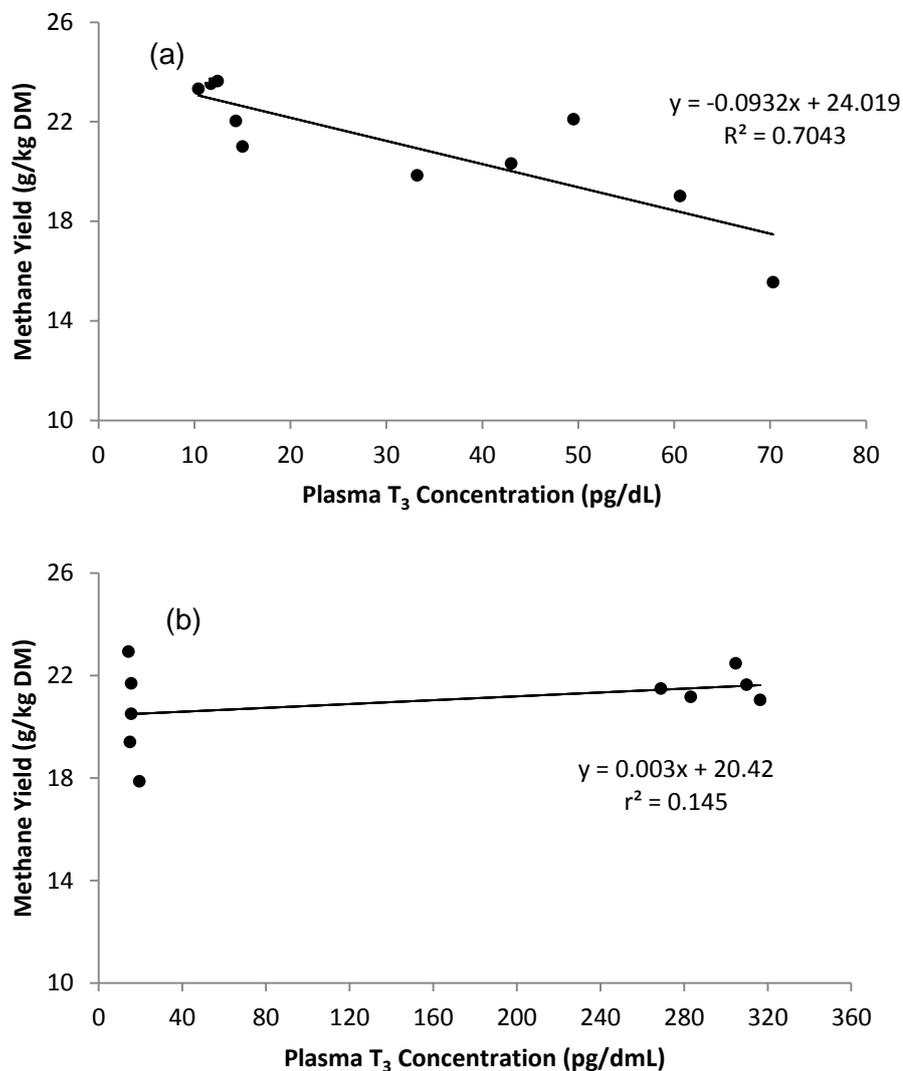


Fig 5 - 2 Relationship between methane yield (g/kg DMI) and plasma T₃ concentration (pg/dL) for sheep injected with T₃ (n=5) or saline (n=5) every second day (period 1 – Fig 5 - 2a) and sheep injected with T₃ (n=5) or saline (n=5) daily (period 2 – Fig 5 - 2b)

5.4 *Discussion*

Modifying kinetics within the digestive tract, particularly the rumen, has long been considered a method of mitigating MY (Okine *et al.* 1989), and a mechanism by which ruminants differ in MY (Hegarty 2004). This study used administration of the thyroid hormone T₃ as a model strategy to change MRT and study its association with enteric methane production without changing substrate supply to the rumen. This was only possible when plasma T₃ concentrations were within normal physiological boundaries. T₃ levels associated with hypo- and hyper-thyroidism vary with age, sex and species although published literature suggests for sheep, the physiological range is approximately 1pg/mL to 6pg/mL for free T₃ and around 500pg/mL to 5ng/mL for total T₃ (Blaxter 1948a; Blaxter 1948b; McBride and Early 1989; Fowden and Silver 1995; Nazifi *et al.* 2003; Sano and Takebayashi 2003; Munoz *et al.* 2008; Badiei *et al.* 2009; Eshratkhah *et al.* 2010).

When T₃ concentrations are increased to non-physiological levels, negative feed-back loops are initiated to stop further production of the elevated hormone and reinstate homeostasis. The hypothalamus is the main synthesis regulator of T₃ and other thyroid hormones as well as controlling the pituitary thyrotrophs through inhibitory factors such as somatostatin and dopamine. Utiger (1995) stated that increased plasma levels of thyroid hormones T₃ and T₄ exert a negative feedback control on both the pituitary and hypothalamus. This negative feedback loop associated with suprasaturated plasma levels of thyroid hormones could be the reason why daily injections of T₃ (Period 2) resulted in no change in any of the parameters measured except CO₂, yet injections of the same amount of T₃ every second day (Period 1) resulted in important physiological changes in tissue metabolism.

Period 1 showed the small increase in circulating T₃ concentration resulting from exogenous T₃ injections every second day caused a reduction in total MRT. The reduction in MRT was

mainly in the hindgut and was probably due to faster emptying of the post-ruminal part of the digestive tract of treated animals compared to control animals. The omasum is known to be able to control digesta flow from the reticulo-rumen by regulating reticulo-omasal orifice contractions (Afzalzadeh and De B. Hovell 2002) and a faster release of digesta through the orifice could have facilitated a faster rate of passage through the abomasum and intestines. The observed faster rate of passage through the hindgut could also be due to the T_3 effect on intestinal activity and motility (Hodin *et al.* 1992; Wegener *et al.* 1992; Vassilopoulou-Sellin and Sellin 1995).

Modifying digesta kinetics within the digestive tract was also associated with a significant reduction in MY, but only when T_3 levels experienced were within the physiological range. Period 1 demonstrated that a change in T_3 plasma concentrations similar to that experienced by an animal in a state of hyperthyroidism (Fallah-Rad *et al.* 2001) could result in a highly significant reduction in methane yield. A natural state of hyperthyroidism occurs during times of thermal stress such as extended periods of cold weather. During the colder seasons of late autumn, winter and early spring, plasma concentrations of T_3 naturally elevate to increase basal metabolic rate in an attempt to elevate body temperature and return to a state of thermal homeostasis (Silva 2005). The results from this study suggest that during these periods of naturally elevated thyroid activity, a divergence from normal digesta kinetic patterns may occur and a decreased MY could be expected. When plasma T_3 levels were elevated to that in excess of the normal physiological range of the animal, as in Period 2, no changes to digesta kinetics or MY were recorded.

Along with reduced MY, the strong inverse relationship between MY and plasma T_3 concentration only occurred when the concentration of T_3 was within a physiological range (Period 1). This response to elevated T_3 plasma concentrations of decreased total MRT in Period 1 is similar to studies conducted in the past. Kennedy *et al.* (1977) were able to

reduce MRT of digesta in sheep with elevated T_3 plasma concentrations within a physiological range. This suggests that T_3 does play some form of regulatory role in digesta MRT and, therefore, leads to a direct or indirect effect on methane yield. In contrast, a high non-physiological increase in plasma T_3 levels (Period 2) yielded a static response in methane yield. This demonstrates two things. Firstly, as mentioned previously, by increasing the rate of digesta flow through the hindgut, a non-significant reduction of digesta retention time within the rumen was sufficient to mitigate methane yield. Secondly, this response to T_3 administration on methane yield only occurs when the hormonal concentration is within the physiological range.

Another possible reason for T_3 mitigating MY is that by modifying the rate of passage through the hindgut, there is a change in the environment within the rumen that could be less conducive to methane production by methanogens. The significant reductions in the concentration of VFAs within the rumen suggest that a change may have occurred to the conditions inside the rumen and reticulum, resulting in less total production of VFA. When comparing the ratios of acetate:propionate and acetate + $\frac{1}{2}$ butyrate:propionate in the rumen, neither treatments in Period 1 nor 2 had any significant impact. A reduced amount of acetate, butyrate and propionate with no change in production ratios would suggest that less fermentation occurred within the rumen and reticulum. With less fermentation occurring, the amount H_2 available for H_2 -utilisers; such as methanogens; would decrease resulting in less interspecies hydrogen transfer (Section 2.2.1). A lower acetate formation would result in less formate being produced through the pyruvate-formate lyase system leading to decreased concentrations of CO_2 and H_2 in the rumen for CH_4 production. This fits well with the hypothesis that less fermentation of digesta within the rumen would yield less methane but, with fast rates of passage and less residence time in the rumen, decreased digestibility would also be expected but not observed. It is known that many animals like sheep, cattle, pigs and horses are capable of producing VFAs in the large intestines, particularly the

caecum (Faichney 1968; Imoto and Namioka 1978). Murray *et al.* (1976) calculated that CH₄ production in sheep large intestines equates to 13 ± 1.3% of total enteric CH₄ production. The effect of modifying the digesta kinetics could be that less VFA production is occurring within the fermentative chambers of the rumen and reticulum resulting in less methane production, while an increase in gastric digestion within the abomasum and VFA production within the caecum are allowing the animal to maintain similar levels of digestion. A decrease in MRT within the large intestines/caecum would also have a significantly negative effect on the production rate of CH₄ from this region. An increase in rumen volume (not measured), however, cannot be discounted as a possibility contributing to differences in VFA concentration given the increased water intake in Period 1. An increase in water flux through the RR due to increased water intake during Period 1 could have resulted in a dilution of the VFA produced.

Colucci *et al.* (1982), feeding dairy cattle both low and high forage diets, deemed that a faster rate of passage of digesta through the digestive tract could be the primary cause of lower digestibility of the consumed feed. A significant increase in water intake as experienced by the sheep receiving T₃ injections every second day (Period 1) could also reduce DM digestibility by increasing ROF through the RR. In the current study though, DM digestibility did not change even though a significant reduction in the total retention time of digesta within the digestive tract of the animal occurred due to the treatment. The observation that DM digestibility did not significantly change even though both hindgut and total MRT were reduced is also in contrast to studies conducted by Miller *et al.* (1974) and Kennedy *et al.* (1977). In their research, both found a strong positive correlation between total MRT and the DM digestibility. Kennedy *et al.* (1977) also noted that the decrease in DM digestibility and total MRT observed in ruminants during cold conditions could be mimicked by administering injections of T₃. Okine *et al.* (1989), in contrast, observed a result similar to our study. They noted a significant decrease in both MRT and MY with no change in the

digestibility of DM when they inserted weights into the rumen of 4 cattle and measured the effect of ruminal distension on MRT and MY. Feed supplied to those animals consisted of a 50:50 mix (DM basis) bromegrass (*Bromus inermis*) and alfalfa (*Medicago sativa*), similar to the feed in our own study. In another experiment, Lu and Jorgenson (1987) found that by adding saponins to roughage diets they were able to decrease MRT in the rumen and total tract, while not changing DMD. They also discovered that a greater proportion of organic matter digestion had shifted from the reticulo-rumen to the SI. This suggests a shift in the site of digestion which could effectively decrease methane and VFA production in the rumen with increase fermentation in the hindgut (caecum). This could mean an increase in post-ruminal digestion and VFA production in the caecum, allowing the animal to maintain an unchanged DMD through the whole tract. This requires further investigation.

Hyperthyroidism is known to be associated with an increase in metabolic activity (Todini 2007) which is often measured by rate of O₂ consumption and CO₂ production by an animal (Whitelaw *et al.* 1972). An increase in metabolic activity in times of hyperthyroidism results in an increase in energy expenditure and heat production (Whitelaw *et al.* 1972). In Period 2, CO₂ production increased significantly with daily T₃ injections suggesting an increase in metabolic activity. Studies have shown that high states of hyperthyroidism have a direct impact on increasing myocardial contractions, increasing both the shortening velocity and tension development in the left ventricle of the heart, greatly increasing respiratory rates, without affecting metabolic activity (Blaxter 1948b; Buccino *et al.* 1967). This is similar to our results for Period 2 where excessive plasma concentrations of T₃ resulted in an increase in CO₂ production without any noticeable change in digesta kinetics and MY. In contrast, sheep receiving T₃ injections every second day (Period 1) recorded no increase in CO₂ production even though digesta kinetics were altered significantly and MY was reduced. This suggests that mildly elevated thyroid hormones in the plasma resulted in Period 1 while an extreme state of hyperthyroidism occurred in Period 2. Another effect of elevated levels of thyroid

hormones is the significant increase in water intake and urine output recorded in Period 1. This effect is similar to a condition referred to as polyuria where alteration in systemic haemodynamics leads to non-osmotic suppression of arginine vasopressin and increased solute excretion resulting from hyperthyroidism (Wang *et al.* 2007).

