The guts of alpaca nutrition:
Understanding energy and protein metabolism

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Summary

Alpacas are unique animals in terms of their digestive capabilities and energy and protein metabolism. Nutritional information pertaining to alpacas has been extrapolated from data based on true ruminant nutritional requirements and is therefore inaccurate and misleading for alpaca producers. The general hypothesis tested in this thesis was that alpacas would be better at utilising feedstuffs and be more efficient at obtaining glucose and amino acids that are essential for both maintenance and fibre production than sheep. Two experiments in this thesis (Chapters 3 and 5) evaluated the potential of using un-degradable dietary protein (UDP) in alpaca diets as a means of optimising fibre growth. Chapter 4 of this thesis reports a training method and the design of a special metabolism pen for alpacas which was developed to conduct the experiments described in Chapters 5 and 7. The next experiment (Chapter 6) determined whether alpacas could utilise calcium propionate as a source of glucose. The last experiment (Chapter 7) examined how intakes of different proportions of energy and protein influenced nitrogen metabolism in alpacas compared to sheep.

The hypothesis tested in Chapter 3 was that alpacas fed a diet containing canola meal high in UDP to meet maintenance requirements would produce more fibre and spend less time urinating than peers fed a similar amount of canola meal with a low proportion as UDP. Alpacas were fed diets of similar metabolisable energy (ME) content at a level calculated to maintain body weight with the following ratios of UDP: rumen degradable dietary protein (RDP); 0:100 (0% UDP), 30:70 (30% UDP), 60:40 (60% UDP) or 100:0 (100% UDP) from canola meal protein. The fibre characteristics of the alpacas were analysed to determine whether fibre production was affected by the different proportions of UDP in the diet. The behaviour of the alpacas in the 100% and 0% UDP

protein groups was also monitored. The alpacas fed the 0% UDP diet produced fibre of finer diameter than the alpacas fed diets containing higher levels of UDP, but the 0% UDP group spent more time urinating. This suggests that when fed RDP, which should increase the ammonia concentration in the fermentative organs, the excess ammonia is converted to urea in the liver and excreted in urine. Thus the proportion of dietary protein as RDP may influence the pathways of nitrogen metabolism in alpacas.

In light of the results from Chapter 3, the experiment in Chapter 5 aimed to determine if protein degradability influenced nitrogen retention or energy balance and whether alpacas utilise nitrogen more efficiently than sheep. It was hypothesised that alpacas fed a diet containing RDP in the form of canola meal would excrete more nitrogen than those fed UDP and, that alpacas fed the same diet as sheep at maintenance would retain more nitrogen. Alpacas and sheep were fed the same diets as used in the experiment for Chapter 3 while they were housed in metabolism pens. Nitrogen and energy balances were measured to determine whether alpacas metabolised nitrogen more efficiently than sheep and whether protein degradability influenced the ability of alpacas to retain nitrogen. The degradability of the protein in the diet did not influence the amount of nitrogen retained in either species and both the sheep and the alpacas retained similar amounts of nitrogen. However, the alpacas tended to retain less nitrogen as a percentage of the nitrogen absorbed from their food than did sheep fed the same diet. The results suggested that sheep and alpacas probably obtain their energy from different components of their food and utilise protein in different ways.

In Chapter 6, the ability of alpacas to spare amino acids for fibre growth by utilising a gluconeogenic precursor was determined. It was hypothesised that alpacas supplemented with calcium propionate would produce more fine fibre than un-

supplemented animals. Although the diets supplemented with calcium propionate should have provided more energy, the ME intake of all animals was similar. It appears that rather than sparing amino acids, the alpacas regulated their energy intake by refusing to consume additional energy as calcium propionate.

Whether alpacas do moderate their energy intake and prefer to utilise protein as their source of glucose for maintenance was examined in Chapter 7. It was hypothesised that irrespective of their energy intake, alpacas would progressively retain more nitrogen as their intake of dietary protein increased. Conversely, it was expected that sheep would retain less nitrogen than alpacas when their intake of dietary protein increased because they rely on gluconeogenic precursors such as propionate, rather than protein, to meet their energy requirement. The alpacas responded to the dietary treatments in a similar manner to sheep by retaining a similar proportion of the dietary nitrogen that they absorbed. However, there was a trend for the alpacas to retain more of the absorbed nitrogen than the sheep when fed a diet that provided almost twice their maintenance requirement of protein. There was some evidence to suggest that alpacas do not regulate their protein intake as they appear to do with their energy intake.

The results from these studies have shown that alpacas obtain glucose for energy predominantly from the protein component of their diet as part of an adaptation to the harsh conditions of their native environment. Our understanding of the ability of alpacas to metabolise energy and nitrogen, compared to sheep, will enable producers to be informed of appropriate ways in which to feed their animals to promote productive and reproductive efficiency.

Declaration

The work presented in this PhD thesis is, to the best of the candidate's knowledge and belief, original and is the candidate's own work, except as acknowledged in the text. The material has not been submitted, either in whole or in part, for a degree at this or another university.

A version of Chapter 3 has been published as Lund et al. (2012) "Undegradable dietary protein in alpaca diets affects fibre diameter and time spent urinating." Animal Production Science 52: 959-963. This paper was prepared for inclusion in this thesis, with contributions by Dominique Blache, Shane Maloney, John Milton, Kristy Glover and Jane Vaughan.

A version of Chapter 4 has been published as Lund et al. (2012) "Gradual training of alpacas to the confinement of metabolism pens reduces stress when normal excretion behaviour is accommodated." Institute for Laboratory Animal Research E-Journal 53: E31-E42. This paper was prepared for inclusion in this thesis, with contributions by Dominique Blache, Shane Maloney and John Milton.

Signed

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Table of contents

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Publications

Journal papers

Lund, K.E., Milton, J.T.B., Maloney, S.K., Robertson, K.M., Vaughan, J., Blache, D. (2012) Un-degradable dietary protein in alpaca diets affects fibre diameter and time spent urinating. *Animal Production Science*, **52,** 959-963 doi: 10.1071/AN11367 (Chapter 3; Appendix 1).

Lund, K.E., Maloney, S.K., Milton, J.T.B., Blache, D. (2012) Gradual training of alpacas to the confinement of metabolism pens reduces stress when normal excretion behaviour is accommodated. *Institute for Laboratory Animal Research Journal* **53,** E31-E42 (Chapter 4; Appendix 2)

Lund, K.E., Milton, J.T.B., Maloney, S.K., Blache, D. (2012) Alpacas fed calcium propionate seem to moderate their energy intake. *Journal of Animal Physiology and Animal Nutrition* **Submitted** (Chapter 6)

Conference proceedings

Lund, K., Milton, J.T.B, Blache, D., Maloney, S. 2011. Dietary levels of un-degradable dietary protein affects fibre diameter and nitrogen excretion in alpacas. In 'Advances in Animal Biosciences, Proceedings of the 8th International Symposium on the Nutrition of Herbivores', p 278. (ISNH, Aberystwyth, Wales). Appendix 3.

Chapter 1

General Introduction

"The practice of feeding animals is considered part science and part art. Unfortunately, when it comes to basic feeding practices for llamas and alpacas, we are short on the science and long on the art." – Van Saun 2009.

Australian alpaca producers feed their animals according to what they think their animals need without really understanding the alpaca's nutritional requirements for particular physiological states. This hit-and-miss approach may result in a number of nutritional diseases and disorders and, overall, is probably the main factor responsible for limiting the efficiency of fibre production and animal reproduction. Clearly, there is a need to inform producers of appropriate ways to feed their animals to promote productive and reproductive efficiency. Dissemination of such information is hampered because there are very little scientific data about the nutritional requirements of alpacas and how alpacas metabolise energy and protein.

The current nutrient requirement recommendations for alpacas have been extrapolated from data pertaining to the energy and protein requirements of true ruminants, such as sheep and goats (Costa and Vaughan 1998). Alpacas, however, are pseudo-ruminants and have evolved in the altiplano region of the Andes mountains where the rainfall is erratic and ambient temperatures are seasonally low. These conditions culminate in feed availability being highly variable, resulting in large fluctuations in nutrient supply. Consequently, alpacas have a number of adaptations that enable them to survive these

harsh conditions. For example, alpacas have a different digestive capacity compared to that of true ruminants and it has been suggested that they metabolise energy and protein with a different efficiency than true ruminants like sheep (San Martin and Bryant 1989; Van Saun 2009). It has also been shown that the digestive efficiency of alpacas increases as the quality of the diet decreases (Van Saun 2006a). Indeed, when alpacas consume forage with a crude protein content that is less than 7.5%, their digestive efficiency is superior to that of sheep (San Martin and Bryant 1989). This phenomenon, and therefore the ability of alpacas to survive on poor quality feed, makes them particularly suited to environments like Australia where forages are typically low in energy and protein content. The alpaca's digestive ability perhaps exacerbates the problem of producers not feeding their animals appropriate diets for their physiological state.

The general objective of this thesis was to determine how alpacas obtain and use energy from their food and how those processes might differ from true ruminants like sheep. It was hypothesised that alpacas would be better than sheep at utilising feedstuffs and be more efficient at obtaining glucose and amino acids that are essential for both maintenance and fibre production. By understanding the processes by which alpacas metabolise glucose, we aim to determine the energy and protein requirements of alpacas when they are fed appropriate feeds that producers can use to optimise the productive and reproductive performance of their animals. The experimental chapters of this thesis compare the energy and protein metabolism of alpacas to that of Merino sheep, a fibre producing species for which a solid understanding of nutritional requirements and digestive functions exists. Several chapters also highlight the effects that certain supplement feeds, chosen for their efficacy for improving fibre growth in sheep, have on the energy and protein metabolism of alpacas.

Chapter 2

Literature review

Introduction

Alpaca fibre has a number of characteristics, such as being strong and lightweight with a high lustre, that makes it unique from other natural fibres and therefore highly desired by the textile and fashion industries (Wuliji *et al.* 2000). Due to the demand for alpaca fibre, the alpaca industry in Australia has considerable potential to grow and produce high quality fibre. For the industry to grow and become more commercially viable, animals that have a high genetic ability to produce large quantities of high quality fibre must be identified so that the genes of those individuals can be selected and disseminated.

Along with genetic potential, nutrition is an important input to consider in a fibre producing system as it can have an obvious impact on the quality of the product produced (Black and Reis 1979; McGregor 2006). Nutritional strategies can alter fibre quality independently of the genetic potential of the animal. Alpaca producers should feed their livestock a diet that promotes the animal's true genetic ability to produce fibre, otherwise they may, in fact, select sub-optimal gene cohorts. Unfortunately, there are misconceptions and gaps in species specific knowledge about nutrition that have resulted in conditions, such as obesity, that restrict the growth of the alpaca industry. There are numerous negative impacts on the economic viability and the productivity of an alpaca herd as a result of obesity. In Australian herds, those impacts of particular note are a susceptibility to heat stress and a consequent reduction in reproductive

efficiency (Van Saun 2006b). Obesity masks the true genetic ability of the animals and consequently, the reproductive potential of alpacas is not being recognised.

Obesity has most likely resulted from producers over-estimating the energy requirements of alpacas and over-feeding their alpacas' with energy and protein supplements (Van Saun 2006b). Alpacas have a pseudo-ruminant digestive system and consequently utilise energy and protein differently from true ruminants. In the past, alpaca diets have been formulated based on the energy requirements of true ruminants which are higher than that of alpacas. The excess energy in these diets that does not get used by alpacas is deposited as fat. It is clear that diets based on true ruminant nutritional information are a poor framework for devising alpaca nutritional requirements. To reduce the incidence of nutrition related diseases such as obesity, and to realise the genetic potential of individual alpacas, producers need to be informed of accurate energy and protein requirements that pertain specifically to alpacas.

This review of the literature is divided into two main parts. Part A describes the digestive system and the current understanding of energy and protein metabolism of alpacas, compared to true ruminants. The energy and protein requirements of alpacas are compared to true ruminant nutritional requirements to highlight the apparent efficiency of the alpaca's digestive system. Part B examines the behaviour of alpacas, particularly in confinement, as this plays an important role in acclimatising alpacas to metabolism crates needed for some of the experiments in this thesis. Overall, this review highlights the current standing of alpaca production in Australia and the apparent need for a greater understanding of alpaca nutrition, with particular focus on the unique aspects of the camelid digestive system that enable alpacas to metabolise energy and protein differently from true ruminants.

PART A. NUTRITION OF ALPACAS

Alpaca production

Alpacas (*Lama pacos)* originate from South America and occur primarily in Peru, Bolivia, Argentina and Chile. The largest population of alpacas is located in the Bolivian and Peruvian Altiplano of the Andes (San Martin and Bryant 1989). Together with llamas, vicuna, and guanaco, alpacas are members of the South American Camelid (SAC) group and are of great economic and social importance to the people of Andean communities. The livelihood of many local people depends on fibre and meat production from alpacas and llamas.

Alpacas were successfully introduced into Australia in 1989 after a previously failed attempt in the mid 1800's. The popularity of alpaca production has increased due to the recognition of their suitability to the Australian environment. Originating from the arid regions of South America, alpacas are well adapted to dry environments and pastures that offer limited nutritional value (Wensvoort *et al.* 2001). The alpaca population has increased rapidly over a short amount of time and there has been a general shift in the industry from breeding to gaining financial returns from fibre (McGregor 2006). Today, Australian producers raise alpacas mainly for fibre production. Alpaca fibre is uniquely lightweight and strong and has several characteristics that are desired by textile processors (Wuliji *et al.* 2000). Although alpaca fibre is highly desired by the textile and fashion industry, realistically the alpaca fibre industry is limited due to there being only a small handful of producers currently able to consistently produce fibre of the quality and quantity required by processors. Advances in reproductive technology, such as embryo transfer programs, and improved management techniques will allow the movement of superior genetics across the nation and therefore the alpaca fibre industry

stands in good stead to become a successful industry in Australia (Becker 1993). Research is needed, however, to broaden the existing knowledge and correct some misleading information, particularly that relevant to the nutritional requirements of alpacas, which is currently holding back the growth of the industry.

Digestion in alpacas versus sheep

1. Anatomy

Alpacas, along with other camelid species, belong to a group called pseudo-ruminants which have a different gut anatomy compared to that of true ruminant species, such as sheep and cattle. Although alpacas chew cud and their ingested food undergoes the same fermentation process, they only have three stomach compartments compared to the four in true ruminants (Figure 2.1; San Martin and Bryant 1989; Van Saun 2006a). Compartment one (C1) is the largest in terms of volume and occupies 83% of the stomach, with 6% and 11% occupied by compartment two (C2) and compartment three (C3) respectively (San Martin and Bryant 1989). Most microbial fermentation occurs in C1 which is similar to the rumen of sheep. Buffering agents and more digestive enzymes are added to the digesta in C2 before it enters C3 where the microbial protein starts to be digested to amino acids for subsequent absorption from the small intestine (Costa and Vaughan 1998).

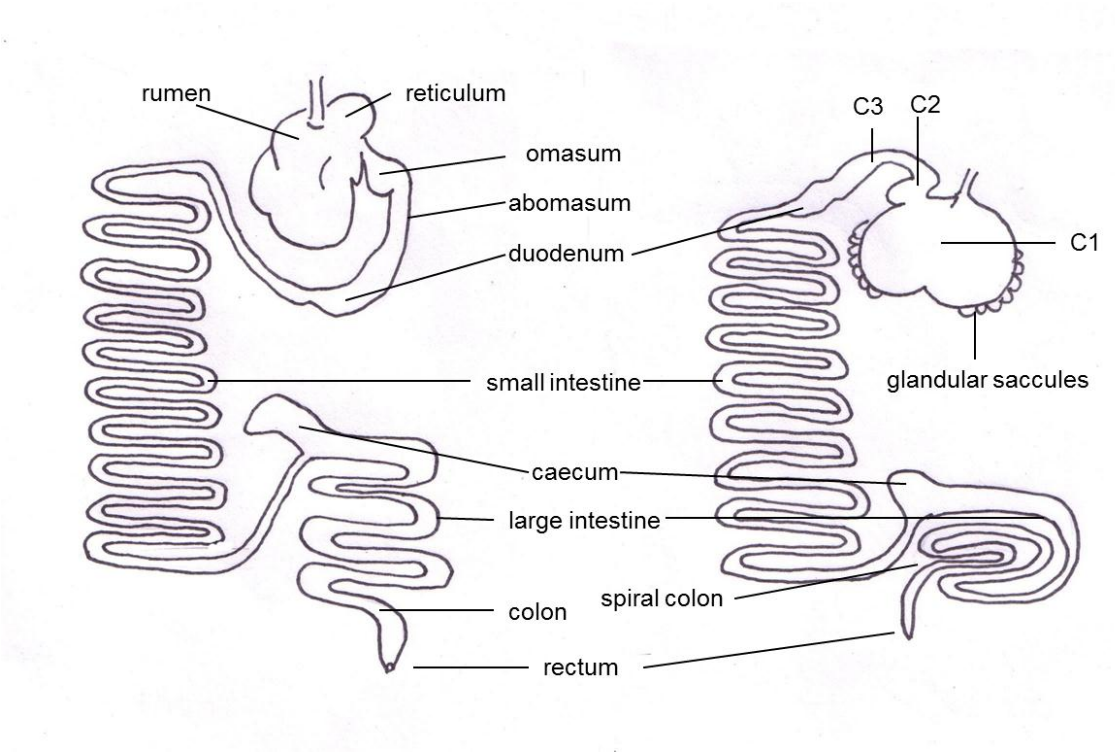

Figure 2.1. The gastrointestinal tract of the alpaca (right) only has three stomach compartments (C1, C2 and C3) compared to four (rumen, reticulum, omasum and abomasum) of true ruminants (left). Adapted from Anon. 2002 and Van Soest 1982.

2. Digestive efficiency

There is some discrepancy in the reported figures for the digestive efficiency of SAC's, mainly due to the differences in the quality of the feed used in these trials. In alpacas, the digestibility of dry matter (DM), for example, when they were fed fescue hay was 55.9% compared to 48.4% when fed wheat straw (Lopez *et al*. 1998). A number of studies conclude that the SAC's have a higher digestive efficiency compared to true ruminant species and are therefore better suited to extracting energy from their food (Genin *et al.* 1994; Dulphy *et al.* 1997; Lopez *et al.* 1998; Sponheimer *et al.* 2003; Robinson *et al.* 2005; Davies *et al.* 2007). It is also agreed that the SAC's higher digestive efficiency is more pronounced when the diet consumed is low in energy and protein (less than 10% crude protein and about 10 MJ digestible energy (DE)/kg DM; (San Martin and Bryant 1989). For alpaca production in Australia, this may be of

benefit. When there is little or low quality feed available, that is during the feed gap seasons, a lower performance from ruminant production animals tends to correspond (Black and Reis 1979). As alpacas are highly adapted to arid environments, like those in Australian agricultural regions, they are a suitable species that can optimise their environment. Alpaca digestive efficiency has been attributed to several physiological characteristics of the digestive system that are summarised in Table 2.1.

Table 2.1. Summary of camelid digestive adaptations that promote greater digestive efficiency than true ruminants (San Martin and Bryant 1989)

Adaptation	How it enhances digestive efficiency
Longer retention of digesta	Increases exposure of digesta to fermentative process
Stable pH	Promotes microbial fermentation
Forestomach motility	Continuous exposure of digesta to microbes
Nitrogen recycling	Produces more microbial protein that can be used for energy

2.1 Retention time

The digestive efficiency of SAC species has been linked to the retention time of the digesta (Sponheimer *et al.* 2003). In a review on SAC nutrition, alpacas were reported to have a mean retention time of 50 to 60 hours compared to about 40 hours in sheep (San Martin and Bryant 1989). As food particles are exposed to sites where microbial action takes place for a longer period, there is greater degradation of the food and therefore more efficient absorption of the nutrients contained in the feed (Genin and Tichit 1997). Robinson *et al.* (2005) found that larger food particles remained in the digestive tract for longer, thus particle size could be a critical factor when determining an appropriate diet for optimising the production of Australian alpacas. The passage rate of liquid is more rapid in camelids than in sheep, which results in a high dilution rate or

proportion of total volume leaving the rumen per hour (Warner 1981; Heller *et al.* 1986). There is a positive correlation between increased dilution rate and improved microbial growth (Stern and Hoover 1979), thus the host animal benefits from improved availability of microbial protein. Both the prolonged retention of food particles and the rapid passage of liquid allow alpacas to be more efficient in obtaining energy from their diet, which can be beneficial for them when they are required to survive periods where feed is scarce.

