Effect of different nitrogen sources on dry matter intake and digestibility of a low-quality roughage fed to sheep

by

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Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae Agriculture

MSc (Agric) Animal Science: Animal Nutrition

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Pretoria

November 2015

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DECLARATION

I hereby declare that this thesis submitted for the MSc (Agric) Animal Science: Animal Nutrition degree at the University of Pretoria is my own work and has not been previously submitted by me for a degree at any other university.

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K. Hendriks

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November 2015

ACKNOWLEDGEMENTS

I would like to acknowledge the following individuals for their contribution in completing this study:

- My supervisor, Prof. W.A. van Niekerk for his inputs and mentoring not only for this postgraduate degree but also his continued involvement in my undergraduate studies;
- My co-supervisor, Prof. J. Jansen van Ryssen for his kind-hearted approach and willingness to contribute valuable inputs;
- Mr. R. Coertze for his efforts and time put into the statistical analyses of the data, as well as his assistance with regards to the trial of this study on the Hatfield Experimental Farm;

Additionally I want to express my sincerest appreciation for the love and support provided by my family and friends, especially when the trial became extremely challenging.

To my husband, without whom I would never have had the courage and determination to complete this degree. His unconditional support and enthusiastic approach made even the worse days seem survivable. Words cannot express my appreciation for his help which made it possible to continue and complete the trial of this study.

Most of all, I want to thank my Heavenly Father for the opportunity and ability which allowed me to fulfil in my goal of completing this degree.

SUMMARY

Effect of different nitrogen sources on dry matter intake and digestibility of a low-quality roughage fed to sheep

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Extensive sheep farming in South Africa generally imply circumstances where roughage is the primary food source. However, when roughages form the main component in ruminant diets these diets are typically characterised by high fibre, low energy and low nitrogen values. Adding nitrogenous compounds to low-quality roughage not only promote the growth of fibrolytic bacteria, but also enhance digestion rates and microbial protein synthesis. Urea is the nitrogen source most often utilised for supplementing nitrogen as it is relatively inexpensive and proven to be effective. However, the rapid hydrolysis of urea to ammonia poses a large risk in that it can cause ammonia toxicity due to the accumulation of ammonia. Therefore it was the aim of this study to determine if using urea in smaller quantities but in combination with other N-supplements, would prove effective with regards to the following parameters: dry matter intake, rumen ammonia nitrogen, blood urea nitrogen, rumen pH, volatile fatty acid concentration, as well as dry matter and neutral detergent fibre digestibility. Four Merino wethers fitted with rumen cannulae were used in a 4 x 4 Latin square design and given a basal diet of *ad libitum* low-quality *Eragrostis curvula* hay. Supplements consisted of a mineral mixture, a nitrogen source (depending on treatment), as well as starch to ensure that the diets

were iso-nitrogenous and iso-energetic. It was imperative to maintain a constant nitrogen:sulphur ratio throughout all treatments and therefore feed grade sulphur was included in the formulation of the treatments (where applicable). The required nitrogen was supplied as follows:

Treatment 1 as control (**U**) - 100% urea;

Treatment 2 - 50% urea and 50% $(NH_4)_2SO_4$ (**UAS**);

Treatment 3 – 50% urea and 50% lucerne (**UL**); and

Treatment 4 – 8% urea, 84% $(NH_4)_2SO_4$ and 8% lucerne (**UASL**).

The results showed that animals fed UASL had a rumen ammonia concentration which was significantly ($P < 0.05$) higher compared to those fed the U treatment. In addition, the U treatment also proved to have the lowest DM and organic matter digestibility which differed significantly from the UASL and UAS treatments. At every measurement, the pH of the rumen fluid for the U treatment was higher compared to the UASL treatment, although these differences were significant at only a few intervals. No significant differences were found between treatments for dry matter intake, blood urea nitrogen, NDF digestibility, mean nitrogen retention, VFA concentration or the effective degradability of DM and NDF as determined by the *in situ* technique. These results did not reveal a clear effective partial replacer for urea as no single treatment performed better across all measured parameters compared to the control treatment (urea only).

LIST OF ABBREVIATIONS

$(NH_4)_2SO_4$	ammonium sulphate
AA	amino acid
ADF	acid detergent fibre
ADIN	acid detergent insoluble nitrogen
BUN	blood urea nitrogen
CF	crude fibre
CO_2	carbon dioxide
CP	crude protein
DM	dry matter
DMI	dry matter intake
DOMI	digestible organic matter intake
ED	effective degradability
FME	fermentable metabolisable energy
MCP	microbial protein
ME	metabolisable energy
MJ	mega joules
N	nitrogen
NDF	neutral detergent fibre
NFC	non-fibre carbohydrates
NH_3	ammonia
NH_3-N	ammonia nitrogen
Nmic	microbial synthesis of nitrogenous compounds
NPN	non-protein nitrogen
NSC	non-structural carbohydrates
OM	organic matter
RDP	rumen degradable protein
RFC	readily fermentable carbohydrates
S	sulphur
SCFA	short chain fatty acid
SE_m	standard error of the mean
TDN	total digestible nutrients
VFA	volatile fatty acid

LIST OF PRODUCTS

Product	Manufacturer	Description
Ammonium sulphate	Protea Animal Feeds Omnia House, 13 Sloane Street Bryanston South Africa	Ammonium sulphate containing at least 210 g N / kg with a protein equivalent of at least 1330 g / kg. Chemical formula: $(NH_4)_2SO_4$
Flower of sulphur	KK Animal Nutrition (Pty) Ltd Umbogintwini Industrial Complex Moss Kolnik Drive Umbogintwini, Kwa-Zulu Natal South Africa	Feed grade sulphur containing at least 995 g S / kg.
Premix	Pennville Simba Business Complex Richards Bay Road Pretoria West South Africa	Standard sheep premix.
Starch	Tongaat Hulett Starch (Pty) Ltd 2 Dick Kemp Street Meadowdale Germiston South Africa	Amyral Corn Starch; contains approximately 13% moisture and supplies 15.99 MJ ME per kg on a dry matter basis.
Urea	KK Animal Nutrition (Pty) Ltd Umbogintwini Industrial Complex Moss Kolnik Drive Umbogintwini, Kwa-Zulu Natal South Africa	Urea with a protein equivalent of at least 2870 g / kg.

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CHAPTER 1
GENERAL INTRODUCTION

Extensive sheep farming in South Africa generally imply circumstances where roughage is the primary food source. Fortunately, not only do ruminants require roughage to ensure normal rumen function (Mulligan *et al*., 2001) but it is also known that ruminants have the ability to ingest and retain large quantities of fibrous plant material where it is subjected to fermentation by the rumen microbes, allowing the nutrients present in roughage to become available to the host animal (Dewhurst *et al*., 2000).

However, when roughages (such as veld or crop residues from agriculture) form the main component in ruminant diets, these diets are typically characterised by high fibre, low energy and low nitrogen (N) values (Dryhurst and Wood, 1998; Huyen *et al*., 2012). Currier *et al*. (2004) qualified low-quality roughages as those containing less than 6% crude protein (CP). The result is that lower quality roughages are very slowly digested within the rumen and the N requirement for microbial protein (MCP) synthesis is seldom met purely by the N intake from the basal diet (Giri *et al*., 2000).

Adding nitrogenous compounds to low-quality roughage not only promote the growth of fibrolytic bacteria, but also enhance digestion rates and MCP synthesis. Consequently forage intake and digestibility are elevated (Salisbury *et al*., 2004) and the energy extraction from the fibre and nutrient-flow to the small intestine, as well as the volatile fatty acid (VFA) production is improved (Sampaio *et al*., 2010).

Urea is the nitrogen source most often utilised for supplementing N as it is relatively inexpensive and proven to be effective. However, the rapid hydrolysis of urea to NH_3 (ammonia) poses a large risk in that it can cause NH_3 toxicity due to the accumulation of NH_3 (Shete and Hol, 2007).

Many alternative compounds to urea have been investigated but the majority has not been as advantageous as urea since most of these compounds are more expensive and not as cost-effective. This fact provided a basis for conducting experiments in which urea is only *partially* replaced by alternative N sources instead of being completely removed from the diet.

The purpose of this research was thus to determine if an ideal combination of urea plus another source (or sources) of N exists as supplementation for sheep farmed under extensive conditions and grazing low-quality roughage. This supplement should be relatively

inexpensive and be effective in terms of animal production (compared to urea alone) whilst also minimising the risk of stock losses due to toxicity.

The parameters tested to determine the efficacy of these supplements were rumen ammonia nitrogen (NH_3-N), blood urea nitrogen (BUN), rumen fluid pH, VFA concentration within the rumen, as well as the effect of the supplements on the dry matter intake (DMI), dry matter (DM) digestibility and neutral detergent fibre (NDF) digestibility of the basal roughage.

CHAPTER 2
LITERATURE REVIEW

When low-quality roughages are fed alone, nutrient intake is low and it cannot support growth. Ruminants on unsupplemented diets need to mobilise energy and protein from their tissues leading to significant weight loss (Bonsi *et al*., 1996) especially during winter when the CP value of the roughage is very low (Krehbiel *et al*., 1998). This merits supplementing sheep farmed under extensive circumstances because when wanting to improve the efficiency by which a low-quality roughage is utilised, it is necessary to increase the microbial activity by means of supplying the required nutrients to the microbes (Haddad, 2000).

2.1   Nitrogen supplementation

Nitrogen is usually supplied in the form of a protein or as a non-protein nitrogen (NPN) which comprises the main component of a supplement (Chenost, 2001). Supplements in the form of protein should contain a fraction of slowly degradable protein as this will increase the quantity of protein that reaches the small intestine (Çetinkaya and Özcan, 1994). In a study conducted by Sampaio *et al*. (2010) it was found that supplementing N-containing compounds in amounts that elevate the CP content in the diet to 100 $g \cdot kg^{-1}$ DM optimise the use of low-quality forage.

2.1.1   Urea

Urea is a NPN supplement produced by chemical synthesis when ammonium carbamate is produced by the reaction of CO_2 (carbon dioxide) and NH_3. Thereafter urea is formed through the dehydration of the ammonium carbamate (Aquilina *et al*., 2012). It has the chemical formula of $(NH_2)_2CO$ and its molecular structure is given in Figure 2.1.
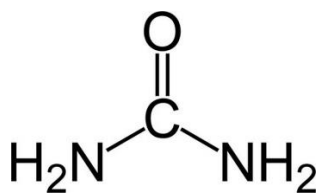
Figure 2.1 Molecular structure of urea

A number of N sources is commercially available in the form of supplements of which urea is the most widely used (Shete and Hol, 2007). However, other compounds such as those classified by Cherdthong *et al*. (2011) as ruminal slow release compounds (e.g. biuret and urea phosphate), have not proved to be as beneficial as urea due to the fact that a part of the NPN may exit the rumen without conversion to NH_3, resulting in a less efficient incorporation into MCP. In addition, compared with most natural proteins, urea is a favourable N supplement due to its relatively lower cost (Currier *et al*., 2004; Cherdthong *et al*. 2011). It is highly degradable in the rumen allowing its rapid hydrolysis to NH_3 and CO_2 by means of urease, a bacterial enzyme (Shete and Hol, 2007). Urease is required for ureolysis and the produced NH_3 serves as the main source of N for the majority of the rumen microbes (Zhao *et al*., 2015).

When carbohydrates are fermented within the rumen, keto-acids are produced. In combination with these acids, NH_3 is used by the rumen microorganisms to produce amino acids (AA's) which are subsequently converted into microbial protein. This microbial protein bears similarity to natural proteins in terms of AA profile and become available to the host animal through digestion and absorption in the lower digestive tract (Shete and Hol, 2007).

Decades ago German workers discovered that urea could be a useful partial replacer of protein in ruminant diets (Kertz, 2010). It was also determined that the conversion of urea to protein is performed by the microbes within the rumen and reticulum and that this held an advantage for the host animal in terms of protein supply (Kertz, 2010). Urea is often fed to ruminants consuming low-quality roughage and the combination of urea and straws generally results in improved digestibility and consequently intake (Cherdthong *et al*., 2011). As mentioned, NH_3 contains the N utilised for MCP production (Aquilina *et al*., 2012). It was reported by Shete and Hol (2007) that the utilisation of urea is dependent upon the level and type of carbohydrate in the ration and that greater utilisation of urea occurs when starch is present compared to when cellulose is the sole carbohydrate.

In the 1980's it was recognised by Owens *et al*. (1980) that the rapid hydrolysis of urea to NH_3 can cause toxicity due to the accumulation of NH_3. Rapid hydrolysis is attributed to its high solubility (Currier *et al*., 2004). According to Zhao *et al*. (2015) the rate of urea hydrolysis is approximately four times greater compared to NH_3 assimilation and this leads to the accumulation of NH_3. This is undesirable not only due to the potential toxicity, but also as it causes poor N utilisation of the diet and contributes to environmental pollution due to excessive N excretion. This is the major reason to limit the utilisation of urea as a NPN source in ruminants for MCP production. It was found that if urea is ingested in large volumes over a limited period of time, it will result in NH_3 toxicity as the rate of NH_3 production will exceed

detoxification by the liver (Currier *et al*., 2004; Cherdthong *et al*., 2011). When NH_3 is absorbed into the bloodstream in concentrations that surpass the safe-threshold it leads to the death of the animal (Shete and Hol, 2007). It has been shown by Dixon (2013) that toxicity is more common when hungry animals are allowed access to the supplement or when animals are permitted to drink rainwater which accumulated on the supplement. Toxicity is common amongst ruminants fed urea-supplemented licks and for this reason alternative N sources should be considered, especially with regards to the effect on DM digestibility, as this will determine whether its use is beneficial to the animal.