5.5 Conclusions

This study has shown that the thyroid hormone T_3 is capable of modifying digesta kinetics within ruminants, primarily through modifying motility through the intestines, resulting in a significant reduction in methane yields as long as T_3 is within a physiological range experienced by the animal. Unlike other studies though, there was no significant reduction in DM digestibility even though VFA concentration was reduced within the rumen. This could suggest that physiological and microbial ecology changes could be occurring within the digestive tract, allowing the ruminant to decrease its retention time of digesta while maintaining DM digestibility. A potential explanation of this could be an impact on interspecies hydrogen transfer occurring within the RR from microbial ecology changes due to the influence of T_3 . A reduction in the concentration of CO_2 and H_2 in the RR for CH_4 production while sustaining DM digestibility possess significant ecological and economical benefits. What direct or indirect role thyroid hormones may have on this interaction, particularly ruminal contractions, is still unclear and requires further investigation. The fact that T_3 concentrations within physiological range were associated with differences in MY suggests that circulating T_3 concentration may have application as a potential indicator of MY in sheep. Further studies are required where plasma T_3 concentrations exist within a normal, physiological range to assess the full impact of the hormone on digesta kinetics and the role in modifying methane yields. Seasonal variations in plasma T_3 concentrations are known to exist in animals, including ruminants, and this seasonal divergence, particularly cold ambient temperatures, needs to be investigated for its potential to vary methane yields.

Chapter 6: Experiment II Impact of Ambient Temperature on Plasma T_3 Concentrations, Digesta Kinetics, and Methane Yield in Sheep

6.1 Introduction

It was shown in the previous chapter that MY and digesta kinetics could be modified through the administration of exogenous T_3 but only when the plasma T_3 concentrations were within a physiological range. Animal plasma T_3 concentrations vary in accordance with seasons, influencing VFI, metabolism and nutrient uptake (Todini 2007). During periods of cold weather, animals increase their VFI and metabolic rate in order to maintain core body temperature (Morris *et al.* 2000). This rise in metabolism is primarily due to the fact that one of the major energy losses from metabolism is heat, with heat production and retention during colder months essential for an animal's survival. Also, when the ambient temperature is low, rumen motility increases significantly causing a decrease in DMD (Young 1981).

In contrast, during periods of hotter weather, animals decrease their VFI and rate of metabolism in response to increased body temperature (Christopherson and Kennedy 1983; Lu 1989). Increasing the environmental temperature to which ruminants are exposed also causes a significant decrease in both amplitude and frequency of muscular contractions in the RR, even if feed is restricted (Attebery and Johnson 1969; Hirayama *et al.* 2004).

Thyroid hormone activity appears to have an inverse relationship with ambient temperature such that a rise in temperature leads to a decrease in plasma T_3 concentrations but a fall in ambient temperature results in a rise in plasma T_3 (Todini 2007). During heat stress, T_3

concentration, metabolic rate, VFI, growth and milk production decrease (Valtorta *et al.* 1982; Christopherson and Kennedy 1983; Lu 1989; Todini 2007) while cold stress induces a rise in T₃ concentration, metabolic rate and VFI (Hocquette *et al.* 1992; Morris *et al.* 2000). During the coldest months of the year (winter), plasma T₃ values are often at their highest and, conversely, lowest during the hotter months of summer (Todini 2007).

Daylight affects T₃ concentrations with highest plasma T₃ concentrations occurring during periods of increasing daylength (spring), with levels lowest during autumn which has decreasing daylength (Buys *et al.* 1990; Souza *et al.* 2002; Todini *et al.* 2006; Eshratkhan *et al.* 2010). This suggests a photoperiod as well as temperature influence on thyroid activity, possibly mediated by oestrus activity or availability of feed. It would appear that temperature and photoperiod have an effect on rumen motility through a change in feed intake.

With temperature and seasonality being natural ways to induce changes in T₃ and the previous experiment (Chapter 5) showing that administration of T₃ impacts on MRT and MY, it may also be possible to influence MY via T₃, and so manipulate rumen kinetics, by varying environmental temperature. The purpose of this study was to determine if exposure of ruminants to low ambient temperatures below their thermal neutral zone could cause a physiological increase in plasma T₃ concentrations, inducing a change in the animal's digesta kinetics and a reduction in methane yield.

6.2 *Material and methods*

6.2.1 *Animals, facilities & feeding*

Six freshly shorn mature Merino wethers with an entry weight of 47 ± 3 kg (refer Section 3.1.2) were fed a ration of 50% lucerne/50% wheaten chaff diet at a rate of 1.35 times maintenance (1318±66g DM/47±3kg BW; 9.40MJ ME/kg DM, 15.0 % CP, 88.3 % DM). After acclimatising to surroundings and fed for two weeks in individual pens, the sheep were

randomly allotted into two equal sized groups and relocated to controlled temperature rooms set at either $9 \pm 1^\circ\text{C}$ (cold treatment; $n=3$) or $26 \pm 1^\circ\text{C}$ (warm control; $n=3$) and placed into individual metabolism crates. Nine degrees celcius was below the animals thermal neutral zone in accordance with the Standing Committee on Agriculture and Resource Management – Ruminant Subcommittee (CSIRO 1994). Another period of two weeks was used to accustom the sheep to surroundings and temperature. This period allowed the animals to adjust to receiving their feed via automatic feeding apparatuses (Section 3.1.4).

Once all animals were adjusted to their environment and feeding regime (consuming at least 90% of their daily ration), each animal was fed 1.35 times maintenance over a 24h period delivered at regular 2h feeding intervals by an overhead automated feeder until completion of the experiment. Any refusals were collected, weighed and recorded. After the adaption period, methane production rate was measured (4 days) then sampling of rumen fluid immediately occurred, followed by a 6 d collection of faecal and urine output, and another 4 days of CH_4 measurements. After measurements were completed, treatments were swapped between groups, with all sheep being re-shorn and adapted as previous with the same parameters measured again in a crossover design.

6.2.2 Blood samples and analysis

Blood samples (10mL) were taken from all sheep twice a week between 1000 and 1100hr using sodium heparin vacutainers (BD Vacutainer, USA) and centrifuged at 1400 g for 15 mins (Beckman TJ-6 tabletop centrifuge) to separate plasma. Plasma from each animal was aliquoted into two 5mL screw top plastic vials and stored at -20°C while awaiting analysis.

Total and FT_3 concentrations present in the plasma were analysed using a competitive sandwich enzyme immunoassay ELISA with colourimetric detection (Abnova Corporation, Taiwan; Total T_3 Ref No. KA0198, Lot No. RN-44172; FT_3 Ref No. KA0199, Lot No. RN-44291).

6.2.3 Methane and rumen fluid collection

6.2.3.1 Methane collection and estimation

Enteric methane emissions and CO₂ production rates for each animal were measured over a 22h period using open circuit respiration chambers (Section 3.2). This procedure was repeated eight times per animal during the experiment – four times under cold conditions and four times during warm conditions. These collections occurred immediately prior to (twice) and after (twice) the 6-d total collection required for measurement of digesta kinetics.

6.2.3.2 Rumen Fluid Sampling

A sample of rumen fluid was collected from each animal (Section 3.3.1) and used for determination of VFA concentrations (Section 3.3.2) and protozoa abundance (Section 3.3.3).

6.2.4 Measurement of digesta kinetics

To enable estimation of digesta kinetics within the animals, the model devised and selected in Chapter 4 (MB2) and utilised in the previous chapter (Chapter 5) was applied to the concentrations of the administered digesta markers found in the faeces.

6.2.4.1 Administration of markers

Immediately after collecting a rumen sample, each sheep was administered a measured dose of both Co and Cr markers via oesophageal intubation. Each animal received 3g Cr mordanted NDF and 2g CoEDTA in 50mL of Milli-Q water via intubation directly into the rumen. To aid in the introduction of the markers, approximately 20mL Milli-Q water was used to help flush the markers into the rumen.

6.2.4.2 Faeces collection

Faeces passed by each animal was collected over the 6-d of total collection (Section 3.5.1) and used for estimation of digesta kinetics.

6.2.4.3 Faeces sample treatment and marker analysis

After collection of faeces, samples were prepared for marker analysis as described in Section 3.4.3. Samples were weighed, dried, ground, digested and analysed for presence of Co and Cr markers using ICP-OES as described in Section 3.4.4.

6.2.4.4 Estimation of digesta passage rates

On completion of marker analysis, digesta kinetics for each animal were determined by simultaneously fitting concentrations of both Co and Cr in the faeces against sampling time using the double-marker multicompartamental sheep digestive tract mathematical model developed in Chapter 4 (MB2). Fitting was conducted using the non-linear curve fitting algorithms in WinSAAM (Stefanovski *et al.* 2003) in order to obtain rate constants of the markers in the reticulo-rumen and hindgut in the digestive tract. Reciprocals of the reticulo-rumen and hindgut rate constants represented the MRT of digesta in the reticulo-rumen (rumen MRT) and hindgut MRT respectively. Total MRT was calculated as the sum of rumen MRT and hindgut MRT.

6.2.5 Estimation of microbial protein outflow from rumen

Microbial protein production was calculated from the mean daily excretion of the purine derivative allantoin (Section 3.7), in the urine collected over the 6-d periods (Section 3.5).

6.2.6 Apparent dry matter digestibility (DMD)

Apparent DMD of digesta for each animal was determined in accordance with Section 3.6.

6.2.7 Wool growth measurement

A 10 x 10 cm mid side patch was also clipped on each sheep using a Wahl Pro Series small animal clipper, type 1247 (240v) with a 45mm, 1/8" cut, clipper blade (Wahl Clipper Corp., Sterling, Illinois, USA) at the beginning of the treatment period. At the end of each period, the area was clipped again with the same clippers and the length of the wool fibres measured (Reis and Schinckel 1961).

6.2.8 Rectal temperature

Measurements of body temperature were conducted by inserting a glass clinical thermometer into the rectum a depth of approximately 8cm (Piccione *et al.* 2002).

6.3 Statistical method

A two sample Student t-test (assuming equal variance) using R statistical software (R Development Core Team 2008) was used to assess effect of different treatments on each animal over the two treatment periods. To account for any possible period effect, the Hills Armitage approach (Hills and Armitage 1979; Armitage and Hills 1982) of statistically comparing the changes between the different treatment periods for each animal was applied to prevent the over inflation of period effect resulting in a loss of power due to inflation of error proportional to period effect squared. The resulting mathematical model used for analysis was:

$$Y_{ijk} = \mu + s_{ij} + \pi_j + \tau_d + e_{ijk}$$

whereby the fixed effects are period (π_j) and treatment (τ_d) and random effects are both subject (s_{ij}) and error (e_{ijk}) (Jones and Kenward 1989; Diaz-Uriarte 2002). The Hills Armitage approach allows testing for treatment differences of multiple periods by computing the difference between the first and second period for each individual, and then using a two-sample *t* test to compare these values between the two sequences (Diaz-Uriarte 2002).

Similar to the previous chapter (refer 5.2.7), a 2 week time span with no treatments between periods was incorporated into the experimental design to eliminate any possibility of carry-over effects. As in experiment 1, it was anticipated that this treatment-free period would be sufficient to eliminate any impact on results in Period 2 due to treatments in Period 1 (Kennedy *et al.* 1977). Results were again reported as significant when $P \leq 0.05$ and as trends when $P \leq 0.10$.

6.4 Results

Both free and total T₃ sheep plasma concentrations increased during cold treatment (9±1°C) (FT₃ – 4.4 v 3.0 pg/mL; Total T₃ – 2.6 v 1.4 ng/mL) compared to warm conditions (26±1°C) (Table 6 - 1). Low temperature reduced MY (P<0.01) by 1.6g methane/kg DM intake; an 8% reduction in MY compared to the same animals when controls (warm). Colder temperatures increased CO₂ production (P=0.01) and rate of wool growth (P<0.01) but water intake decreased (P<0.001) while urine output was unaffected by treatment (P=0.89). Body temperatures of animals, once acclimatised to the conditions, did not change due to treatment as indicated by rectal temperatures measured.

Table 6 - 1 The effect of exposure to decreased ambient temperature (9°C±1 v 26°C±1) on plasma thyroid hormone levels, methane yield, CO₂ production, dry matter intake (DMI), digestibility (DMD), daily wool growth, water intake, urine output, and rectal temperatures of sheep fed every two hours (means ± SEM). There were no statistical period effects found.