2.2 Stable pH

The forestomach pH, particularly its stability, also contributes to the digestive efficiency of a species because it promotes greater microbial fermentation (Van Saun 2006a). Within the fermentative chambers of the alpaca forestomach, C1 and C2, there are unique saccules which are lined with glandular epithelium that secrete bicarbonate and phosphate ions (San Martin and Bryant 1989; Van Saun 2006a). The secretion of these ions aids in the buffering capacity of the forestomach and results in a more stable pH of about 6 or 7 compared to that of true ruminants which tend to have acidic forestomach conditions (San Martin and Bryant 1989). In addition, the chemical and physical conditions in the forestomach, including pH, are more stable in alpacas than sheep (Dulphy *et al.* 1997). In terms of energy requirements, acidic stomach conditions, such as those in true ruminants, increase the maintenance energy requirement of the forestomach bacteria, thus a low microbial protein yield is obtained (San Martin and Bryant 1989). In alpacas, stable forestomach conditions are beneficial because the microbial population requires less energy for maintenance and can therefore make more microbial protein that can be utilised by the host animal.

2.3 Forestomach motility

Motility aids the fermentation process by ensuring that the ingested feed is exposed to microbes that degrade the feedstuff. Like true ruminants, camelid forestomach motility occurs in two phases, however the pattern of motility is very different. The first wave involves the contraction of C2 followed by the distal part of C1. After this, the cranial area of C1 contracts followed by C2 and the caudal portion of C1. The second phase may repeat three to six times before resting for a short period (Van Saun 2006a). This sequence of contractions is repeated two to seven times per contraction cycle (an average cycle is 1.8 ± 0.2 minutes long) thus camelids have more continuous activity in the forestomach compared to true ruminants (Vallenas and Stevens 1971; San Martin and Bryant 1989). It is likely that the higher activity of the forestomach contributes to the homogenous nature of the stomach contents whereas the stomach contents of true ruminants have a more stratified, layered nature (Genin *et al.* 1994; Van Saun 2006a).

In terms of digestive efficiency, the enhanced foregut motility is a benefit for alpacas and other camelids because they can utilise their feed to a greater extent. Continuous motility of the foregut ensures that the digesta is constantly exposed to microbial attachment and degradation (Van Saun 2006a). As they can extract more energy and nutrients from their ingested feed, alpacas appear better able to survive periods of limited feed availability.

2.4 Nitrogen recycling

Like true ruminants, alpacas are capable of recycling nitrogen to their fermentative organs, which is beneficial for protein synthesis (Dulphy *et al.* 1997). It is generally agreed that SAC's lose less nitrogen in their faeces and, in particular, their urine, compared to true ruminants. Llamas fed various diets of hay excreted 25% of the

nitrogen ingested in their urine compared to 49% in sheep fed the same diets (Dulphy *et al.* 1997). Instead of excreting nitrogen in the form of urea, alpacas recycle urea to the forestomach where microbial bacteria convert it into protein (Genin *et al.* 1994; Dulphy *et al.* 1997; Van Saun 2006a). In turn, microbial protein provides the alpaca with a source of amino acids that can be used for glucose for energy. The capacity of alpacas to recycle nitrogen offers an avenue for producers to manipulate diet and feeding strategies to improve food conversion efficiency. The nitrogen metabolism of alpacas is reviewed more closely in a later section.

Glucose metabolism

Little is understood about glucose metabolism in alpacas. As a foregut fermenter, one may presume that alpacas have a similar blood glucose concentration to that of true ruminants. Camelids, however, have a higher blood glucose level of approximately 7 mmol/L, which is similar to non-ruminant species, and higher than the 2.5 – 4.2 mmol/L in ruminants (Van Saun 2006a). Alpacas are also reported to have a poor glucose tolerance and can show a hyperglycaemic response to minimal stress which is partially the result of a weak insulin response and moderate insulin resistance (Cebra *et al.* 2004; Van Saun 2006a).

It is not understood why alpacas have elevated blood glucose levels and such an extreme hyperglycaemic response, particularly when they originate from regions where dietary glucose and glucose precursors such as propionate are minimal. It is possible that stress hyperglycaemia, elevated blood glucose concentration and poor glucose clearance are physiological characteristics of camelids rather than pathological observations (Cebra *et al.* 2001). It is likely that rather than there being a single explanation for the observed high blood glucose concentration in camelids, a number of

factors play contributing roles. Insulin resistance may be a potential factor contributing to high blood glucose concentration (Van Saun 2006a). Camelids and true ruminants may process carbohydrates differently, and rather than using propionate as a main energy source, camelids might utilise alternative substrates, such as amino acids, for gluconeogenesis. Alpacas may derive energy from amino acids but, as an adaptation to their native environment and low feed availability, may have a high blood glucose concentration as a reserve of energy. The concept of alternative energy substrates is discussed in more detail in the following section.

Nitrogen metabolism and energy production

As previously mentioned, alpacas are thought to have a great ability to recycle nitrogen in the form of urea. Compared to true ruminants, camelids have a greater ration of salivary flow to stomach compartment size, providing a larger buffering capacity from the saliva (San Martin and Bryant 1989). Several studies have also found that SAC's retain about five times more nitrogen compared to sheep because they have lower nitrogen excretion in the faeces and urine (San Martin and Bryant 1989; Dulphy *et al.* 1997; Sponheimer *et al.* 2003; Robinson *et al.* 2005).

It is thought that the nitrogen recycling capability of SAC's increases in efficiency when the diet is of lower quality (Costa and Vaughan 1998). Alpacas and llamas originate from the arid rangelands of South America which are characterised by poor-quality forages, and therefore a greater ability to utilise energy from these plants has probably been an important selection pressure during their evolution. Studies in South America have found that llamas will graze 15 to 20% more of the coarse, less digestible grasses than sheep (Genin *et al.* 1994). This feeding behaviour, coupled with a higher level of urea recycling, which results in greater production of microbial protein, suggests that

camelids, unlike true ruminants, obtain energy from amino acid deamination rather than relying upon the breakdown of volatile fatty acids (or short-chain fatty acids) like propionate (Figure 2.2).

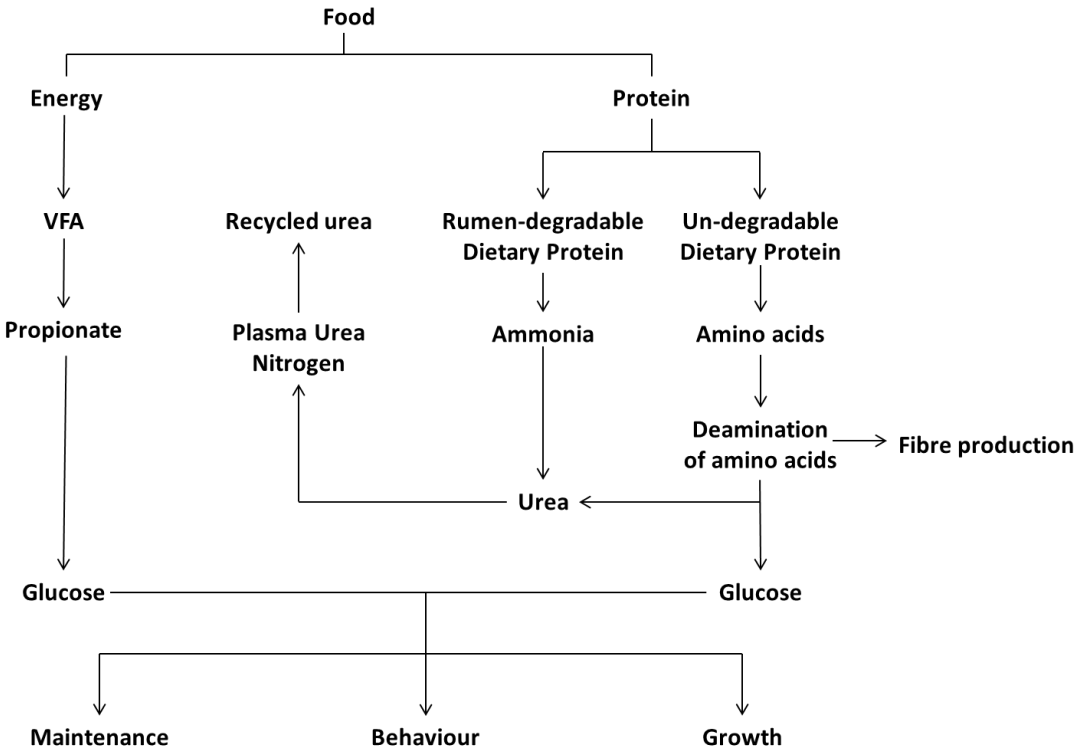

Figure 2.2. The possible pathways that alpacas use to obtain energy from their food are either from the production of glucose from volatile fatty acids (VFA) or the deamination dietary protein. Note that amino acids are also required for fibre growth and therefore a limitation of available amino acids results in a decline of fibre production. Adapted from (McDonald *et al.* 2002; Blache *et al.* 2007).

True ruminants obtain their energy from glucose via hepatic gluconeogenesis from propionate. Propionate is a volatile fatty acid that is a product of fermentation. In normal feeding conditions, propionate provides about 70% of the glucose requirement of true ruminants (McDonald *et al.* 2002). According to Van Saun (2006a), alpacas produce very little glucose from propionate because their typical diet has a low sugar and starch content. Also, due to the alpaca's low intake of generally low-quality roughage, the amount of microbial protein that reaches the small intestine is likely to be

limited (Van Saun 2006a). The alpaca's emphasis on using a unique pathway poses a problem for producers of alpaca fibre because amino acids are also required for the production of fibre and becomes even more important for producing high-quality fibre. The apparent conflict between amino acids required for gluconeogenesis and for protein assemblage probably means that a diet with a limited amount of amino acids could result in lower production levels. To overcome this conflict in amino acid use, it may be possible to provide enough amino acids for maintenance and fibre production by supplementing their diets with amino acids that bypass digestion in the rumen and instead reach the small intestine.

Using un-degradable dietary protein to enhance production

The concept of feeding production animals a source of ruminally un-degradable dietary protein (UDP) to boost amino acid absorption in the small intestine has been researched in other ruminants and it seems applicable in the case of the alpaca, particularly because of their unique digestive capability. Wool growth in sheep is often limited by a lack of amino acids (McDonald *et al.* 2002). By providing a supplement of UDP to sheep, amino acids can be absorbed in the small intestine and used for wool growth. If alpacas are given a supplement of UDP, then it is possible that there will be a sufficient supply of amino acids to be put towards fibre production as well as the provision of energy. Conversely, if alpacas can utilise energy supplements such as calcium propionate and obtain glucose for maintenance, there may be more amino acids available for fibre production. Further research in this area will provide an understanding of this unique capability and allow producers to manipulate their animals' diet accordingly so that they may optimise their production and maintain healthy livestock. Although the manipulation of the diet may be beneficial in optimising fibre production, the actual

energy and protein requirements of alpacas remains controversial and therefore needs to be better understood in order to formulate diets that will promote production.

Energy and protein requirements

The recommended energy requirements for alpacas in Australia are based on assumptions and extrapolations from true ruminant and other camelid species data. The maintenance energy requirement for sheep is about 0.4 MJ metabolisable energy (ME)/kg $BW^{0.75}$ (Costa and Vaughan 1998; Van Saun 2006a), which is higher than figures reported for other camelids. There are few studies that have investigated camelid energy requirements and there is variation between the reported estimates, largely due to differences in methodologies used and the energy content of the feed. von Engelhardt and Schneider (1977) determined the maintenance energy requirement for llamas to be 0.256 MJ ME/kg $BW^{0.75}$ whereas Carmean *et al.* (1992) and associates estimated a requirement of 0.353 MJ ME/kg $BW^{0.75}$. The latest reported figure for the maintenance energy requirement of alpacas is 0.305 MJ ME/kg $BW^{0.75}$, which is an average of the von Engelhardt and Schneider (1977) and Carmean *et al.* (1992) estimates (Van Saun 2006a). Van Saun (2006a) considers this estimate to be appropriate for North American feeding conditions and takes into account the high prevalence of obesity in the North American camelid herd.

During 2008, the University of Western Australia conducted a study on alpaca fibre production response to a protein supplement. Throughout this experiment, the alpacas maintained body mass when fed about 20% below the maintenance energy requirement estimate recommended by Van Saun (2006a). Again, differences in methodology and feed may account for the difference between both energy requirement estimates, however, for Australian alpaca producers, the local energy requirement estimate may be

more appropriate to adopt. As mentioned, the high incidence of obesity in the Australian alpaca herd may be indicative of producers over-feeding their livestock and the study from 2008 highlighted the importance of producers maintaining livestock management techniques, such as regular body condition scoring.

There is limited information about camelid protein requirements and most alpaca diets are formulated using models and extrapolated data from true ruminants. Costa and Vaughan (1998) recommend that alpacas be provided with feed with 8-10% crude protein, which is similar to the estimate for sheep. Although alpacas are more efficient at nitrogen recycling than sheep when fed low protein diets, it appears that their efficiency becomes similar to that of sheep as the protein density in their food increases. Therefore the recommendation by Costa and Vaughan (1998) would be enough to supply crude protein for maintenance plus enough for a safety buffer.

Van Saun (2006a) considered a study by Huasaquiche (1990) that estimated that alpacas require 3.5 g CP/kg $BW^{0.75}$ for maintenance, which is lower than estimates for sheep (4.74 g CP/kg $BW^{0.75}$). Van Saun (2006a) used this value as a guide in developing additional protein requirement models, such as for maintenance plus pregnancy, but stated that more research into camelid protein nutrition is needed to better define protein requirements.

It is clear that to feed alpacas to promote optimal reproduction and fibre production, a greater understanding of their energy and protein metabolism is needed. To obtain more information about energy and protein metabolism, we need to conduct experiments where the animals are housed in metabolism crates for a certain amount of time so that

total faecal and urine output can be determined in order to calculate the energy and nitrogen balance of alpacas.

PART B. ALPACA BEHAVIOUR IN METABOLISM PENS

Alpaca behaviour

Although animals housed in metabolism crates appear to have all their physical needs met, they have no opportunity to have physical contact with their peers, graze, or express locomotor behaviour. The restriction of such activities not only influences the validity of scientific results but changes in these factors can indicate a response to stress experienced by the animal (Bowers *et al.* 1993). Consequently, from a welfare standpoint, the use of metabolism crates in research is a controversial practise. Behaviour can however be used as a measure of an animal's well-being. According to Mal *et al.* (1991), an animal's perception of a new environment is often expressed through changes in their behavioural patterns. By understanding and identifying certain behavioural indicators that are specific to alpacas we can measure their response to stress and thereby minimise the impact of housing alpacas in metabolism crates.

1. Activity of camelids

Alpacas are social animals that typically herd (Fowler 1998). In an effort to establish and control group dynamics, alpacas use body language, such as ear and tail position, with scent and vocalisations to communicate with other group members (Hoffman 2006). The vocalisations of alpacas may be particularly useful in determining distress during experiments. Alpacas are known to make a wide range of distinct sounds. "Humming", for example, is a common sound that is often made during bonding or when alpacas are separated from their social group (Fowler 1998). When particularly

distressed or frightened, alpacas will scream and when they perceive a threat, they will emit a high-pitched alarm call to warn the rest of the group of the danger (Fowler 1998; Hoffman 2006). Alpacas also spend time grooming which involves rolling in dust baths. Often when an alpaca chooses to roll the rest of the group will also roll (Hoffman 2006). It is not fully understood why alpacas groom themselves, however it is possible that it is a method of relief from parasites or tension. Overall, unlike wild populations of camelids where communication within the herd is vital for survival, body language and vocalisations within managed herds of alpacas where producers control stocking rates and resources are the main tools for maintaining social order for trivial daily activities such as feeding.

Although the daily activity of alpacas has not been studied extensively, there appears to be a distinct pattern to their behaviour throughout the day that is similar to other camelid species. Alpacas present a daily rhythm of behaviour, being more active in the early morning and, after resting around noon, show another peak of activity in the evening (Scheibe *et al.* 1991). These observations are in accordance with those from a study where camels spent the majority of a 24 hour period resting and ruminating (von Engelhardt *et al.* 2006). According to von Engelhardt *et al.* (2006), the camels used in their study spent about ten hours resting periodically throughout the day and night and rumination occupied about eight hours. Like other ruminants, the majority of rumination took place in the early morning or late evening hours and eating was done during daylight hours (von Engelhardt *et al.* 2006; Davies *et al.* 2007). Llamas have been observed to have a nocturnal rumination period. Therefore, because alpacas are from the same family as camels and llamas, it is reasonable to expect similar behaviour (Dulphy *et al.* 1997). An interesting observation about camels is that they exhibit a distinct lag between feeding and rumination and it is thought that this could be further evidence of

promoted utilisation of low quality feed. It is important to emphasise that behavioural patterns can be affected by other factors such as season, food quality, and confinement, and these factors should be considered when comparing behavioural studies.

2. Behavioural responses to confinement and isolation

Studies into biological rhythms have shown that animals have a sense of time and that they recognise confinement or the reduction of available space. If an animal is given a choice it may choose to stand in a place where it spends less time in confinement (Houpt 2005). The natural behaviour of an alpaca may change when they are in a confined environment. Behaviour is commonly used as a stress indicator in conjunction with measurable physiological characteristics such as cortisol and heart rate. Alpacas are social animals and isolation from their peers can be a stress (Baldock and Sibly 1990). Considering the social similarities between the alpacas and other herd species such as sheep, it is possible that they exhibit similar behavioural indicators of stress and we can therefore use sheep as a model for alpacas.

Food intake and the amount of time that an animal spends eating can decrease when an animal is confined or isolated, either due to the isolation stress or the increased chance of food becoming soiled in the reduced space (Houpt and Houpt 1988; Romney *et al.* 1996). Sheep that were newly introduced to an animal house environment displayed a significant change in the time spent eating (Done-Currie *et al.* 1984). Initially, the sheep ate very little of their daily ration, however as the experiment progressed, they began to consume their entire ration. The authors suggested that this change in behaviour was evidence of an adaptation to the environment. In experiments where sheep were housed in confined cages, the researchers noted that the animals initially ate less feed but later adjusted their behaviour and therefore their feed intake after adapting to confinement

(Houpt 2005). Like sheep, alpacas have a structured group hierarchy that dictates their behaviour in grazing systems and in the wild, therefore it is reasonable to assume that alpacas will undergo a similar adjustment period when they are restricted to a confined area.

Sheep that are moved from a paddock to an animal house environment have been observed to withdraw and spend more time in non-alert states compared to sheep that are accustomed to an animal house environment (Done-Currie *et al.* 1984). It is possible that this withdrawal behaviour demonstrated by the sheep may be an attempt to alleviate the stress of being in a new environment (Done-Currie *et al.* 1984). Prolonged time in the animal house seemed to provoke the beginning of stereotypic behaviours such as weaving, standing on the pen bars, and licking (Done-Currie *et al.* 1984). Similar stereotypic behaviours have been observed in other species that have been exposed to prolonged isolation or confinement, such as pigs and horses (Chen and Orksov 2003; Lund *et al.* 2012; Orellana-Boero *et al.* 2012). Stereotypic behaviours are thought to evolve out of boredom and frustration caused by the inability to behave as they would in their natural environment, such as in terms of movement and social contact (Lund *et al.* 2012). Although there is a lack of information pertaining to stereotypic behaviour in alpacas, it is reasonable to assume that they could develop such behaviour when housed in the confinement of metabolism crates. Therefore it is important to consider ways in which to alleviate the problem. To reduce the incidence of stereotypic behaviours, it is preferable to address the cause of the behaviour rather than prevent the behaviour itself. The metabolism pens used in this thesis were specially designed for alpacas. They have a greater floor space than conventional metabolism crates thereby enabling the animals to turn around and lie with their neck fully extended in front of them and the sides of the pen are designed to allow the animals to remain in contact with the other animals in

their group. We anticipated that the design of the metabolism pens would alleviate the stress of confinement and reduce the incidence of stereotypic behaviours.