2.2 Alternative protein sources

Alternative N sources to be seriously considered as partial replacers of urea can be divided into two main categories: 1) protein sources like seed oilcakes/meals, soybean hulls, cottonseed hulls and legume hay; and 2) NPN sources like ammonium sulphate $((NH_4)_2SO_4)$ and ammonium chloride, both of which are considered non-organic compounds. According to Oltjen (1969), a comparison between these two categories resulted in growth rates of ruminants fed diets containing only NPN being lower than those ruminants fed protein-containing diets. It was found that when NPN completely replaces protein in purified diets, the growth rate and feed efficiency are only approximately 65% of that achieved on the protein-containing diets (Oltjen, 1969). These findings warrant the further investigation of partially replacing urea with at least one protein source in each of the mentioned categories since the study of Oltjen (1969) was performed decades ago and more recent research could be useful to the animal feed industry. With regards to the possible alternatives it was the focus of this study to test a protein source, i.e. a legume hay (lucerne) and $(NH_4)_2SO_4$ (an NPN source).

2.2.1 *Legume hay*

Lucerne (*Medicago sativa*) is known as a legume with high nutritional value and a good source of natural protein (Robinson, 1998). In addition, it is known that lucerne (as with other legumes) have higher rates of ruminal degradation than grasses and when this is coupled with good CP levels can increase the concentration of rumen ammonia. This in turn leads to elevated levels of the cellulolytic rumen microbes which will improve the extent of digestion (Kariuki *et al*., 1999). Generally lucerne hay contains between 25% and 30% NSC which contribute greatly to the total digestible nutrients (TDN) in the hay. Additionally, the NDF component is rapidly digested by microorganisms in the rumen, also contributing to TDN (Robinson, 1998). The protein is also highly digestible at 80% - 90% digestibility further increasing its TDN

value. Lucerne is known to have a high CP level, but an additional benefit provided by this protein source is the proportion of CP (25% - 30%) which is regarded as undegradable intake protein, supplying the lower digestive tract with natural protein. Based on nutritional value, lucerne provides a great alternative as N supplement (Robinson, 1998).

The beneficial effects of lucerne (e.g. improved DM intake and digestibility) have been ascribed to its provision of readily fermentable cell wall carbohydrates and essential nutrients such as N and minerals for the rumen microbes. According to Bird *et al*. (1994) increases in digestibility could be due to increased availability of AA's together with the generation of additional NH_3 from the degradation of the soluble N part of lucerne. According to Mirzaei-Aghsaghali *et al*. (2008) the rapid digestion of its structural fibre in the rumen allows lucerne to serve as an intake stimulator. In addition, stimulation of rumination and chewing results in lucerne having a high buffering capacity. Also, apart from supplying undegraded digestible protein to the lower digestive tract, lucerne is also rich in mineral content, including calcium, phosphorous and magnesium (Higginbotham *et al*., 2008).

Kariuki *et al*. (1999) found in their study by looking at parameters such as intake and ruminal degradation of the feed, that lucerne hay improved the quality of the test diets (napier grass *Pennisetum purpureum*) and resulted in higher animal performance. The test diets had a CP content of 11.7% These results were supported by Baloyi *et al*. (2006) who conducted a study on the use of legumes as protein supplements in sheep grazing medium quality veld hay. It was found that N-retention was improved due to the increased availability of protein and energy together with the higher digestibility of the supplemented legume hay. Dry matter and N utilisation were also enhanced coupled with an improvement in fermentation (Baloyi *et al*., 2006). In a study conducted by Bird *et al*. (1994) lucerne was given as supplement to wheat straw diets and it was found that not only did the lucerne greatly increase the total DMI, it also increased the N-retention to a considerable extent. This was attributed to a higher level of MCP synthesis within the rumen. In addition, when lucerne was included at 150 $g \cdot day^{-1}$ in the ration, the *in situ* degradabilityy of the wheat straw was significantly improved.

In a study designed to measure N utilisation, rumen fermentation parameters and abomasal N flow, it was determined by Bowman and Asplund (1988) that supplementing with lucerne increased rumen NH_3-N levels (compared to diets without N supplements) without resulting in excessively high NH_3-N concentrations. Coupling lucerne supplementation with urea infusion led to more efficient utilisation of the supplied N which minimised NH_3-N losses. The importance of adding N in the form of a natural protein or urea to a low-quality roughage diet is seen in the considerable elevation of rumen NH_3-N levels. This fact is important in high

fibre low protein diets as these generally demonstrate low rumen NH_3-N levels which in turn reduce the quantity and efficiency of MCP synthesis owing to a N-deficiency.

2.2.2 Ammonium sulphate

Non-protein N compounds are attractive alternatives to natural protein sources owing to a lower cost per unit of N (Currier *et al*. 2004; Ali *et al*., 2008). Ammonium sulphate is a well-known source of NPN within in the ruminant feed industry, although it is not as frequently utilised as urea. Many commercially formulated feeds do however include both urea and $(NH_4)_2SO_4$. A disadvantage of feed-grade $(NH_4)_2SO_4$ is the potential difficulty in dissolving as this is required for its incorporation into a supplement (Dixon, 2013).

One of only a few recorded studies was conducted by Bolsen *et al*. (1973) to compare trials where steers and lambs were fed urea or urea plus $(NH_4)_2SO_4$. It was concluded from these trials that the animals receiving urea plus $(NH_4)_2SO_4$ had slower weight gains, consumed less feed and required more feed per unit of gain compared to animals fed rations supplemented with only urea. However, these results were obtained when high levels of maize served as basal diet and different results may thus be obtained when low-quality roughage comprises the basal diet. Further investigation in this regard is needed to confirm or contradict these findings. It is important to keep in mind that the addition of sulphur (S) in experimental diets will only lead to improved animal performance if these diets are deficient in S (Bolsen *et al*., 1973).

2.2.2.1 Sulphur consideration

It is known that ruminants have a S requirement for MCP production since S is an important element required in the synthesis of AA's methionine, leucine and cysteine. Dietary S fed as an AA or in inorganic form is either reduced to sulphide by microbial organisms in the rumen after ingestion and is then incorporated into MCP, or it is oxidised to sulphate by the liver (Fron *et al*., 1990; Binta *et al*., 2012). When animals were fed diets low in S, supplementation improved DM intake, weight gain and wool growth (Fron *et al*., 1990). It was reported by the NRC (1976) that the requirement for S by sheep as a ratio of N:S is 10:1.

However, S toxicity can also occur when S is consumed and metabolised at high levels. Bioavailability of S is dependent on the form in which it is fed − organic S sources have a higher S bioavailability than inorganic S (such as $(NH_4)_2SO_4$) sources. It is important to consider intake levels of S when $(NH_4)_2SO_4$ is fed as a source of NPN. One of the few recorded studies was performed by Graham *et al*. (1976) to investigate this specific question. These authors investigated the merit of feeding $(NH_4)_2SO_4$ alone or in combination with urea as

supplement to low N roughages. Blood NH_3 levels were significantly lower with $(NH_4)_2SO_4$ dosing compared to urea dosing. In addition, when 15 g of N was fed as a single dose of $(NH_4)_2SO_4$ the wethers could tolerate it but the equivalent of urea proved to be toxic to these animals. However, when $(NH_4)_2SO_4$ was fed as sole NPN source a lack of appetite and rumen stasis were observed which the authors attributed to possible sulphide toxicity. Graham *et al.* (1976) concluded that $(NH_4)_2SO_4$ fed in combination with urea was well utilised as a source of both S and N – even when the $(NH_4)_2SO_4$ supplied 50% of the supplemented N.

2.3 Lag time

Lag time is regarded by Huhtanen *et al.* (2008) as the time necessary for inoculation and elaboration of microbial attachments to feed substrates and generally refers to the time that passes before microbial degradation commences. Lag time can be altered by various dietary factors – most relevant for this study is the addition of small quantities of soluble carbohydrates to a forage diet to improve the digestion of fibre through the enhancement of bacterial attachments to feed particles (Huhtanen *et al.* 2008).

An elongated lag time usually occurs due to asynchronous availability of the nutrients required by the rumen microbes - most commonly these nutrients are fermentable metabolisable energy (FME) and N (Varga, 1986). More recently it was stated by Belanche *et al.* (2012) that the availability of energy and N is regarded as the most important factors limiting the growth of the rumen microbes. Rapidly released N from NPN sources is usually not synchronised with the slower release rate of FME from forages. Apart from the resultant lag time, this generally contributes largely to the toxicity found with urea as a NPN supplement (Belanche *et al.* 2012).

2.4 Rumen NH_3-N

Within the rumen, nitrogenous compounds (NPN and true protein) is broken down to peptides and AA's which will ultimately be deaminated to result in NH_3-N. In addition, NH_3-N in the rumen fluid also originates from the breakdown of urea recycled from the liver (Abdoun *et al.*, 2005) after detoxification to NH_3 (Abdoun *et al.*, 2007). It was concurred by Wang *et al.* (2012) that there is an inverse relationship between the concentration of NH_3-N and the rate at which urea is transferred into the rumen. Most of this NH_3-N is incorporated in the production of MCP (Bach *et al.*, 2005) since ruminal NH_3-N is one of the main sources of N for MCP synthesis (Wang *et al.*, 2012). As a consequence, the outflow of digesta from the rumen consists predominantly of MCP, NH_3-N and undegraded protein – from dietary or

endogenous origin (Bach *et al*., 2005). It can thus be said that the proportion of rumen degradable protein (RDP) in the diet is the major factor determining the production of NH_3-N (Wang *et al*., 2012).

More than 50% and commonly as much as 75% of the AA's which are absorbed by ruminants, is from MCP origin (Aquilina *et al*., 2012). For this reason MCP is regarded as a significant source of protein (Dewhurst *et al*., 2000; Bach *et al*., 2005). Cellulolytic microbes which are responsible for the degradation of structural carbohydrates have relatively low requirements for maintenance as these microorganisms grow slowly, but NH_3-N is utilised as main N source. In contrast, microorganisms fermenting non-fibre carbohydrates (NFC) derive up to 66% of protein from peptides or AA's whilst only the remaining 34% is derived from NH_3-N (Russell *et al*., 1992). It was suggested by Bach *et al*. (2005) that NH_3-N accumulation in the rumen is the result of microbes in the rumen preferring peptides or AA's as sources of N or energy.

However, NH_3-N in excess of what is utilised by rumen microorganisms is absorbed into the blood where it is metabolised within the liver to form urea and is then lost via the urine (Cherdthong *et al*., 2011). This is an undesirable occurrence as a potentially large part of the N is wasted. It was found by Tamminga (1996) that under normal feeding conditions the most effective approach to reduce these N losses is either by manipulating the rumen protein degradation (by decreasing it) and/or by increasing the N use by rumen microbes.

The entire quantity of MCP flowing to the small intestine is dependent on the availability of nutrients and the efficiency with which it is used by the rumen bacteria (Bach *et al*., 2005). A number of studies showed that infusing increased amounts of readily fermentable carbohydrates (RFC) decreased the NH_3-N concentrations as a result of improved uptake of N by the rumen microorganisms leading to increased ruminal MCP passage in addition to improved N balance (Löest *et al*., 2001). This was also supported by Alvarez Almoraa *et al*. (2012) that reported the ruminal NH_3 concentration declined in reaction to factors that decreased NH_3 production, e.g. intake of less degradable N source, or by factors known to promote NH_3 assimilation, e.g. synchronisation of N and fermentable energy. For these reasons feed grade starch was included in this study in each of the formulated supplements as a source of RFC in an attempt to minimise lag time and reduce NH_3 toxicity where possible.

Griswold *et al*. (2003) reported that bacteria responsible for degrading structural carbohydrates only utilise NH_3, compared to the bacteria degrading non-structural carbohydrates (NSC) which prefer N from AA's and peptides. Satter and Slyter (1974) emphasised that the availability of NH_3 is an important determinant of MCP production as the

greatest part of the rumen bacteria population utilise it as N source. In a different study conducted by Jones *et al*. (1998) it was indicated that when diets contain more than 40% NSC, additional N from AA's and peptides exceeding the requirement of the NSC-degrading bacteria would not be utilised for the production of NH_3. This resulted in a reduction of rumen NH_3-N below what was regarded by Satter and Slyter (1974) as a critical level of 5 mg·dL^{-1}. From the study of Satter and Slyter (1974) it was concluded that 5 mg dL^{-1} of rumen NH_3 was adequate for the degradability of fibre and that no additional benefit would be gained by a higher level of N supplementation.