	Warm at 26°C (n=6)	Cold at 9°C (n=6)	SEM	Significance
T ₃ concentrations				
- FT ₃ (pg/mL)	3.0	4.4	0.5	*
- Total T ₃ (ng/mL)	1.4	2.6	0.4	*
MY (g CH ₄ /kg DMI)	19.3	17.7	0.6	**
CO ₂ Production (mL day ⁻¹ /kg DMI)	374.1	395.7	8.8	*
DMI (g/day)	1100	1138	16	NS
DMD (%)	61.8	60.8	0.4	#
Wool Growth (µm/day)	221	286	26	**
Water Intake (mL/day)	3294	1994	531	***
Urine Output (mL/day)	857	868	8	NS
Rectal Temperature (°C)	38.9	38.9	0.1	NS

Rows annotated with asterisk or hash significantly differ between treatments (#P≤0.1, *P≤0.05, **P≤0.01, ***P≤0.001)

Conditions within and outputs from the rumen (Table 6 - 2) were affected by ambient temperature, with MP outflow (P=0.03) and total VFA concentrations (P=0.03) increasing

while protozoa abundance decreased ($P<0.05$) in cold conditions. An increase in total ruminal VFA concentrations was principally due to a significant increase in ruminal acetate concentration ($P=0.03$) and a slight increase in propionate ($P=0.10$). Ruminal protozoal numbers decreased due to a reduction in small entodiniomorph numbers ($P<0.01$).

Table 6 - 2 The effect of exposure to decreased ambient temperature ($9^{\circ}\text{C} \pm 1$ v $26^{\circ}\text{C} \pm$) on microbial protein from the rumen, ruminal VFA concentrations and protozoal abundance of sheep fed every two hours (means \pm SEM). There were no statistical period effects found.

	Warm at 26°C (n=6)	Cold at 9°C (n=6)	SEM	Significance
Microbial Protein (g N/day)	7.6	8.6	0.3	*
VFA Concentrations (mM)				
Acetate	57.6	65.6	3.3	*
Butyrate	6.2	7.2	0.5	NS
Propionate	18.7	21.0	1.0	NS
Total	85.0	96.6	4.7	*
Acetate:Propionate	3.1	3.2	0.1	NS
(Acetate+1/2Butyrate):Propionate	3.3	3.4	0.1	NS
Protozoa (count/mL)				
Small Entodiniomorphs ($\times 10^5$)	18.6	14.6	1.6	**
Large Entodiniomorphs ($\times 10^4$)	2.6	1.4	0.6	NS
Holotrichs ($\times 10^5$)	2.0	2.2	0.1	NS
Total ($\times 10^5$)	20.8	16.9	1.5	*

Rows annotated with asterisks or hash significantly differ between treatments (# $P\leq 0.1$, * $P\leq 0.05$, ** $P\leq 0.01$)

Digesta kinetics were significantly modified by exposure to cold temperatures (Table 6 - 3). Total MRT was reduced by 3.4h ($P<0.01$), an 8% reduction in MRT compared to warm conditions. This was due to MRT decreasing by 1.2h in the rumen ($P<0.01$) and 2.2h in the hindgut ($P=0.01$), reductions of 4% and 15% respectively. The estimated amount of NDF material in the rumen (Indigestible DM mass) also decreased ($P=0.01$).

Table 6 - 3 The effect of exposure to decreased ambient temperature ($9^{\circ}\text{C} \pm 1$ v $26^{\circ}\text{C} \pm$) on digesta kinetic parameters of sheep fed every two hours (means \pm SEM). There were no statistical period effects found.

	Warm at 26°C (n=6)	Cold at 9°C (n=6)	SEM	Significance
Rumen MRT (h)	26.4	25.2	0.5	**
Hindgut MRT (h)	14.2	12.0	0.7	*
Total MRT (h)	40.6	37.2	1.2	**
Rumen Indigestible DM mass (g)	605	579	11	*

Rows annotated with asterisks or hash significantly differ between treatments ($\#P \leq 0.1$, $*P \leq 0.05$, $**P \leq 0.01$)

Change in MY due to exposure to cold temperatures showed a strong linear association with changes in rumen MRT ($y=0.541x + 0.364$, $r^2=0.69$; $P < 0.01$), hindgut MRT ($y=1.045x + 0.146$, $r^2=0.55$; $p < 0.05$) and total MRT ($y=1.586x + 0.510$, $r^2=0.87$; $P < 0.001$) (Fig 6 - 1).

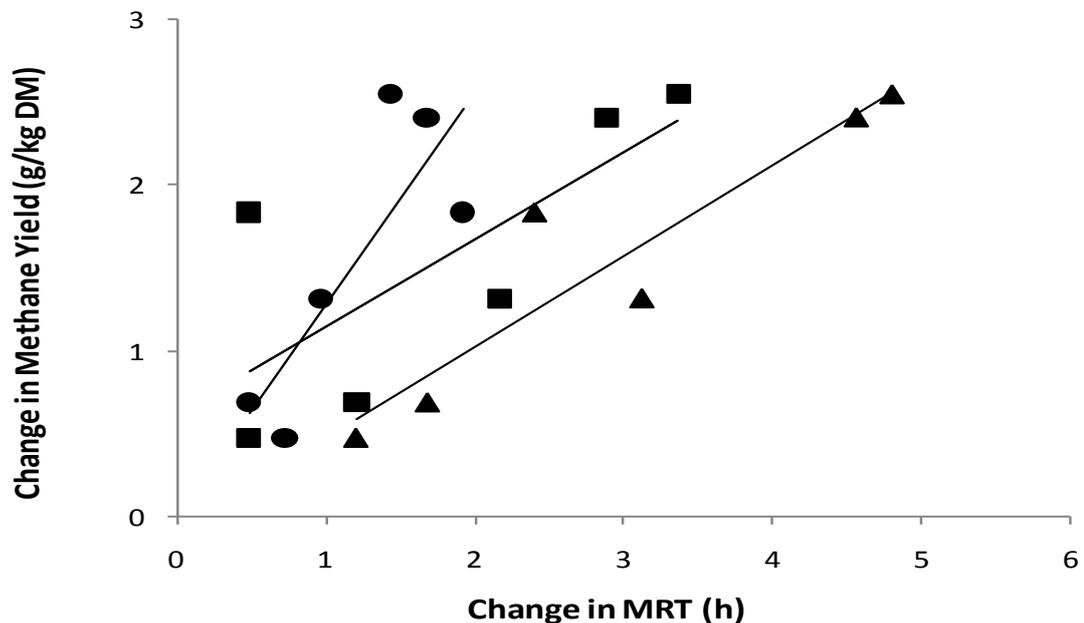


Fig 6 - 1 Relationships between change in methane yield (g/kg DMI) and change in mean retention times in the rumen (●), hindgut (■) and total tract (▲) of sheep when exposed to both warm ambient temperatures ($26^{\circ}\text{C} \pm 1$) compared to when exposed to cold ambient temperatures ($9^{\circ}\text{C} \pm 1$)

6.5 Discussion

Reduction of MY through modification of ruminant digestive kinetics is possible due to the strong link between MY and MRT of digesta (Pinares-Patiño *et al.* 2003; Barnett *et al.* 2012). There is also an increasing understanding of the roles hormonal and neural systems play in the control of digesta kinetics in ruminants (Bueno and Ruckebusch 1974; Hauffe and Von Engelhardt 1975; Onaga *et al.* 2008; Onaga *et al.* 2011; Oh-ishi *et al.* 2013). In the first experiment (Chapter 5), digesta MRT was reduced through the exogenous administration of the thyroid hormone, triiodothyronine (T_3), leading to mitigation of ruminant MY. That study suggested that digestive kinetics is a significant mechanism in controlling the production of ruminant enteric methane, and physiological factors which influence MRT of digesta in the GIT play a key role in the abundance of methane produced.

Exposure of ruminants to cold ambient temperatures induced a natural elevation in plasma T_3 concentrations. This is because immediately after the onset of exposure to cold, the pituitary gland increases the release of thyroid stimulating hormone (TSH), elevating thyroid releasing hormone (TRH) concentrations which stimulate T_3 and T_4 secretion from the thyroid gland (Reichlin *et al.* 1972). Unlike in Chapter 5 where daily T_3 injections caused FT_3 concentrations to far exceed physiological limitations (29.3 ± 1.0 pg/mL), the cold temperatures in the current study only increased plasma FT_3 levels to within the naturally occurring range (Chapter 5), giving levels similar to those measured with injections every second day in experiment 1. Seasonal variations in plasma T_3 concentrations have been reported, with heat stress conditions reducing (Todini *et al.* 2007; Koluman and Daskiran 2011) and cold stress increasing concentrations (Todini 2007). Results from this experiment also indicated that colder ambient temperatures can induce a natural decrease in MY and MRT, with MRT reduction being recorded in both the ruminal and post-ruminal regions of the GIT. In the previous chapter, injections of T_3 every second day only modified hindgut MRT and not ruminal MRT, possibly due to T_3 effects on intestinal motility and activity (Hodin *et al.*

1992; Wegener *et al.* 1992; Vassilopoulou-Sellin and Sellin 1995; Zhang *et al.* 2012). While our results support the notion that exposure to cold ambient temperatures elevates plasma T_3 concentrations within a physiological range, it is unclear whether this elevated T_3 concentration is the main instigator of change in MRT and MY, or if other physiological mechanisms triggered by exposure to cold temperatures have an important role.

Several studies have shown that cold ambient temperatures can cause reductions in rumen MRT (Westra and Christopherson 1976; Kennedy *et al.* 1977; Kennedy *et al.* 1982). Cold acclimatised sheep increase duration of quiescence of the ROO across different phases of the feeding cycle (Okine *et al.* 1995) and increase RR contraction frequency, amplitude and duration (Okine *et al.* 1989) to allow increased passage of digesta through to the omasum. An increase in nutrient passage from the forestomach to the intestines is presumably related to enhanced forestomach motility and relaxation of the ROO (Miaron and Christopherson 1992; Okine *et al.* 1995), but Li *et al.* (2000) suggested it may also be an effect of increased feed intake induced by the energy demands associated with the environmental conditions. Restricted feeding, though, was applied to our experiment (1.35 x maintenance) avoiding confounding with possible indirect impact of temperature through increased VFI on ruminal MRT.

It has been suggested that during periods of exposure to cold temperatures, there is an increase in rumination activity (LaPlace 1970; Dikmen *et al.* 2011; Graunke *et al.* 2011) and that an increase in digesta flow from the omasum to the abomasum occurs during periods of increased rumination (Gonyou *et al.* 1979). This physiological effect relating to colder temperatures could help explain the increase in digesta rate of flow along with triiodothyronine's known impact on intestinal motility (Hodin *et al.* 1992; Wegener *et al.* 1992; Vassilopoulou-Sellin and Sellin 1995; Zhang *et al.* 2012). Increased passage of digesta from the omasum to the abomasum due to increased rumination in association with increased amplitude, frequency, and duration of RR contractions and quiescence of the ROO would

have significant impact in reducing rumen MRT. Reduced rumen MRT, along with increased post-ruminal digesta ROF through the intestines due to autocrine/paracrine/endocrine influences, could have a significant impact on total digesta MRT and MY.

Feed consumed by ruminants that are exposed to lower ambient temperatures is digested to a reduced extent compared to animals in thermoneutral conditions, with reduced digestion ruminally but no shift in intestinal extent of digestion (Westra and Christopherson 1976; Kennedy *et al.* 1977; Kennedy and Milligan 1978; Westra and Hudson 1981; Kennedy *et al.* 1982; Li *et al.* 2000). However, results from this experiment demonstrated no significant reduction in apparent digestibility due to cold temperatures. While extent of digestion in the rumen was not measured, rumen MRT was reduced by exposure to cold ambient temperatures yet total apparent DMD did not change. Kelly and Christopherson (1989) also found that sheep exposed to cold ambient temperatures had reduced OM digestibility in the rumen associated with reduced rumen MRT but, total apparent digestibility did not change. They, therefore, concluded that intestinal digestion must have increased to compensate for the reduced digestibility in the rumen. Okine *et al.* (1989) reported similar results using cattle with weights inserted into the rumen to measure effect of ruminal distension on MRT and MY while Lourenço *et al.* (2010) recorded no temperature effect on apparent digestibility when exposing two different breeds of sheep to different diets and ambient temperatures. This suggests that exposing animals to low ambient temperatures may cause an improvement in the efficiency of utilisation of dietary energy.

Shorter rumen MRT often improves synthetic efficiency of ruminal bacteria (Kennedy and Milligan 1978; Mathison *et al.* 1995; Li *et al.* 2000). Urine samples tested from the animals in this experiment pointed to an increase in MP outflow due to exposure to cold ambient temperatures. Meng *et al.* (1999) recorded similar results *in vitro* by increasing dilution rates. They found that by increasing dilution rates, representing a faster ROF of digesta from the

rumen or shorter rumen MRT, both microbial growth and efficiency of microbial growth increased.