Separation from peers causes sheep to increase their activity and spend more time vocalising. Vocalisation is also displayed by other species such as horses and goats and is seen as a sign of distress and the animal looking to reinstate its social unit (Price and Thos 1980; Mal *et al.* 1991). Goats spend even more time vocalising and rearing in their pens than sheep. It is thought that the goats' increased vocalisation when separated from their peers is explained by their preference to browse scrub habitat, which imposes sight barriers and therefore a reliance on auditory, rather than visual, communication is needed (Price and Thos 1980). As alpacas have a broad vocal repertoire (Fowler 1998) and are known to rely heavily on vocalisation as a means of communication within the herd, it is likely that separation from their peers will invoke an increase in their vocalisations and can therefore be used as a measure of the animal's response to stress.

3. Habituation and training

Although confinement and restraint can invoke behavioural and physiological stress responses, it is possible to train and habituate animals to a confined environment (Grandin 1989). As a social animal, alpacas are likely to form a stronger bond with the trainer than animals that are considered to be solitary, such as cats. Consequently, social animals are able to use this bond as a motivating factor in overcoming fear (Barboza and Parker 2006). The process of habituation, when an animal is repeatedly exposed to empty threats so that their fear response becomes weaker, is highly effective in allowing the animal to determine whether their defence reactions of freeze, flight or fight are relevant towards novel stimuli. Sheep that are acclimatised to a procedure or routine generally display reduced physiological and behavioural responses compared to sheep

that experience the same procedure for the first time (Grandin 1989). In reviewing behavioural principles of sheep handling, Hutson (2000) concluded that gradual training and handling reduced some metrics of the response to handling in sheep and that food rewards were a particularly useful incentive when training sheep. It is possible that the alpacas used in the experiments in this thesis could be trained to the confinement of the metabolism pens to reduce the welfare compromise of the animals.

By understanding the behaviour of alpacas and learning to identify and monitor the potential signs of stress in alpacas, we may develop methods that will minimise the stress imposed on the alpacas used in this research. By designing a metabolism pen with the welfare of the animal as a foremost consideration and by developing a training protocol to gradually habituate the animals to the metabolism pens, it may be possible to reduce the negative impact on the welfare of the animals whilst obtaining scientifically valid data on the energy and protein requirements of alpacas.

PART C. CONCLUSIONS AND SCOPE OF THESIS

Alpacas are unique animals in terms of their digestive capabilities and energy and protein metabolism. Extrapolations and estimates based on true ruminant nutritional requirements appear to be inaccurate and are misleading for alpaca producers, thus giving rise to problems such as obesity within the Australian herd. It is obvious from the lack of information in the literature that more nutritional research specific to alpacas is needed, particularly that which will enable the alpaca's energy and protein requirements to be quantified. The primary aims of this thesis are to determine the ability of alpacas to metabolise energy and nitrogen compared to that of sheep and to identify potentially useful energy and protein supplements that enhance fibre growth and fully capitalize on

the alpaca digestive abilities. To accomplish these aims, alpacas will need to be housed in metabolism crates that may potentially invoke a response to stress similar to that seen in sheep and other domestic animals housed in similar conditions. However, to reduce to welfare compromise of the animals used in this research and to ensure that scientifically valid results are obtained from these studies, an understanding of alpaca behaviour and response to stress is also required.

Understanding alpaca energy and protein metabolism will provide a strong foundation upon which future research can be built and will ultimately help producers feed their animals in a way which promotes the growth of the alpaca industry.

Chapter 3

Un-degradable dietary protein in alpaca diets affects fibre diameter and time spent urinating

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Introduction

Alpacas were introduced to Australia to establish a new fibre industry with a focus to produce large quantities of quality fibre (Fysh 2003; McGregor 2006). In sheep, most of the glucose requirement is derived from propionate and fibre production is markedly influenced by the supply of dietary protein (Black and Reis 1979; McDonald *et al.* 2002). There is evidence that alpacas obtain most of their glucose from the deamination of dietary amino acids rather than from propionate (Van Saun 2006a). Consequently, when alpacas are fed to meet their energy requirements for maintenance they may utilise most of the amino acids absorbed from the small intestine to meet their needs for glucose, especially since they maintain a blood glucose level higher than sheep (Kaneko *et al.* 2008). Therefore, under these conditions, alpacas may have an inadequate supply of amino acids to meet their requirements for fibre growth and it may be necessary to supply them with supplemental protein to optimise fibre production.

In ruminants, the extent to which ingested protein is degraded depends upon its solubility and the time it is retained in the rumen. The protein that enters the rumen is either degradable (RDP) or un-degradable (UDP; Bach *et al.* 2005). In the rumen, RDP can be degraded to varying degrees from peptides through to ammonia and these

products can all be utilised for the synthesis of microbial protein (Bach *et al.* 2005). Ammonia that is not captured for microbial protein synthesis passes from the rumen and is converted to urea by the liver. This urea can be recycled back to the rumen, either via saliva or across the rumen wall, or excreted in urine (McIntyre 1970; McDonald *et al.* 2002). Alpacas are thought to be particularly efficient at recycling nitrogen which would be advantageous in environments where the feed that is available is of low nitrogen content (Genin *et al.* 1994; Genin and Tichit 1997). If there is adequate fermentable carbohydrate in the diet, the nitrogen recycled by alpacas, in the form of urea, may be the main source of nitrogen for microbial protein synthesis occurring in the first and second chambers of the alpaca stomach (fermentative chambers). Excess nitrogen, for example from RDP when there is inadequate fermentable carbohydrate for microbial protein synthesis, will be liberated as ammonia, absorbed from the fermentative chambers and converted to urea in the liver. Urea is known to be a diuretic agent (Owen *et al.* 1943) and when the level of plasma urea is high diuresis will occur. When alpacas are fed protein high in RDP this is likely to lead to diuresis and the frequency of urination may be considerably higher than usual.

Un-degradable dietary protein that passes from the rumen is digested in the abomasum and small intestine to amino acids, which are then absorbed and used for synthetic processes, including fibre growth, or can be converted to glucose. Un-degradable dietary protein can be produced from protein meals by heating the meal in the presence of reducing sugars to promote a mild Maillard reaction, without over-heating to render the protein insoluble in the lower digestive tract. Masters *et al.* (1999) demonstrated that the wool growth of sheep supplemented with UDP as expeller canola meal (about 50% UDP) was 11% higher than that of sheep fed a similar level of protein as lupins (about

25% UDP). Based on these results, canola meal protein was considered an appropriate supplement to investigate the responses in fibre growth with alpacas.

In this study, we fed alpacas a maintenance diet and tested whether the proportion of RDP to UDP from canola meal in the diet influenced fibre growth. We hypothesised that alpacas fed at maintenance a diet containing canola meal high in UDP would produce more fibre and spend less time urinating than peers fed a similar amount of canola meal with a low proportion as UDP.

Materials and methods

Alpacas were fed diets of similar metabolisable energy (ME) content at a level calculated to maintain body weight (Van Saun 2006a) with the following ratios of UDP:RDP; 0:100 (0% UDP), 30:70 (30% UDP), 60:40 (60% UDP) or 100:0 (100% UDP) from canola meal protein. The behaviour of the alpacas in the 100% and 0% UDP protein groups was monitored using a video recorder. The fibre characteristics of the alpacas were analysed to determine whether fibre production was affected by the different proportions of UDP in the diet.

Animals

Castrated male Huacaya alpacas (n = 32) aged 24 – 35 months were transported to Shenton Park Research Station at the University of Western Australia and housed randomly in bare, outdoor individual pens, approximately 3 m x 10 m. The alpacas were allowed to become acclimatised to their new environment, feeding routine and handlers for approximately two months prior to the commencement of a three month feeding period during June to August (winter). Each alpaca had *ad libitum* access to fresh water, a feed shelter and a shaded area. They were fed once daily at around 0800 h and their

refusals from the previous day were recorded. All animals were weighed to the nearest 0.5 kg and their body condition was scored at the same time each week. The amount of feed offered to each animal was adjusted over the three month period as necessary to ensure that all animals were fed to maintain body weight and condition.

Feed treatments and analyses

The 32 alpacas were randomly allocated to four dietary treatments, each with eight animals of similar mean live weight (48.0 ± 0.22 kg, range of 37 – 62 kg) and body condition score (2.3 ± 0.05 units, scale 1 = emaciated to 5 = obese; Fysh 2003). The weight of each feedstuff fed to the individual alpacas was calculated based on the individual's metabolic weight. The alpacas received a basal diet of milled barley straw at 22 g/kg$^{0.75}$, a roughage-based pellet at 9.7 g/kg$^{0.75}$ (Macco 101 pellet, Macco Feeds Australia, Williams, Western Australia), 25 g/head.day of a complete mineral mix and 25 g/head.day of dried sugar cane molasses (Palabind, Probiotec, Laverton North, Victoria, Australia). A supplement of 4.6 g/kg$^{0.75}$ of canola meal was added to the basal diet as either untreated, flaky cold-pressed canola meal (0% UDP), or the same canola meal finely milled to facilitate the passage from the rumen and mixed with a water solution to provide 2.5% dextrose and 0.5% sodium hydroxide (by weight) before being heated to about 85ºC in a paddle mixer fitted with a thermostatically controlled heating belt that renders it un-digestible at rumen pH (100% UDP). Two treatment groups were fed mixes of the treated and untreated canola meal to give two treatments of 30% and 60% UDP. The untreated and treated canola meal were analysed for acid detergent insoluble nitrogen (ADIN) to determine the extent of non-enzymatic browning due to overheating and buffer soluble nitrogen to determine the overall solubility of the canola meal protein (Licitra *et al.* 1996). The canola meal in all treatments provided about 50% of the total protein in each diet.

Sampling and analysis of fibre

Each animal had a mid-side patch approximately 10 x 10 cm clipped to skin level prior to the start of the dietary treatments using a Laube clipper with a size 40 blade (Kim Laube and Co., Oxnard, California, USA). At the end of the three month feeding period the same mid-side area was clipped and the fibre was removed. The fibre that was removed at the start and the end of the treatment period was dried and weighed. At both time points, the dimensions of the mid-side patch were measured to accurately determine the area from which the fibre was removed in order to calculate dry fibre growth per square centimetre of the area sampled. The diameter of a representative sample of the fibre clipped from the mid-side of each animal was measured by an accredited commercial company (MicronMan, Bibra Lake, Western Australia) using an Optical Fibre Diameter Analyser (OFDA) 2000 (BSC Electronics, Perth, Australia; number of counts ranged from 800 to 1250 per sample).

Blood collection and analysis

Blood samples were taken via jugular venipuncture into vacuette EDTA tubes prior to feeding on one day each week. After centrifugation at 3000 rpm for 10 minutes, the plasma was removed and frozen at -20ºC for subsequent analysis. Plasma urea nitrogen was measured in plasma samples taken the week prior to the start of the experiment and in weeks two, eight and nine of the feeding period using a Kinetic UV test with an Olympus test kit OSR6134 on an Olympus AU400 analyser. These four time points coincided with the weeks when it was necessary to adjust food intake to ensure the animals were always fed to maintain live weight and body condition.

Behavioural observations

The behaviour of each of the eight alpacas fed the 0% and 100% UDP treatments was recorded using four closed-circuit television (CCTV) cameras and digital surveillance system software (Kguard DVR7134 version 1.1, Kguard Security, Taipei, Taiwan, 2005). Each animal was recorded for eight hours over five days commencing at the start of feeding each morning. The video footage was analysed using Interact software (Interact, version 8, Mangold International GmbH, Arnstorf, Germany, 2005) to determine the total time each animal spent performing each activity. Behaviour was classified into 8 categories (eating, lying, standing, walking, grooming, defecating, drinking, and urinating).

Statistical analyses

The mean fibre diameter and the dry fibre growth of the eight animals in each treatment group were compared using an ANOVA (GenStat®, 11th edition, VSN International Ltd., Hemel Hempstead, United Kingdom, 2008). The fibre diameter value at the beginning of the treatment was used as a covariate. The mean change in live weight, body condition and plasma urea nitrogen concentration of the eight animals in each treatment were compared using an ANOVA (GenStat®, 11th edition, VSN International Ltd., Hemel Hempstead, United Kingdom, 2008). The live weight and body condition score from the first week of the treatment period were used as a covariate. Pairwise comparisons between the four treatment groups were also conducted with Dunnett's test using the statistical program R (R, version 2.14.0, The R Foundation for Statistical Computing: http://www.r-project.org/, 2011). The mean time spent in each behaviour for the eight animals in the two extreme treatment groups was arcsine transformed. A proportion of video footage was used for analysis due to several video recordings stopping before the eight hour period had concluded. For most animals, around 90% or

more of the video footage was used, except for one animal in the 100% UDP group where only 66% of the footage was suitable for analysis. The transformed data were analysed using ANOVA with repeated measures (GenStat® 11th edition, VSN International Ltd., Hemel Hempstead, United Kingdom, 2008).

Results

All alpacas consumed the pellets and canola meal offered each day over the 3 month feeding period. On a few occasions some animals did not eat a small amount of the straw offered. The mean metabolisable energy (ME) intake did not differ between groups ($p = 0.985$). The change in live weight ($p = 0.662$) and body condition ($p = 0.278$) did not differ between groups (Table 3.1).

The weight of fibre grown per unit area over the 14 week feeding period was similar between all treatment groups ($p = 0.313$). The mean diameter of the fibre grown by the animals in each group was less than the mean diameter of their fibre at the start of the 14 week experiment. The alpacas fed the diet with 0% UDP grew fibre of smaller diameter than the alpacas fed the three diets with higher levels of UDP ($p = 0.039$; Table 3.1).

Table 3.1. Mean (±s.e.) metabolisable energy (ME) intake, change in live weight, body condition, fibre growth and fibre diameter of alpacas fed diets containing different proportions of UDP over 14 weeks

	Proportion of UDP from canola meal in diet			
	0%	30%	60%	100%
ME intake (MJ/d)	4.2 ± 0.22	4.3 ± 0.21	4.2 ± 0.27	4.2 ± 0.26
Change in live weight (kg)	1.7 ± 0.28	1.5 ± 0.85	2.9 ± 1.11	1.5 ± 1.03
Change in condition score (1-5)	-0.6 ± 0.15	-0.2 ± 0.16	0.0 ± 0.19	-0.2 ± 0.16
Fibre growth (mg/cm^2)	33.8 ± 2.42	39.6 ± 3.29	42.2 ± 3.97	37.7 ± 3.10
Fibre diameter (μm)	18.1 ± 0.50[a]	20.4 ± 0.93[b]	21.4 ± 0.63[b]	20.4 ± 0.82[b]

[ab] Values within a row with different superscripts are different ($p < 0.05$).

There was no effect of treatment on plasma urea nitrogen ($p = 0.530$), however the group that received 0% UDP had the lowest plasma urea nitrogen concentration with a mean of 4.2 ± 0.19 mmol/L compared to the 30%, 60% and 100% UDP treatment groups with mean concentrations of 4.8 ± 0.25, 4.7 ± 0.12 and 4.6 ± 0.16. The mean plasma urea nitrogen concentration for all animals in the week prior to the start of the experiment was significantly higher than the mean values for weeks 2, 8 and 9 of the experiment ($p = 0.004$).

There were no differences between the 0% and 100% UDP treatment groups for any of the observed behaviours ($p > 0.05$; Table 3.2), except that the alpacas fed the diet containing 0% UDP spent a significantly greater proportion of time urinating compared to alpacas fed the 100% UDP diet ($p = 0.027$). However, the frequency of urination was not different between the two groups (0% diet: 2.0 ± 0.5 urinations/day; 100% diet: 2.5 ± 0.9 urinations/day; $p > 0.05$).

Table 3.2. Time budget of alpacas fed a diet containing either 0% UDP or 100% UDP for each behaviour category observed. Times for each behaviour category are expressed as percentage of total time

	Proportion of UDP from canola meal in diet	
	0%	100%
Eating	27.1 ± 4.68	32.6 ± 3.71
Lying	20.2 ± 4.27	17.1 ± 1.65
Standing	75.5 ± 3.91	76.4 ± 2.26
Walking	4.1 ± 0.77	6.4 ± 1.79
Grooming	0.8 ± 0.10	1.6 ± 0.57
Defecating	0.4 ± 0.13	0.2 ± 0.06
Drinking	0.4 ± 0.13	0.2 ± 0.11
Urinating	0.4 ± 0.13[a]	0.1 ± 0.04[b]

[ab] Values within a row with different superscripts are different ($p < 0.05$).

Discussion

The hypothesis that alpacas fed canola meal with a high proportion of UDP would produce more fibre and spend less time urinating than peers fed a similar amount of canola meal with a low proportion of UDP was partially supported. The fibre from the alpacas fed 0% UDP was finer than that of the alpacas in the other groups. This result is consistent with those of Masters *et al*. (1999) where sheep supplemented with UDP in the form of canola meal grew more wool of greater diameter than those supplemented with a lower proportion of UDP as lupins. While the fibre diameter was smaller in the alpacas fed only RDP (0% UDP), the alpacas fed higher proportions of UDP did not produce more fibre. This result suggests that if fibre production is limited, then the availability of nitrogen was lower in the alpacas fed 0% UDP. While fibre growth appeared to be limited by nitrogen availability in the 0% UDP group, the alpacas in all treatment groups maintained their live weight throughout the experiment, suggesting

that protein was probably not limiting and that the mechanisms for retention and utilisation of nitrogen for fibre growth may differ between alpacas and sheep. The apparent difference in the fate of nitrogen between alpacas fed 0% UDP and those fed higher levels of UDP could be partly explained by the behavioural observations.

The general characteristics of the behavioural attributes measured for the two extreme groups were similar, except that the alpacas in the 0% UDP group spent four times as much time urinating as those in the 100% UDP group. One interpretation of these results is that the latter group retained more nitrogen. However, as urea is a diuretic agent it is possible that the alpacas in the 0% UDP group excreted more urea in their urine with less being recycled to the fermentative chambers. Under these conditions, nitrogen may have limited microbial protein synthesis in the fermentative chambers with the result that less amino acids were available to be absorbed and used for fibre production.

Llamas fed a low protein diet were found to efficiently recycle urea and utilised about 85% of the urea recycled for microbial protein synthesis (von Engelhardt and Schneider 1977). Although similar conclusions have been drawn for alpacas, they appear to be less efficient at utilising high levels of dietary nitrogen than llamas (Davies *et al.* 2007). In our experiment, it is possible that the canola meal low in UDP was mostly degraded in the fermentative chambers and ultimately excreted as urinary urea. Nevertheless, the increase in plasma urea nitrogen concentration observed two weeks after the alpacas began to receive the experimental diets, and that persisted in weeks 8 and 9, may indicate that the microbes in the fermentative chambers adapted to the experimental diets by increasing protein degradation. Although not significantly different, the mean plasma urea nitrogen concentration of the alpacas fed 0% UDP tended to be lower than

in the alpacas fed higher proportions of UDP. Taken together, our results lend support to the view that nitrogen metabolism in alpacas may differ from that of true ruminants such as sheep and that alpacas might have a limited ability to recycle nitrogen to their fermentative chambers.

To help elucidate these possible differences, it would be useful to compare the proportion of nitrogen apparently absorbed that is excreted in the urine of alpacas and sheep fed 0% and 100% UDP from canola meal. The most appropriate method of determining the fate of absorbed nitrogen is to house alpacas and sheep in metabolism pens so that their energy and nitrogen balance can be measured. Relatively little work has been done with alpacas being housed in metabolism pens. In the next chapter, I examine the effectiveness of a training protocol that is designed to introduce alpacas to metabolism pens, minimise the stress of being in a confined space and, thereby reduce the potential compromise in the validity of the results from nutritional experiments.

Chapter 4

Gradual training of alpacas to the confinement of metabolism pens reduces stress when normal excretion behaviour is accommodated

A modified version of this chapter is published in Institute for Laboratory Animal Research E- Journal (2012) ***53****: E31-E42.*

Refer to Appendix 2 (p 119) for published version.

Introduction

The confinement of an animal is known to alter normal behaviour and can be a significant stressor (Bowers *et al.* 1993). The use of metabolism crates in research, where the animal is confined for an extended period, is a controversial practise as both the welfare of the animal and the validity of scientific results can be compromised (Fraser 2008). Little has been reported with respect to the reaction to stress of alpacas in metabolism crates. While there are some reports on confinement in other species, it is important to bear in mind that there can be large interspecies variability in the capacity to tolerate and adjust to confinement (Bowers *et al.* 1993).