When rumen bacterial growth is optimised through improvement of rumen NH_3-N concentration, it leads to elevated microbial populations in turn improving the digestibility and degradability of the roughage and potentially DMI. This necessitates defining the optimal level of rumen NH_3-N concentration which will maximise animal production when grazing low-quality roughage. Detmann *et al*. (2009) performed a study on heifers specifically aimed at investigating the ruminal degradation of NDF of a low-quality hay at various levels of N supplementation. The supplement was a combination of urea, sulphate and albumin at fixed ratios of inclusion. These authors stated that although many parameters have been proposed to assess the availability of N compounds in the rumen, rumen NH_3-N has been the qualitative reference in evaluating the sufficiency of the rumen environment. Detmann *et al*. (2009) suggested that strategic supplementation of nitrogenous compounds is the best option to manage ruminants grazing low-quality forage, as it stimulates the activity of the fibrolytic microbes in the rumen which in turn improves the breakdown of the low-quality fibrous carbohydrates. In their study, NDF degradation was measured with regards to ED and their results is given in Figure 2.2. Intake as a function of rumen NH_3-N concentration (given as RAN in Figures 2.2, 2.3 and 2.4) is portrayed in Figure 2.3 and microbial synthesis of nitrogenous compounds (Nmic) is showed in Figure 2.4. Detmann *et al*. (2009) found that the maximum response for MCP synthesis (given as Nmic in Figure 2.3) was reached when rumen NH_3-N concentration was 14.5 mg·dL^{-1}. The rumen NH_3-N concentration necessary to optimise NDF degradation was 8 mg·dL^{-1} and for optimum feed intake it was 15 mg·dL^{-1}. The authors ascribed the difference in optimum values to a better adequacy of the ratio of metabolisable protein:metabolisable energy in the metabolism of the animal. This increased the feed intake even after optimisation of NDF degradation. Although supplementation with nitrogenous compounds led to a positive effect on the rumen NH_3-N concentration, it is important to emphasise that an optimum level exists and supplementation above this level is wasteful (Detmann *et al*., 2009).
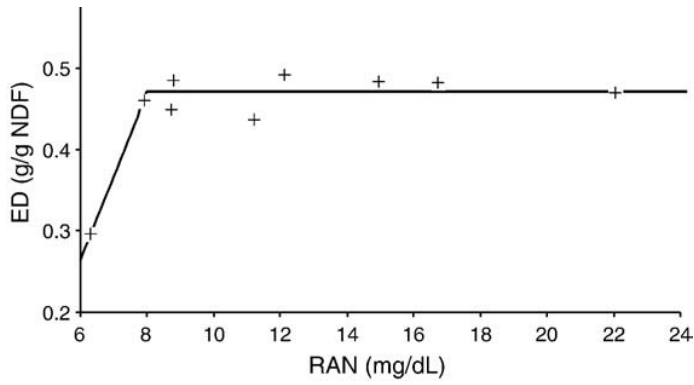
Figure 2.2 Relationship between effective degradability of NDF and rumen NH3-N concentration (Detmann *et al.*, 2009)
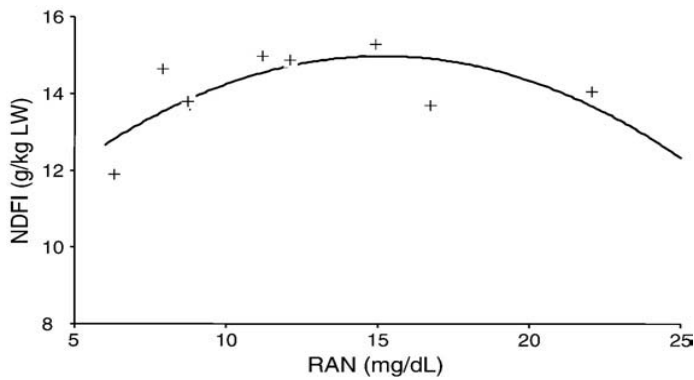
Figure 2.3 Relationship between NDF intake NDFI and rumen NH_3-N concentration (Detmann *et al.*, 2009)
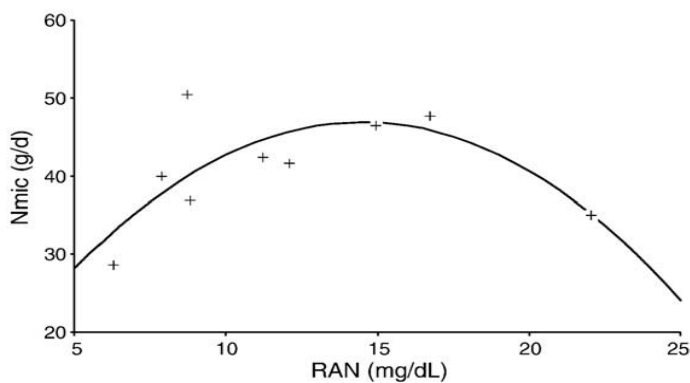
Figure 2.4 Relationship between intestinal flow of microbial nitrogen and rumen NH_3-N concentration (Detmann *et al.*, 2009)

With regards to the treatment supplements of this study, it should be noted that the three supplements under investigation (urea, $(NH_4)_2SO_4$ and lucerne) differ with regards to rate of N release and the effect thereof will hopefully become evident in the results. The rate of $(NH_4)_2SO_4$ breakdown is not as rapid as urea hydrolysis and N is released at a slower rate. This might be the reason why it has been reported that ruminant organisms utilise $(NH_4)_2SO_4$ to a greater extent compared to urea (Graham *et al*., 1976; NRC, 1976). Lucerne, on the other hand has a slower initial degradation and N release rate, but it continues for a longer period of time providing a more consistent supply of N (Repetto *et al*., 2003).

2.5 Apparent nitrogen balance

Nitrogen balance is usually calculated as the difference between the quantity of N intake and N output (Spanghero and Kowalsi, 1997). When no corrections are made for N excreted not from dietary origin, this balance is regarded only as apparent since it does not represent the true value of N retained (McDonald *et al*., 2011). The losses of N from endogenous origin in animal production are considered important although it is difficult to measure. It was reported by the NRC (1985) that in sheep as much as a third of N in the faeces could originate from endogenous N and that the remaining N is of microbial origin. These losses usually originate form digestive enzymes, lymph, epithelial cells, mucins, saliva, bile and other degradable products from the lining of the gastrointestinal tract (NRC, 1985; Tamminga *et al*., 1995).

The balancing of N is important as it is an indicator of the synchrony between carbohydrate and N compounds found within the rumen. The better the synchrony, the lower will be the urinary-N output which leads to an improved N-balance (Jetana *et al*., 2010). A great loss of N leads to a poor N-balance which is indicative of a diet lacking carbohydrates and/or oversupplying N.

Roughage with a low to moderate CP level is generally considered to be limiting in RDP which will lead to an increased N-recycling to the rumen resulting in low urinary-N loss (Lawler-Neville *et al*., 2006). As a source of RDP is added, more N will be absorbed as NH_3 or more AA's will be deaminated which increases the loss of N in urine (Lawler-Neville *et al*., 2006). This loss can be reduced by ensuring that sufficient carbohydrate sources are supplied to the rumen. With N-recycling, most of the absorbed NH_3, as well as the greatest part of N from AA's are converted to urea in the liver which is released to the blood circulation. This urea follows one of two fates – it is either excreted into urine or it is re-introduced into the digestive tract by directly crossing the rumen wall or via saliva (Rémond *et al*., 1993).

Abdoun *et al*. (2007) referred to N-recycling as a salvage mechanism and stated that it is vital to the ability of ruminants to survive on low protein diets as the N is re-directed for use in MCP production which is more productive than elimination in urine. Furthermore, recycling and reuse of N could cover up to 80% of a ruminant's N requirement and this allows ruminants to survive in a wide range of habitats (Abdoun *et al*., 2007). This was confirmed by Obitsu and Taniguchi (2009) when it was stated that N-recycling in ruminants play a crucial role in the adaptation of these animals to diverse nutritional conditions, which can range from extensive systems characterised by poor quality feed to intensive systems with high quality feed. Obitsu and Taniguchi (2009) also reported that N-recycling will differ even between breeds of the same species, probably due to variation in body composition and endocrine status affecting the variation of body protein turnover during growth. An example was that *Bos indicus* cattle and associated cross-breeds have greater urea production plus recycling, compared to *Bos taurus* cattle. This was attributed to the ability of *Bos indicus* to adapt to low-quality feed and high environmental temperatures. Obitsu and Taniguchi (2009) concluded that urea production, excretion and re-entry are directly related to the composition of the diet and the intake.

In a study conducted by Lawler-Neville *et al*. (2006) it was found that urea supplementation of poor quality grass hay led to a very poor N-retention value and although this could have been due to a number of factors e.g. lag time, FME and solubility of protein, the authors attributed it to the decreased forage and supplement intake leading to a poor N intake. However, in that study a urea solution was mixed with the forage which was different to this study and therefore different results could be expected. The rate of N release from the breakdown of $(NH_4)_2SO_4$ compared to urea is reported to be slower due to the fact that urea is rapidly hydrolysed and $(NH_4)_2SO_4$ is degraded without enzymes (NRC, 1976). In terms of lucerne, the release of N is even slower as microbes have to adhere to the cellular components first, after which degradation of the plant material will commence. However, the degradation of lucerne occurs for a longer period of time resulting in a more consistent supply of N (Repetto *et al*., 2003). As a result of these characteristics of the supplements to be tested in the current study, N-balance could differ significantly due to the fact that synchrony of nutrients will not occur at the same level throughout the treatments.

2.6 Production of volatile fatty acids

The majority of energy in plant carbohydrates is retained in the end products of fermentation and only a small part is lost as heat. These end products of fermentation are mainly organic VFA's and become accessible to the host after considerable fermentation by the

resident microorganisms. Acetic acid is the main short-chain fatty acid (SCFA) produced with propionic and butyric acids also present in large concentrations, even though the amounts can fluctuate significantly depending on the diet (van Houtert, 1993; Tagang *et al*., 2010). Ruminants rely on SCFA's for as much as 70%-80% of maintenance energy requirements (van Houtert, 1993; Tagang *et al*., 2010).

The conditions residing within the rumen determine to a great extent the proportions of the various VFA's produced by favouring or diminishing certain groups of rumen microbes (van Houtert, 1993). The anaerobic conditions prevailing within the rumen has been regarded as a limiting factor in terms of the amount of MCP that can be produced. This limitation exists due to the fact that anaerobic microbes have the ability to metabolise feed substrates to VFA's with only 10%-20% efficiency while the aerobic microorganisms are able to complete the same metabolism with 60%-70% efficiency (Oltjen, 1969). This limitation will increase in importance as the NPN percentage in the diet increases since lower quantities of dietary AA's would be available for the synthesis of MCP. This implies that larger quantities of AA's would thus have to be synthesized from carbon skeletons and NH_3 (Hvelplund, 1991). It was reported by Chiou *et al*. (1997) that the effect of the dietary protein source was significant on the total VFA concentration in the rumen. The source of protein fed also had a significant influence on particularly the acetate and propionate concentrations.

The production of VFA's originate mainly from carbohydrates in the feed, but proteins as well as lipids also contribute to the pool. According to the NRC (2007), almost all of the produced acetate and propionate are removed from the digestive tract and transported to the liver. Following this removal, each VFA has a different fate with regards to its purpose. Ruminants consuming diets high in concentrates tend to produce more propionate compared to roughage based diets when more acetate is produced (Herring, 2014). Acetate is needed mainly for the synthesis of cholesterol and long-chain fatty acids and is utilised mostly by muscle and adipose tissue (NRC, 2007). Propionate is comprehensively metabolised in the liver in the process of gluconeogenesis. This is of great importance as very little glucose is directly absorbed from the gastrointestinal tract in ruminants (Tagang *et al*., 2010). The process involving glucose production from propionate is a very energy-efficient pathway (Herring, 2014). Butyrate is extensively metabolised within the epithelial cells of the omasum to form ketone bodies and any butyrate found in the blood circulation is oxidised or also used for fatty acid synthesis. The formation of ketone bodies has been suggested to be the mechanism of supplying energy to the rumen epithelium (van Houtert, 1993).

2.7 Importance of NDF

Roughages are important as ruminants have the ability to utilise large quantities and have developed attributes to aid in the digestion of fibre as it is slow and incomplete. These attributes include swallowing of large forage particles and selectively retaining it in the rumen to allow subjection to fermentation. Importantly, digestion is enhanced by rumination (Mertens, 2002). In addition, the inclusion of fibre in the diet prevents metabolic disorders such as acidosis caused by high levels of grains or finely chopped forages.

Mertens (2002) stated that there are three routinely used methods of measuring fibre content i.e. crude fibre (CF), acid detergent fibre (ADF) and NDF, of which only NDF isolates all insoluble fibre components found within plants (hemicellulose, cellulose as well as lignin). Neutral detergent fibre represent the residues remaining after feed is digested in a neutral detergent solution. These residues are predominantly hemicelluloses, cellulose and lignin.