With an increase in microbial growth and reduced OM and DM digestion in the rumen due to cold ambient temperatures (Li *et al.* 2000), increased microbial efficiency would be expected. Microbial growth efficiency is the grams of microbial N produced for each kg of organic matter digested. Meng *et al.* (1999) proposed three possible reasons for the changes in microbial growth and efficiency. First, a high dilution rate would select for faster growing microbial species. Secondly, as ROF increases more microbial flora would be in the exponential growth phase, reducing the requirement for energy and N to be used for microbial maintenance and more for growth. Thirdly, they proposed that increased ROF would result in a depression of the protozoal population, reducing predation of bacteria by protozoa. Total protozoal numbers for this experiment were reduced by treatment, primarily due to a smaller population of small entodiniomorphs present in ruminal fluid suggesting that the third theory offered by Meng *et al.* (1999) may be operational (protozoa abundance).

Changing the microbial population in the rumen would be reflected not only in changed MY and microbial N outflow, but also in VFA concentrations. Total VFA concentrations in the rumen increased at the colder temperature, primarily due to an increase in acetate. The increase in VFA concentrations may be affected by a possible decrease in ruminal volume, especially with the reduced amount of water intake during treatment. Degen and Young (1980) reported a reduction in sheep RR fluid volume when exposed to cold ambient temperatures. Decreased water intake in sheep when exposed to cold temperatures and reductions in rumen temperatures have been reported in other studies (Westra and Christopherson 1976; Bewley *et al.* 2008). Alternatively, changes in the microbial populations within the rumen due to modified MRT could explain the increase in VFA concentrations, particularly acetate, with decreased MY. Nollet *et al.* (1998) demonstrated that the addition of *Lactobacillus plantarum* 80 supernatant and *Peptostreptococcus*

productus ATCC35244 to ruminal fluid *in vivo* and *in vitro* significantly increases acetate and total VFA concentrations in the rumen while reducing CH₄ emissions by 18 to 30% (Nollet *et al.* 1998). Romero-Pérez *et al.* (2011) alternatively found that cattle exposed to extreme cold ambient temperatures (-4.6 to -30°C) had a reduction in *Lactobacillus* numbers. Examination of microbial population changes reported by Romero-Pérez *et al.* (2011) though show fluctuations in microbial population numbers instead of a steady decline and, at some colder temperatures, greater microbial numbers than at the warmest temperature.

Changes to the microbial population within the rumen would also impact on the level of interspecies hydrogen transfer occurring between the H₂ – producers and H₂ – utilisers. As acetate is a major precursor in the formation of formate, an essential component in CH₄ production within the rumen, any increase in acetate concentrations would therefore result in increases in CH₄ emissions. This was not the case for this study even though acetate concentrations increased by 14% when animals were exposed to reduced ambient temperatures. This suggests that a possible threshold or limitation may be occurring; restricting the activities of the methanogens even though conditions would be more conducive to CH₄ production. Zhou *et al.* (2012) noted that the addition of fumarate, an alternative electron acceptor in the rumen (Morgavi *et al.* 2010), to a sheep diet significantly reduced methanogen and protozoa numbers in the rumen fluid even though acetate and total VFA concentrations increased. Zhou *et al.* (2012) also found that modifying the ruminal conditions through the addition of fumarate improved rumen fermentation in sheep fed high-forage diets as suggested by the increased total VFA concentrations, similar to this study. As formate oxidation requires efficient interspecies hydrogen transfer (Schink 1997), it is suggested that a close physical contact between formate oxidising bacteria (syntrophs) and methanogenic archaea is considered indispensable for efficient VFA oxidation (De Bok *et al.* 2004). Coaggregation of H₂ – producers and H₂ – utilisers has been demonstrated to facilitate interspecies hydrogen transfer (Ishii *et al.* 2005). Disruption of this coaggregation

could result in decreased CH₄ production as alternative electron acceptors are utilised in the rumen. This requires further investigation.

An increase in microbial proliferation and an increased ROF from the rumen to the intestines would allow a greater supply of amino acids for absorption from the alimentary tract, a major limiting factor in wool production (Hogan and Weston 1967). Increasing microbial growth and efficiency due to exposure to cold temperatures results in increased amino acid flow to the SI (Christopherson and Kennedy 1983). An increased ROF from the rumen also allows a greater proportion of undegraded dietary protein and non-ammonia N to pass through to the SI (Hogan and Weston 1967). Hogan and Weston (1967) also noted that sheep fed diets containing either a high or low crude protein content were able to produce similar masses of wool by varying their ruminal digesta kinetics. In agreement with the statement, our study showed sheep in cold conditions had reduced ruminal MRT and protozoal abundance while increasing MP N outflow from the rumen. A result of this was a significant increase in wool growth ($P < 0.01$). Smuts *et al.* (1995) found that not only was retention time of digesta in the rumen negatively correlated with wool growth, it is a repeatable characteristic which is reasonably heritable ($h^2 = 0.45$). Peripheral blood flow also increases due to cold temperature exposure (Bell *et al.* 1976; Will *et al.* 1978). An increase in blood flow to the wool follicles carrying increased quantities of amino acids would increase wool follicle growth. Therefore, chronic exposure to cold ambient temperatures does appear to significantly increase wool production but, whether the level of increased growth measured in this experiment could be maintained continuously requires further investigation. If similar levels of wool growth due to modified digesta kinetics could be maintained, this would be a great financial benefit for the producer.

6.6 **Conclusions**

This study has shown that exposure of sheep to cold ambient temperatures can cause a physiological modification to digesta MRT, particularly in the rumen, leading to a reduction in total MRT. The cold exposure also caused an elevation of the thyroid hormone T_3 , a known endocrine involved in MRT modification associated with metabolism, similar to previous studies (Westra and Christopherson 1976; Kennedy *et al.* 1977; Nazifi *et al.* 2003). Therefore, T_3 is an influential hormone concerning digesta kinetics and further studies are required to determine the extent of association T_3 has with digesta MRT and MY (Chapter 7).

This experiment confirms the findings of experiment 1 that the influence of physical factors on MRT (e.g. particle size) can be moderated by animal hormonal factors. Okine *et al.* (1998) suggested that ruminants can influence reticular contraction characteristics and duration of ROO quiescence in response to various stimuli including diet and physiological demands for optimal transfer efficiency of digesta from the rumen. Goopy *et al.* (unpublished) suggested naturally divergent sheep demonstrating variations in digesta MRT without affecting DMD could yield a more efficient digestive system by increasing the production and outflow of MP. MP outflow from the rumen in our experiment did increase suggesting treatment induced a more efficient digestive system. Coupled with no change in DMD, increased VFA concentrations in the rumen, reduced digesta MRT, and increased wool growth, physiological modifications to the ruminant digestive tract as a result of environmental influences may produce a more efficient and advantageous digestion system. Understanding the key mechanisms behind digesta MRT could lead to physiological markers for indirect genetic selection for reduced MY while sustaining or even improving productivity. This area requires further investigation.

Chapter 7: *Triiodothyronine May be a Useful Predictor of Methane Yield in Sheep*

7.1 Introduction

Discovering and developing processes which reduce enteric methane production without compromising animal productivity has become critical for sustainable livestock production. There is an increase in the understanding of the role hormonal and neural systems play in the control of digesta kinetics (Onaga *et al.* 2011) with Sections 2.4 and 2.5 demonstrating a strong hormonal link of gut kinetics to MY. With the discovery of hormones which regulate digesta MRT including CCK, SRIF, and serotonin; it is realised the impact of physical properties of the diet on digesta MRT (e.g. digestibility and particle size) can be modified by the physiological and metabolic state of the animal (Kennedy *et al.* 1977; Zaenuri and Godwin 1997; Okine *et al.* 1998; Barnett *et al.* 2012). This means that hormones associated with regulating gut kinetics can explain or modify some of the nutritional impacts on MRT. Understanding this, it may be possible to develop indirect genetic selection tools for reduced MY based on the detectable levels of hormones involved in regulating digesta MRT.

T₃ is a hormone known to be involved in digesta MRT modification through metabolism (Kennedy *et al.* 1977; Barnett *et al.* 2012). Experiment I (Chapter 5) demonstrated that the administration of exogenous T₃, within physiological boundaries, induced a reduction in digesta MRT leading to a decrease in MY. Experiment II (Chapter 6) showed that exposing sheep to low ambient temperatures resulted in an elevation in plasma T₃ concentrations and a reduction in digesta MRT and MY. This confirms that changes in plasma T₃ concentrations are associated with changes in digesta MRT and MY. What is uncertain though, is whether T₃ asserts a direct influence on MY within the RR and if plasma T₃ concentrations are useful indicators of MY. The purpose of this assessment was to determine if plasma T₃

concentrations are useful predictors of MY in sheep and, therefore, have the potential to be used as indirect animal genetic selection tools for MY.

7.2 *Materials & methods*

7.2.1 *Animals, facilities & feeding*

Blood samples were collected from 52 Merino or Border Leicester crossbred sheep from four different experiments to determine plasma concentrations of FT₃ and Total T₃. These experiments are summarised below. MY from all animals was also measured in these studies to ascertain whether T₃ potentially could be used as a predictor of MY.

Thesis Experiment I

Ten Merino wethers were housed in individual metabolism crates and fed a diet of 50% lucerne/50% oaten chaff continuously (1/12th every two hours) at a daily rate of 1.2 x maintenance (Chapter 5). Only data collected (blood samples taken and MY measured) when each animal was a control were included in analysis in this chapter.

Thesis Experiment II

Six Merino wethers were housed in individual metabolism crates and fed a diet of 50% lucerne/50% oaten chaff continuously (1/12th every two hours) at a rate of 1.35 x maintenance (Chapter 6). Treatment involved exposing each animal to low ambient temperatures to induce a natural change in T₃ plasma concentrations. As the plasma T₃ concentrations were naturally induced and not a result of exogenous administration, all measurements obtained during exposure to cold and warm temperatures were used in this chapter.

Department of Primary Industries NSW (DPI) Sheep

One hundred and sixty Merino and Border Leicester crossbred ewes being part of the SheepGENOMICS flock were measured for MY by open circuit calorimetry (Section 3.2). Twenty of these animals were selected for further analysis on the basis of displaying either high (MY >1 sd above mean) or low (MY >1 sd below mean) MY. The animals were housed in individual metabolism crates and fed a diet of 50% lucerne/50% oaten chaff once daily (0800 h) at a rate of 1.2 x maintenance (Experiment designed and managed by J. Goopy (DPI) who made data available. Blood samples from the 20 high or low MY sheep were collected and analysed by M. Barnett).

After two weeks adaptation, MY for each of the animals was measured (Section 3.2). This was followed immediately by sampling of rumen fluid and the commencement of a 6 day total collection of faeces and urine, with another period of methane measurements at the close of the study.

University of New England (UNE) Sheep

Twenty Merino wethers from the Trangie Agriculture Research Centre, Trangie NSW were selected for either high or low fleece weight, based on estimated breeding values (Sheep Genetics; University of New England, Armidale). The animals were housed in individual metabolism crates and fed a diet of 50% lucerne/50% oaten chaff once daily (0900 h) at a rate of either 1.0 kg (n=10) or 1.5 kg DM (n=10). One animal was injured during the adaptation period (2 weeks prior to measurements) and three others had daily feed refusals in excess of 20% of that offered. These animals were removed from the experiment and had no measurements taken. Measurements were taken from the remaining 16 animals (Experiment designed and managed by I. de Barbeiri. Blood samples collected and analysed by M. Barnett).

In a similar manner to the DPI sheep, UNE sheep were adapted to feed and surroundings over 2 weeks then each animal's MY was measured twice (Section 3.2), followed immediately by sampling of rumen fluid then a 6 day total collection of faecal and urine output, and another period of replicated methane measurements .

7.2.2 Blood samples and analysis

Blood samples (10mL) were taken from each animal once, at the completion of each associated experiment using sodium heparin vacutainers (BD Vacutainer, USA) and centrifuged at 1400 g for 15 mins (Beckman TJ-6 tabletop centrifuge) to separate plasma. Plasma from each animal was aliquoted into two 5mL screw top plastic vials and stored at -20°C while awaiting analysis.

Total and FT₃ concentration present in the plasma were analysed using a (Human) competitive sandwich enzyme immunoassay ELISA with colourimetric detection (Abnova Corporation, Taiwan; Total T₃ Ref No. KA0198, Lot No. RN-44172; FT₃ Ref No. KA0199, Lot No. RN-44291) at an absorbance of 450nm with a sensitivity of 0.05pg/mL.

7.2.3 Calculating methane yield

Enteric CH₄ emissions and CO₂ production rates for each animal were measured over a 22h period using open circuit respiration chambers (Section 3.2). This procedure was repeated four times per animal during the DPI experiment and eight times during the UNE experiment. In the sheep studies, these collections occurred immediately prior to and after the 6 d total collection required for measurement of digesta kinetics.