The effect of confinement has been studied in other farm animals such as sheep, pigs and horses (Mal *et al.* 1991; Bowers *et al.* 1993). Acclimatisation periods and habituation training can assist sheep in adapting to confinement and restraint during handling (Grandin 1989). Sheep that are acclimatised to a procedure or routine generally display reduced physiological and behavioural responses associated with the stress response compared to sheep that experience the same procedure for the first time

(Grandin 1997). Alpacas are social animals with a strong group hierarchy (Fowler 1998) therefore, the act of placing them in confinement where they are exposed to a novel environment and have limited social contact is likely to induce changes in behaviour and physiology associated with the 'stress response', as seen in other social species such as sheep (Done-Currie *et al.* 1984; Bowers *et al.* 1993).

The main impetus that led to this study was the need to conduct nutrition experiments, which require alpacas to spend around seven days in a metabolism pen so that energy and nitrogen balance can be determined. The aim of this study was to design a protocol to successfully train alpacas to remain in a specially designed metabolism pen that would be used for future nutritional studies (Figure 4.1). The training protocol and metabolism pens were developed to decrease the welfare compromise associated with the transition from a paddock and group situation to the semi-isolation in the metabolism pen (the pen is not total isolation because an alpaca in the pen could see and communicate with other alpacas). For ethical reasons and safety concerns, we did not compare the behavioural stress response of trained and untrained alpacas but instead compared the same animals when they were naïve to following training. It was expected that the alpacas would gradually show fewer stress-associated behaviours as training progressed and that they would adapt to the confinement of the metabolism pen.

Figure 4.1. The metabolism pen designed for alpacas with sufficient floor space and high sides.

Material and Methods

These methods were approved by the University of Western Australia Animal Ethics Committee (RA/3/100/877).

The alpacas were gradually trained to the confinement of a metabolism pen. During the training, the alpacas' behavioural response to this stress was monitored and used as a measure of the amount of strain the animal was experiencing.

Preliminary Halter and Lead Training

Ten alpacas underwent halter training and were used in this study. They were sourced from local breeders where they had been housed in a paddock environment and were rarely yarded. The alpacas had received little to no training prior to being used in the experiment. Upon arrival at Shenton Park Research Station at the University of Western

Australia, the alpacas were given a week to become accustomed to their new environment. They were kept in a paddock with an open-sided shelter. This paddock was the location for the training periods and when they were not in the metabolism pens the alpacas were allowed to roam freely in the paddock. Prior to the commencement of the metabolism pen training protocols, the alpacas were familiarised with the handlers and underwent halter and lead training. The same method of halter training was used for all the alpacas (Table 4.1). Each training session lasted approximately 15 – 20 minutes though the time taken for each step of the protocol varied according to the progress made by each individual alpaca. Generally, a new step was introduced every 2 days. Some sessions were modified according to the progress made by the individual alpaca. Within ten days, most alpacas were accustomed to the halter and would walk on the lead without pulling. Food rewards were offered when a training step was successfully completed and this method of positive reinforcement was used through the entire training process.

Table 4.1. General method of halter and lead training for alpacas

Training step	Description of method
1	Alpaca caught and held by handler. Gently rubbed over neck and shoulders.
2	Alpaca introduced to the halter – allowed to sniff, halter gently placed in front of nose, nosepiece gradually eased into place and removed.
3	Once nosepiece was in place, strap behind ears was fastened. Alpaca taught to stand quietly and allow halter to be removed.
4	Alpaca was led in straight line, encouraged not to pull. Introduced to "walk on" and "stand" commands.
5	Alpaca was led in a more complicated trajectory e.g. weaving, over obstacles.

Metabolism Pen Training

1. Monitoring during training

The level of stress that an animal perceives can be assessed using either or both physiological and behavioural indicators (Baldock and Sibly 1990; Grandin 1997). For the present study, behavioural indicators were chosen to assess the response to stress as they are non-invasive and/or impose no additional stress on the animal. They also provide an immediate measure of the response to stress and were therefore a more useful and practical assessment during this experiment. Alpacas display a number of behavioural reactions to stressful situations, such as holding their tail above their back, spitting, rolling their head, making loud screaming vocalisations, and flaring their nostrils (McGee-Bennett 2001). During the introduction of an alpaca to a metabolism pen, possible behavioural indicators of stress such as repetitively lying and getting up over short time intervals, attempts to escape, kicking towards the pen, restlessness or

pacing around the pen, loud vocalisations, excessive alertness defined by the ears pricked forward and flared nostrils, or pushing against the sides of the pen were considered to define a stressed alpaca. The incidence of these behavioural responses was quantified during the training of the alpacas and used as a guide to the degree of success and subsequent progression through the training. During constant monitoring, the alpaca's behaviour was recorded every 5 min or when the alpaca showed signs of distress. If an alpaca showed three or more of the stress signals over two monitoring periods (every 5 min to every 2 h, depending on the training step), the alpaca was removed from the metabolism pen and retrained from the previous training step. Throughout the study, whenever one alpaca was in the metabolism pen, its companions were kept in a small yard that was constructed around the pen, or in other metabolism pens placed close by, so that all animals remained in contact with each other.

2. Training protocols

The study consisted of two parts. Firstly, a preliminary study was conducted to assess a training protocol designed from knowledge of the social behaviour of alpacas. Four alpaca wethers (2 – 2.5 years old) were used and were successfully accustomed to the metabolism pens using the following training protocol (see Protocol 1 below) within 11 days. During step 6, the metabolism pen was constructed under the open –sided shelter so that the alpacas had sufficient cover whilst in the pen. The alpaca's water bucket and daily food ration of milled barley straw, a roughage based pellet (Macco 101 pellet, Macco Feeds Australia, Williams, Western Australia) and dry, granulated sugar cane molasses (Palabind, Probiotec, Laverton North, Victoria, Australia) were left in the pen. Dry molasses was also used as a food reward during the other steps of the protocol.

Protocol 1

1. An area of ground with the same dimensions as the metabolism pen (1.6 x 1.6 m) was marked out using metal pickets ~ 1.5 m high and rope. The alpacas were individually confined in this makeshift pen for 30 min while being constantly monitored by a handler standing several metres away. This was repeated two or three times until the animal settled and showed no stress-associated behaviours.
2. The alpacas were left in the makeshift pen for 2 h under constant supervision.
3. The flooring of the metabolism pen was placed in a small yard with the alpacas, where they spent half a day exploring and familiarising with it.
4. The alpacas were restricted to the flooring for 2 h with constant monitoring. For practical reasons, this step was modified during the training such that the handler led the alpacas over the flooring and left them standing on the floor for longer periods (10, 30, 60 sec).
5. Gradually, the sides of the metabolism pen were added and the alpacas were left in the constructed pen for 30 min.
6. The time that the alpacas spent in the metabolism pen was gradually increased from 2, to 4, 8 and 24 h.

The behaviour of the four alpacas indicated that the gradual construction of the metabolism pen sufficiently desensitised the alpacas to confinement and allowed the animals to become familiar with the pen and to remain, apparently unconcerned, in the pen for long periods (Table 4.2).

Table 4.2. Assessment of training success achieved with each step of Protocol 1

Training step	Degree of success and observations
1	All of the alpacas stayed in pen. Animals had to remain haltered because the rope fence did not always prevent them from leaving the defined area. No signs of any perceived stress; escape initiated out of boredom rather than fear.
2	Alpacas remained haltered. No signs of any perceived stress.
3	Repeated over 3 days. All alpacas observed exploring the flooring; were content to lie down near it and eat food from it.
4	All alpacas were comfortable with being led on and off the flooring. No signs of any perceived stress.
5	Two opposing sides added; all alpacas led over with no signs of perceived stress. Third side added; alpacas accepted gradual confinement with no signs of perceived stress. Fourth side added; some signs of perceived stress were evident. One alpaca circled the pen, looking for a way out, and attempted to escape several times by pushing and banging on the sides of the pen. This animal was removed from the metabolism pen and calmed down before starting again from the beginning of the training step. Second attempt was more successful; alpaca discovered his food and ate, which resulted in less circling and no attempts at escape.
6	All alpacas appeared comfortable. As time increased, the alpacas were observed to refrain from urinating and defecating until they were released. When required to stay in pen for $\geqslant$ 8 hours. the alpacas urinated and defecated. Some animals appeared to experience some discomfort, evident by small humming noises and sniffing at the flooring. When the animals did defecate, their faeces were solid and packed together unlike the animals' normally excreted individual pellets. Consequently, the faeces did not fall through the flooring of the metabolism pen to be trampled and spread over the floor, making collection for nutritional studies difficult and inaccurate.

Following the preliminary study, a more flexible, modified protocol (Protocol 2) was developed where some of the training steps originally used were altered to suit the progress of an individual alpaca and the degree of success achieved with each step. A second study using six different alpaca wethers of similar age was conducted to test the following Protocol 2 (see below). The alpacas completed Protocol 2 within 6 -7 days.

Protocol 2

1. The flooring of the metabolism pen was placed in a small yard where the alpacas spent half a day exploring and familiarising with it.
2. The alpacas were led across the flooring until no behavioural indications of stress were observed.
3. Gradually, the sides of the metabolism pen were added and the alpacas were led through the constructed pen.
4. The alpacas spent a full day in the metabolism pen, in sight of peers, under constant supervision.

Design of the Metabolism Pens

The metabolism pens used in this study were specially designed for alpacas, but are suitable for other production animals such as sheep and goats. The welfare of the animal in the pen was a high priority, thus the metabolism pen had a larger floor space (1.6 x 1.6 metres) than most conventional metabolism crates and accommodated an alpaca lying down with its neck stretched out in front, as is their habit (Figure 4.2). The flooring was situated as low as possible to the ground so that the animal was only required to take a small step up to enter, but high enough to permit urine and faeces collection apparatus under the flooring. The flooring was made of non-slip plastic material that is commonly used in piggeries (Stepper flooring, MIK International,

Germany). The sides of the crate were made high (1.2 m) but allowed the animal see its peers.

Figure 4.2. The metabolism pen has sufficient floor space to accommodate an alpaca lying down with their neck stretched out in front of them.

Results

Five of the six alpaca wethers were successfully trained to the metabolism pen using Protocol 2. One alpaca was excluded from the training phase because he showed no improvement during basic handling procedures such as being haltered and walked on the lead. It was decided that any attempt to put him near the metabolism pen would impose a large stress and be dangerous to the animal and to the handler.

The alpacas did not show any behavioural signs of stress during step 1 of the training protocol when they explored the flooring of the metabolism pen. Initially, most of the

animals sniffed at the floor then ignored its presence. After approximately one hour, one alpaca walked over the floor.

During step 2, stress behaviours were observed within the first ten minutes of the training sessions. Three out of five alpacas progressed to the next training step after one, twenty minute session (Figure 4.3). The other two animals that repeated the training step showed five incidents, collectively, of excessive alertness in the first 10 min of the session. On three occasions, restlessness in the form of pulling on the lead rope was also recorded (Table 4.3).

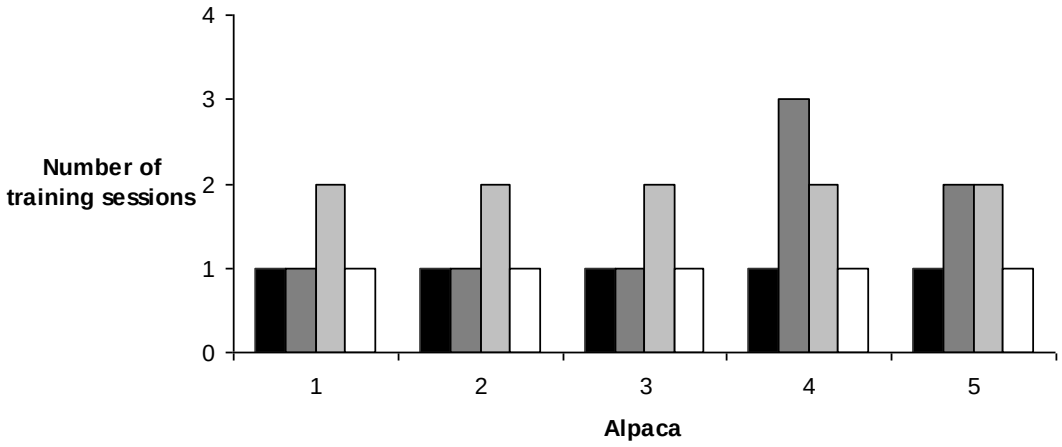

Figure 4.3. Number of training sessions required by individual alpacas for each training step. Black bars = training step 1 (half day), dark grey bars = training step 2 (sessions 15 – 20 mins in duration), light grey bars = training step 3 (15 – 20 mins in duration), white bars = training step 4 (full day).

Table 4.3. Collective number of incidents of stress-associated behaviours expressed by all alpacas during each step of Protocol 2*

Step	Time (mins)	Stress indicators							Total no. of incidents
		Unsettled	Escape attempt	Kicking at pen	Pushing on pen	Loud vocals	Restlessness (pacing)	Excessive alertness	
1	Half day	-	-	-	-	-	-	-	0
2	5	1	-	-	-	-	1	3	5
	10	1	-	-	-	-	2	2	5
	15	-	-	-	-	-	-	1	1
	20	-	-	-	-	-	-	-	0
3	5	-	-	-	-	-	2	1	3
	10	-	-	-	-	-	2	-	2
	15	-	-	-	-	-	-	-	0
	20	-	-	-	-	-	-	-	0
4	Whole day	2	2	-	1	-	7	-	12

*Dashes indicate that no behavioural response was observed.

During the first 10 min of step 3 of the training protocol, some alpacas demonstrated restlessness and excessive alertness. However, only two training sessions were needed for all the animals to complete this step (Table 4.3).

Initially, the alpacas were restless when they were left in a metabolism pen for a day (step 4). One animal initially attempted to escape by pushing on the sides of the pen, however, all animals settled within an hour and ate their daily food ration. Throughout the day some animals made soft vocalisations, often in response to another animal. After 9 h, most of the alpacas were unsettled and restless and observed to be repeatedly standing up and lying back down, pacing around their pen and sniffing at the floor. It was evident that they were uncomfortable. Alpacas defecate and urinate at communal dung heaps and their behaviour suggested that they needed to defecate and urinate but were unwilling to do so. One animal attempted to escape by rearing. On the second attempt at escape the alpaca seemed to accidently urinate which apparently prompted him to stand in the body position associated with excretion, and he did urinate and defecate. After urinating and defecating the alpaca reverted back to being calm and showed no further overt behaviours associated with perceived stress. The behaviour of this animal suggested that the presence of faeces in the pen could be used as a stimulus to trigger defecation. The faeces from that animal's pen were transferred into the other pens in an attempt to trigger a response from the other animals (see Discussion). This method was successful in changing the alpaca's learned behaviour of using communal dung heaps and after they had all urinated and defecated they showed no further behaviours associated with perceived stress and continued to urinate and defecate normally.

Discussion

The protocol developed in this study was successful at introducing alpacas to specially designed metabolism pens and led to a progressive decline in the occurrence of behavioural responses to stress that can be experienced by animals when subject to confinement. The alpacas adapted to the confinement of the metabolism pens and showed fewer behaviours associated with a response to stress as the training progressed. The success of the protocol may be attributed to the progressive nature of the training where each alpaca was presented with one stressor at a time and given time to become comfortable with that stressor before proceeding to the next training step. In a review of behavioural principles of sheep handling, Hutson (2000) concluded that gradual training and handling can reduce the stress of handling in sheep, and it is apparent from our results that his conclusions pertain also to alpacas. Gradual exposure to novelty can allow animals to become accustomed to stimuli that may otherwise prompt stress-associated behaviours (Grandin 1997). In this study, care was taken to ensure that the alpacas were apparently comfortable with each training step before progression to the next step. For example, the alpacas were not introduced or led over the flooring of the metabolism pen until they walked calmly on the lead and responded to verbal commands from their handler.

The training protocol was also tailored to alpacas by using information and observations of alpaca behaviour. Alpacas are naturally inquisitive and social animals and should find exploring novel objects a positive experience when kept in a stable group (Tennessen 1989; Fowler 1998). Therefore, the alpacas were allowed to explore the metabolism pen flooring in their own time when it was left in their paddock. By keeping them together as a group and letting them approach the flooring by themselves, the stress of having a novel object in their yard seems to have been reduced. Similarly,

because of their social nature, the metabolism pen was designed to allow the alpacas to see each other, even when they were lying down as they do in the field. The freedom to express normal behaviour is regarded as one of the 'five freedoms' used to assess animal welfare (FarmAnimalWelfareCouncil 1992). The metabolism pen was designed to be more accommodating of the normal behaviour of alpacas than conventional metabolism crates, which tend to completely isolate the animal by blocking visual contact with conspecifics and restricting the amount of floor space to such a degree so that the animal can not easily turn around. It appears that in this study, the welfare of the animals was not compromised.

Although the training protocol was developed for alpacas, it was evident during the study that some behaviours that are characteristic of the species were more difficult to overcome. Alpacas defecate and urinate on communal dung heaps (Fysh 2003). When the alpacas were required to remain in the metabolism pens for extended periods they initially displayed no signs of an agitated state, but later became agitated and restless. Given the subsequent behaviour of the alpacas we conclude that this restlessness was due to them resisting the micturition and defecation reflexes because they did not have access to a communal heap. It was possible, however, to modify this learned behaviour and train the alpacas to defecate and urinate within a few hours of being in the pen by transferring fresh faeces from a pile either in the paddock or another metabolism pen, into the pen. This action seemed to act as a stimulus to release the inhibition of the excretion reflexes. After the initial excretion, the alpacas continued to defecate and urinate in the pens, regardless of the amount of time that they spent out of the metabolism pens between training sessions. This was an important outcome because the object of keeping animals in a metabolism pen is usually so that faeces and/or urine can be collected. It also highlights the importance of the relationship that animals have

between olfactory cues and behaviour. It is clear from this experiment that, rather that having clean and disinfected pens, the animals required the presence of some faeces in the metabolism pens for them to perform natural functions. From a regulation and ethical view, keeping animal facilities very clean or sterile may likely impact the behaviour of the animal and inadvertently cause stress.

The training protocol may also have been successful because positive reinforcement, mainly in the form of food, was used. Sheep can be trained to voluntarily accept restraint with the assistance of grain as a reward (Grandin 1989). Similarly, Hutson (2000) has recommended that the aversive and stressful nature of handling could be reduced by using food rewards. In the present study, the alpacas appeared to remember that when they behaved in a particular way, they would be rewarded. The gradual absence of behavioural responses to stress suggested that the animals had a positive experience.

Although, according to our measures, the training protocol was successful in introducing alpacas to the metabolism pens and reducing the potential stress of being confined within the pen, it has to be noted that we used only behavioural indicators to assess the response to stress. Other authors have suggested that it is best to use both behavioural and physiological indicators, such as cortisol or heart rate (Grandin 1997; Arzamendia *et al.* 2010). However, behavioural indicators alone were a practical and immediate measure of the response to stress in this study. As cortisol concentration was not immediately available, it was not an appropriate stress indicator in this study. Cortisol concentrations may have confirmed the response to stress by the animal, although there is evidence to suggest that cortisol may not always show a response during handling particularly when the animals are habituated (Lay *et al.* 1992; Andrade

et al. 2001). Likewise, heart rate measurements can be confounded by activity and may not provide an accurate measure of stress during the training. Moreover the training protocol was spread over a few weeks so it was not practical to equip each alpaca daily with devices to measure heart rate or with indwelling cannulae to take serial blood samples. Both of these procedures can themselves act as stressors. Changes in behaviour are the first symptoms that can indicate the state of an animal's well-being (Dellmeier 1989). The nature of the training relied heavily upon the handler recognising when an alpaca showed reluctance to the task he was being asked to do and therefore being able to remove the animal from the stressful situation. Studies on the response to confinement in other species, such as horses, have also used behaviour as the sole indicator of animal well-being (Mal *et al.* 1991). Likewise, a correlation between behavioural observations and physiological measures that reflect the physiological response to stress has been identified in cattle (Jefferies 1961).