The NDF component is considered as an important part of ruminant diets since it provides substrates for microbial fermentation within the rumen and also regulates intake of DM (Mertens, 2002; Natel *et al*., 2013). The low digestibility of NDF can however limit DMI when it is included in high levels in the diet. Stanton and LeValley (2010) stated that the NDF content in the feed directly influences the DMI, as well as the time of rumination; however, it is negatively correlated with the available energy concentration. These measures relate more directly to animal performance and for this reason using NDF could prove more useful as a predictor of animal performance compared to CF. However, Lazzarini *et al*. (2009) explained that the reason why the greatest proportion of the energy substrates from the potentially degradable NDF of roughage low in quality cannot be used, is due to the deficiency of enzymatic systems in the rumen brought on by the fact that N is insufficient. Also, feed with lower NDF values result in decreased rumination and chewing time which lead to a depressed flow of saliva to the rumen causing a lower rumen fluid pH (Mooney and Allen, 1997). This is due to decreased levels of buffer reaching the rumen and in turn affects the acetate:propionate ratio (Natel *et al*., 2013).

2.8 Rumen fluid pH

The maintenance of the pH within the rumen is pivotal for the state of the microbiota in terms of stability, as well as persistence. The pH can usually vary between 5.5 and 7.5 depending not only on the diet type but also on the frequency of feeding (Franzolin and Dehority, 2010). It was also found by Rémond *et al*. (1993) that when the pH of the rumen fluid is around 7.0, the absorption of NH_3 is increased. Abdoun *et al*. (2007) explained this by

means of Figure 2.5 in which it is illustrated that with an increase in pH from 6.4 to 7.4 the total NH_3 concentration increases by a factor of 10. This indicates that the permeability of the rumen wall with regards to NH_3 absoption is significantly increased at pH above 7.0. In research by Rémond *et al*. (1993) it was suggested that movement of NH_3 across the rumen epithelium occurred primarily in the unionized form via diffusion and that some NH_4^+ may also be absorbed with VFA anions. In addition, it is known that the transepithelial flux of NH_3 is a passive process and the permeability of the rumen wall favours the undissociated form of NH_3 above NH_4^+. Rémond *et al*. (1993) determined that when pH was decreased, the concentration of undissociated NH_3 declined which contributed to a reduced absorption of NH_3. This was confirmed by Abdoun *et al*. (2007) when these authors stated that at lower ruminal pH (6.6 and below) the rumen wall permeability for NH_3 is depressed, causing absorption of NH_3 to remain stable regardless of an increase in the rumen NH_3-N concentration.
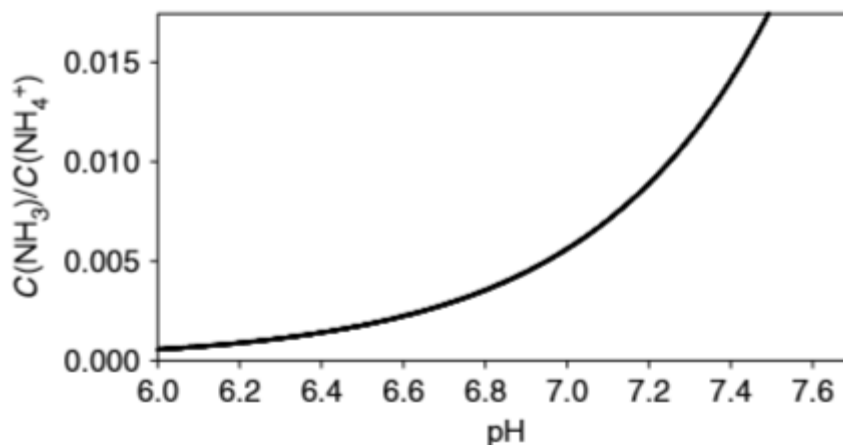
Figure 2.5 Relationship between rumen NH_3 concentration and rumen fluid pH (Abdoun *et al*., 2007)

The pH of the rumen fluid is important as it is one of the most crucial factors governing the incidence of NH_3 toxicity. Evidence of NH_3 toxicity was observed by Abdoun *et al*. (2007) when the rumen pH was above 7.3 and the liver could no longer detoxify the increased quantity of NH_3 caused by the higher absorption rate due to the high pH. It is therefore important to measure the pH of the rumen fluid in order to monitor if it is lower when urea is partially substituted with other N sources when the goal is to minimise the incidence of NH_3 toxicity.

In addition, the ruminal pH is an important parameter to monitor throughout digestibility studies as it is an essential factor governing optimal microbial activity. When the pH within the rumen is below 6.2, fibre digestion will necessarily decrease drastically (Ranilla *et al*., 2000). This is due to a reduction in the digestion of cellulose by cellulolytic microorganisms as a low pH causes a drop in both the efficiency and growth of these species (Owens and Goetsch, 1986). This concept was supported both by Doyle (1987) and Ranilla *et al*. (2000) who found that a reduction in cellulase activity occurs with a decreasing pH until negligible cellulose digestion is found below pH 6.0.

2.9 Blood urea nitrogen

Nitrogen in the blood is the result of diffused NH_3-N across the wall of the rumen, as well as the transport of AA's and peptides from the small intestine. Absorbed AA's and peptides which are not utilised in the gastrointestinal tract are deaminated by the liver and converted to urea. This urea forms part of the BUN pool (Kohn *et al*., 2005). Plasma urea concentration is therefore considered a realistic predictor of both N intake and N utilisation in sheep as rumen NH_3-N concentration is highly correlated with plasma urea concentration (Sunny *et al*., 2007).

Although a close relationship between N intake and BUN levels exists, it was pointed out that many factors can modify this relationship, most notably starvation and consumption of diets low in N which increases BUN levels as body protein is catabolised (Torell *et al*., 1974). Conversely, diets high in N can also be associated with high BUN levels due to the fact that N in excess of what is required by the animal is taken up by the blood after conversion to urea by the liver (Aliyu *et al*., 2012). In literature, BUN levels regarded as normal usually fall between the values in a range. According to Eshratkhah *et al*. (2008) this range is $10.9 - 21.2$ mg·100 mL^{-1} for sheep, resembling the range reported by the Research Animal Resources (RAR, 2009) of $8 - 20$ mg·100 mL^{-1}.

2.10   Hypotheses

H_0: Replacing urea partially with $(NH_4)_2SO_4$ will have no effect on certain rumen parameters, digestibility, disappearance of DM and NDF, N-balance, DMI and body weight (BW).

H_1: Replacing urea partially with $(NH_4)_2SO_4$ will have an effect on certain rumen parameters, digestibility, disappearance of DM and NDF, N-balance, DMI and BW.

H_0: Replacing urea partially with lucerne (*Medicago sativa*) hay will have no effect on certain rumen parameters, digestibility, disappearance of DM and NDF, N-balance, DMI and BW.

H_1: Replacing urea partially with lucerne hay will have an effect on certain rumen parameters, digestibility, disappearance of DM and NDF, N-balance, DMI and BW.

H_0: Replacing urea partially with both $(NH_4)_2SO_4$ and lucerne hay will have no effect on certain rumen parameters, digestibility, disappearance of DM and NDF, N-balance, DMI and BW.

H_1: Replacing urea partially with both $(NH_4)_2SO_4$ and lucerne hay will have an effect on certain rumen parameters, digestibility, disappearance of DM and NDF, N-balance, DMI and BW.

2.11   Objectives of the study

The objectives of this study were to determine whether replacing urea partially by $(NH_4)_2SO_4$, lucerne (*Medicago sativa*) hay or by a combination of $(NH_4)_2SO_4$ and lucerne hay will increase efficiency of low-quality roughage digestibility, improve intake and N-balance.

CHAPTER 3
MATERIALS AND METHODS

3.1 Facility and experimental animals

A comprehensive project protocol was submitted to and approved by the Animal Ethics Committee of the University of Pretoria (project number: EC036-14). Following approval, the research trial was conducted at the University of Pretoria Experimental Farm which is situated in Hatfield, Pretoria. Four cannulated Merino wethers with an average age of 24 months were used in this trial. The wethers had an average body weight of approximately 43 kg at commencement of the trial.

A veterinarian from the Faculty of Veterinary Science at the University of Pretoria surgically fitted each of the wethers with an 11C 2" Rubber Cannula (Bar Diamond, Inc. [®], 29865 Sharp Lane, Idaho, USA). The cannulas had a 5 cm centre diameter and a 12 cm outer diameter.

3.2 Intake trial

An intake trial was conducted on the low-quality hay to calculate approximate roughage intake. This was done over a 14-day period before the study commenced. Every day the sheep were fed a weighed amount of roughage of which the orts was weighed back at the start of the next feeding. A commercial protein lick (Table 3.1) was supplied together with *ad libitum* water to ensure that the basal metabolic demands of the sheep were met.

Table 3.1 Nutrient composition of the commercial protein lick fed during intake trial $(g \cdot kg^{-1})$

Nutrient	Inclusion $(g \cdot kg^{-1}$ on DM basis)
Protein (min)	440.0
NPN as % of protein (max)	90 %
Urea (max)	138.0
Fibre (max)	100.0
Phosphorous (min)	15.0
Calcium (max)	45.0

The intake values obtained in the last 7 days of this intake trial were used to determine average intake per sheep. This served as the approximate amount of roughage that was fed to each wether in the main study and was also used to determine the amount of supplement to be fed with commencement of the trial.

3.3 Experimental design

The experiment was a 4 x 4 Latin Square Design and each wether was randomly assigned to a different treatment during each period. The study consisted of four 18-day periods and lasted for a total of 72 days. This was divided into 10 days for adaptation (Wickersham *et al*., 2008) followed by 5 days during which different samples were collected for analyses and the final 3 days were allocated to the *in situ* digestibility trial. The wethers were kept together in a pen for the first 7 days of the adaptation period to minimize stress after which they were placed in separate metabolism crates. The body weight of each wether was recorded before each new experimental period.

3.4 Experimental diets

The sheep had *ad libitum* access to low-quality *Eragrostis curvula* hay (Table 3.2). Fresh water was supplied daily and the supplements were inserted directly into the rumen via the cannulae three times a day (07:00, 12:00 and 16:00).

Table 3.2 Chemical analysis of the basal *Eragrostis curvula* hay (g·100 g^{-1})

	DM	Ash	CP	NDF	ADIN
As is basis	92.51	4.33	5.77	73.50	1.42
DM basis	100.00	4.68	6.24	79.45	1.53

3.4.1 *Nutrient supplementation*

In this study all formulations of the supplements were done according to the nutrient requirements (NRC, 2007) of a 40 kg ewe fed for breeding purposes (Table 3.3). This was done to simulate conditions persisting within the rumen when specific requirements exist according to which feed is given.

Table 3.3 Nutrient requirements (per day) of a 40 kg ewe fed for breeding purposes (NRC, 2007)

Nutrient	Requirement (DM basis)
Energy	6.82 MJ ME
Crude protein	69.0 g
Calcium	2.1 g
Phosphorous	1.5 g

3.4.2 Adaptation period

For the 10-day adaptation period (Wickersham *et al*., 2008) treatments were formulated according to the average roughage intake determined through the intake trial and consisted of a mineral mixture, an N source (depending on treatment), as well as starch to ensure that the diets were iso-nitrogenous and iso-energetic. It was imperative to maintain a constant N:S ratio throughout all treatments and therefore feed-grade S was included in the formulation of the treatments (where applicable). For both treatments 2 and 4, $(NH_4)_2SO_4$ was included at 3.0% of total DMI (Graham *et al*., 1976).

The required N was supplied as follows:

Treatment 1 - 100% urea;

Treatment 2 - 50% urea and 50% $(NH_4)_2SO_4$;

Treatment 3 – 50% urea and 50% lucerne; and

Treatment 4 – 8% urea, 84% $(NH_4)_2SO_4$ and 8% lucerne (Table 3.4).

Table 3.4 Composition of the control and test treatments on as is basis ($g·d^{-1}$)

Treatment		Urea	$(NH_4)_2SO_4$	Lucerne	Starch	Premix	Feed grade sulphur
1	Urea (Control diet)	12.9	-	-	156.6	1.2	5.5
2	Urea + $(NH_4)_2SO_4$	2.1	23.1	-	156.6	1.2	-
3	Urea + Lucerne	6.5	-	113.6	91.1	1.2	5.5
4	Urea + $(NH_4)_2SO_4$ + Lucerne	1.1	23.1	18.6	154.8	1.2	-

During the first adaptation period it was found that the pH of the rumen fluid of the wethers decreased at an undesirable rate due to the amount of starch fed as part of the supplements and as a result it was decided to feed sodium bicarbonate in order to ensure the pH does not reach a critically low value. Sodium bicarbonate was included at a rate of 1.5% of total DM intake during the adaptation period and at 1.0% of total DM intake during the sampling and *in situ* periods.

3.4.3   *Sampling period*

During the 5-day sampling and 3-day *in situ* periods supplement quantities were altered daily according to roughage intake of the previous day. This was done for each sheep individually to ensure the stated requirements were met as closely as possible.

3.5 Sampling and data collection

The wethers were placed in separate metabolism crates on day 8 of the adaptation period and a faecal bag was attached to each animal. Calculation of intake of the roughage, as well as the collection of faeces, urine and rumen fluid took place during the 5-day sampling period. All samples collected were frozen as soon as possible until laboratory analyses commenced (Osuji *et al*., 1993).

3.5.1   *Roughage sampling and intake calculations*

Roughage intake was estimated by subtracting the orts which remained the following morning from the initial amount fed the previous day. The fresh roughage, as well as the orts was thoroughly mixed before separate subsamples of approximately 3% of both was taken (Olson *et al*., 1999). Respective roughage and orts samples were pooled over the 5-day period for each wether.