7.3 Statistical methods

A regression analysis using Minitab (v15.1.30.0; Minitab Inc., USA) was used to assess relationships between plasma T₃ concentrations and measurements of MY and digesta MRT in ruminants. All sheep measurements were pooled together to provide regression analysis. The coefficient of determination, r², was presented as a value between 0 and 1 with regression analysis indicating the relationship between MY and thyroid hormones, measured by ANOVA, presented as significant when P≤0.05 and as a trend when P≤0.10. Residual plots were used to assess the quality of a regression and a Ryan-Joiner test (similar to Wilk-Shapiro W-test) in Minitab was used for testing normality with P-value greater than 0.10 providing no evidence against the normality hypothesis and a P-value less than 0.01 indicating the residuals do not follow a normal distribution.

7.4 Results

A total of 58 sheep MY and blood samples were compared using regression analysis. Plasma FT₃ concentrations were found to have a slight linear association with MY, accounting for 16% of the variance (MY = 24.1 - 0.765 FT₃; r²=0.16, P-value<0.01; Fig 7 - 1) with normal probability of standardised residuals being linear and the scatter of standardised MY residuals against plasma FT₃ concentrations being randomly distributed around zero. A Ryan-Joiner test gave a P-value greater than 0.10 providing no evidence against the normality hypothesis (Fig 7 - 2).

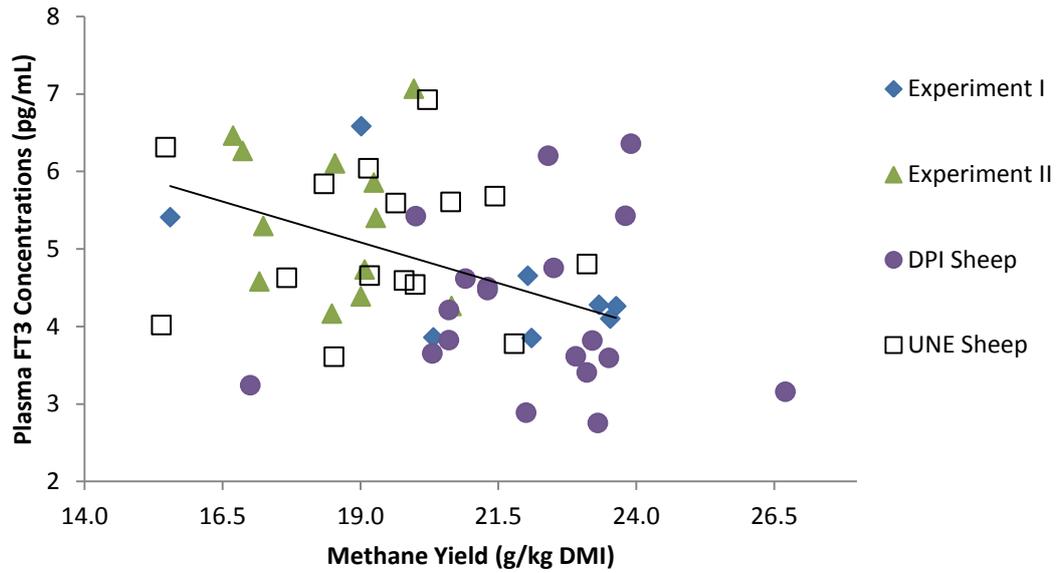


Fig 7 - 1 Relationship between methane yield (g/kg DMI) and plasma free T₃ concentrations (pg/mL) in sheep. Data are pooled results from 4 different sheep experiments conducted between 2010 and 2012

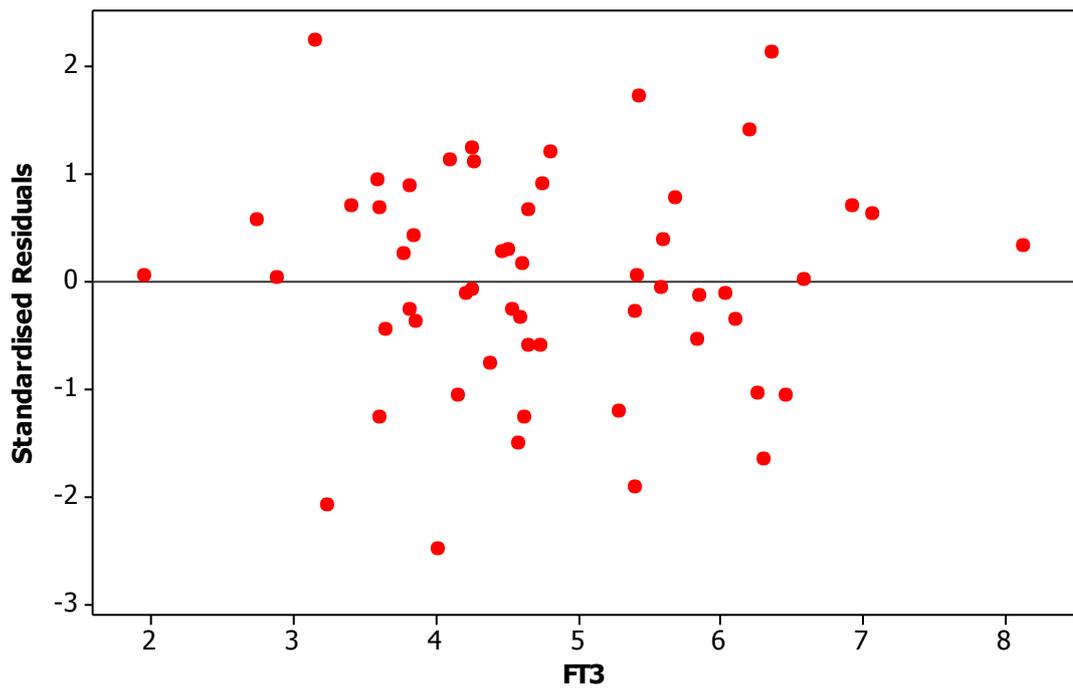


Fig 7 - 2 Plot of methane yield standardised residuals distribution against plasma free T₃ concentrations (pg/mL) in sheep

Plasma total T₃ concentrations (ng/mL) were regressed against sheep MY (g/kg DMI) and found to also show a weak linear association, accounting for 13% of variance (MY = 22.2 - 0.818 Total T₃; r² = 0.13, P-value<0.01; Fig 7 - 3). Normal probability of standardised residuals were found to be not linear and the scatter of standardised MY residuals against plasma total T₃ concentrations showed a decreasing trend. A Ryan-Joiner test gave a P-value less than 0.01, providing further evidence the residuals do not follow a normal distribution.

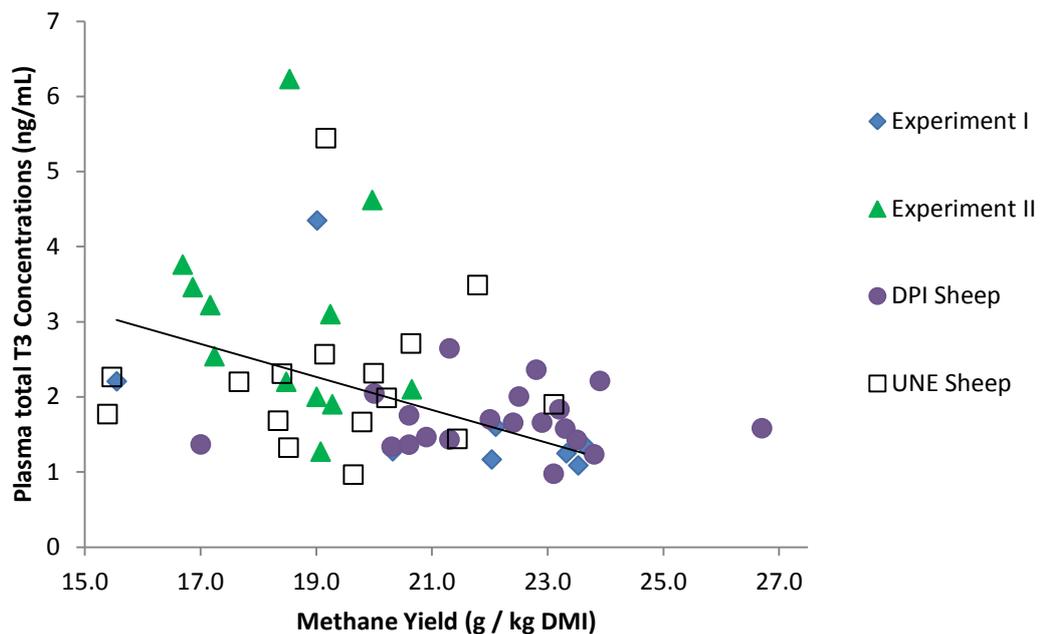


Fig 7 - 3 Relationship between methane yield (g/kg DMI) and plasma total T₃ concentrations (ng/mL) in sheep

7.5 Discussion

Sustainable livestock production requires the development of processes which reduce enteric methane production without compromising animal productivity. Methane produced by ruminants is not only a GHG but is also energy lost from production purposes (IPCC 2007; Lassey 2008). The role that hormonal and neural systems play in the control of digesta kinetics in ruminants is becoming increasingly understood (Onaga *et al.* 2011) with digesta

MRT now positively linked to MY (Pinares-Patiño *et al.* 2003). Significant correlations have been observed between plasma concentrations of the endocrine regulator T_3 , at physiological levels, and the MRT of digesta and MY in ruminants (Barnett *et al.* 2012). This study demonstrated a link between MY and circulating T_3 concentration does exist for sheep, with FT_3 accounting for 16% of variability in sheep MY. While the proportion of variation explained is small, only a limited number of animals were available for analysis in this study (52 sheep). Also, the relationship between sheep FT_3 and MY gave a P-value of less than 0.01 indicating the relationship is highly significant while the Ryan-Joiner test provided no evidence against normality of the residuals. Total T_3 also demonstrated similar suitability for being a predictor of MY in sheep by accounting for 13% of variability and also giving a P-value for regression analysis less than 0.01, although the Ryan-Joiner test was significant indicating possible error in residual normality.

Differences in feeding level and pattern, and the ambient temperature occurring between the four experiments may account for some of the error and improve the regression model between T_3 and MY in sheep. Diet between experiments, while similar in type (1:1 ratio wheaten/lucerne chaff), were given to the animals in different quantities. Animals in experiment I and DPI sheep were fed at 1.2 x maintenance while those in experiment II were fed at 1.35 x maintenance. UNE sheep received either 1.0 kg or 1.5 kg as fed. Ambient temperature also may have contributed to variation in the model as both experiment I and II sheep were housed in temperature controlled environments but DPI and UNE sheep were not. Seasonality may have also impacted on the model as DPI and UNE sheep were measured for MY at different times of the year compared to the other sheep. To improve the model, it would be necessary to incorporate diet, temperature and seasonal variations in T_3 concentrations as an additional source of variation.

7.6 Conclusion

Okine *et al.* (1998) suggested that not just the physical properties of feed but also the physiological and metabolic state of the animal determines the MRT of digesta. Digesta MRT has been positively linked to MY (Pinares-Patiño *et al.* 2003) and heritability for MY has recently been demonstrated through investigations into physiological variances between-animals (Pinares-Patiño *et al.* 2011). T_3 being a naturally derived hormone which is associated with changes in digesta MRT and MY (Kennedy *et al.* 1977; Barnett *et al.* 2012) potentially could be useful as an indicator of enteric methane emissions from ruminants. While the cohort on animals used in this study was limited (52 sheep) and diet and seasonal factors may have also modified T_3 levels and MRT independently, there is enough indication to suggest T_3 , in particular FT_3 , may have potential as a predictor of MY in sheep. Further investigation of any association between T_3 and MY would require a much larger number of animals to be measured but should this association be validated, indirect genetic selection for reduced MY based on measured T_3 concentration may be possible.

Chapter 8: *General Discussion*

Agriculture is a major contributor to GHG, accounting for approximately two-thirds of global anthropogenic CH₄ sources (IPCC 2007). A by-product of the fermentative process of digestion predominating in ruminants, enteric CH₄ has a global warming potential 25 times CO₂ (IPCC 2001). Mitigation of enteric methane emitted from ruminant livestock is being pursued through modification of diet composition and quality, dietary supplements, utilising plant secondary compounds, and employing biological or chemical methane-inhibiting additives to kill or reduce the activity of CH₄ producing Archaea (Beauchemin *et al.* 2008; Morgavi *et al.* 2010; Nolan *et al.* 2010; Buddle *et al.* 2011; Tan *et al.* 2011; Lin *et al.* 2013). Discovering and developing systems which reliably reduce enteric methane emissions without compromising animal productivity has become critical in attempts to mitigate agricultural livestock emissions intensity. Emissions intensity (EI) is the amount of CH₄ emissions produced per unit animal product or profit (Hegarty and McEwan 2010). Both measuring and mitigating emissions from grazing ruminants is difficult, so discovering a proxy for MY would enable animals to be phenotyped and selected for the MY trait.