This study highlights that, although the training protocol worked for most of the alpacas, some animals may not become accustomed to handling or novel experiences regardless of the amount of time spent by the handler in taming the animal. During this study, one alpaca was excluded from the training to the metabolism pen because he continued to show signs of stress during basic handling sessions. This animal's continued adverse reaction may be attributed to its previous life experiences or its genetic makeup or, an interaction between the two. Often, an animal's previous experience can influence their response to stress (Grandin 1997). In addition, genetic factors, such as temperament, may also influence an animal's resistance to stressors and their ability to be trained (Grandin 1997).

Overall, the training protocol that was developed to train alpacas to the confinement of metabolism pens was successful in reducing the incidence of stress-associated behaviours whilst in the pens. This success was largely attributed to the gradual progression of the training steps and the protocol being tailored to suit alpacas using positive reinforcement. This study demonstrated that when confinement is necessary, such as during metabolic experimentation, adequate procedures can be developed to introduce large experimental animals to confined spaces while imposing minimum stress. By implementing such procedures, both animal welfare and the validity of the scientific outcomes can be maximised.

Chapter 5

Alpacas fed rumen degradable and un-degradable dietary protein tend to excrete more nitrogen in their urine than Merino sheep but have similar nitrogen retention

Introduction

In Chapter 3, alpacas fed RDP in the form of expeller canola meal spent more time urinating, and produced fibre of finer diameter, than those fed the same canola meal with a higher level of UDP (Lund *et al.* 2012; Chapter 3). This suggests that when fed RDP, which should increase the ammonia concentration in the fermentative organs, the excess ammonia is converted to urea for excretion in urine. Thus, the proportion of dietary protein as RDP may influence the pathways of nitrogen metabolism in alpacas.

Little is known about nitrogen metabolism in alpacas, especially how nitrogen is partitioned in the body. In sheep however, nitrogen metabolism is quite well understood and experiments similar to that in Lund *et al.* (2012; Chapter 3) where fibre growth has been improved by adding protein of different levels of degradability to the diet have been done (Masters *et al.* 1999). Alpacas are thought to have adapted to efficiently utilise forages that are limited in dietary nutrients by using protein as their primary source of energy and by recycling nitrogen, rather than relying upon volatile fatty acids such as propionate for glucose for energy like true ruminants (San Martin and Bryant 1989; Van Saun 2006a). As a fibre producing species like the alpaca, sheep provide a suitable model to compare the fate of absorbed nitrogen.

In the present study, we compared the nitrogen and energy balance of alpacas and sheep when they were fed a maintenance diet containing UDP or RDP. We aimed to determine if protein degradability influences nitrogen retention or impacted energy balance, and whether alpacas utilise dietary nitrogen more efficiently than sheep. For this experiment, mature animals of both species were fed at maintenance so that both energy and nitrogen metabolism could be compared without being influenced by growth or fat deposition. We hypothesised that alpacas fed a diet containing RDP in the form of canola meal would excrete more nitrogen than those fed UDP. We also expected alpacas fed the same diet as sheep at maintenance to retain more nitrogen and energy.

Materials and methods

Alpacas and sheep were fed the same diets as used in the experiment for Chapter 3 whilst housed in metabolism pens. The diets contained either UDP or RDP from canola meal which had been prepared using the same methodology used in Lund *et al.* (2012; Chapter 3). Using a randomised block design, each animal was its own control and therefore received each treatment diet during the experiment. Nitrogen and energy balances were measured to determine whether alpacas metabolised nitrogen more efficiently than sheep and whether protein degradability influenced the ability of alpacas to retain nitrogen. These methods were approved by the University of Western Australia Animal Ethics Committee (RA/3/100/935).

Animals

Huacaya alpaca wethers (n = 7) and Merino sheep wethers (n = 7) of similar body weight (51.1 ± 1.04 kg) and mature in age were transported to Shenton Park Research Station at the University of Western Australia. All animals were acclimatised and housed in individual metabolism pens that were constructed under an open-sided shed

in their outdoor paddock during the treatment period. The metabolism pens were specially designed for alpacas with the welfare of the animals as a foremost consideration. Each metabolism pen had sufficient floor space for the animals to lie down with their neck stretched out in front of them and they could maintain visual contact and touch noses with other individual animals housed in pens next to them. Before commencing the experiment, each animal was introduced to the metabolism pen using a gradual training protocol (Lund *et al.* 2012; Chapter 4). Each animal was provided with *ad libitum* water and was fed daily between 0700 h and 0800 h. The animals' body condition and live weight were measured weekly before feeding, using the same set of scales, to ensure that they were being fed at a maintenance level. All animals were weighed to the nearest 0.5 kg and body condition was scored on a scale from 1 = emaciated to 5 = obese (Fysh 2003).

Feed treatments

Both sheep and alpacas were fed a basal diet at maintenance of milled barley and oat straw, a roughage based pellet (Macco 101 pellet, Macco Feeds Australia, Williams, Western Australia), 20 g/head/day of a complete mineral mix and 20 g/head/day of dried, granulated sugar cane molasses (Palabind, Probiotec, Laverton North, Victoria, Australia). Canola meal was added to the basal diet as a protein supplement (Table 5.1). The canola meal was either cold pressed into flakes which provided RDP or had been heat treated so that it supplied UDP. To increase the chance of absorption in the small intestine, the UDP canola meal was ground into fine particles to minimise the time during which the protein could be exposed to possible degradation in the rumen (Lund *et al.* 2012; Chapter 3). The diet consisted of the same components and was formulated in a similar way to that previously used in Lund *et al.* (2012; Chapter 4). The amount of pellets offered to the alpacas in this experiment was slightly lower at 8.8 g/kg$^{0.75}$

compared to 9.7 g/kg$^{0.75}$ as offered in Lund *et al.* (2012; Chapter 4) due to small differences in the nutritive content between batches of pellets. Representative samples of each feedstuff were analysed for gross energy content, using a Ballistic Bomb Calorimeter (Gallenkamp ®, Loughborough, United Kingdom) that was calibrated using benzoic acid standards, and nitrogen content was determined using a Vario Macro Elemental CHN analyser (Elementar Analysensysteme GmbH, Hanau, Germany), which was calibrated for nitrogen analysis using glutamate as the standard.

Table 5.1. Amount of each feedstuff offered to alpacas and sheep to maintain live weight and condition

Feedstuff	Alpacas	Sheep
Straw	22.0 g/kg$^{0.75}$	24.6 g/kg$^{0.75}$
Pellets	8.8 g/kg$^{0.75}$	9.8 g/kg$^{0.75}$
Canola meal	4.6 g/kg$^{0.75}$	5.1 g/kg$^{0.75}$
Nitrogen offered	0.56 g/d.kg$^{0.75}$	0.62 g/d.kg$^{0.75}$
Energy offered	0.72 MJ/d.kg$^{0.75}$	0.80 MJ/d.kg$^{0.75}$

The animals were randomly allocated to a dietary treatment and then acclimatised to the treatment feed for seven days in individual, sand yards before being housed in metabolism pens for seven days during which data were collected. After seven days, the animals were removed from the metabolism pens and acclimatised to the other treatment feed for a further seven days before being returned to the metabolism pens. At the conclusion of the experiment, each animal had been fed both the RDP and the UDP diet. Feed refusals were recorded daily so that total food intake could be calculated. Water intake was also measured daily.

Urine collection and analysis

Urine was collected daily when the animals were housed in the metabolism pens (see pen design in Lund *et al.* (2012; Chapter 4)). Concentrated hydrochloric acid was added to the urine collection trays to ensure that the pH of the urine remained below 3 and thus nitrogen was retained in the urine. The total volume of urine excreted by each individual animal was measured daily. A 10% sub-sample was then taken and pooled over the seven day treatment periods and frozen for later analysis. The gross energy content of the urine was determined by freeze drying the samples and combusting them in a Ballistic Bomb Calorimeter (Gallenkamp ®, Loughborough, United Kingdom). Nitrogen content was determined by the Kjeldahl method using a Nitrogen analyser (Kjeltec 8400, FOSS, Hillerød, Denmark).

Faecal collection and analysis

The faeces from individual animals housed in metabolism pens were collected daily. The total amount eliminated daily was measured and a 10% sample was kept for analysis. The 10% samples were pooled for each animal, stored and frozen at -20ºC. At the end of the collection period, two replicates of the pooled samples were dried in an oven at 60ºC until constant weight was recorded. The dry faeces were ground into 0.5 mm particles using a sample grinder (Retsch, Haan, Germany) and stored in plastic vials to be used for gross energy and nitrogen content analysis. Faecal energy was measured by combusting the samples in a Ballistic Bomb Calorimeter (Gallenkamp ®, Loughborough, United Kingdom). Faecal nitrogen was measured using a Vario Macro Elemental CHN analyser (Elementar Analysensysteme GmbH, Hanau, Germany).

Blood sampling and analysis

Blood samples were taken via jugular venipuncture into vacuette EDTA tubes five hours after the animals had been fed. All the animals were accustomed to being handled and gently restrained for this procedure. The concentration of glucose in the blood was measured using a blood glucose meter (Accu-chek Advantage, Roche Diagnostics, Basel, Switzerland). After centrifugation at 3000 rpm for 10 minutes, the plasma was decanted and frozen at -20ºC for later analysis. Plasma urea nitrogen (PUN) was measured using a Kinetic UV test with an Olympus test kit OSR6134 on an Olympus AU400 analyser (Olympus, Tokyo, Japan).

Determining nitrogen and energy use for fibre growth

The partitioning of nitrogen and energy to wool growth was determined for the sheep using information and data pertaining to Merino sheep and the specific flock of sheep from which the animals used in this experiment originated. For the alpacas, fleece growth data from other Huacaya alpacas was used. It was estimated that the sheep used in this experiment produced 5 kg of greasy wool per year, of which 60% is protein (Cottle 2010). The alpacas produce about 2.5 kg of fibre per year, however, as alpaca fibre contains less wax substances than sheep wool, about 90% of their fibre is protein (Wang *et al.* 2003). This information was used to estimate the amount of nitrogen and energy used for fibre or wool growth by each species during the experiment.

Statistical analysis

One alpaca lost significant live weight and condition, despite eating all of his maintenance ration and had a water intake about double that of the other alpacas. This animal was therefore excluded from the experiment for health and welfare reasons. The

sheep that was the match in live weight for that alpaca was also removed so that n = 6 for both species.

T-tests were used to determine whether there was a change in live weight and body condition of the alpacas and the sheep when they were fed the UDP and the RDP diets (GenStat®, 11th edition, VSN International Ltd., Hemel Hempstead, United Kingdom, 2008).

Multivariate analysis of variance (MANOVA) tests and Student Newman-Keuls multiple comparisons were used to compare treatment and species effects on all of the parameters measured during the experiment (Statistica for the Macintosh V1, StatSoft Inc., Tulsa, Oklahoma, USA, 1994). The correlation between water intake and the volume of urine output for sheep and alpacas fed both treatment diets was calculated.

Results

All animals consumed their entire daily ration of food during both dietary treatments. The alpacas and sheep maintained live weight (p = 0.206 for alpacas and p = 0.452 for sheep; Table 5.2) and body condition (p = 0.363 for alpacas; Table 5.2) during the experimental period. There was no difference in metabolic body weight in sheep or alpacas for either the UDP or RDP diets (p = 0.102), although both species seemed to lose more weight when fed the UDP diet.

Table 5.2. Mean (±s.e.) change in live weight (kg) and condition score of alpacas and sheep when fed the two diets

		RDP	UDP
Live weight (kg)	Alpacas	-0.3 ± 0.17	-0.9 ± 0.44
	Sheep	-0.6 ± 0.58	-1.3 ± 0.58
Condition score	Alpacas	0.0 ± 0.00	-0.1 ± 0.08
	Sheep	0.0 ± 0.00	0.0 ± 0.00

Water intake did not differ between species ($p = 0.691$) or diets ($p = 0.234$). On average, the animals consumed 2.4 ± 0.16 kg of water per day. Likewise, the volume of urine output did not differ between species ($p = 0.79$) or diet ($p = 0.54$; Table 5.3). The urine output was strongly correlated with water intake for both diets: sheep fed RDP $r^2 = 0.81$, alpacas fed $r^2 = 0.90$, sheep fed UDP $r^2 = 0.94$, alpacas fed UDP $r^2 = 0.80$.

Table 5.3. Mean (±s.e.) urine output (mL) of sheep and alpacas fed diets containing either rumen-degradable dietary protein (RDP) or un-degradable dietary protein (UDP)

	RDP	UDP
Alpacas	689 ± 240.9	737 ± 202.6
Sheep	848 ± 181.7	733 ± 186.3

Nitrogen metabolism

Nitrogen intake was higher for the sheep than for the alpacas due to the sheep having a higher requirement than alpacas ($p < 0.001$; Table 5.4) however there was no difference in nitrogen intake between the diets ($p = 0.054$; Table 5.4) for either species. The sheep absorbed more nitrogen than the alpacas ($p < 0.001$) and both species absorbed more nitrogen when fed the RDP diet compared to the UDP diet ($p = 0.039$; Table 5.4).

The weight of faeces eliminated did not differ between species ($p = 0.131$) or between the diets ($p = 0.061$). The sheep had a lower percentage of nitrogen in their faeces compared to the alpacas ($p < 0.001$), however, there was no difference between alpacas and sheep in the amount of nitrogen eliminated in the faeces with respect to metabolic body weight ($p < 0.070$; Table 5.4). Dietary treatment did not influence nitrogen eliminated in faeces ($p = 0.192$).

The concentration of nitrogen in the urine did not differ between diets ($p = 0.394$) or species ($p = 0.803$). The amount of nitrogen excreted in the urine per day did not differ with diet ($p = 0.710$) and was similar for both alpacas and sheep ($p = 0.891$; Table 5.4). Overall there was no difference in the daily amount of nitrogen excreted in the urine with respect to absorbed nitrogen for either treatment diet ($p = 0.302$) or species ($p = 0.137$; Table 5.4), however alpacas excreted about 1.3 times more absorbed nitrogen in their urine than sheep.

Alpacas retained less nitrogen than sheep ($p = 0.042$; Table 5.4). There was no difference in the amount of nitrogen retained between UDP or RDP diets ($p = 0.118$). Although the alpacas retained less nitrogen as a percentage of nitrogen absorbed than sheep, the difference between the two species was not significant ($p = 0.137$; Table 5.4).

Table 5.4. Nitrogen balance of sheep and alpacas fed diets containing either rumen-degradable dietary protein (RDP) or un-degradable dietary protein (UDP)

	Alpacas		Sheep	
	RDP	UDP	RDP	UDP
N intake (g/d.kg$^{0.75}$)	0.56 ± 0.01^{a}	0.55 ± 0.00^{a}	0.63 ± 0.01^{b}	0.61 ± 0.01^{b}
N in faeces (g/d.kg$^{0.75}$)	0.26 ± 0.01	0.27 ± 0.004	0.24 ± 0.01	0.26 ± 0.01
N absorbed (g/d.kg$^{0.75}$)	0.30 ± 0.01^{ax}	0.28 ± 0.01^{ay}	0.39 ± 0.01^{bx}	0.36 ± 0.01^{by}
N in urine (g/d.kg$^{0.75}$)	0.19 ± 0.02	0.22 ± 0.01	0.21 ± 0.03	0.19 ± 0.05
N retained (g/d.kg$^{0.75}$)	0.11 ± 0.02^{a}	0.06 ± 0.01^{a}	0.18 ± 0.03^{b}	0.16 ± 0.04^{b}
N in urine: N absorbed	0.65 ± 0.07	0.77 ± 0.04	0.54 ± 0.09	0.53 ± 0.12
N retained: N absorbed	0.35 ± 0.07	0.23 ± 0.04	0.46 ± 0.09	0.47 ± 0.13

^{ab} Superscripts within rows represent differences between species. ^{xy} Superscripts within rows represent differences between diets

In both alpacas and sheep the sum of nitrogen output in the faeces, urine and wool was similar to their nitrogen intake (Figure 5.1).

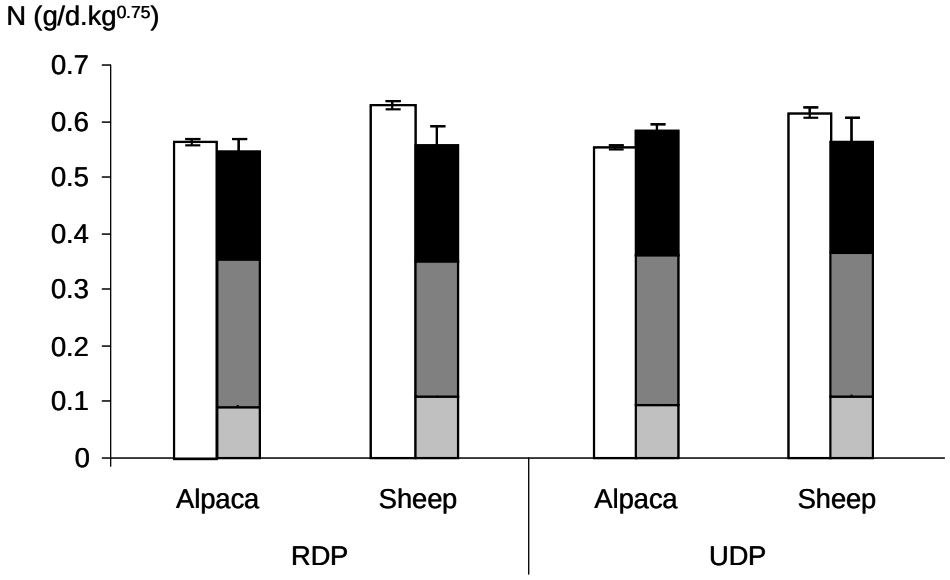

Figure 5.1. Partitioning of nitrogen intake (g/d.kg$^{0.75}$) (white bars) between urine output (black bars), faeces output (dark grey bars) and wool/fibre growth (light grey bars).

Energy metabolism

The energy intake was higher in sheep than alpacas due to their greater energy requirement ($p < 0.001$), however both the UDP and RDP diets were isocaloric (Table 5.5). There was no difference in the amount of gross energy eliminated in the faeces of the sheep and the alpacas ($p = 0.125$), however both species excreted more energy when fed the UDP diet ($p = 0.041$; Table 5.5). The sheep absorbed more energy from their food than alpacas ($p = 0.007$) and both species absorbed more energy on the RDP diet ($p = 0.034$; Table 5.5).

There was no difference in the amount of gross energy excreted in the urine between species ($p = 0.485$) or diets ($p = 0.274$), even when expressed in terms of the energy absorbed from the food (species: $p = 0.264$ and, diet: $p = 0.132$). The sheep retained more energy than the alpacas ($p = 0.007$) and both species retained more energy when fed the RDP diet ($p = 0.039$; Table 5.5). The energy retained from the energy absorbed was not different between species ($p = 0.207$) or diets ($p = 0.234$; Table 5.5).

Table 5.5. Energy balance of sheep and alpacas fed diets containing either rumen-degradable dietary protein (RDP) or un-degradable dietary protein (UDP)

	Alpacas		Sheep	
	RDP	UDP	RDP	UDP
E intake (MJ/d.kg$^{0.75}$)	0.72 ± 0.00^{a}	0.72 ± 0.00^{a}	0.80 ± 0.01^{b}	0.80 ± 0.01^{b}
E in faeces (MJ/d.kg$^{0.75}$)	0.34 ± 0.01^{x}	0.37 ± 0.02^{y}	0.35 ± 0.01^{x}	0.41 ± 0.02^{y}
E absorbed (MJ/d.kg$^{0.75}$)	0.38 ± 0.01^{ax}	0.35 ± 0.02^{ay}	0.45 ± 0.01^{bx}	0.40 ± 0.02^{by}
E in urine (MJ/d.kg$^{0.75}$)	0.004 ± 0.001	0.006 ± 0.001	0.004 ± 0.001	0.004 ± 0.001
E retained (MJ/d.kg$^{0.75}$)	0.38± 0.01	0.34 ± 0.02	0.45 ± 0.01	0.39 ± 0.03
E in urine: E absorbed	0.01 ± 0.002	0.02 ± 0.003	0.01 ± 0.002	0.01 ± 0.004
E retained: E absorbed	0.99 ± 0.002	0.98 ± 0.003	0.99 ± 0.002	0.99 ± 0.004

^{ab} Superscripts within rows represent differences between species. ^{xy} Superscripts within rows represent differences between diets

Metabolites in blood and plasma

The plasma urea nitrogen concentration of sheep was lower when they were fed the RDP diet than when they were fed the UDP diet (p = 0.045; Table 5.6). The plasma urea nitrogen concentration of the alpacas did not differ between diets. There was no difference in plasma urea nitrogen concentration between the two species (p = 0.525; Table 5.6).