3.5.2   *Faecal sampling*

The attached faecal bags were emptied every morning before the sheep were fed and total faecal output after 24 hours was weighed. Thereafter a representative sample of approximately 10% was taken daily (Chen and Gomes, 1995) and pooled separately for each of the wethers over the 5-day sampling period.

3.5.3   Rumen fluid sampling

Rumen fluid composition exhibits diurnal variation and for this reason it was necessary to collect samples at different times during the course of the sampling period in order to collect representative data for a 24-hour period (Table 3.5). Fluid was obtained from different regions within the rumen using a suction strainer and strained through two layers of cheese cloth. The fluid was used to determine ruminal NH_3-N, VFA concentration, as well as rumen pH:

1) 5 mL of 50% H_2SO_4 as preservative was added to 30 mL of rumen fluid for NH_3-N analysis;

2) 4 mL of 25% H_3PO_4 as preservative was added to 20 mL of rumen fluid for VFA analysis;

3) pH was measured immediately after the fluid was strained through two layers of cheese cloth using a pH meter (Oakton Waterproof EcoTestr pH 2 Pocket Tester, Oakton Instruments®, Vernon Hills, Illinois, USA). The meter was re-calibrated daily with standard buffers (Buffer solution pH 4.00 and Buffer solution pH 7.00, Merck® (Pty) Ltd, Modderfontein, Gauteng, SA).

Table 3.5 Rumen fluid and blood collection times

Day in sampling period	Morning	Afternoon
Day 1	06:00	18:00
Day 2	03:00	15:00
Day 3	00:00	12:00
Day 4	09:00	21:00

Respective rumen fluid samples were pooled for VFA concentration and NH_3-N determination for each wether over the treatment periods.

3.5.4   Urine sampling

A collection bottle with 5 mL of 10% H_2SO_4 as preservative was placed underneath the urine pans of the metabolism crates to collect urine. The urine was collected to analyse the N concentration. The 10% H_2SO_4 was added to decrease the pH to below 3 in order to avoid N loss (Osuji et al., 1993; Chen and Gomes, 1995). Total volume output was recorded after which a subsample of 50 mL was taken and frozen (Jetana et al., 2010) and all urine samples were pooled over each sampling period for each wether respectively.

3.5.5 *In situ trial*

The NDF and DM degradability over time were determined for the hay by means of an *in situ* trial after the 5-day sampling period. A representative sample of the dry roughage (Hvelplund and Weisbjerg, 2000) were milled through a 1 mm sieve (Kitessa *et al*., 1999) and 5.0 g was weighed off into rumen Dacron bags which were then suspended in the rumen for incubation. The bags were 10 x 10 cm in size with 50 micron porosity (ANKOM Technology®, O'Neil Road, New York, USA). The samples were prepared in duplicate and incubated for 0, 3, 6, 9, 12, 18, 24, 48 and 72 hours, respectively (Ørskov *et al*., 1980). Bags were retrieved by means of the stocking method as described by Cruywagen (2006). After retrieval the bags were washed under running tap water until the water ran clear and thereafter the bags were frozen pending analyses of DM and NDF of the residues in the bags.

3.5.6 *Blood sampling*

During the sampling period blood was drawn from the jugular vein of each wether in order to analyse the BUN. This was done at the same time as rumen fluid collection (Table 3.4) since BUN levels also vary depending on the time of day and a curve needed to be drawn for a 24-hour period. Du Buisson, Kramer, Swart, Bouwer Inc. as part of Ampath, pooled the blood samples for each wether and also performed the analyses. The blood was collected in serum separation tubes as the analyses were done on blood serum.

3.6 Sample preparation and assays

The following laboratory analyses were done at Nutrilab, Department of Animal and Wildlife Sciences at the University of Pretoria. All samples were analysed in duplicate.

3.6.1 Feed, orts and faeces

Total tract digestibility of DM, organic matter (OM) and NDF was determined by using feed, orts and faecal samples after being milled through a 2 mm sieve. For DM determination approximately 2.0 g of the samples were placed in a 105 °C oven overnight after which the samples were weighed back to calculate moisture loss (Hinnant and Kothmann, 1988; Osuji *et al*., 1993). These samples were then placed in a 250 °C oven for 2 hours followed by 600 °C for 4 hours in order to determine the ash concentration. The DM and ash percentages were used to calculate OM percentage. For NDF, filter bags were filled with 0.45 – 0.55 g of sample and

heat sealed for NDF determination with the ANKOM Automated Fiber Analyzer[®]. After the samples dried, they were weighed back and NDF determined on a DM basis.

Digestibility was then determined as follows for OM, DM and NDF respectively (McDonald *et al*., 2011):

$$\text{Apparent digestibility} = \frac{[\text{Nutrient in feed (g)} - \text{nutrient in orts (g)}] - \text{nutrient in faeces (g)}}{[\text{nutrient in feed (g)} - \text{nutrient in orts (g)}]}$$

For N, the values were measured by the TruMac[®] Series Macro Determinator (LECO Corporation). Since endogenous N losses were not accounted for in this study, the mean apparent N-retention values were determined by means of the following equation (Jetana *et al*. 2010):

$$\text{Apparent N-retention (g·d}^{-1}) = \text{Intake N (g·d}^{-1}) - \text{faecal N (g·d}^{-1}) - \text{urine N (g·d}^{-1})$$

3.6.2 Rumen fluid – ammonia nitrogen

Rumen fluid samples collected for analysis of NH_3-N were centrifuged to obtain pure fluid after which it was prepared with phenol and hypochlorite reagents and placed into a 100 °C water bath for 5 minutes (Smith and Murphy, 1993). After the samples cooled down the absorbance was measured by the SPEKOL[®] 1300 UV VIS Spectrophotometer (Analytik Jena AG, Rostock, Germany). The absorbance values for NH_3-N were converted to mg·100 mL^{-1} of fluid by means of known values for the working standards.

3.6.3 Rumen fluid – volatile fatty acids

The rumen fluid collected for determining of the VFA composition was analysed using the Tracera® Gas Chromatography System 2010 (Shimadzu Corporation, Nakagyo-ku, Kyoto 604-8511, Japan). Results for acetic acid, propionic acid, butyric acid, iso-butyric acid and valeric acid were given as percentage per rumen fluid.

3.6.4 *In situ* samples

The nylon bags were dried in an oven for 24 hours at 55° C. Residues of the *in situ* trial were analysed for DM, Ash and NDF. The analytical methods followed, were the same as described for feed, orts and faeces samples (3.6.1).

3.7 Calculations

Supplement quantities were calculated (Table 3.6) based on chemical analyses of the roughage and requirements to be met of a 40 kg ewe fed for breeding purposes (NRC, 2007) for the duration of the trial. The calculations were done based on the average intake of 600 g roughage per sheep per day during the adaptation period. During the sampling and *in situ* periods the roughage intake was changed daily according to the roughage intake of the previous day.

Table 3.6 Calculations to determine the quantity of supplements required for each treatment

Crude protein			Unit
Roughage intake:	(as is basis)	600.00	g / sheep / day
	(DM basis)	552.60	g / sheep / day
Roughage ADIN:		22.67	%
(100-ADIN):		77.33	%
Roughage CP (DM basis):		7.40	%
		40.89	g CP in 552,6 g hay
Roughage CP available:		31.62	g
Required CP:	(DM basis)	69.00	g / sheep / day
Required CP from supplements:	(DM basis)	37.38	g / sheep / day
$(NH_4)_2SO_4$ CP:	(DM basis)	1.34	g CP / g DM
Urea CP:	(DM basis)	290	%
Lucerne CP:	(DM basis)	18.06	g CP / kg DM

Treatment 1: 100% Urea

Urea (g) required/sheep/day	(DM basis)	12.89	g
Urea (g) required/sheep/day	(as is basis)	12.93	g

Total feed intake:	(as is basis)		
Roughage		600.00	g
Urea		12.93	g
Starch		156.56	g
Total		769.49	g

Treatment 2: Urea and $(NH4)_2SO_4$

Total feed intake		769.49	g
3% inclusion of $(NH_4)_2SO_4$:	(as is basis)	23.08	g
$(NH4)_2SO_4$:	(DM basis)	23.32	g
CP from $(NH4)_2SO_4$:	(DM basis)	31.25	g
CP required from urea:	(DM basis)	6.13	g
Thus urea (g) required/sheep/day	(DM basis)	2.12	g
Thus urea (g) required/sheep/day	(as is basis)	2.12	g

Treatment 3: Urea and lucerne

CP required by urea	(DM basis)	18.69	g
Thus urea (g) required/sheep/day	(DM basis)	6.44	g
Thus urea (g) required sheep/day	(as is basis)	6.47	g
CP required by lucerne	(DM basis)	18.69	g
Thus lucerne (g) required/sheep/day	(DM basis)	103.49	g
Thus lucerne (g) required/sheep/day	(as is basis)	113.60	g

Table 3.6 *Continued*

Treatment 4: Urea, lucerne and $(NH4)_2SO_4$

Total feed intake		769.49	g
3% inclusion of $(NH_4)_2SO_4$:	(as is basis)	23.08	g
$(NH4)_2SO_4$:	(DM basis)	23.32	g
CP from $(NH4)_2SO_4$:	(DM basis)	31.25	g
CP required from urea and lucerne:	(DM basis)	6.13	g
CP required by urea	(DM basis)	3.07	g
Thus urea (g) required/sheep/day	(DM basis)	1.06	g
Thus urea (g) required/sheep/day	(as is basis)	1.06	g
CP required by lucerne	(DM basis)	3.07	g
Thus lucerne (g) required/sheep/day	(DM basis)	16.98	g
Thus lucerne (g) required/sheep/day	(as is basis)	18.64	g

Metabolisable energy			Unit
Roughage ME:			
MJ / kg DM		8.4	
MJ in roughage DM consumed		4.64	MJ ME / day
Required ME:		6.82	MJ ME / day
Required ME from supplements:		2.18	MJ ME / day
Starch supplement:	(DM basis)	87	%
ME supplied:		15.99	MJ / kg DM
Lucerne ME:	(DM basis)	8.80	MJ ME / kg DM

Treatment 1: 100% Urea

ME required from supplement	(DM basis)	2.18	MJ ME / day
Starch supplement required	(DM basis)	136.22	g
Starch supplement required	(as is basis)	156.56	g

Treatment 2: Urea and $(NH4)_2SO_4$

ME required from supplement	(DM basis)	2.18	MJ ME / day
Starch supplement required	(DM basis)	136.22	g
Starch supplement required	(as is basis)	156.56	g

Treatment 3: Urea and lucerne

ME required from supplements	(DM basis)	2.18	MJ ME / day
ME supplied by lucerne	(DM basis)	0.91	MJ ME / day
ME required from starch	(DM basis)	1.27	MJ ME / day
Starch supplement required	(DM basis)	79.27	g
Starch supplement required	(as is basis)	91.10	g

Treatment 4: Urea, lucerne and $(NH4)_2SO_4$

ME required from supplements	(DM basis)	2.18	MJ ME / day
ME supplied by lucerne	(DM basis)	0.15	MJ ME / day
ME required from starch	(DM basis)	2.03	MJ ME / day
Starch supplement required	(DM basis)	126.87	g
Starch supplement required	(as is basis)	145.82	g

Table 3.6 *Continued*

Sulphur			Unit
S from (NH$_4$)$_2$SO$_4$:	(DM basis)	0.24	g / g DM
S from Feed grade Sulphur:	(DM basis)	0.995	g / g DM
Treatment 1: 100% Urea			
S required from feed grade sulphur	(DM basis)	5.60	g
Feed Grade Sulphur required	(as is basis)	5.62	g
Treatment 2: Urea and (NH4)$_2$SO$_4$			
S from (NH$_4$)$_2$SO$_4$:	(DM basis)	5.60	g
Treatment 3: Urea and lucerne			
S required from feed grade sulphur	(DM basis)	5.60	g
Feed Grade Sulphur required	(as is basis)	5.62	g
Treatment 4: Urea, lucerne and (NH4)$_2$SO$_4$			
S from (NH$_4$)$_2$SO$_4$:	(DM basis)	5.60	g

3.8 Statistical analyses

All statistical analyses were done as a Latin Square Design using Statistical Analyses Systems (SAS, 2015) and the sampling data obtained following completion of laboratory analyses were subjected to the Proc GLM statistical model (Montgomery, 2009):

$$\gamma_{ijk} = \mu + \tau_i + \rho_j + \varsigma_k + \varepsilon_{ijk}$$

where:

γ_{ijk} is the observation in the ith row and the kth column for the jth treatment

μ is the overall mean

τ_i is the ith treatment effect

ρ_j is the jth period effect

ς_k is the kth animal effect

ε_{ijk} is the random error

Repeated Measures Analysis of Variance with the GLM model (SAS, 2015) was used for repeated measures and testing for no differences in treatment means under the null hypothesis was done, using:

$F_0 = MS_{treatments} / MS_E$ distributed as $F_{p-1, (p-2)(p-1)}$

The Fisher Least Significant Difference Method (SAS, 2015) was used for comparing all pairs of means in order to establish if differences between treatments were significant or not with significance measured as $P < 0.05$:

$$\text{LSD} = t_{\alpha/2,\ N\text{-}a} \sqrt{MS_E \left(\frac{1}{n_i} + \frac{1}{n_j} \right)}$$

3.8.1 Statistical analyses of *in situ* data

In situ DM and NDF data were analysed according to the equation by Ørskov and McDonald (1979):

$$p = a + b\,(1 - e^{-ct})$$

With p = degradation after time "t";

a = the intercept of the degradation curve at time zero (which is the component degraded immediately)

b = the potential degradability over time of the remaining component

c = the rate constant for the degradation of fraction b

The obtained values for a, b, c as well as ED were then analysed statistically by means of the Proc GLM model as described above. However, with regards to the NDF analyses it is important to note that an incomplete data set was used as the data points did not fit the Ørskov model as desired and it was therefore necessary to remove some of the data. Regardless, the removal of data did not significantly affect the values for the mentioned parameters and only served the purpose of improving the coefficient of variation.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 NH$_3$-N and BUN

There were no significant (P < 0.05) differences for the mean BUN concentration between the control (urea only) and the other treatments (Table 4.1). Urea in the blood is directly transferred to the rumen by moving across the epithelial cells where it is degraded to NH$_3$ when the concentration of urea is higher than in the rumen. Also, a high concentration of NH$_3$ in the rumen would lead to diffusion of NH$_3$ into the blood (Reynolds and Kristensen, 2008). Therefore, as could be expected, it was found that the results of BUN and NH$_3$-N followed the same tendency across treatments as a definite correlation exists between BUN and rumen NH$_3$-N (Figure 4.1). Torell *et al*. (1974) reported that BUN levels in ruminants are used for assessing the value of protein in the feed on the premise that BUN levels are directly correlated to the production of NH$_3$ in the rumen.