8.1 *Digesta MRT influences ruminant MY*

The length of time digesta is retained within the digestive tract of the ruminant, particularly the RR, is a key critical control point regulating enteric methane production (Pinares-Patiño *et al.* 2007). Pinares-Patiño *et al.* (2003) found that sheep with longer rumen MRT had larger rumen fills, higher fibre digestibilities, and greater MY. The MRT of DM determines the length of time digesta is exposed to the microbial fermentative processes within the RR which leads to methane production. Repeatability of the relationship between digesta MRT and enteric MY in ruminants has been established, allowing genetic selection of animals for reduced MY (Pinares-Patiño *et al.* 2011).

Since the work of Freer and Campling (1963) it has been considered that maximising rumen fill regulated digesta ROF from the RR and that the physical properties of the diet were the main factor determining digesta MRT (Freer and Campling 1963; Hungate 1966). This hypothesis on regulation of digesta passage has since been modified to also include the physiological and metabolic state of the animal as major factors determining digesta kinetics, with these new additions able to moderate the physical limitations of the diet (Kennedy *et al.* 1977; Zaenuri and Godwin 1997; Okine *et al.* 1998; Oh-ishi *et al.* 2013). Little was known of the between-animal variations in physiological factors which may regulate digesta MRT, leading us to investigate the extent to which digesta kinetics influences the MY of sheep.

When sheep received injections of T_3 every second day (Chapter 5), reductions in MRT (9%) and MY (14%) occurred. This was in contrast to when sheep were injected daily with T_3 , elevating their plasma T_3 concentrations to pharmacologic levels which caused no changes to either digesta MRT or MY. Therefore, it was suggested that thyroid hormones were effective in reducing digesta MRT, resulting in a reduction in enteric MY, but only if plasma concentrations of T_3 were within the normal physiological range experienced by the animal. In Chapter 6, ambient temperature was used to naturally induce a physiological change in thyroid hormone concentrations. Thyroid hormones have an inverse relationship with ambient temperature, with rises in temperature causing decreases in plasma T_3 concentrations while a fall in ambient temperature results in increased plasma T_3 levels (Todini 2007). Our animals, when exposed to cold ambient temperatures, experienced increased plasma T_3 levels in agreement with published literature (Hocquette *et al.* 1992; Morris *et al.* 2000; Todini 2007). Digesta MRT was reduced (9%) and MY decreased significantly (8%), consistent with the hypothesis that reducing digesta MRT in the GIT reduces ruminant MY.

It is important that alterations to digesta MRT regulators are made within the animal's normal physiological ranges, as exceeding these levels can have neutral or negative effects on MRT and MY. Excessive plasma concentrations of T_3 at pharmacologic levels are known to induce a negative feed-back loop to the hypothalamus and pituitary gland, stopping production of T_3 and initiating the release of digesta kinetic inhibitory factors like somatostatin to reinstate homeostasis (Utiger 1995). This negative feed-back loop due to plasma suprasaturation of thyroid hormones could be the reason why daily injections of T_3 resulted in no change to MRT and MY, while injections every second day (Chapter 5) and sheep exposed to cold temperatures (Chapter 6) only elevated plasma T_3 concentrations within physiological limits and decreased MRT and MY. This is an important consideration should the notion of developing hormonal vaccines or feed additives to affect thyroid activity be considered.

8.2 *Production advantages of reduced MY*

In excess of 8% of digested energy for ruminants is lost as eructated CH_4 (Lassey 2008). This energy loss is a loss of potential productivity, a key economic driver for change in the agricultural industry. To divert a portion of this methane energy into productivity, be it weight gain, muscling, fertility, milk production, wool growth, etc, would be highly advantageous to producers. This would result in a reduction in the EI for an agricultural production enterprise. Increasing productivity reduces EI by diverting a greater portion of the feed energy obtained from the animal's diet for production purposes, thereby decreasing the amount of enteric methane produced per unit of productivity (Chagunda *et al.* 2009). Nutrition and management can aid in reducing EI by improving ovulation rate, faster growth rates, earlier joining, delayed culling, and removal of less productive animals to name a few (Cruickshank *et al.* 2009; Hunter and Niethe 2009; Hegarty and McEwan 2010). Genetic selection can also reduce EI indirectly through greater live weight gain, milk production and feed efficiency (Hegarty *et al.* 2007; Hunter and Niethe 2009; Hegarty and McEwan 2010; Wall *et al.* 2010). In wool production, Smuts *et al.* (1995) found that short digesta MRT greatly increased the

probability of obtaining a high wool growth rate. Therefore, increasing productivity and/or reducing MY by reducing digesta MRT would decrease the EI of a wool producing enterprise, providing an economically viable base for reductions in enteric methane emissions.

When sheep were exposed to cold temperatures (Chapter 6), microbial protein outflow increased and total ruminal protozoa numbers reduced in association with reduced digesta MRT. This could be due to increased digesta ROF from the RR resulting in increased microbial growth and efficiency of microbial growth with a depression of protozoan population (Kennedy and Milligan 1978; Mathison *et al.* 1995; Li *et al.* 2000). Reduced numbers of protozoa in the RR would result in reduced predation of bacteria by the protozoa, allowing an increase in bacterial numbers (Meng *et al.* 1999). An increase in microbial output and increased digesta ROF from the RR to the intestines would provide the animal with a greater supply of amino acids for absorption from the alimentary tract, a major limiting factor in wool production (Hogan and Weston 1967). Daily wool growth in Chapter 6 increased due to cold treatment by almost 30%, suggesting this potential increased supply of sulphur amino acids may have occurred. Past studies have shown that feed consumed by ruminants exposed to cold ambient temperatures is digested to a reduced extent due to decreased retention time in the rumen (Westra and Christopherson 1976; Li *et al.* 2000). Our studies (Chapter 5 and 6) showed no change in dry matter digestibility which may be due to increased digestion in the SI to compensate for reduced OM digestion in the RR (Kelly and Christopherson 1989; Okine *et al.* 1989; Lourenço *et al.* 2010). Therefore, increased wool growth and ruminal microbial protein outflow with decreased protozoa abundance and no change in DMD suggests an increase in productivity in coordination with a decrease in MY, result being a reduction in EI. In Chapter 6, each sheep in warm conditions (mean weight 43.5kg) received 1.35 times maintenance (mean daily ration of 1200g) and produced a mean MY of 19.3 g CH₄/kg DMI. This equates to 8.4kg CH₄ emitted for approximately 5kg wool

produced or an EI of 1.7 kg CH₄/1.0 kg wool. An 8% reduction in MY coupled with a 30% increase in wool growth in cold conditions, as seen in experiment II, reduces the EI to 1.2 kg CH₄/1.0 kg wool (Fig 8 - 1). Should it be possible to maintain the reduction in digesta MRT with sustained DMD and increased wool growth, an increase in wool growth with a reduction in MY would provide a significant decrease in the EI of a wool production system.

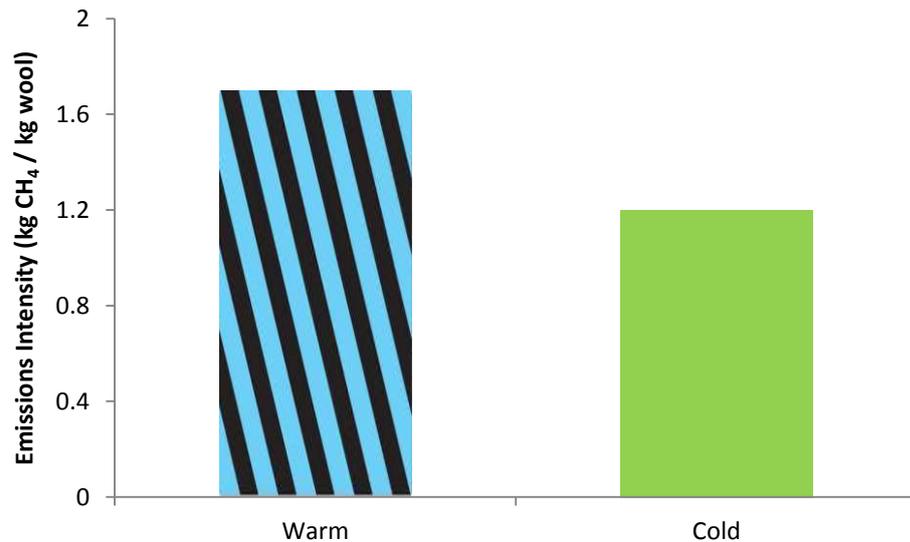


Fig 8 - 1 Estimated change in emissions intensity for a wool production system associated with a 30% increase in wool growth and an 8% reduction in MY resulting from cold ambient conditions

8.3 ***Thyroid hormones as predictors of MY***

Neural and humoral receptors in the epithelial lining of the RR, ROO, and omasum indicate physiological regulators impact on ruminal digestive kinetics. Okine *et al.* (1998) suggested digesta MRT was not only determined by the physical properties of the feed but also the animal's physiological and metabolic state. The thyroid hormone T₃ has been shown to be associated with changes in digesta MRT and MY of ruminants. Thyroid hormones are known to influence digesta kinetics by modifying the animal's metabolic state (Todini 2007) and have been shown in this thesis to modify digesta MRT and MY. What is uncertain though is whether T₃ asserts a direct influence on MRT and MY, and if plasma T₃ concentrations can

be useful predictors of MY. It is important for the agricultural industry to develop low cost and accurate measures of or proxies for MY that are effective predictors of MY and simple to utilise. One of the aims of this thesis was to determine if plasma thyroid hormone concentrations were useful predictors of MY in ruminants and, therefore, have potential application as indirect genetic selection tools.

Chapters 5 and 6 demonstrated that physiological mechanisms are capable of modifying digesta kinetics and confirm that the positive association between digesta MRT and MY as suggested by Pinares-Patiño *et al.* (2003) exists. In Chapter 7, FT₃ showed potential as a possible predictor of MY in sheep after testing the association between its plasma concentrations and MY by regression analysis. FT₃ accounted for 16% of variability in sheep MY, with the relationship between sheep FT₃ and MY deemed significant. While the cohort of animals used was small, there is enough indication to suggest T₃, in particular FT₃, may be useful as an indirect genetic selection tool for reducing MY in sheep. This further emphasises the need to understand the key mechanisms regulating digesta MRT, enabling development of physiological markers with potentially greater covariance than T₃. If heritable themselves, these physiological markers could be used for indirect selection to reduce ruminant MY while sustaining, or even improving, productivity.

8.4 *Other regulatory hormones with potential as MY predictors*

Thyroid hormones, particularly FT₃ have shown potential as proxies for MY to allow animal genetic selection for MY but the correlation between T₃ and MY currently is limited. Kennedy *et al.* (1977) suggested that changes in ruminant digestive function due to cold exposure are not only associated with increases in plasma thyroid hormone concentrations but alterations to other digesta kinetic regulators as well. Immunocytochemical studies have revealed the presence of numerous endocrine and neural cells distributed throughout the

epithelium and glands of the GIT which are known to influence digesta kinetics (Calingasan *et al.* 1984; Kitamura *et al.* 1985; Mimoda *et al.* 1998; Yonekura *et al.* 2002; Münnich *et al.* 2008; Onaga *et al.* 2008). It is possible that one or more of these hormonal regulators may provide a substantive phenotypic proxy for MY, allowing the development of economically viable, robust, and repeatable methods for reducing CH₄ emissions from agricultural ruminants.

Hormonal regulators of digesta kinetics have specific functions for governing digesta MRT, either amplifying or dampening smooth muscle contraction, but are also involved in a complicated system of interaction and regulation of other regulatory hormones. Few regulatory hormones appear capable of increasing amplitude and frequency of contractions within the GIT (Okine *et al.* 1998; Onaga *et al.* 2011; Oh-ishi *et al.* 2013). The main function of most digesta MRT regulators is to decrease smooth muscle contraction, slowing down digesta ROF (Okine and Mathison 1996; Saras *et al.* 2007; Onaga *et al.* 2008). Many of the autocrines, paracrines, and endocrines involved in regulating digesta ROF, whether they be secreted locally in the gut or from the hypothalamic/pituitary axis, interact with others either synergistically to enhance an action or in an inhibitory fashion designed to suppress the release of other hormones (Lippl *et al.* 2004; Saras *et al.* 2007; Lemamy *et al.* 2012). An illustration of the complex series of interactions occurring between digesta kinetic regulatory hormones and other endocrine factors within the GIT and peripheral system during a period of increased digesta ROF has been proposed based on current understanding and is illustrated below (Fig 8 - 2).