The blood glucose concentration of the alpacas was about 1.6 times higher than that of the sheep (p < 0.001; Table 5.6) and did not vary with diet.

Table 5.6. Mean (±s.e.) blood glucose concentration (mmol/L) and plasma urea nitrogen concentration (mmol/L) of alpacas and sheep fed diets containing either un-degradable dietary protein (UDP) or rumen-degradable dietary protein (RDP)

	Alpacas		Sheep	
	RDP	UDP	RDP	UDP
Plasma urea nitrogen (mmol/L)	4.4 ± 0.35	4.7 ± 0.42	3.9 ± 0.19 [a]	4.6 ± 0.40[b]
Blood glucose (mmol/L)	5.1 ± 0.18[a]	5.1 ± 0.24[a]	3.2 ± 0.08[b]	3.1 ± 0.06[b]

[ab] Values within a row with different superscripts are different ($p < 0.05$)

Discussion

In a previous experiment, alpacas fed RDP produced a finer fibre and spent more time urinating than alpacas fed UDP which suggested that the degradability of protein may influence the nitrogen metabolism of alpacas (Lund *et al.* 2012; Chapter 3). The aim of the present study was to determine whether alpacas were more efficient at utilising nitrogen than sheep when they were a fed maintenance diet containing UDP or RDP. The degradability of the protein in the diet did not influence the level of nitrogen retained in either species, therefore the results did not support the expectation that alpacas and sheep would excrete more nitrogen when they were fed a diet containing RDP in the form of canola meal than when fed UDP. It was also hypothesised that alpacas would retain more nitrogen than sheep fed the same diet at a maintenance level because alpacas have been reported to be more efficient at recycling and retaining nitrogen than true ruminants in order to survive in regions where available forages fluctuate in nutrient content (Costa and Vaughan 1998; Van Saun 2006a). This hypothesis was not supported as both the sheep and the alpacas retained similar amounts of nitrogen. However, the alpacas seemed to retain less nitrogen as a percentage of the nitrogen absorbed from their food than did sheep fed the same diet. Our results

suggested that sheep and alpacas probably obtain their energy from different components of their food and utilise protein in different ways.

In this experiment, neither alpacas nor sheep excreted more nitrogen when they were fed a diet containing RDP in the form of canola meal than when they were fed UDP. The addition of UDP in ruminant diets is known to decrease the amount of nitrogen excreted in the urine (Wang *et al.* 2008). Likewise, alpacas fed RDP possibly excreted more nitrogen in their urine due to them spending more time urinating than their peers fed UDP (Lund *et al.* 2012; Chapter 3). Therefore, our result is surprising and it is not clear why similar results to those previously reported were not obtained in this experiment. It is possible that the canola meal protein that provided UDP in this experiment may have had a lower solubility to that used in the experiment where alpacas were observed to spend more time urinating (Lund *et al.* 2012; Chapter 3). However, a great difference in protein solubility seems unlikely since the diets had the same composition and were prepared using the same methods and machinery as the diets used by Lund *et al.* (2012; Chapter 3). Another possible explanation is that climatic conditions may impact on the nitrogen utilisation of the animals. The present experiment was conducted during summer and ambient temperatures reached over 35ºC on some days, whereas Lund *et al.* (2012; Chapter 3) took place during winter. Ambient temperature, particularly within the range of an animals' thermal stress, can influence nutritional parameters, including nitrogen balance and nutrient digestibility (Ames and Brink 1977). Further experimentation needs to be conducted to elucidate this result.

It was interesting however, that both species seemed to lose live weight when consuming the UDP diet. When fed UDP, the alpacas lost about three-fold and the sheep lost about two-fold the amount of weight lost when fed the RDP diet. Body fat

mobilisation has been increased in dairy cows with the addition of UDP in the diet (Orskov *et al.* 1987). Canola meal, the protein used in this experiment, has a high level of methionine, which is known to have a lipotrophic effect and therefore strip body fat off the animal (Wiese *et al.* 2003). In addition to this, the UDP canola meal protein was protected from degradation in the rumen and therefore more amino acids, such as methionine, should have been available for absorption in the small intestine. It may be worthwhile investigating this trend further to provide a greater insight into the energy and nitrogen metabolism of alpacas. The degradability of dietary protein may play an important role in formulating diets for alpacas, particularly considering the prevalence of obesity in the alpaca herd in developed countries such as Australia and the United States of America (Van Saun 2006b).

In this experiment, the amount of nitrogen retained as a proportion of the amount of nitrogen absorbed was not different between the alpacas and sheep, which implied that both species utilise nitrogen with a similar efficiency. Our results and those previously reported where alpacas have a superior ability to retain nitrogen and a greater digestive efficiency, are likely to be different due to the quality of the diet used in individual experiments. Alpacas have evolved in a harsh environment where the quality of available feed can be low and limited in nutrients. This selection pressure has resulted in alpacas having adaptations that allow them to maintain an efficient nitrogen balance when nutrients are limited, hence when they are fed diets with a low crude protein content, they are more efficient than sheep (Costa and Vaughan 1998). In our experiment, the diets for the sheep and alpacas were formulated according to the most recent protein and energy requirements for maintenance and therefore provided sufficient protein. Consequently, there was no need for the alpacas to recycle and retain nitrogen to satisfy their protein requirements. In fact, the crude protein content in our

diets was comparable to that in other studies that have concluded that the digestive efficiencies of alpacas and sheep are similar (San Martin and Bryant 1989). However, despite the alpacas and sheep having statistically similar nitrogen retentions, the alpacas in this experiment retained less of the nitrogen that was absorbed from their food by excreting the nitrogen in their urine. This demonstrates that the alpacas were deaminating amino acids but, having satisfied their requirement for glucose, were excreting waste nitrogen. This result implies that alpacas rely on protein to derive glucose rather than volatile fatty acids like sheep, and have a lower requirement than sheep.

The fibre production performance of both species should be taken into consideration when comparing nitrogen retention in alpacas and sheep. Alpacas and sheep are commercially grown for their fibre and have been selected for their fibre growing attributes (McGregor 2006). Sheep generally produce at least twice the weight of fibre that alpacas do, and indeed, the sheep used in this experiment originate from a flock selected for growing large amounts of fine wool (producing about 5 kg of greasy wool per year). Consequently, we estimate that the sheep use more protein for wool growth than the alpacas (0.68 g/d.kg$^{0.75}$ to 0.58 g/d.kg$^{0.75}$). The fact that sheep partition more nitrogen to wool growth may also explain why the alpacas tended to retain less nitrogen than the sheep.

Although the results from this experiment and those in Chapter 3 differ despite the fact that the feed treatments were the same, it appears that protein is an important component of the diet of alpacas in terms of providing glucose for energy for maintenance and amino acids for fibre growth. In the next chapter, I examine the addition of calcium propionate to alpaca diets as another feeding strategy that may

ensure that alpacas have an adequate supply of amino acids for both glucose production and fibre growth.

Chapter 6

Alpacas fed calcium propionate seem to moderate their energy intake

Submitted for publication in Journal of Animal Physiology and Animal Nutrition

Introduction

The metabolism of glucose in alpacas is not well understood, but it seems to be different to that of true ruminants (Van Saun 2006a). Alpacas maintain blood glucose concentrations at about two and a half times that of true ruminants (Van Saun 2006a), have a weak insulin response and they clear glucose slowly (Cebra *et al.* 2001; Cebra *et al.* 2004; Ueda *et al.* 2004). In true ruminants, glucose is derived primarily from the volatile fatty acid, propionate (McDonald *et al.* 2002). In alpacas, however, propionate production in the fermentative chambers is likely to be low since the native forages of South America, where alpacas evolved, can be of low digestibility and have minimal levels of non-structural carbohydrates such as sugar and starch (Van Saun 2006a).

To meet their glucose requirement, alpacas may need to use an alternative to propionate as their main glucogenic substrate. There is strong evidence to suggest that alpacas derive glucose predominantly from the amino acids absorbed from the small intestine. Therefore, if alpacas use mainly amino acids to meet their requirement for glucose, it is reasonable to expect that there would be less amino acids available for fibre growth. It may be possible to overcome this conflict by using feeding strategies to ensure that fibre production remains at an optimal level. Calcium propionate has been used as an energy supplement for ruminants, such as cattle to improve productivity by enhancing energy

efficiency (Waterman *et al.* 2001). If alpacas were to meet some of their glucose requirement from an alternate source, such as calcium propionate, this should reduce their dependence on amino acids for energy and lead to an improvement in fibre growth.

An experiment was designed to determine if alpacas can utilise calcium propionate as a source of energy and therefore spare amino acids from deamination, making them available for fibre growth. By manipulating both the degradability of the protein and the availability of calcium propionate in the ration it was expected that a strategy to optimise fibre production in alpacas could be formed. It was hypothesised that alpacas supplemented with calcium propionate will produce more fine fibre than un-supplemented animals.

Materials and methods

Calcium propionate was added to the rations fed to alpacas and fibre production was measured to detect changes in fibre attributes in response to calcium propionate. These methods were approved by the University of Western Australia Animal Ethics Committee (RA/3/100/707).

Animals

Castrated Huacaya alpacas (2-3 years old) were housed in individual outdoor pens, approximately 3 m x 10 m. The pens had sand bedding and were bare of vegetation. The alpacas were acclimatised to their environment and feeding routine for several weeks prior to the commencement of the ten week treatment period. Each alpaca had *ad libitum* access to water and a shelter. The alpacas were fed once daily at around 0800 h and feed refusals from the previous day were recorded. All animals were weighed to the nearest 0.5 kg and condition scored (scale of 1 to 5; 1 = emaciated and 5 = obese; Fysh

2003) at the same time each week and the amount of feed was adjusted, if necessary, to ensure they maintained body weight and condition.

Feed treatments

The alpacas were randomly allocated to four dietary treatment groups each with eight animals (n = 8) of similar mean live weight (46.5 kg ± 0.40 kg) and body condition score. The metabolic live weight of each alpaca was used to calculate the amount of each feedstuff fed. Throughout the study, the alpacas were fed a basal diet of milled barley straw at 3 g/kg$^{0.75}$, a roughage based pellet at 8 g/kg$^{0.75}$ (Macco 101 pellet, Macco Feeds Australia, Williams, Western Australia), a high fibre pellet at 20 g/kg$^{0.75}$ (Macco Feeds Australia, Williams, Western Australia), 25 g/head.day of a complete mineral mix and 25 g/head.day of dried sugar cane molasses (Palabind, Probiotec, Laverton North, Victoria, Australia). The animals in two of the treatment groups received a supplement of 4.5 g/kg$^{0.75}$ of 'flaky', cold-pressed canola meal which provided them with RDP. The other two groups received 4.5 g/kg$^{0.75}$ of finely milled, heat-treated canola meal to provide a greater proportion of UDP. Calcium propionate (CALPRONA – C/CA, Verdugt, Netherlands) was added to the diet of the animals in one of the UDP and one of the RDP treatment groups at 25 g/kg$^{0.75}$. The supplement was delivered in a powder form mixed with finely chopped straw. Originally, this mixture was to be pelleted but due to problems producing the pellets, was fed as a loose mix comprising of 20% calcium propionate and 80% straw. Each component of the diet was analysed for metabolisable energy (ME) content and crude protein, as well as a number of other nutritional characteristics, by a commercial feed analysis company (Independent Lab Services, Serpentine, Western Australia) and this information was used to formulate each alpaca's maintenance diet.

Blood collection and analysis

Blood samples were taken from all 32 alpacas via jugular venipuncture in vaccuette EDTA tubes prior to feeding on the same day each week. The concentration of blood glucose was measured using a blood glucose meter (Accu-chek Advantage, Roche Diagnostics, Basel, Switzerland). After centrifugation at 3000 rpm for 10 minutes, the plasma was removed and frozen at -20ºC for later analysis. Plasma was assayed for insulin by double-antibody RIA (Downing *et al.* 1995). The samples were assayed as duplicate 100 μL aliquots and the limit of detection of the insulin assay was 1.3 μU/mL. Intra-assay CV's were 3.7% at 8.8 μU/ml, 5.6% at 4.3 μU/ml and 7.3% at 2.38 μU/ml. The assay was validated for alpaca plasma by checking for parallelism using a serial dilution of pooled samples of alpaca plasma (Figure 6.1). Plasma samples from weeks one, four, seven and ten of the treatment period were analysed for plasma urea nitrogen using a Kinetic UV test with an Olympus test kit OSR6134 on an Olympus AU400 analyser (Olympus, Tokyo, Japan).

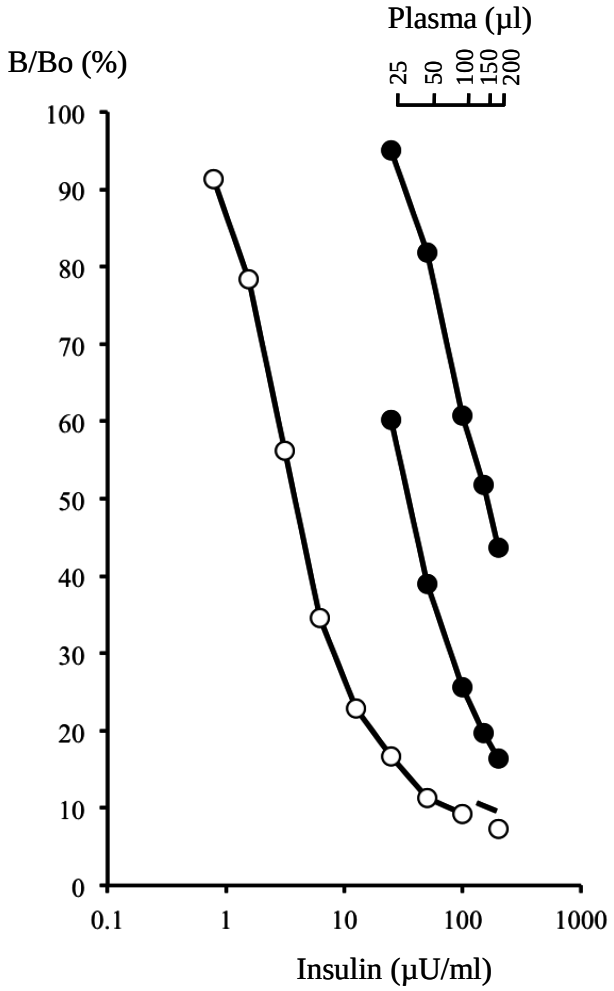

Figure 6.1. Standard curves for radioimmunoassay for insulin showing parallelism with serial dilution of alpaca plasma.

Fibre collection and analysis

Each alpaca had a mid-side patch (approximately 10 x 10 cm) shaved to skin level prior to the start of and, at the end of, the treatment period using a Laube clipper with a size 40 blade (Kim Laube and Co., Oxnard, California, USA). On both occasions, the fibre removed from the patch was dried and weighed and the dimensions of the mid-side patch were measured to calculate dry fibre growth per square centimetre of the area sampled during the treatment period. A representative sample of fibre from each mid-side patch was analysed for fibre diameter using an Optical Fibre Diameter Analyser (OFDA 2000; BSC Electronics, Perth, Australia).

Data analysis

The metabolisable energy (ME) intake for each alpaca was calculated using the ME values for each feedstuff in concert with the amount offered and the refusals for each alpaca. The ME intake for each treatment group was compared by ANOVA with pairwise comparisons using the Student-Newman-Keuls test (GenStat®, 11th edition, VSN International Ltd., 2008). Live weight and condition score were analysed by ANOVA using the initial values as covariates and pairwise comparisons were made using the Student-Newman-Keuls test. All raw data from the OFDA 2000 fibre analysis were analysed by ANOVA with covariate adjustment using the initial fibre samples. Multiple pairwise comparisons were made using a Students-Newman-Keuls test ($p<0.05$) (GenStat®, 11th edition, VSN International Ltd., 2008). The same analysis procedure was used to determine differences in the weight of fibre produced by the animals in each treatment group. An ANOVA with repeated measures and an ANOVA with pairwise comparisons using the Student-Newman-Keuls test was used to compare the plasma urea nitrogen concentration, insulin concentration and glucose concentration of the blood (GenStat®, 11th edition, VSN International Ltd., 2008).

Results

During the experiment, the alpacas fed calcium propionate refused some of their daily ration, and the food remaining was mostly calcium propionate and a small amount of highly lignified straw. Because the total ME offered was slightly higher in the calcium propionate supplemented groups, there was no difference in the total daily metabolisable energy intake between the four groups ($p = 0.278$; Table 6.1).

Table 6.1. Mean (±s.e.) metabolisable energy (ME/d) offered, ME intake, ME refused, ME offered as calcium propionate (CaP), ME intake from calcium propionate, ME refused as calcium propionate, total crude protein (CP) intake and change in live weight and condition score (1-5) over the entire experiment for alpacas fed diets with (+) or without (-) added calcium propionate (CaP) and containing either un-degradable dietary protein (UDP) or rumen degradable dietary protein (RDP)

	Diet			
	+ CaP UDP	+ CaP RDP	- CaP UDP	- CaP RDP
Total ME offered (MJ/d)	6.6 ± 0.3[a]	6.6 ± 0.2[a]	5.3 ±0.3[b]	5.3 ± 0.4[b]
ME refused (MJ/d)	1.4 ± 0.3[a]	1.7 ± 0.4[a]	0.1 ± 0.0[b]	0.1 ± 0.0[b]
ME intake (MJ/d)	5.2 ± 0.2	4.7 ± 0.3	5.2 ± 0.2	5.2 ± 0.1
ME offered as CaP (MJ/d)	3.9 ± 0.14[a]	3.8 ± 0.13[a]	0[b]	0[b]
ME refused as CaP (MJ/d)	1.2 ± 0.3[a]	1.6 ± 0.2[a]	0[b]	0[b]
ME intake from CaP (MJ/d)	2.6 ± 0.2[a]	2.2 ± 0.2[a]	0[b]	0[b]
Total CP intake (g/d)	66.8 ± 2.1[a]	64.2 ± 2.4[a]	75.4 ± 2.2[b]	75.4 ± 2.1[b]
Change in live weight (kg)	-1.5 ± 0.92[a]	-1.9 ± 0.41[a]	0.8 ± 0.52[b]	-0.9 ± 0.32[a]
Change in condition score	-0.2 ± 0.09	-0.2 ± 0.09	-0.1 ± 0.06	-0.1 ± 0.08

[ab] Values within a row with different superscripts are different ($p < 0.05$)

All of the animals, except those that received a diet of UDP without calcium propionate, had similar changes in live weight during the experiment. By the end of the experiment, the alpacas in the group fed the diet of UDP without calcium propionate were of higher live weight than those in the other three groups ($p = 0.005$). However, there was no difference in the change in condition score between the four groups ($p = 0.687$; Table 6.1).

The animals in the two groups fed calcium propionate produced significantly less fibre ($p < 0.001$) that was of smaller diameter ($p = 0.002$) than those that were not fed calcium propionate (Table 6.2).

Table 6.2. Mean (± s.e.) fibre diameter at the start and end of the experiment and weight of fibre produced of alpacas fed diets with (+) or without (-) added calcium propionate (CaP) and containing either un-degradable dietary protein (UDP) or rumen degradable dietary protein (RDP)

	Diet			
	+ CaP UDP	+ CaP RDP	- CaP UDP	- CaP RDP
Weight of fibre (mg/cm^2)	33.1 ± 2.35[a]	41.7 ± 4.67[a]	44.4 ± 1.91[b]	46.1 ± 2.86[b]
Fibre diameter (start) (μm)	21.4 ± 0.64	22.9 ± 0.54	20.6 ± 0.62	19.3 ± 0.74
Fibre diameter (end) (μm)	21.0 ± 0.69	22.3 ± 0.62	21.0 ± 0.75	19.9 ± 0.69
Change in fibre diameter (μm)	-0.4 ± 0.17[a]	-0.6 ± 0.11[a]	0.3 ± 0.24[b]	0.5 ± 0.22[b]

[ab] Values within a row with different superscripts are different ($p < 0.05$)

There was no effect of treatment ($p = 0.300$) or time ($p = 0.239$) on the concentration of urea in the plasma for the four weeks these measurements were made (Table 6.3). The concentration of glucose in blood was not affected by the treatments ($p = 0.300$), however, there were marked fluctuations in blood glucose levels throughout the experiment ($p < 0.001$; Figure 6.2). There was no difference in the concentration of insulin in the plasma between the treatment groups ($p = 0.407$) but, like glucose, insulin levels also fluctuated during the experiment ($p < 0.001$; Figure 6.3).