The total rumen NH$_3$-N measured in the rumen fluid was the result of the degradation of the nitrogenous compounds fed to the wethers, as well as the breakdown of urea recycled from the liver after ammonia detoxification (Abdoun *et al*., 2007). The rumen NH$_3$-N concentration for the UASL treatment (16.5 mg·100 mL^{-1}) differed significantly (P < 0.05) from those of the U and UL treatments at 10.2 and 11.0 mg·100 mL^{-1}, respectively. Baloyi *et al*. (2006) measured the results of legume supplementation for veld hay against what the authors regarded as an optimal concentration of 20 mg·100 mL^{-1} NH$_3$-N for low-quality forages. According to Kanjanapruthipong and Leng (1998) the optimum NH$_3$-N in the rumen fluid is between 5 mg·100 mL^{-1} and 25 mg·100 mL^{-1}. These authors emphasised that it is extremely important to determine an optimum concentration for NH$_3$-N in the rumen fluid especially when animals are fed a low-quality roughage diet since any increase in the ratio of protein to energy could lead to an elevation in the utilisation of the roughage through improved intake and digestibility. The results of this study are in accordance with this statement as values ranged from 10.2 to 16.5 mg·100 mL^{-1}. The highest NH$_3$-N values were obtained with the UASL treatment which could indicate an additive effect with the combination of all three supplements.

Table 4.1 The effect of different nitrogen sources on the mean rumen NH_3-N concentration and mean BUN concentration as measured over a 24-hour period

	Nitrogen supplementation[1]				
	U	UAS	UL	UASL	SE_m[2]
BUN (mg·100 mL^{-1})	14.3	18.2	15.4	18.5	0.78
NH_3-N (mg·100 mL^{-1})	10.2^b	12.7^{ab}	11.0^b	16.5^a	1.89

[1] Supplements: U (urea only); UAS (urea + $(NH_4)_2SO_4$); UL (urea + lucerne); UASL (urea, lucerne + $(NH_4)_2SO_4$)

[2] SE_m = standard error of the mean

[ab] Row values with a different superscript differ significantly ($P < 0.05$)
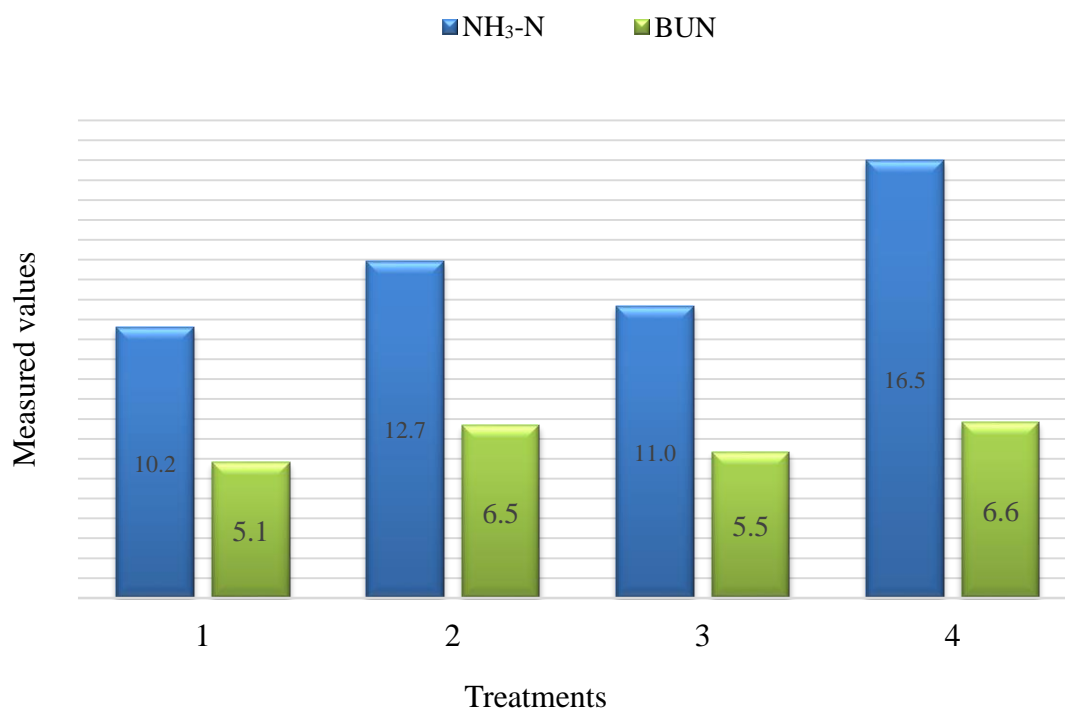

Figure 4.1 Comparison of the tendency for the mean NH_3-N in ruminal fluid and BUN levels across treatments

However, according to a more recent study by Detmann *et al*. (2009) it was found that for low-quality roughage the optimum rumen NH_3-N concentration for NDF degradability was determined to be 8 mg·100 mL^{-1}. In order to obtain optimum intake and optimum MCP

production, respective levels of 15 mg·dL^{-1} and 14.5 mg·dL^{-1} are required. Therefore in the current study, all of the treatments fulfilled in this requirement for NDF degradation but only treatment UASL qualifies for optimum intake and MCP production.

4.2 Rumen fluid pH

At a few of the sampling times the rumen fluid pH showed variation between some of the treatments (Table 4.2). The pH measurements ranged from $6.4 - 7.1$ for the control treatment (urea only), from $6.0 - 7.0$ for UAS, from $6.1 - 7.0$ for UL and from $5.9 - 6.9$ for the UASL treatment. At every measurement, the pH of the rumen fluid for the urea only treatment was higher compared to the UASL treatment, which is evident in the average pH values. The pH measurements together with the times when supplements were inserted in the rumen are illustrated in Figure 4.2.

The average pH values corresponds to the expected NH_3-N concentrations (Table 4.1) of the treatments in that the treatment with the lowest overall pH (UASL) exhibited the highest NH_3-N concentration. This is explained by the fact that at lower ruminal pH, the rumen wall permeability for NH_3 is depressed, causing absorption of NH_3 to remain stable regardless of an increase in the rumen NH_3-N concentration (Abdoun *et al*., 2007). The reverse is also true in that the treatment with the highest average pH (U) revealed the lowest NH_3-N concentration which could indicate an increased absorption of NH_3 due to higher permeability of the rumen epithelium at higher pH (Rémond *et al*. 1993). However, the lower pH explains why no clear signs of acute NH_3 toxicity prevailed as the absorption rate of NH_3 is lower at a low pH (Abdoun *et al*., 2007).

4.3 DM, OM and NDF digestibility

The apparent digestibility of DM, NDF and OM is presented in Table 4.3. The DM digestibility of the control (U) treatment differed significantly ($P < 0.05$) from both the UAS and the UASL treatments with a low digestibility of 45.6%. The digestibility percentages of the tested treatments exceeded 51%. There were no significant differences between the treatments for NDF digestibility as it ranged only from 45.0% to 47.2%. The OM digestibility showed significant difference ($P < 0.05$) between the control treatment and the UAS treatment and there was a tendency for the control to differ from the UASL treatment ($P \leq 0.10$).

The DM digestibility showed similarity to the OM digestibility across treatments (Figure 4.3). However, the same similarity was only apparent for treatments U, UAS and UL with regards to NDF digestibility.

Table 4.2 The variation in rumen fluid pH as an effect of different nitrogen sources as supplements measured at 3 hour intervals

	Nitrogen supplementation[1]				
Time	U	UAS	UL	UASL	SE_m[2]
00:00	6.6	6.6	6.7	6.4	0.15
03:00	6.7[a]	6.8[a]	6.6	6.2[b]	0.15
06:00	7.0	7.0	7.0	6.9	0.11
09:00	7.1[a]	7.0	6.8[b]	6.9	0.06
12:00	6.7	6.5	6.5	6.4	0.15
15:00	6.7[a]	6.1[b]	6.6[a]	6.3	0.15
18:00	6.4	6.2	6.4	5.9	0.19
21:00	6.5[a]	6.0	6.1	6.0[b]	0.16
Average pH	6.7	6.5	6.6	6.4	

[ab] Row means with a different superscript differ significantly ($P < 0.05$)

[1] Supplements: U (urea only); UAS (urea + $(NH_4)_2SO_4$); UL (urea + lucerne); UASL (urea, lucerne + $(NH_4)_2SO_4$)
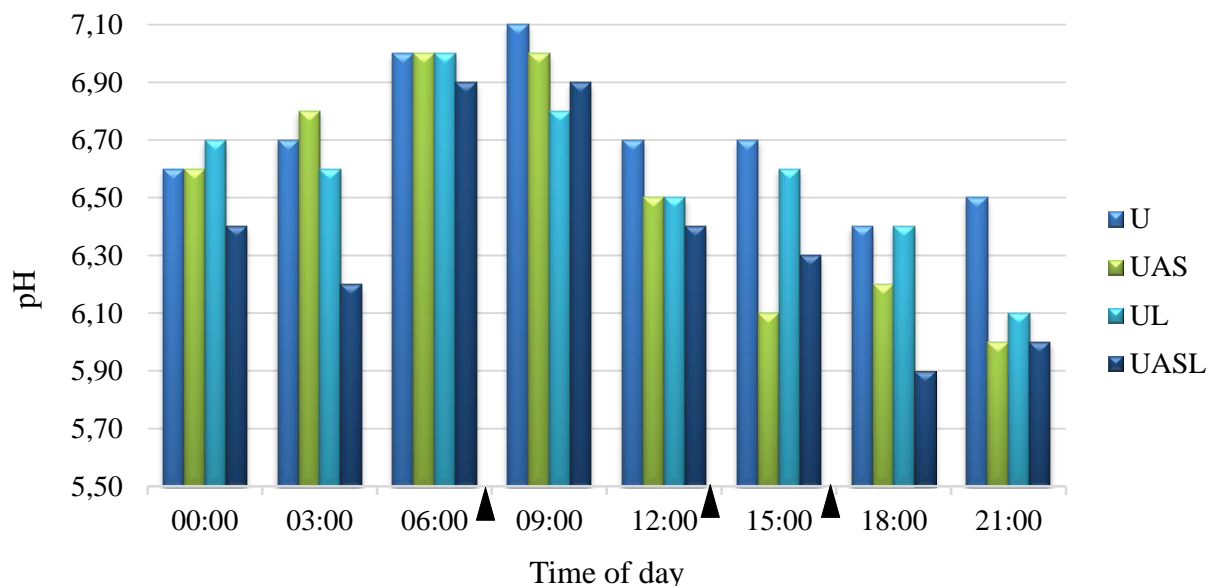
[2] SE_m = standard error of the mean

Figure 4.2 Comparison of pH measurements taken at different sampling times (time of day supplements were given indicated by ▲ at 7:00, 12:10 and 16:00, respectively)

Table 4.3 The apparent digestibility percentages of DM, NDF and OM when *Eragrostis curvula* hay was supplemented with different nitrogen sources

	Nitrogen supplementation[1]				
	U	UAS	UL	UASL	SE_m[2]
DM digestibility (%)	45.6[b]	57.2[a]	51.9	55.3[a]	2.53
NDF digestibility (%)	45.5	47.2	45.2	45.0	2.41
OM digestibility (%)	48.7[b]	58.2[a]	51.4	57.3	2.50