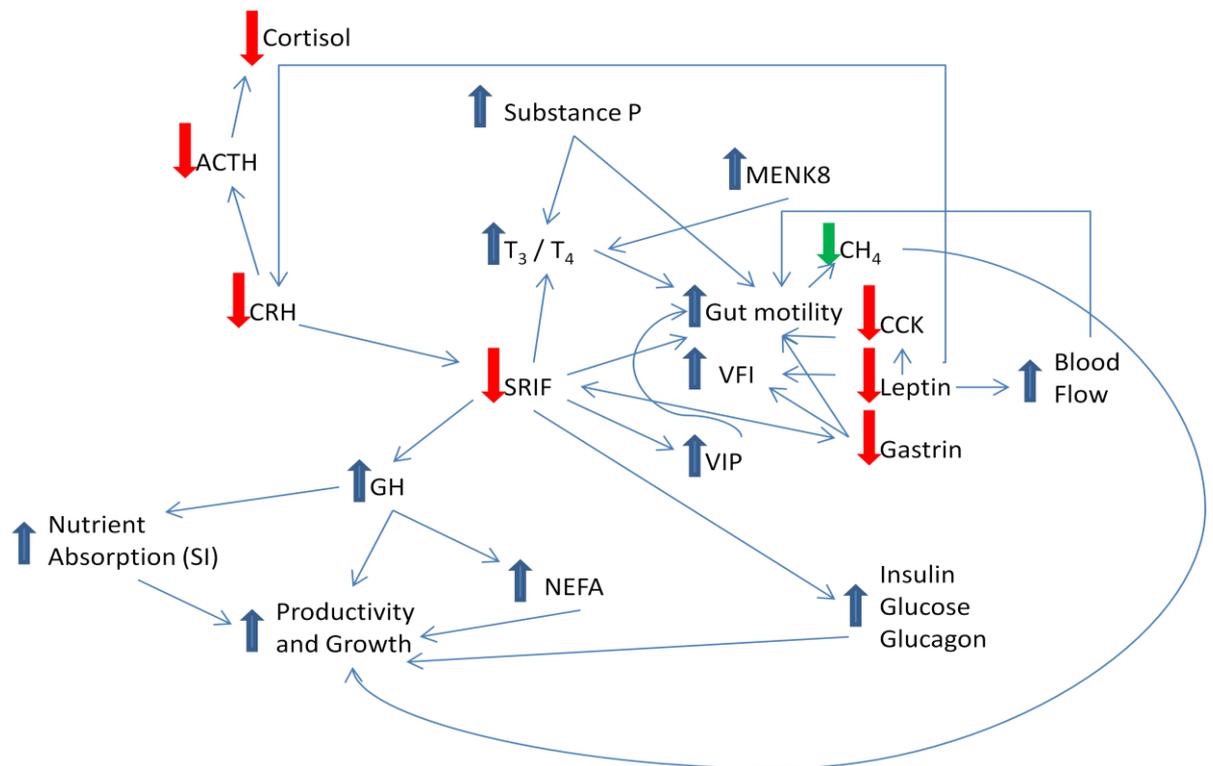


Fig 8 - 2 Schematic of proposed interactions and actions of digesta kinetic regulatory hormones and peripheral hormones to increase digesta ROF within the GIT leading to reduced MY (↓ indicates an induced concentration reduction; ↑ indicates an induced concentration increase; connecting arrows suggest proposed influence a hormone has on other regulatory hormones).

Most hormones associated with regulating digesta kinetics slow down gut motility, but a small number increase smooth muscle contraction in the GIT. Thyroid hormones increase contractions through changes in the animal's metabolic state but, whether they enact a direct influence on GIT muscle or affect a central neural influence on contractions is still uncertain. Two other hormones produced in the GIT which directly increase smooth muscle contraction are substance-P and motilin. Substance-P, a member of the tackykinin family, induces tonic (extended) contractions of RR, omasal, and abomasal muscle (Onaga *et al.* 2011; Oh-ishi *et al.* 2013), and prevent ghrelin's inhibitory actions on thyroid hormones (Khazali and Mahmoudi 2009). Elevated plasma concentrations of substance-P also increase the release of somatotropin (GH) and decreases somatostatin (SRIF) secretion (Lemamy *et al.* 2012). Motilin, a regulatory peptide primarily located in the wall of the duodenum and proximal

jejunum, is thought to be required to initiate the high-amplitude contractile phase III of the migrating myoelectric complex (MMC) in the small intestine (Yokohata and Tanaka 2000; Tanaka 2002).

A suite of regulatory hormones found within the GIT and summarised below, are designed to slow down the smooth muscle contraction of the RR, ROO, omasum, abomasum, and small intestine. VIP, a highly basic octacosapeptide, plays a major role in the relaxation of the omasal body, in particular the ROO, and can lower the amplitude of contractions in the RR (Okine and Mathison 1996; Onaga *et al.* 2009; Lalatta-Costerbosa *et al.* 2011) In contrast to other inhibitory hormones, VIP decreases digesta MRT in the RR by allowing an increased quantity of digesta to pass through the ROO into the omasum (Onaga *et al.* 2009). CCK, a peptide hormone secreted by the duodenal mucosa, is widely accepted as a major regulator of ruminal contractions (Baile *et al.* 1986; Onaga *et al.* 2008). CCK inhibits cyclic RR contractions when animals are consuming diets high in fat or experiencing intestinal acidification like acidosis (Bruce and Huber 1973; Grovum 1981; Ruckebusch 1983; Choi *et al.* 2000). Met-enkephalin (MENK8), a hypothalamic endogenous opioid peptide, also evokes a blockade of the cyclic contractions of the RR by acting as a Ca-channel blocker, causing the localised release of acetylcholine and specific noncholinergic compounds (Mitznegg *et al.* 1977; Dobbins *et al.* 1981; Ruckebusch *et al.* 1984; Kania and Domański 1996). MENK8 is also capable of slowing metabolism in some species, in contrast to the direct actions of T₃ (Krzymowski and Stefanczyk-Krzyszowska 2012). Gastrin, a GIT regulatory peptide, is capable of inhibiting VFI, reducing frequency of reticular contractions, inhibiting abomasal emptying, and seriously affecting the normal MMC (Grovum 1981; Gregory *et al.* 1985; Grovum 1986; Parkins and Holmes 1989). Elevated levels of gastrin also inhibit ghrelin secretion, affecting VFI (Lippl *et al.* 2004).

One of the more broad-acting and potentially desirable smooth muscle controlling factors to target in regulating digesta MRT is somatostatin (SRIF). A tetra-deca peptide hormone secreted in both the GIT and hypothalamus, SRIF is a potent inhibitor of peptide hormone secretion and smooth muscle contraction in the GIT (Saras *et al.* 2007). Potentially one of the most important digesta kinetics regulators, SRIF regulates GIT motility, peptide secretion, nutrient absorption, and metabolic hormones release (Christensen *et al.* 1990; Saras *et al.* 2007; Mushtaq and Cheema 2009; Chen *et al.* 2011; Jin *et al.* 2011). Elevated plasma concentrations of SRIF inhibit the release of numerous peripheral hormones including thyroid hormones, growth hormone, and ghrelin (Christensen *et al.* 1990; Sugino *et al.* 2004; Lemamy *et al.* 2012). As the primary role of SRIF is as an inhibitory peptide, reducing digesta ROF, digestion, absorption, growth and development; the active suppression of or genetic selection for reduced secretion of SRIF could prove highly beneficial in reducing MY and EI while improving productivity.

8.5 Conclusion

This thesis has demonstrated that (1) the evidence by Pinares-Patiño *et al.* (2003) that the retention time period of digesta in the RR is positively correlated to MY can be corroborated, and (2) Okine *et al.* (1998) was correct in suggesting that the physiological and metabolic states of the animal are as important in regulating digesta kinetics as are the physical properties of the diet. By inducing a change in the physiological and metabolic states of the animals in these experiments I was able to induce a small but significant change in their digesta kinetics. These reductions in digesta MRT led to significant reductions (14% and 8%) in MY without any variations to diet or intake. It should be noted though that each experiment was conducted utilising restricted feeding. Restricted feeding has been shown to impact on aspects such as MY, metabolism, and the physiological state of the animal (Zinn *et al.* 1995; Maekawa *et al.* 2002; Beauchemin *et al.* 2008). Future research needs to address any possible interaction effects which may occur from hormonal and feeding levels on MRT and MY.

I have also revealed a possible link between physiological regulations of digesta MRT and the essential ruminal interspecies hydrogen transfer between H₂ – producers and H₂ – utilisers. When I was able to maintain rumen MRT but decrease total MRT, acetate production decreased resulting in a lower MY; possibly through physiological limitations on the actions of the H₂ – producers. When digesta MRT was reduced in all areas of the GIT, acetate production increased but MY still decreased. This suggests another possible limitation on the interspecies hydrogen transfer actions in the rumen but, this time, most likely impacting on the H₂ – utilisers such as methanogens. This may be a result of disruptions to the coaggregations between producers and utilisers essential to facilitate interspecies hydrogen transfer.

My studies also identified the possibility plasma concentration of triiodothyronine, a key mechanism involved in regulating digesta kinetics, could have the potential to be a predictor of MY in sheep. Pinares-Patiño *et al.* (2011) showed that MY in sheep was a repeatable trait and that physiological variances between animals in digesta MRT potentially could provide an efficient, cost-effective, and sustainable mitigation option for methane production in agricultural livestock. While T₃ has shown some potential as a selection tool for reducing MRT, further understanding the key regulators of digesta kinetics and the complex interactions between them could lead to identifying superior physiological markers for indirect genetic selection of reduced MY with improved productivity.

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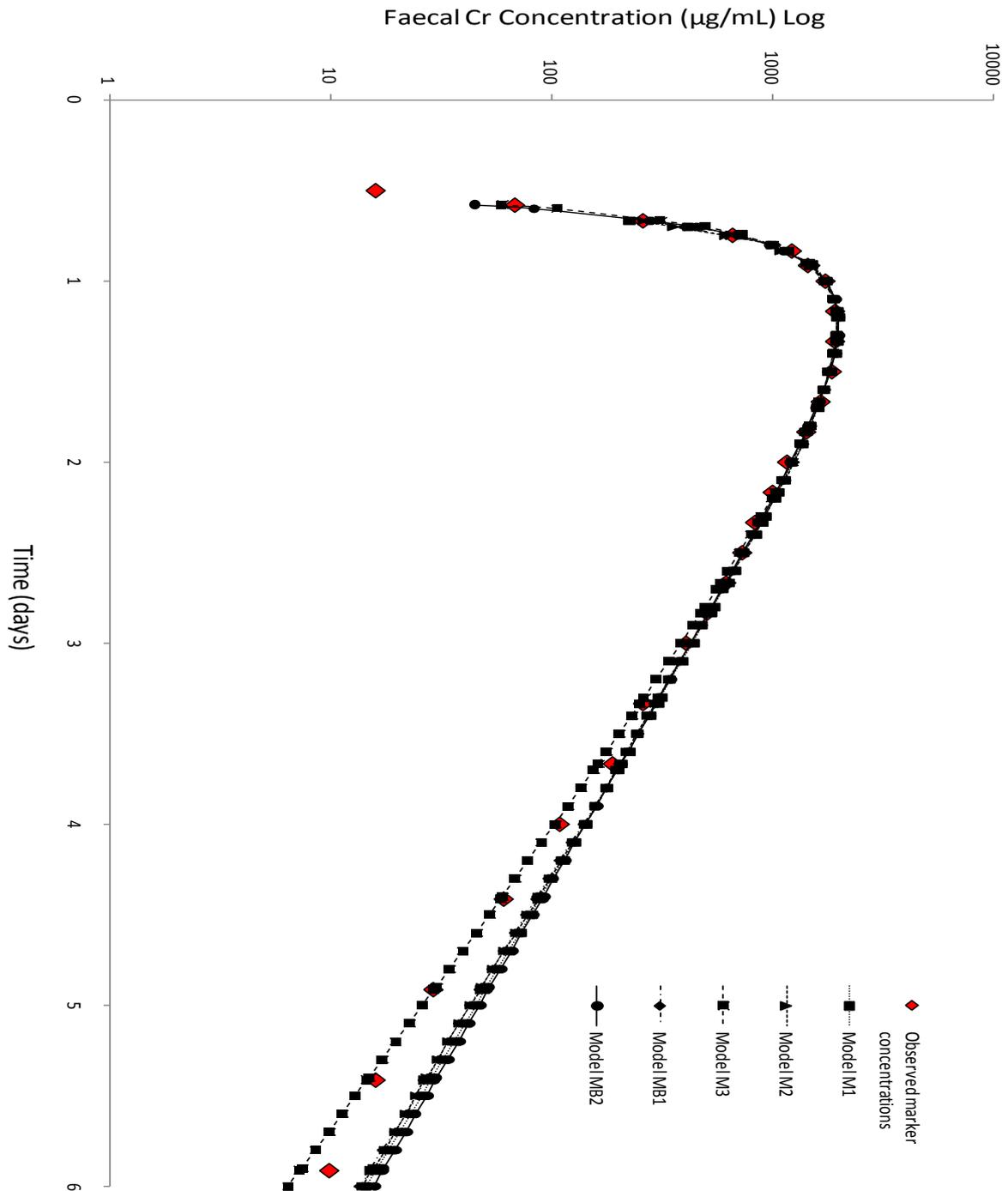
Appendix 3 - "Faecal Cr and Co concentrations for the six sheep used to develop new digesta kinetics model in chapter 4"