Table 6.3. Mean (±se) blood glucose, plasma insulin and plasma urea nitrogen concentrations of alpacas fed diets with (+) or without (-) added calcium propionate (CaP) and containing either un-degradable dietary protein (UDP) or rumen degradable dietary protein (RDP)

	Diet			
	+ CaP UDP	+ CaP RDP	- CaP UDP	- CaP RDP
Blood glucose concentration (ng/mL)	4.9 ± 0.10	4.9 ± 0.10	4.8 ± 0.09	4.8 ± 0.09
Insulin concentration (uU/mL)	2.2 ± 0.13	2.0 ± 0.10	2.2 ± 0.08	2.4 ± 0.07
Plasma urea nitrogen concentration (mmol/L)	4.7 ± 0.12	5.6 ± 0.16	5.2 ± 0.13	4.9 ± 0.03

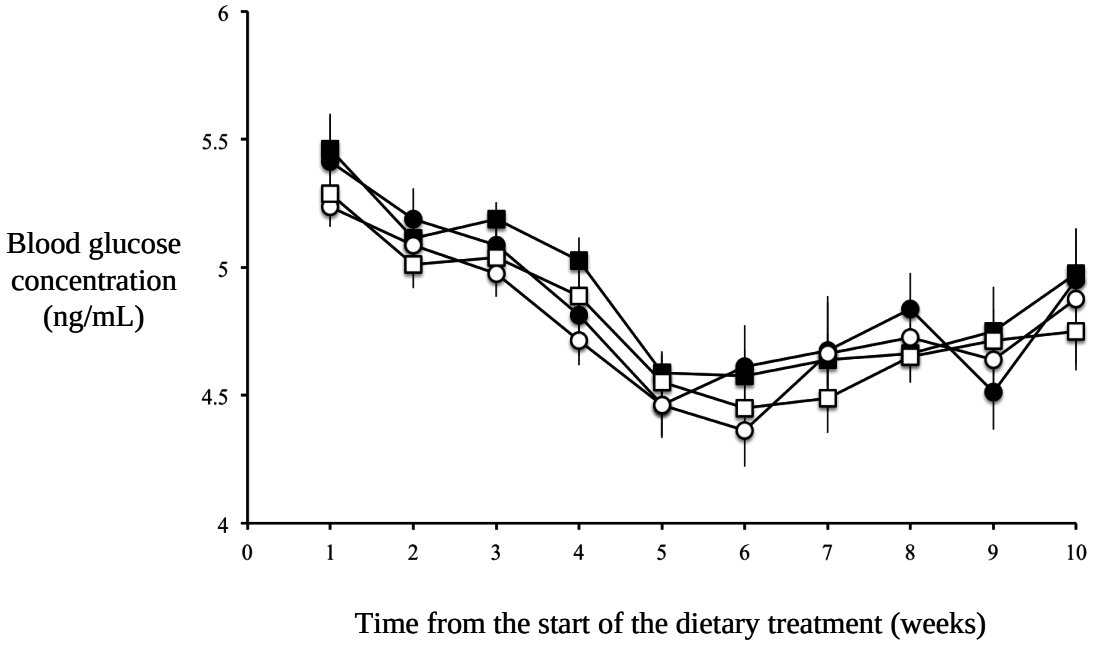

Figure 6.2. Concentration of glucose in blood (mean ±se) of alpacas fed a diet containing either un-degradable dietary protein (UDP - circle) or rumen degradable dietary protein (RDP - square) and without (open symbol) or with calcium propionate (closed symbol) during 10 week of feeding the experimental diets.

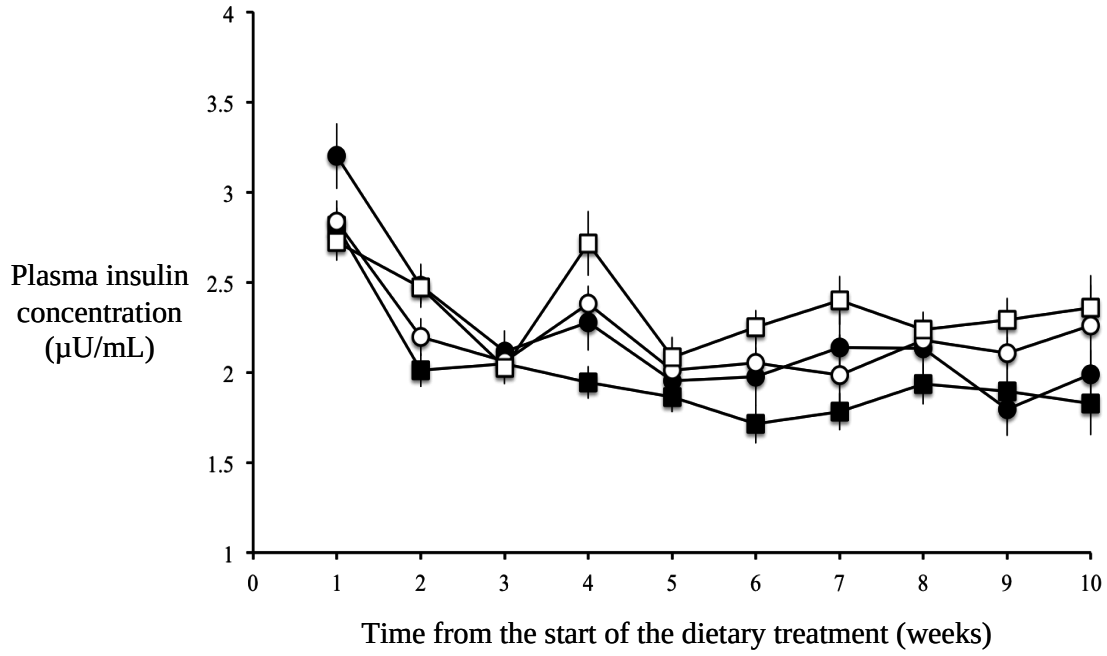

Figure 6.3. Plasma concentration of insulin (mean ±se) in alpacas fed a diet containing either un-degradable dietary protein (UDP - circle) or rumen degradable dietary protein (RDP - square) and without (open symbol) or with calcium propionate (closed symbol) during 10 week of feeding the experimental diets.

Discussion

The objective of this study was to determine whether alpacas fed calcium propionate would increase their fibre yield by sparing amino acids from being used for glucose production. The alpacas were expected to derive glucose from calcium propionate and therefore spare amino acids from gluconeogenesis and so make them available for fibre growth. This hypothesis was not supported, since although the diets supplemented with calcium propionate should have provided more energy, the metabolisable energy intake of all animals was similar. It seems that rather than sparing amino acids, the alpacas regulated their energy intake by refusing to consume additional energy as calcium propionate.

The alpacas fed diets containing calcium propionate seemed to moderate their energy intake. The amount of protein consumed from the diets containing calcium propionate was less than that from the diets without calcium propionate meaning that there should have been less amino acids available for absorption from the diets with calcium propionate. Fibre production is strongly influenced by the amount of amino acids available for absorption (Reis and Sahlu 1994). By regulating their energy intake, the alpacas in the calcium propionate treatments were probably denied the amino acids they required for fibre production. Consequently, they produced less fibre and the diameter of their fibre decreased during the experiment, while the diameter of the fibre produced by the alpacas that were not fed calcium propionate increased during the experiment.

Interestingly, the live weight of the alpacas fed the diet containing UDP without calcium propionate increased during the experiment. The most likely explanation for this result is that more amino acids from the UDP diet reached the small intestine and were absorbed and converted to glucose, rather than being fermented in the rumen and converted to urea in the liver. Consequently, these alpacas would have used less metabolisable energy to excrete nitrogen as urinary urea and instead, gained live weight. The metabolism and regulation of glucose in alpacas is an enigma (Van Saun 2006a) and the results from this experiment lend further support to this statement. The concentration of blood glucose of the animals in the four treatment groups gradually declined over the duration of the experiment. It is interesting, however, that the concentration of plasma insulin also dropped initially before becoming relatively stable towards the end of the treatment period. Alpacas are reported to have a low response to insulin which may account for the trend observed between blood glucose concentration and insulin concentration in this experiment (Cebra *et al*. 2004). In true ruminants, such as sheep, insulin and glucose are known to be related to feed intake and body condition

(Woods and Porte 1976; Sibbald and Rhind 1997). In the present experiment, feed intake was controlled at a maintenance level and the alpacas did not lose a significant amount of body condition. It appears that the response in the concentration of blood glucose and plasma insulin was not attributed to any factor pertaining to the treatments imposed in this experiment. This further highlights the perplexity of glucose metabolism in alpacas. It has been suggested that the weak response of alpacas to insulin may be an adaptation for survival at high altitudes and under conditions of variable feed availability (Araya *et al.* 2000).

Whether alpacas do moderate their energy intake and prefer to utilise protein as their source of glucose for maintenance is examined in the next chapter.

Chapter 7

Alpacas do not retain more nitrogen than sheep when fed a diet containing twice their maintenance requirement of protein

Introduction

Much of this thesis has highlighted the importance of protein in the diet of alpacas as an important nutrient to meet both the protein and energy requirements of alpacas. Nevertheless, energy intake itself is important in terms of nitrogen retention (Black and Griffiths 1975; Mould and Robbins 1981; Bach *et al.* 2005). Some studies have examined the effect of changing the intake of either protein or energy in alpaca diets, but these studies have not examined the impact on nitrogen metabolism when both the energy and protein components of the diet are manipulated concurrently. Because protein seems to serve as an important energy source in alpacas, as highlighted in the preceeding chapters of this thesis, information on the balancing of energy and protein intake is essential if we are to develop appropriate diets for alpaca production systems.

The optimal utilisation of energy and protein in alpacas requires that these nutrients be fed in the correct ratios. For example, when intake of dietary energy is inadequate, body protein may be catabolised to meet the animal's energy requirements. Therefore the deposition of protein in tissues is likely to be affected by the availability of non-protein energy-yielding substrates (Poppi and McLennan 1995). Also when animals consume a diet with excess protein, but deficient in energy, the recycled nitrogen may not be efficiently retained as there would be insufficient energy for the microbial population to utilise this recycled nitrogen in the rumen (Mould and Robbins 1981).

Alpacas, unlike sheep, rely on the protein portion of their diet to obtain glucose for energy. In chapter 6, the energy component of the diet was manipulated by providing diets with or without calcium propionate. The alpacas appeared to down-regulate their energy intake when they were fed extra energy in the form of calcium propionate. The animals relied on the amino acids from the protein in their diet to derive glucose, at the cost of fibre growth. Thus, some pertinent questions that arise are 1) do alpacas regulate their protein intake when the amount of protein offered exceeds their requirements and, 2) how much energy do alpacas require for the efficient retention of nitrogen?

In this experiment, we examined how intakes of different proportions of energy and protein influenced nitrogen metabolism in alpacas and sheep. It was hypothesised that irrespective of their energy intake, alpacas would progressively retain more nitrogen as their intake of dietary protein increased. Conversely, it was expected that sheep would retain less nitrogen than alpacas when their intake of dietary protein increased because they rely on gluconeogenic precursors such as propionate, rather than protein, to meet their energy requirement.

Materials and methods

While they were housed in metabolism pens, alpacas and sheep were fed diets containing various levels of energy and protein in a Latin square design. Nitrogen and energy balance was measured to determine whether alpacas metabolise nitrogen more efficiently than sheep and whether alpacas regulate their protein intake. These methods were approved by the University of Western Australia Animal Ethics Committee (RA/3/100/935).

Animals

Upon arrival at Shenton Park Research Station at the University of Western Australia, Huacaya alpaca wethers (n = 6) and Merino sheep wethers (n = 6) of similar body weight (52.2 ± 1.30 kg) and mature in age were acclimatised and housed in individual metabolism pens in an outdoor paddock. All animals underwent a training protocol and a phase of introduction to the metabolism pen so that they were familiar with confinement before the start of the experiment (Lund *et al.* 2012; Chapter 4). Each animal was provided water *ad libitum* and was fed in the morning between 0700 and 0800 hours. The animals' weight and body condition were monitored once each week prior to being fed by weighing the animals to the nearest 0.5 kg and condition scoring them using a scale where 1 = emaciated to 5 = obese (Fysh 2003).

Feed treatments

During the experiment each alpaca and sheep was fed three treatment diets containing milled barley straw, an oat husk pellet (Macco Feeds Australia, Williams, Western Australia), canola meal protein and 20 g/head.day of a complete mineral mix (Table 7.1). The canola meal was heat treated using the same procedures as outlined in Chapters 3 and 5 to reduce the solubility at rumen pH. The treatment diets provided different amounts of energy and protein as follows: Treatment 1 (ME/MP) provided a maintenance level of energy and protein, Treatment 2 (1.4ME/MP) provided 1.4 times both the animal's maintenance energy and protein requirement and Treatment 3 (1.4ME/HP) provided 1.4 times the energy required for maintenance by increasing the amount of canola meal protein that was fed (Table 7.2). The energy and protein requirements for maintenance were taken from the current recommendations for alpacas (Van Saun 2006a). The sheep were also fed on a metabolic body weight basis according to the maintenance requirements of alpacas and each of the alpacas and sheep were fed

all three treatment diets over the course of the experiment in a Latin square design. The animals were acclimatised to each feed treatment for 1 week in individual, sand yards before being housed in metabolism pens for 1 week while the balance studies were conducted. Representative samples of each feedstuff were analysed for gross energy content, using a Ballistic Bomb Calorimeter (Gallenkamp ®, Loughborough, United Kingdom), which was calibrated using benzoic acid standards, and nitrogen content was determined using a Vario Macro Elemental CHN analyser (Elementar Analysensysteme GmbH, Hanau, Germany), which was calibrated for nitrogen analysis using glutamate as the standard. While the animals were housed in the metabolism pens, the feed refusals were recorded daily so that total food intake could be calculated and sub-samples were taken for energy and nitrogen analysis. After a week of measurement, the animals were removed from the metabolism pens and acclimatised to another feed treatment for a week before being returned to the metabolism pens for the conduct of the balance studies. Water intake was also measured daily by measuring the volume of water consumed from a known quantity provided the previous day.

Table 7.1. The amount of each feedstuff fed to alpacas and sheep on each treatment diet

Feedstuff	Treatment 1 (ME/MP)	Treatment 2 (1.4ME/MP)	Treatment 3 (1.4ME/HP)
Straw	20.7 $g/kg^{0.75}$	29.0 $g/kg^{0.75}$	16 $g/kg^{0.75}$
Pellets	16.5 $g/kg^{0.75}$	23.1 $g/kg^{0.75}$	23.1 $g/kg^{0.75}$
Canola meal	4.3 $g/kg^{0.75}$	6.0 $g/kg^{0.75}$	11.7 $g/kg^{0.75}$

Table 7.2. The average (±s.e.) amount of energy and protein offered to alpacas and sheep fed the three treatment diets

	Treatment 1 (ME/MP)	Treatment 2 (1.4ME/MP)	Treatment 3 (1.4ME/HP)
Energy (MJ ME/d)	6.0 ± 0.1	8.3 ± 0.2	8.0 ± 0.2
Crude protein (g/d)	68.0 ± 1.3	95.5 ± 1.8	124.5 ± 2.3

Urine collection and analysis

Urine was collected daily during the week the animals were in the metabolism pens. To prevent the volatilisation of nitrogen as ammonia from the urine, 10 mL of concentrated hydrochloric acid was added to the collection trays of the metabolism pens. The total amount of urine voided by each animal was measured and a 10% sub-sample was bulked over each seven day treatment period. Sub-samples of the bulked urine were stored and frozen until analysed to determine the nitrogen and energy content. Nitrogen content was measured via the Kjeldahl method using a Nitrogen analyser (Kjeltec 8400, FOSS, Hillerød, Denmark). The energy content of the urine was determined by concentrating the urine by freeze drying and then combusting the dried sample in a Ballistic Bomb Calorimeter (Gallenkamp ®, Loughborough, United Kingdom).

Faecal collection and analysis

The faeces voided by the animals when housed in the metabolism pens were collected and the total amount eliminated daily was measured. A 10% sub-sample was bulked from each day over each 1 week collection period when the three feed treatments were fed and stored and frozen at -20ºC until analysed. At the end of the collection period, duplicate samples were taken from the bulked samples and dried in an oven at 60ºC for four days until there was no further change in weight. The dry faeces were ground in a sample grinder (Retsch, Haan, Germany) to pass through a 0.5 mm screen and then

stored in plastic vials until analysed for energy and nitrogen content. Faecal energy was measured by combusting the dried samples in a Ballistic Bomb Calorimeter (Gallenkamp ®, Loughborough, United Kingdom). Faecal nitrogen was measured using a Vario Macro Elemental CHN analyser (Elementar Analysensysteme GmbH, Hanau, Germany).

Blood sampling and analysis

Blood samples were taken via jugular venipuncture into vacuette EDTA tubes five hours after the animals had been fed. The concentration of glucose in the blood was immediately measured using a blood glucose meter (Accu-chek Advantage, Roche Diagnostics, Basel, Switzerland). Plasma was removed after centrifugation at 3000 rpm for 10 minutes and frozen at -20ºC until analysed. Plasma urea nitrogen (PUN) was measured using a Kinetic UV test with an Olympus test kit OSR6134 on an Olympus AU400 analyser (Olympus, Tokyo, Japan).

Statistical analysis

T-tests were conducted to determine if there was any change in live weight or body condition during the week when each animal received each dietary treatments. All other parameters that were measured in this experiment were statistically analysed using a Latin square analysis of variance. When the ANOVA indicated a significant effect, Tukey's test was used to compare the differences between species and between treatments (GenStat®, 11th edition, VSN International Ltd., Hemel Hempstead, United Kingdom, 2008).

Results

There was no difference in the change in live weight or body condition score of the alpacas (p = 0.960 and 0.152) and the sheep (p = 0.118 and 0.100) over the duration of the experiment even though some alpacas did refuse a small amount of coarse straw material (Table 7.3).

Table 7.3. Change (±s.e.) in live weight (kg) and body condition score of alpacas and sheep over the experiment

		Treatment 1 (ME/MP)	Treatment 2 (1.4ME/MP)	Treatment 3 (1.4ME/HP)
Live weight (kg)	Alpacas	0.4 ± 0.40	0.3 ± 0.25	0.3 ± 0.54
	Sheep	2.9 ± 1.07	-0.7 ± 1.01	-1.1 ± 1.92
Condition score	Alpacas	0.2 ± 0.11	-0.1 ± 0.08	0.2 ± 0.11
	Sheep	-0.1 ± 0.08	0.0 ± 0.00	0.2 ± 0.11

The alpacas and the sheep had a similar apparent water intake of approximately 2 kg/day (p = 0.348). There was no effect of treatment on water intake (p = 0.386). There was no difference in the amount of urine excreted by the alpacas and the sheep (p = 0.493). The alpacas excreted, on average, 637 ± 75.7 mL/day and the sheep excreted 687 ± 30.6 mL/day. Urine output did not differ across treatments (p = 0.273). The sheep digested the dry matter of their diet with an efficiency of 61%, compared to about 53% for the alpacas (p = 0.026; Table 7.4).

Table 7.4. Dry matter (DM) digestibility for sheep and alpacas fed three treatment diets; maintenance energy and protein (ME/MP), 1.4 x maintenance energy (1.4ME/MP) and 1.4 x maintenance energy using increased protein (1.4ME/HP). Values are means ± s.e.