[1] Supplements: U (urea only); UAS (urea + $(NH_4)_2SO_4$); UL (urea + lucerne); UASL (urea, lucerne + $(NH_4)_2SO_4$)

[2] SE_m = standard error of the mean

[ab] Row values with a different superscript differ significantly (P < 0.05)
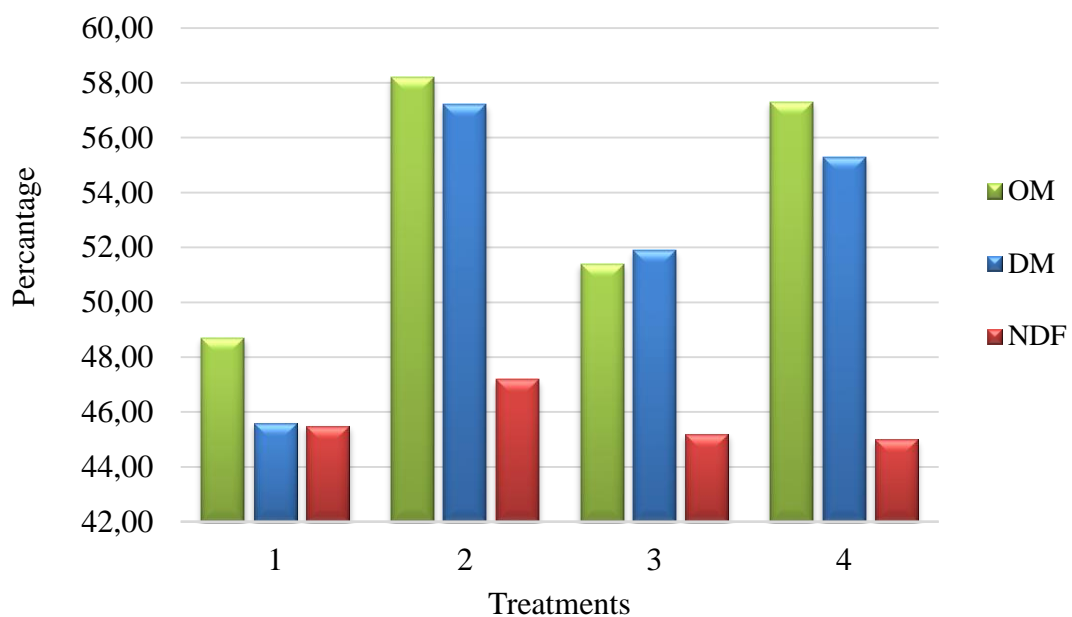
Figure 4.3 The apparent digestibility percentages of *Eragrostis curvula* hay for OM, DM, and NDF across treatments

Research by Haddad (2000) found an increase in both the DM and OM digestibility of barley straw when low levels of lucerne were supplemented compared to only urea inclusion. Also, in a report by Heuzé *et al*. (2015) the authors supported the premise that supplementing low-quality *Eragrostis curvula* hay with tree lucerne (*Chamaecytisus palmensis*) led to an

increase in the digestibility of OM. This could be a possible explanation for the finding of the current study where DM and OM digestibility were the highest when lucerne was included in small quantities.

Mouriño *et al*. (2001) did an in-depth study to determine the influence of pH on the digestion of cellulose and stated that substantial evidence proves that at pH below 6.0 the fermentation of NDF decreases *in vitro*. In addition, Grant and Mertens (1992) found from an *in vitro* digestion study that not only was there a considerable lag time with NDF digestion, but the rate of fermentation was also markedly decreased when pH was below 6.2. These results are supported by Grant (1994) when it was evident that regardless of the absence or presence of different types of starch, NDF digestion was poor at a pH below 6.2. It was concluded by Russell and Wilson (1996) that the principal microbial species in the rumen responsible for digesting cellulose did not proliferate at a low pH and Ranilla *et al*. (2000) stated that when the pH of the rumen fluid decreased from 6.8 to 6.0 a depression in the digestion of fibre became apparent and a further reduction to below 6.0 could lead to severe inhibition. A few years later, it was accepted by Cajarville *et al*. (2006) that the optimum pH for cellulolytic activity is 6.7 ± 0.5. This could possibly explain why in the current study the lower NDF digestion was correlated with the treatment (UASL) exhibiting the lower average rumen fluid pH (Table 4.2).

The treatments with the $(NH_4)_2SO_4$ supplementation had the highest apparent DM and OM digestibility. This could not, however, be attributed to the S it contains since all treatments were supplemented with flower of sulphur in order to have all treatments on an iso-sulphur basis. No recorded studies have been done to determine the effect of $(NH_4)_2SO_4$ on digestibility and it is suggested from the current study that $(NH_4)_2SO_4$ had a positive effect on the apparent digestibility of DM, OM and NDF with regards to the treatments with the $(NH_4)_2SO_4$ inclusion (i.e. UAS and UASL). Further research should be conducted to determine the optimum level of $(NH_4)_2SO_4$ inclusion with regards to digestibility as it was the aim of this research to only fulfil in maintenance requirements with regards to CP. One explanation could be that the rate of $(NH_4)_2SO_4$ breakdown is not as rapid as urea hydrolysis and therefore N is released at a slower rate. In addition, it has been reported that ruminant organisms utilise $(NH_4)_2SO_4$ to a greater extent compared to urea (NRC, 1976).

As mentioned previously, efficient microbial growth and optimal MCP synthesis is highly dependent on the synchronised availability of both N and FME (Das *et al*., 2014). Therefore, it can be postulated that the treatments with the highest digestibility percentages (UASL and UAS) had the best synchrony of these nutrients in the rumen, allowing for efficient microbial activity. In contrast, treatment U portrayed the poorest digestibility indicating that

the hydrolysis of urea occurred too rapidly for the rumen microbes to utilise the released N effectively.

4.4 Volatile fatty acids

The total VFA concentration, as well as the molar concentration and percentages of the important VFA's are summarised in Table 4.4. There was no significant (P < 0.05) difference for the total concentration of VFA's between the four treatments. In the study of Haddad (2000) it was determined that the total rumen VFA concentration increased when lucerne was supplemented (inclusions were 150, 300 and 450 g·day^{-1}) compared to only or none urea inclusion which was attributed to the higher fermentability of the diet. According to Bird *et al*. (1994) this higher fermentability is the result of an increased availability of AA's, as well as RFC leading to improved synchrony of energy and N. This was also confirmed by Baloyi *et al*. (2006) who stated that the rate of fermentation was improved by the additional fermentable substrate. These support the tendency of the current study where the treatments containing lucerne (UL and UASL) had higher total VFA concentrations (68.8 mM·L^{-1} and 67.9 mM·L^{-1}, respectively) compared to the U and UAS treatments.

Table 4.4 Effect of different nitrogen supplements on VFA concentrations and percentages

	Nitrogen supplementation[1]				
	U	UAS	UL	UASL	SE$_m$[2]
Total VFA production (mM·L^{-1})	64.2	63.2	68.8	67.9	3.22
Acetic acid (mM·L^{-1})	48.2	48.1	54.0	52.0	3.08
Propionic acid (mM·L^{-1})	13.8	12.2	11.9	13.4	1.28
Butyric acid (mM·L^{-1})	1.2	1.5	1.4	1.2	0.13
Isobutyric acid (mM·L^{-1})	0.7	1.2	1.1	1.0	0.18
Valeric acid (mM·L^{-1})	0.3	0.2	0.4	0.3	0.04
A : P ratio	3.5	4.1	4.6	4.2	0.51
Acetate (%)	74.5	76.0	69.0	76.7	4.22
Propionate (%)	22.2	19.5	19.5	19.7	1.98
Butyrate (%)	1.8	2.3	2.2	1.8	0.12

[1] Supplements: U (urea only); UAS (urea + $(NH_4)_2SO_4$); UL (urea + lucerne); UASL (urea, lucerne + $(NH_4)_2SO_4$)

[2] SE$_m$ = standard error of the mean

There were no significant differences (P < 0.05) between the individual molar concentrations or the percentages of the three main VFA's (Table 4.4). This finding was also reported by Haddad (2000) who tested the influence of supplementing sheep with urea only or lucerne when feeding a low-quality basal diet.

4.5 *In situ* disappearance of DM and NDF

The *in situ* DM degradation for the low-quality hay is presented in Table 4.5. For the *a* fraction, there was a significant (P < 0.05) difference between the UAS and UL treatments. This indicates that a shorter lag phase was present for the disappearance of the hay when urea combined with lucerne was supplemented, compared to when urea and $(NH_4)_2SO_4$ were the supplements. Lucerne (as sole supplement) is generally accepted to have a longer lag phase (a smaller *a* value) but degradation continues for a longer period (larger *b* value) due to the more consistent supply of N, as well as energy (Repetto *et al*., 2003) compared to quickly degradable supplements such as urea and $(NH_4)_2SO_4$. However, since urea and lucerne were combined it is possible that the urea assisted in a quick initial degradation (Shete and Hol, 2007) after which the lucerne had its longer lasting effect on degradation due to lucerne also supplying FME. This is due to the enhanced proliferation of the rumen microbes as a result of an increased availability of AA's and RFC supplied by lucerne (Bird *et al*., 1994).

In terms of the *b* component, as well as the ED, there were no significant differences although the UL treatment tended to be the highest. This is supported by Bird *et al*. (1994) who found that lucerne supplementation proved to increase the DM disappearance after 24 hours. The outflow rate constant (*c*) also showed no significance between treatments.

The NDF degradation of the *in situ* trial is given in Table 4.6. There were no significant differences (P < 0.05) for any of the degradation parameters calculated. However, treatment UL which had the highest inclusion of lucerne tended to have the highest value for both the *b* and ED components. In a study by Detmann *et al*. (2009) it was suggested that for optimum ED of low-quality roughage NDF, a rumen NH_3-N concentration of 8 mg·100 mL^{-1} is required. All of the treatments fulfilled in this requirement which explains why no significant difference existed between treatments.

According to Ørskov *et al*. (1980) the ED component represents the amount of the DM/NDF which will be degraded in the rumen and is defined by the time for which the DM/NDF is present in the rumen. In a recent study on dairy cows, Tesfayohannes *et al*. (2013) reported that the ED of the DM component of low-quality *Eragrostis curvula* hay was 44.2%.

Table 4.5 Effect of treatments on the DM degradability of the *Eragrostis curvula* hay determined by the *in situ* method

	Nitrogen supplementation[1]				
	U	UAS	UL	UASL	SE$_\text{m}$[3]
Degradation parameters[2]					
a	7.3	6.9[b]	8.9[a]	7.3	0.49
b	31.4	33.0	40.0	35.7	6.85
c	0.05	0.05	0.02	0.03	0.014
ED	30.2	31.4	35.1	31.1	3.36

[1] Supplements: U (urea only); UAS (urea + $(NH_4)_2SO_4$); UL (urea + lucerne); UASL (urea, lucerne + $(NH_4)_2SO_4$)

[2] Parameters: a – soluble fraction, b – potentially degradable fraction, c – rate of degradation of b fraction

[3] SE$_\text{m}$: standard error of the mean

[ab] Row values with a different superscript differ significantly ($P < 0.05$)

Table 4.6 Effect of treatments on the NDF degradability of the *Eragrostis curvula* hay determined by the *in situ* method

	Nitrogen supplementation[1]					
	U	SE$_\text{m}$[3]	UAS	UL	UASL	SE$_\text{m}$[4]
Degradation parameters[2]						
a	2.6	0.71	2.2	1.8	2.1	0.41
b	33.9	12.39	36.2	42.2	36.8	7.15
c	0.03	0.006	0.03	0.02	0.02	0.003
ED	26.6	5.9	25.7	30.2	26.4	3.38

[1] Supplements: U (urea only); UAS (urea + $(NH_4)_2SO_4$); UL (urea + lucerne); UASL (urea, lucerne + $(NH_4)_2SO_4$)

[2] Parameters: a – soluble fraction, b – potentially degradable fraction, c – rate of degradation of b fraction

[3] SE$_\text{m}$: standard error of the mean of treatment U only due to incomplete dataset

[4] SE$_\text{m}$: standard error of the mean of treatments UAS, UL and UASL

Ribeiro *et al.* (2011) performed a study on beef steers to examine the effects of NPN supplements on low-quality (2.49% CP) on various parameters including DM and NDF degradability. It was found that no significant differences existed between the control (hay

only) and treatments for ED of both DM and NDF and values for ED of NDF ranged from 36% - 38% and between 41% and 43% for the ED of DM. In both of these cases the ED of the current study was lower than the abovementioned reported values. This could be attributed to the use of different animals (cattle vs. sheep) as feed utilisation between species differ (van Soest, 1994).

For both DM and NDF degradability the treatment with the highest lucerne inclusion (UL) exhibited the best ED of all the treatments. This is supported by Bird *et al*. (1994) where a study was performed on low-quality wheat straw to investigate the effect of lucerne supplementation on *in situ* degradability. Lucerne was supplemented at 150 g·day^{-1} and it was found that an increase in the degradability occurred with the lucerne addition. The authors attributed the improvement to an increased availability of AA's, as well as RFC which in turn improved the synchrony of energy and N due to the slower degradation and therefore release rate, assisting in the enhancement of the cellulolytic microbes. This is also supported by Haddad (2000) who found in his *in situ* study on degradability in ewes fed low-quality roughage that the rate and extent of NDF degradability was the same with urea and without urea, but meaningfully differed when lucerne was added as nitrogen supplement.