Collection Time (h)	<u>Faecal Cr Concentrations ($\mu\text{g/mL}$)</u>					
	Sheep ID					
	547	6012	6058	6131	6245	6506
8	0.0000	0.0000	0.0000	15.3246	0.0000	0.0000
10	0.0000	0.0000	0.0000	12.4888	0.0000	0.0000
12	5.6909	0.0000	0.0000	66.8872	0.9207	4.5869
14	58.2064	4.8625	0.0000	168.1337	18.3736	
16	188.8522	47.9056	13.7809	442.9324	90.1458	54.0780
18	932.9524	211.5780	161.3401	904.8114	311.0173	269.4816
20	1392.5213	458.7458	281.1929	1692.3430	844.2174	689.0130
22	1725.8220	834.3365	471.8961	2126.0389	1339.0814	1207.9369
24	2106.0334		730.5318	2471.7356	2036.0795	1336.7326
28	2193.1666	1405.0789	1019.3113	2349.6017	2185.0657	1687.9298
32	2277.8756	1650.6688	1312.4119	2303.9125	2339.4218	1857.2885
36	2182.9577	1901.1496	1475.5087	1929.0081	2119.6217	2143.2660
40	1843.8124	1836.6951	1636.1626	1558.0639	1952.4490	1972.5569
44	1524.4007	1822.5542	1417.6061	1658.4551	1744.0774	1816.3860
48	1261.1874	1600.1197	1369.4892	1202.2511	1222.6451	1538.2075
52	973.5225	1537.5011	1306.8709	1049.8447	1015.8486	1180.8971
56	859.0509	1294.8597	1210.4092	807.2885	843.7194	1057.5554
60	647.6035	1129.0978	1144.3750	571.6133	691.2432	889.4850
64	564.2929	1016.5113	1088.6493	397.5067	620.9119	678.3167
68	462.6868	869.7515	781.1337	338.3503	441.1667	586.0010
72	351.3074	752.8108	759.2362	256.1255	351.3249	505.3149
80	182.3842	467.6687	430.4578	153.0719	165.5493	310.7938
88	158.1056	376.3901	271.4109	82.5961	122.3507	211.3257
96	79.2762	315.3857	186.3776	32.3852		133.3009
106	45.7858	147.9172	110.2637	11.7941	34.8839	82.5209
118	23.7207		71.5725	9.4420	13.0647	32.1288
130	6.2242	53.5090	28.5812	6.1876	5.5452	18.3573
142	3.9900	27.4119	15.1361	1.3918	1.7703	6.6479

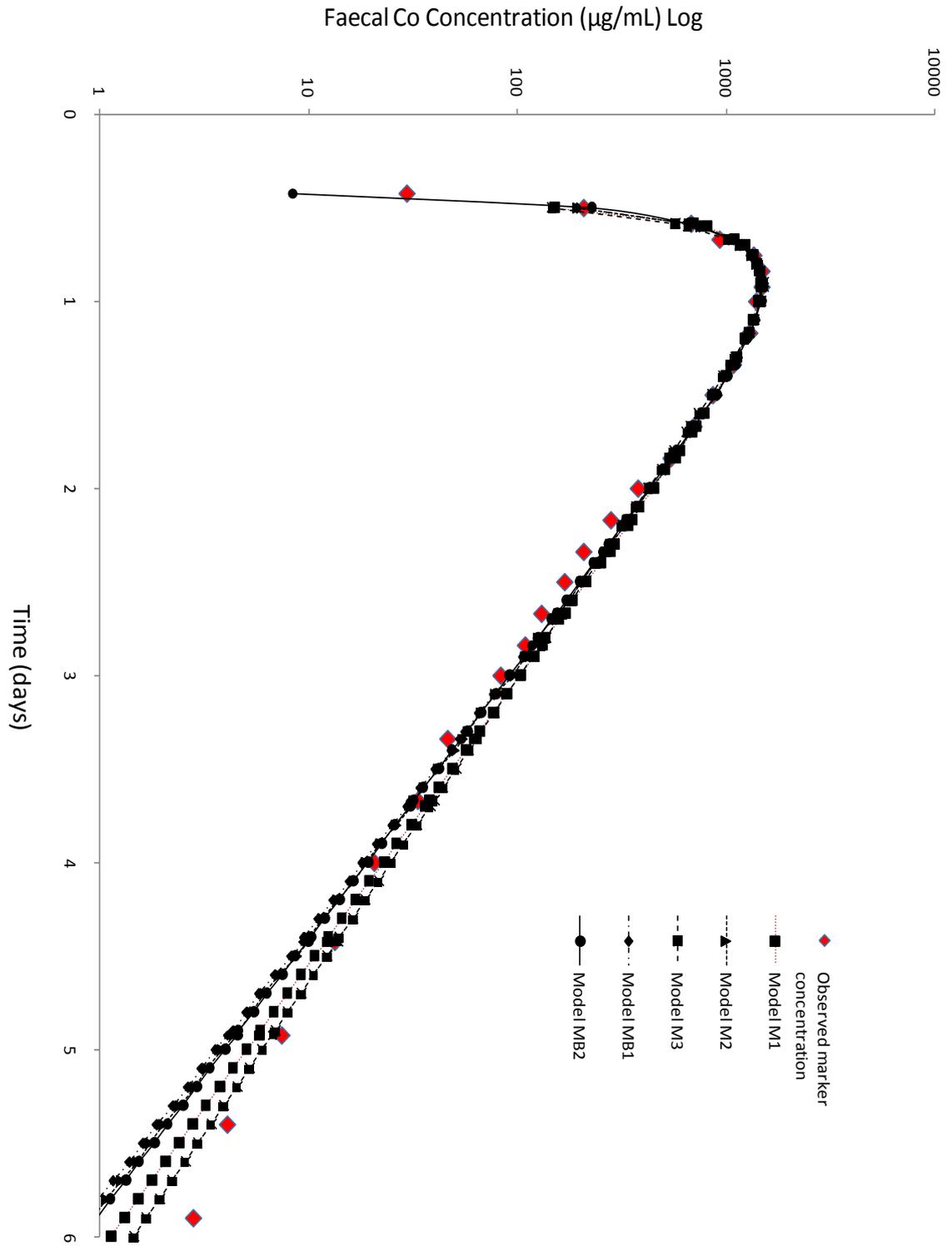
Collection Time (h)	Faecal Co Concentrations ($\mu\text{g}/\text{mL}$)					
	Sheep ID					
	547	6012	6058	6131	6245	6506
8	1.5047	0.0000	0.0000	0.1000	1.7782	9.2435
10	2.7188	0.0000	0.0000	7.0797	19.1423	15.6337
12	345.4967	0.2283	0.0000	416.1130	212.7787	356.3313
14	991.0640	399.9596	2.8442	1047.7189	361.1729	
16	1371.9678	1012.9151	481.4078	1191.5579	1046.2750	748.7570
18	1867.7673	1182.6136	1055.9320	1423.5651	1418.3495	1220.6224
20	1641.3714	1544.2831	1357.0028	1566.2915	1543.2971	1618.3020
22	1681.9544	1713.6083	1456.2117	1747.2015	1566.7036	1704.2708
24	1628.1449		1417.6279	1646.8109	1522.8994	1723.4672
28	1343.1982	1644.1883	1264.5757	1475.6944	1400.0761	1685.8833
32	1102.1332	1444.4699	1162.6538	1238.1385	1164.9099	1386.2564
36	833.6036	1160.5025	959.6439	815.1850	921.0900	1176.0933
40	652.0875	962.1897	842.2845	530.7309	737.9862	885.7210
44	521.2496	717.6345	632.2203	381.5292	534.2942	659.6274
48	354.4953	674.6594	524.1342	253.8922	352.8267	573.4274
52	262.0928	484.2539	440.4457	187.0937	283.9419	400.9925
56	176.5317	273.0932	333.8020	137.2297	201.0079	290.8598
60	118.6863	215.0033	234.6196	76.8480	166.6432	229.0755
64	108.4243	135.4969	195.2032	58.3569	152.2449	169.6404
68	90.9893	113.2792	146.2219	45.3182	88.2238	129.4100
72	64.5934	109.0341	117.6553	37.4554	70.8856	93.5661
80	42.4471	48.3426	54.2225	18.2919	33.7445	54.5351
88	29.1330	34.7752	31.7892	10.9524	25.0341	32.7361
96	14.2918	27.6556	20.2730	6.0124		26.4101
106	10.5862	14.3791	12.0132	4.5870	9.3822	13.4753
118	5.2410		8.0352	3.3095	5.9232	9.9323
130	2.7305	5.3582	4.1747	1.9994	3.8172	6.4685
142	1.7549	3.9034	2.6611	1.8032	2.3842	3.7924

Appendix 4 - "Comparison of fitted marker curves for all five models tested in chapter 4"

Log value of faecal Cr concentrations against time



Log value of faecal Co concentrations against time



Appendix 5 - "WinSAMM working file of model MB2 developed in chapter 4 and used throughout thesis"

A SAAM31 MODIFIED M2 MODEL WITH TWO RUMEN POOLS & HG MIXING POOL-
Group Sheep (control)

2 25

H PAR

L(2,1) 1.016131E+00 9.999998E-03 1.000000E+04

L(3,2) 2.058836E+00 9.999998E-04 1.000000E+01

L(4,3) 24

L(5,4) 9.341373E+00 9.999998E-04 9.999998E+03

L(6,5) 24

DT(3) 5.898174E-01 1.000000E-01 1.000000E+01

DN(3) 6.000000E+00

P(1)=730

P(2)=p(1)/2

C Digestibility %

P(5) 5.920000E+01

P(6)=P(5)

K(5)=100/(100-P(5))

L(12,11) 1.933349E+00 1.000000E-01 1.000000E+01

L(13,12) 2.865086E+00 1.000000E+00 1.000000E+02

L(14,13) 24

L(15,14) 1.260559E+01 9.999998E-04 9.999998E+03

L(16,15) 24

DT(13) 4.347589E-01 1.000000E-01 4.000000E+00

DN(13) 6.000000E+00

K(15)=K(5)

H INI

C Dose of Cr particulate marker (units as for faecal data)

IC(1)=p(10)

P(10) 9.904626E+05 7.000000E+05 1.100000E+06

C Dose of solute marker Co

IC(11)=P(11)

P(11) 5.512761E+05 4.000000E+05 5.700000E+05

H STE

U(1)=P(1)-P(2)

U(11)=P(2)

H DAT

X G(5)=(F(5)+F(25))/(M(5)+M(15))

X G(15)=F(15)/(M(5)+M(15))

G(10)=((M(1)+M(2))*M(13))/(M(3)*(M(11)+M(12)))

G(11)=(M(3)*M(14))/(M(4)*M(13))

c Rumen contents - Solids

g(30)=m(1)+m(2)

c MRT Rumen

g(31)=(1/l(2,1))+1/l(3,2))

c MRT Hindgut

g(32)=dt(3)+1/l(5,4))

107
 G(10) 1 .1
 G(11) 1 .1
 p(10) 990000 20000
 p(11) 550000 20000
 g(30) 500 100
 g(31)
 g(32)
 p(1)
 p(2)

c
 m(1)
 m(2)
 m(4)
 m(11)
 m(12)
 m(14)

C *** Chromium faecal concentrations
 C hours ug Cr/gDM
 105 G(5) /24 sd=100
 c 8 1.02061095
 c 10 0.900641835
 12 10.57165514
 14 42.12341907
 16 105.9522107
 18 410.4815795
 20 819.4357254
 22 1191.342958
 24 1562.479717
 28 1727.170167
 32 1907.29555
 36 1930.076086
 40 1793.323325
 44 1684.551374
 48 1415.80318
 52 1202.968696
 56 1032.084052
 60 879.9896195
 64 737.5423025
 68 596.8501623
 72 499.8351377
 80 311.221001
 88 213.4799763
 96 149.6882897
 106 83.63997954
 118 36.53408618
 130 22.52857288
 142 12.0381361

105 g(5)
 0
 2 .1 60
 C *** Co faecal concentrations
 115 G(15) /24 sd=100
 c 8 1.6220
 10 5.0751

	12	162.7170	
	14	425.1120	
	16	836.6663	
	18	1229.0492	
	20	1408.8559	
	22	1574.2602	
	24	1517.5550	
	28	1436.3929	
	32	1259.7596	
	36	1002.4734	
	40	813.2286	
	44	634.9079	
	48	504.2299	
	52	389.6188	
	56	279.3134	
	60	211.5894	
	64	159.1277	
	68	123.0817	
	72	93.8473	
	80	54.9090	
	88	33.9918	
	96	22.5319	
	106	13.0193	
c	118	7.6213	
c	130	4.7101	
c	142	3.6004	
115 g(15)			
	0		
2	.1		60
106			
	0		
2	.01		600
116			
	0		
2	.01		600
C ***** END DATA			