4.6 Intake of dry matter and digestible organic matter

The mean DMI and digestible organic matter intake (DOMI) expressed as g DM·kg^{-1} BW$^{0.75}$ for each treatment were determined from the average intakes of the four animals and are presented in Table 4.7. Animals receiving the UL supplements tended to have higher DM intakes ($P < 0.10$) compared to animals receiving the UASL supplements, although differences were not significant. The effects of supplementing a low-quality basal diet with a legume hay (e.g. lucerne) are well researched and the conclusion is generally a notable increase in DMI (Bird *et al*., 1994; Foster *et al.*, 2009). This supports the finding of the current study where the supplement with lucerne and urea had the higher DMI. Haddad (2000) and Wang *et al.* (2008) also reported that the inclusion of lucerne hay to the diet led to increases in DMI in their respective studies.

The DOMI showed no significant difference ($P < 0.05$) across treatments (Table 4.8). Although not significant, the DOMI was higher for the UAS and UL treatments. Kariuki *et al.* (1999) studied the effects of supplementing grass hay with lucerne on production parameters and found an increase in OMI.

Table 4.7 The effect of different nitrogen sources as supplements to *Eragrostis curvula* hay on total dry matter intake (DMI) and digestible organic matter intake (DOMI) of sheep

	Nitrogen supplementation[1]				
	U	UAS	UL	UASL	SE_m[2]
Mean DMI (g DM·kg^{-1} BW$^{0.75}$ per day)	45.8	44.7	50.7	43.9	2.28
Mean DOMI (g DM·kg^{-1} BW$^{0.75}$ per day)	20.5	21.9	21.9	20.8	1.10

[1] Supplements: U (urea only); UAS (urea + $(NH_4)_2SO_4$); UL (urea + lucerne); UASL (urea, lucerne + $(NH_4)_2SO_4$)

[2] SE_m = standard error of the mean

Additionally, in a recent study by Schnaider *et al.* (2014) it was found that the inclusion of a legume hay in a basal diet consisting of dwarf elephant grass (*Arachis pintoi*) hay positively affected the DOMI. Although not significant, the treatments which included $(NH_4)_2SO_4$ tended to have the lower DMI. It was reported by Graham *et al*. (1976) that $(NH_4)_2SO_4$ inclusion in the diet possibly caused a depressed appetite resulting in lower feed intakes. However, there were no significant differences (or notable tendencies) between treatments in terms of the DOMI which is considered a more accurate measurement of usable matter consumed by the animal.

It was found by Detmann *et al*. (2009) that when the digestibility of fibrous compounds are improved it results in an increase in intake of low-quality roughage. Conversely, this is associated with elevated forage degradation brought on by N supplementation. This corresponds to the current study where the treatment with the highest ED for both DM and NDF *in situ* degradation (UL) also had the highest DMI.

4.7 Apparent nitrogen balance and body weight

In this study, no corrections were made for endogenous N losses therefore the N-balance is only regarded as apparent values (McDonald *et al*., 2011). The mean apparent N-retention values of the wethers is given in Table 4.8. There were no significant ($P < 0.05$) differences between the values across treatments, although the UAS treatment showed more than double the value obtained with the UL treatment and tended to have a higher apparent N-balance. According to Jetana *et al*. (2010) a better N-balance could indicate better synchrony between the carbohydrate and N compounds within the rumen. This is the result of more efficient utilisation of the N by the rumen microbes.

Table 4.8 The effect of different nitrogen sources as supplements on apparent N-retention ($g \cdot d^{-1}$)

	Nitrogen supplementation[1]				
	U	UAS	UL	UASL	SE_m[2]
Apparent N-retention ($g \cdot d^{-1}$)	2.3	2.4	1.0	1.9	1.13

[1] Supplements: U (urea only); UAS (urea + $(NH_4)_2SO_4$); UL (urea + lucerne); UASL (urea, lucerne + $(NH_4)_2SO_4$)

[2] SE_m = standard error of the mean

However, in contrast to these results Foster *et al*. (2009), as well as Bird *et al*. (1994) found that N-retention was increased by legume supplementation. It is important to note that in the current study the statistical analysis of the N-retention revealed an extremely high coefficient of variation which leads to questioning the validity of these results. In addition, previous studies on N-balance in cows revealed that the N-retention values calculated from intake and excretion via urine and faeces are usually higher than the true value due to the loss of NH_3 from the urine and faeces when collected and analysed (Spanghero and Kowalski, 1997). Furthermore, Reynolds and Kristensen (2008) stated that even when corrections are made for these (and endogenous) losses, in most situations the measurements of the N-retention are still too high.

The average loss in body weight of the wethers as a percentage of total BW for each treatment is depicted in Figure 4.4. Throughout all of the treatments (including the control) the wethers lost body weight which further questions the legitimacy of a positive N-balance since BW loss is usually correlated with a negative N-balance. In a study performed by Fincham *et al*. (2009) on feeding medium quality hay (grass only compared to a grass-legume mixture) to lambs with a BW of 38 kg ± 4.3 kg, it was found that the lambs lost an average of 7 kg during the study. This loss was reflected in a negative apparent N-balance and the authors stated that the negative value could be partially explained by loss of BW from muscle proteins during the study. Other authors (Swanson *et al*., 2004) also reported a loss in BW when wethers consumed low-quality hay and attributed the loss to a deficiency in CP as the BW losses decreased with increasing CP supplementation. In addition, Swanson *et al*. (2004) concluded that the negative apparent N-retention and BW loss could have resulted from loss of muscle mass as a result of the confinement of the animals in the metabolism crates.
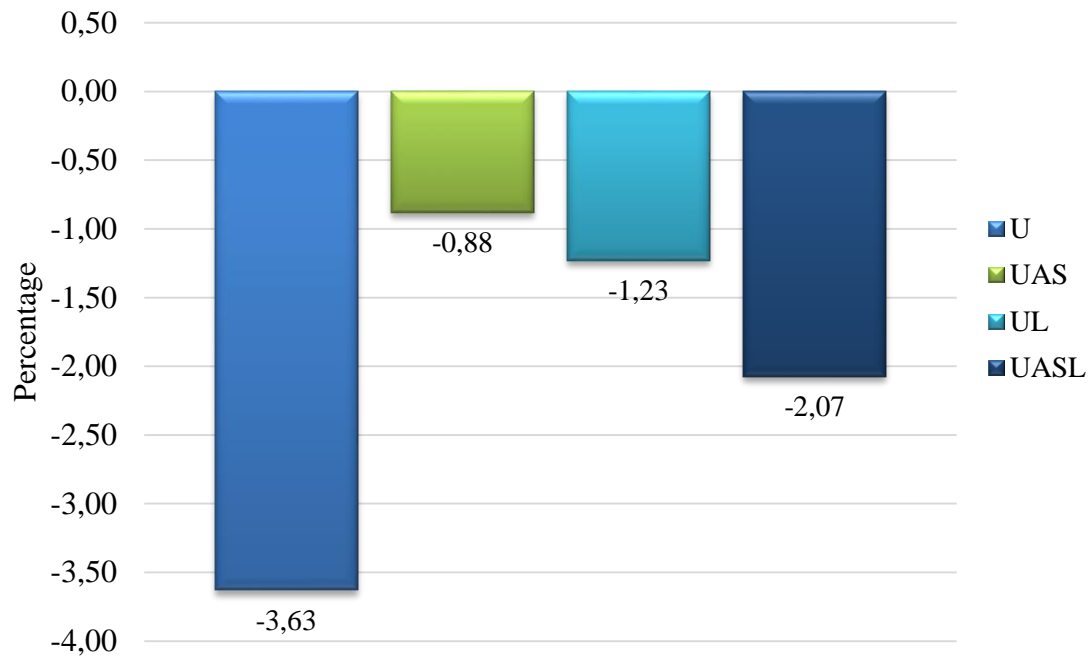
Figure 4.4 Average weight loss per treatment as a percentage of sheep body weight

CHAPTER 5

CONCLUSION

It was the aim of this study to determine if ammonium sulphate and / or lucerne (*Medicago sativa*) could be considered as effective partial replacers of urea in an attempt to provide sheep farmers throughout South Africa with an alternative nitrogen supplement with a lower potential NH_3 toxicity risk. The objectives were to measure the effects of three alternative N supplements (compared to the control) on important production parameters with a low-quality *Eragrostis curvula* hay as basal diet. Although all of the treatments were formulated on an iso-energetic, iso-nitrogenous and iso-sulphur basis, significant differences did exist for some of the measured parameters. The UASL treatment had the higher value for rumen NH_3-N concentration but the value was low enough to disregard the possibility of acute NH_3 toxicity – this was also attributed to the fact that UASL exhibited the lowest pH values measured at the different time intervals which reduced the rate of NH_3 absorption. In contrast, between treatments there were no significant differences for BUN. Dry matter and OM digestibility were higher for UASL compared to the control (U) although no significance was found between treatments for NDF digestibility. The UAS treatment also proved more efficient than the control with regards to DM and OM digestibility. The UL treatment tended to have the highest value for the ED (for both DM and NDF) determined by means of the *in situ* trial, and also exhibited the highest mean DMI (measured as $g \cdot kg^{-1}$ $BW^{0.75}$ per day), although this was not significant. The remaining production parameters (mean N-retention, total VFA concentration, and individual molar concentrations of the three main VFA's), as well as the degradation parameters determined *in situ* showed no mentionable differences.

It is important to note that these results did not reveal a clear effective partial replacer for urea as no single treatment performed better across all measured parameters compared to the control treatment (urea only). It can therefore not be conclusively stated that replacing urea (even partially) with either of the tested alternative N sources will be efficient and economically viable based solely on this study. In addition, it must be mentioned that even though $(NH_4)_2SO_4$ is a well-known N supplement, it was difficult to compare results in this experiment to other studies as none has been done in the last two decades to evaluate the value of ammonium sulphate as an effective NPN source.

CHAPTER 6
CRITICAL EVALUATION

The results as discussed in the previous chapters did not necessarily yield the outcome which was hoped for - to conclusively prove that the nitrogen sources tested could be used successfully as urea replacers. Success being defined as a source which could significantly reduce the rumen NH_3-N concentration, as well as the BUN levels, whilst improving DM and NDF digestibility, as well as DMI.

However, it is possible that the same research design and treatments could yield different results if conducted with different animals. The animals used in this study had non-ideal body weights with great variation between the wethers. It is suggested to use animals with a uniform body weight of at least 50 kg in order to obtain more reliable results. In addition, the animals should be given a proper recovery period after being cannulated in order to gain weight lost after the operation, as well as to become accustomed to a cannula in the rumen. This is especially necessary if animals were bought elsewhere and transported to a new facility which necessitates adapting to both the new environment and their new physical state, i.e. being rumen cannulated. In a review by Harmon and Richards (1997), the authors recommended that animals should be adapted to their environment even prior to the operation. This will not only improve recovery but animals which are adapted fully in a new environment will perform better. Adding to these stress factors was the additional stress placed on animals not used to human interaction and handling. This was especially evident in this trial as it took quite some time for the sheep to become even slightly accustomed to human presence in a close vicinity.

In terms of the supplements, it was initially intended to divide the supplements into 2 which were to be given in the morning and again in the afternoon at feeding time. However due to the low body weights of the wethers and the high requirement for starch to be supplemented it was found during the preliminary adaptation period to cause rumen acidosis. As a result it was necessary to increase the number of supplement feedings to 3 times daily in an attempt to lower the highly fermentable starch given at a time. A problem arising from this amendment to the initial experimental design was that the rumen environment was disturbed 3 times a day by opening the cannula. It is already unnatural to disturb the rumen environment by allowing the entrance of oxygen and this was exacerbated by increasing the number of times the rumen was subjected to air. As stated by Rode (2000), the conditions persisting within the rumen are strictly anaerobic and the presence of oxygen is toxic to many of the rumen

microorganisms. Consequently rumen activity could not accurately portray that found in an undisturbed rumen and the accuracy of the results can definitely be improved by decreasing the number of supplement feedings.

In addition, using cannulated animals is not a flawless method and it is common to experience difficulty with regards to the correct functioning of rumen cannulae. With gas build-up in the rumen the plug would sometimes be pushed out. In some instances, it also happened that the entire cannula would fall out. This was problematic due the fact that rumen fluid would lose fluid and also be exposed to air for different time periods depending on when the plug was reinserted into the cannula. All of abovementioned interferes with the normal workings within a rumen which will negatively influence results obtained throughout the trial.

In terms of the supplements used, lucerne inclusion could have been different. Robinson (1998) classified lucerne hay into different categories based on nutritional characteristics. Of importance is CP, which is categorised from fair (CP 18%) to extra premium (24%). The lucerne hay used in the current study contained 18.06% CP and was therefore of fair quality. If a better quality lucerne hay was used (good – 20.5% CP or premium – 22.0% CP) or alternatively if lucerne could have been included at higher levels, it is possible that the treatments with the lucerne inclusion could have resulted in more significant results as both the degradable and undegradable protein, as well as FME components would have been higher.

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