	Alpacas			Sheep		
	ME/MP	1.4ME/MP	1.4E/HP	ME/MP	1.4ME/MP	1.4E/HP
DM intake (g/d.kg$^{0.75}$)	38.6 ± 0.42^{a}	51.8 ± 1.19^{c}	47.7 ± 0.51^{b}	40.6 ± 0.41^{a}	56.9 ± 0.57^{d}	50.0 ± 0.51^{bc}
DM in faeces (g/d.kg$^{0.75}$)	18.6 ± 0.55^{ab}	23.8 ± 1.04^{c}	21.3 ± 0.56^{bc}	15.9 ± 0.83^{a}	22.3 ± 1.65^{bc}	19.7 ± 1.15^{abc}
DM absorbed (g/d.kg$^{0.75}$)	20.0 ± 0.49^{a}	28.0 ± 0.54^{bc}	26.4 ± 0.40^{bc}	24.7 ± 0.90^{b}	34.5 ± 1.41^{d}	30.3 ± 1.18^{c}
DM digestibility (g/d.kg$^{0.75}$)	0.52 ± 0.01^{a}	0.54 ± 0.01^{ab}	0.55 ± 0.01^{ab}	0.61 ± 0.02^{b}	0.61 ± 0.03^{b}	0.61 ± 0.02^{b}

^{ab} Superscripts within rows represent differences ($p < 0.05$)

Nitrogen metabolism

In accordance with the design of the experiment, nitrogen intake increased ($p < 0.001$) as the amount of protein offered increased. There was a difference between species ($p = 0.021$) and diet ($p = 0.024$) in nitrogen digestibility. The nitrogen apparently absorbed increased ($p = 0.001$) as the nitrogen offered in the diets increased (Table 7.5). The sheep absorbed more nitrogen than the alpacas on all three diets ($p = 0.009$). There was an effect of diet ($p = 0.015$) and species ($p = 0.025$) on the amount of nitrogen eliminated in the faeces. The sheep eliminated less nitrogen in their faeces when fed Treatment 1 compared to either species when fed Treatments 2 and 3. When the alpacas were fed Treatment 1 they eliminated less nitrogen than when they were fed Treatments 2 and 3.

There was no effect of treatment ($p = 0.171$) or species ($p = 0.952$) on the amount of nitrogen excreted in the urine with respect to the amount of nitrogen absorbed (Table 7.5). However, the alpacas fed Treatment 1 excreted about 38% more of the absorbed nitrogen in the urine than when they were fed Treatment 2 and 28% more than when fed Treatment 3. The alpacas and sheep retained similar amounts of nitrogen as a percentage of the nitrogen absorbed ($p = 0.952$) on all three treatments ($p = 0.171$; Table 7.5).

Table 7.5. Nitrogen balance of sheep and alpacas fed three treatment diets; maintenance energy and protein (ME/MP), 1.4 x maintenance energy (1.4ME/MP) and 1.4 x maintenance energy using increased protein (1.4ME/HP). Values are means ± s.e.

	Alpacas			Sheep		
	ME/MP	1.4ME/MP	1.4ME/HP	ME/MP	1.4ME/MP	1.4ME/HP
N intake (g/d.kg$^{0.75}$)	0.65 ± 0.004^{a}	0.89 ± 0.01^{b}	1.15 ± 0.01^{c}	0.67 ± 0.01^{a}	0.95 ± 0.01^{b}	1.20 ± 0.01^{c}
N in faeces (g/d.kg$^{0.75}$)	0.30 ± 0.01^{ab}	0.40 ± 0.02^{c}	0.40 ± 0.01^{c}	0.25 ± 0.01^{a}	0.36 ± 0.02^{bc}	0.34 ± 0.02^{bc}
N absorbed (g/d.kg$^{0.75}$)	0.34 ± 0.01^{a}	0.50 ± 0.01^{b}	0.75 ± 0.01^{c}	0.42 ± 0.02^{d}	0.58 ± 0.02^{e}	0.86 ± 0.02^{f}
N digestibility (g/d.kg$^{0.75}$)	0.53 ± 0.02^{a}	0.56 ± 0.02^{ab}	0.65 ± 0.01^{cd}	0.62 ± 0.02^{bc}	0.62 ± 0.02^{bc}	0.72 ± 0.02^{d}
N in urine (g/d.kg$^{0.75}$)	0.17 ± 0.01	0.16 ± 0.01	0.27 ± 0.03	0.18 ± 0.01	0.18 ± 0.01	0.37 ± 0.02
N retained (g/d.kg$^{0.75}$)	0.17 ± 0.01	0.34 ± 0.01	0.48 ± 0.03	0.24 ± 0.02	0.41 ± 0.03	0.49 ± 0.03
N in urine: N absorbed	0.50 ± 0.03^{a}	0.31 ± 0.01^{b}	0.36 ± 0.04^{b}	0.43 ± 0.03^{ab}	0.31 ± 0.03^{b}	0.43 ± 0.02^{ab}
N retained: N absorbed	0.50 ± 0.03	0.69 ± 0.01	0.64 ± 0.04	0.57 ± 0.03	0.69 ± 0.03	0.57 ± 0.02

^{ab} Superscripts within rows represent differences ($p < 0.05$).

Energy metabolism

The energy intake (p = 0.010) and the energy absorbed (p = 0.015) differed between the three feed treatments. The alpacas absorbed less energy from their diets than the sheep on all three treatments (p = 0.020; Table 7.6). When the animals were fed Treatment 2, they eliminated more energy in their faeces than when they were fed Treatment 1 (p = 0.005; Table 7.6). The alpacas had a lower digestibility of energy than the sheep (p = 0.010). There was no difference in the amount of energy excreted in the urine relative to the energy absorbed between the three treatments (p = 0.397) for either the sheep or the alpacas (p = 0.294; Table 7.6). The alpacas and the sheep retained similar amounts of energy on all three treatments (p = 0.397; Table 7.6). When they were fed Treatments 1 and 2, the sheep retained more energy than when they were fed the Treatment 3 diet.

Table 7.6. Energy (E) balance of sheep and alpacas fed three treatment diets; maintenance energy and protein (ME/MP), 1.4 x maintenance energy (1.4ME/MP) and 1.4 x maintenance energy using increased protein (1.4ME/HP). Values are means ± s.e.

	Alpacas			Sheep		
	ME/MP	1.4ME/MP	1.4E/HP	ME/MP	1.4ME/MP	1.4E/HP
E intake (MJ/d.kg$^{0.75}$)	0.93 ± 0.01^{a}	1.25 ± 0.03^{b}	1.17 ± 0.01^{c}	0.98 ± 0.01^{a}	1.36 ± 0.01^{b}	1.22 ± 0.01^{c}
E in faeces (MJ/d.kg$^{0.75}$)	0.39 ± 0.01^{a}	0.53 ± 0.02^{b}	0.47 ± 0.01^{b}	0.35 ± 0.02^{a}	0.50 ± 0.04^{b}	0.44 ± 0.03^{b}
E absorbed (MJ/d.kg$^{0.75}$)	0.54 ± 0.01^{a}	0.72 ± 0.02^{b}	0.70 ± 0.01^{b}	0.63 ± 0.02^{a}	0.86 ± 0.03^{c}	0.79 ± 0.03^{bc}
E digestibility (MJ/d.kg$^{0.75}$)	0.58 ± 0.01^{a}	0.58 ± 0.01^{a}	0.60 ± 0.01^{a}	0.64 ± 0.02^{b}	0.63 ± 0.03^{b}	0.64 ± 0.02^{b}
E in urine (MJ/d.kg$^{0.75}$)	0.05 ± 0.01	0.03 ± 0.01	0.06 ± 0.01	0.02 ± 0.01	0.02 ± 0.00	0.07 ± 0.01
E retained (MJ/d.kg$^{0.75}$)	0.48 ± 0.01	0.69 ± 0.02	0.64 ± 0.01	0.61 ± 0.02	0.83 ± 0.03	0.72 ± 0.03
E in urine: E absorbed	0.10 ± 0.01	0.05 ± 0.01	0.08 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.08 ± 0.01
E retained: E absorbed	0.90 ± 0.01^{ad}	0.95 ± 0.01^{bcd}	0.92 ± 0.01^{acd}	0.98 ± 0.01^{b}	0.97 ± 0.01^{b}	0.92 ± 0.01^{d}

^{ab} Superscripts within rows represent differences ($p < 0.05$)

Metabolites in blood and plasma

The concentration of glucose in the blood was higher for the alpacas than the sheep on all three treatments (p = 0.001; Table 7.7). The concentration of urea nitrogen in the plasma did not differ between sheep and alpacas but was higher in both species on Treatment 3 than on Treatment 1 and 2 (p = 0.010; Table 7.7).

Table 7.7. Mean (±s.e.) blood glucose concentration (mmol/L) and plasma urea nitrogen concentration (mmol/L) for alpacas and sheep fed three treatment diets

		Treatment 1 (ME/MP)	Treatment 2 (1.4ME/MP)	Treatment 3 (1.4ME/HP)
Blood glucose (mmol/L)	Alpacas	5.8 ± 0.13[a]	5.8 ± 0.13[a]	5.7 ± 0.07[a]
	Sheep	3.4 ± 0.16[b]	3.5 ± 0.09[b]	3.5 ± 0.10[b]
Plasma urea nitrogen (mmol/L)	Alpacas	4.4 ± 0.32[a]	4.1 ± 0.20[a]	6.3 ± 0.23[b]
	Sheep	3.8 ± 0.25[a]	3.3 ± 0.11[a]	5.2 ± 0.44[b]

[ab] Values within rows with different superscripts are different (p < 0.05)

Discussion

The alpacas responded to the three dietary treatments in a similar manner to sheep by retaining a similar proportion of the dietary nitrogen that they absorbed. It was expected the sheep would retain less nitrogen than alpacas when fed increasing amounts of protein because alpacas are reported to be good at recycling nitrogen (Van Saun 2006a) and they deaminate amino acids to meet their requirement for glucose (Chapter 6). It was also hypothesised that alpacas would retain more nitrogen as nitrogen intake increased. The results from this experiment do not support either hypotheses, however, there was a trend for the alpacas to retain more of the absorbed nitrogen than the sheep when fed a diet that provided almost twice their maintenance requirement of protein. The results provide further evidence that the maintenance requirement of alpacas is

lower than that of sheep as the sheep absorbed more energy and nitrogen than the alpacas.

Contrary to our expectations, the alpacas did not retain more nitrogen than the sheep for any of the three treatment diets. This result is similar to that reported in Chapter 5 and also by Pinares-Patino *et al.* (2003) who found that there was no difference between alpacas and sheep in their partitioning of nitrogen between the urine and faeces when the animals were fed lucerne hay or grazed a pasture of perennial rye grass and white clover. As reported in the previous experiments in this thesis (Chapter 5) and those of Pinares-Patino *et al.* (2003), the nitrogen content of the diet probably influences whether or not there are differences in nitrogen retention. Had the diets been of low nitrogen content, we may have found that the alpacas are more efficient at conserving nitrogen than sheep.

It is noteworthy that when the alpacas were fed the diet with the highest level of protein (Treatment 3), they seemed to retain slightly more of the absorbed nitrogen than the sheep largely by excreting less in their urine. It appears that when alpacas are fed a diet with a high level of protein and they have sufficient amino acids to meet their requirement for glucose, they probably deposit muscle protein which they can subsequently catabolise to supply glucose when the protein in the diet is low. This, to some extent, supports the view that alpacas may deposit protein rather than fat as a survival mechanism in a harsh climate. Protein deposition as a survival mechanism has been studied in reindeer and caribou and allows these animals to tolerate diets that are low in nitrogen without impairing normal functions and reproduction (Barboza and Parker 2006). The apparent importance of protein for alpacas also lends some support to the speculative statement made in light of the results in Chapter 6 as to whether alpacas

regulate their protein intake. In this case, alpacas do not appear to regulate their protein intake in the same way that they appear to regulate their energy intake.

One feature of nitrogen metabolism common to both alpacas and sheep was that the concentration of urea nitrogen in the plasma tended to be higher when they were fed the high protein diet (Treatment 3). According to McIntyre (1970), plasma urea nitrogen is related to the rumen ammonia concentration and tends to remain constant unless the diet has high levels of soluble protein, in which case excretion of urea in urine increases. In the current experiment, despite the higher PUN values when the animals were fed Treatment 3, the water intake and urine volume did not differ between the three treatments for either the sheep or the alpacas. The higher PUN result appeared anomalous because with the nitrogen available to the animals being higher for Treatment 3, the animals should excrete more nitrogen as urinary urea as they would not need to recycle nitrogen in order to meet their requirements. In sheep fed a dry roughage of high nitrogen content the electrolyte content of the feed can be important to ensure satisfactory renal excretion of urea to maintain the concentration of urea nitrogen in the plasma near the maximum value (Godwin and Williams 1984). It is possible that the electrolyte content of the feed used in our experiment was not high enough to initiate diuresis and therefore the concentration of urea nitrogen in the plasma was maintained.

The results from this experiment also provide evidence to support the view that alpacas have a lower energy and protein requirement than sheep. Firstly, the alpacas generally had a lower dry matter intake than the sheep. Dry matter intake is related to the time that feed particles are retained in the reticulo-rumen, termed the retention time. If the feed is of good quality, in order to satisfy the nutrient requirements of the animal the rumen does not fill to the same extent as when the quality of feed is poor. Therefore, the

energy demands of the animal should ultimately control dry matter intake (Thornton and Minson 1972). In the current experiment the sheep were fed according to the most current recommendations for the maintenance requirement of alpacas (Van Saun 2006a) and the sheep tended to utilise dietary nitrogen and energy more efficiently and had a higher digestibility than the alpacas. On all three treatment diets, the sheep absorbed more nitrogen and energy than the alpacas, probably in order to meet their higher requirements.

Chapter 8

General Discussion

Animals evolve, adapting to their environment and facilitating survival in the face of natural challenges. For thousands of years, alpacas have been able to survive the harsh environment of the altiplano region of the Andes mountains and they have adaptations that enable them to cope with variable feed availability and fluctuating nutrient supplies (San Martin and Bryant 1989; Van Saun 2006a). Prior to the work reported in this thesis, alpacas were reported to have a superior ability to digest feed of poor quality compared to true ruminants (San Martin and Bryant 1989). However, the mechanisms employed for the metabolism of energy and protein were not clear. The aim of the work in this thesis was to help us understand the processes by which alpacas obtain and use energy and protein for metabolic processes, compared to true ruminants, such as sheep. The alpacas were expected to retain more nitrogen from their food than sheep through the adaptations they have evolved, however, both species had similar nitrogen and energy balances. The need to feed the animals to meet their requirements for maintenance resulted in the animals under study not having a need to employ nitrogen and energy conservation mechanisms that may normally be associated with the handling of a limited supply of nutrients. This thesis builds a picture of how alpacas utilise energy and protein from their food and also how they obtain glucose for energy, predominantly from the protein component of their diet.

In all of the experiments reported, the animals were fed at a maintenance level according to the most current recommendations in the literature (Van Saun 2006a). In

Chapter 3, fat deposition was not apparent, thus live weight and body condition were maintained by progressively decreasing the amount of metabolisable energy offered per day to a level around 20% less than that currently recommended by Van Saun (2006a). In later experiments, the difference between the recommended and the actual maintenance metabolisable energy intake was not as large. The diet in the later experiment contained calcium propionate and the alpacas may have regulated their intake. The experiment was also conducted during summer, not winter as was the experiment in Chapter 3, and this variable may have some influence on the maintenance requirement of alpacas. Nevertheless, it was necessary to feed the animals at around maintenance so that the nitrogen and energy balance of the animals could be compared to sheep without being influenced by growth or fat deposition.

In the experiments reported in Chapters 5, 6 and 7, it was apparent that the alpacas used protein to supply their needs for glucose rather than volatile fatty acids (Chapters 5, 6 and 7). In other ruminants, supplementation with a gluconeogenic precursor such as calcium propionate has been shown to improve energy efficiency and production (Waterman *et al.* 2001), whereas the alpacas appeared to down-regulate energy intake when the diet was supplemented with calcium propionate (Chapter 6). It is possible that alpacas regulated their energy intake on the basis of the intake of gluconeogenic precursors. They therefore have a strong reliance upon protein to supply energy for both maintenance of blood glucose as well as for fibre production. The mechanism may be part of an adaptation for survival in an environment that has variability in nutrient supply and the available forages are low in non-structural carbohydrates that would normally be fermented to propionate.

The adaptations of alpacas to their native environment were a motivating factor for the introduction of alpacas into Australia. It was reasoned that alpacas should suit the quite arid Australian conditions that would favour the rise of an alpaca fibre industry (Fysh 2003). However, the feeding systems currently used for alpaca production in Australia have implications for obesity associated with the regulation of metabolic pathways (or preference between metabolic pathways) for glucose production. Obesity is considered a prevalent nutritional disease in alpacas living in developed countries like Australia and the United States of America (Van Saun 2006b). Alpacas may store fat and muscle protein as an adaptation to periods of limited nutrient availability like other species, such as camels and Brahman cattle, to allow them to survive the harsh environment in which they live (Schmidt-Nielsen 1964). The hump of the camel consists mostly of fat that can be oxidised to yield energy for metabolism, as well as water. Similarly, Brahman cattle store fat in the hump on their shoulder (Schmidt-Nielsen 1964). Alpacas may reduce their energy expenditure by utilising nitrogen efficiently and storing energy as fat or muscle tissue, to be used in periods when the availability of quality forage is limited. In a production system however, feeding alpacas beyond their nutritional requirements, particularly with high energy-yielding supplements, is probably the foremost cause of obesity. The results in this thesis suggest that the type, not just the quantity, of nutrients offered to alpacas may be significant in promoting obesity and inappropriate supplements that are high in protein may be enhancing this problem.

The fact that the quality of the food on offer seems to be important for alpacas, more so than the quantity available, could play a major role in determining appropriate feeds and forages for alpacas farmed in Australian production systems. In Chapter 5, there was a trend for both alpacas and sheep to lose live weight when they were fed UDP, possibly because the methionine from the canola meal supplement was protected from

degradation in the rumen and therefore able to impart a lipotrophic effect on the fat stores in the body. Also, in Chapter 6, the alpacas exhibited more non-opportunistic behaviour and refused to consume the extra energy that was offered in the diets that contained calcium propionate. Whereas they have previously been regarded as fairly opportunistic feeders that are able to make use of a wide variety of forages (San Martin and Bryant 1989), in this experiment they did not avail themselves of the extra energy supplied as calcium propionate. It is possible that the non-opportunistic behaviour of alpacas is a mechanism for maintaining their advantage in digesting the array of different types of food available to them year round. Goats from areas characterised by forages that are high in tannins have a higher digestive efficiency than other ruminants and show similar non-opportunistic behaviour when vegetation of high protein content and digestibility is available (Silanikove 1997). In these regions where the high quality vegetation is short lived, it is thought that the short-term benefits of switching to a high quality diet could be lost because such a switch involves selecting for specific micro-organisms and thus altering the rumen ecology. Regaining the microbial balance of the rumen when the quality forage is no longer available becomes an issue when the goats again have to consume forages containing high levels of tannins (Silanikove 1997).

In Chapters 5 and 7, it was expected that alpacas would retain more nitrogen than sheep because alpacas are reported to recycle and retain nitrogen, a characteristic that helps them survive in their native environment (Dulphy *et al.* 1997; Robinson *et al.* 2005). However, the alpacas retained similar amounts of nitrogen as the sheep. It is possible that the alpaca's enhanced digestive ability is only apparent when they are challenged for nutrients, as is typical of their native environment. Although feeding all animals for maintenance only was a strength of the work in this thesis, this did not afford the opportunity to examine the true capacity of alpacas to recycle and retain nitrogen,

because the level of nitrogen that we fed the animals was adequate. If we had fed the alpacas a diet with crude protein at a level below that used in this thesis, and more closely aligned with the feed in the environment in which they evolved, we may have revealed an efficiency for nitrogen retention superior to that of sheep.

It is also important to note that in comparing alpacas to sheep, we were comparing one species that has only been farmed in Australia since 1989 with another that has undergone selection for fibre growth for more than two hundred years. The genetic base of the Merino sheep flock in Australia is therefore much narrower than the genetic base of alpacas. Nevertheless, the sheep that were used in the experiments reported in this thesis, were from the same flock with a tight genetic relationship. On the other hand, the alpacas were loaned to us by two studs that regularly import new genetics from South America. The animals we used were wethers, and so probably lacked the genetics for the desirable attributes of uniform, fine fibre production. Thus the genetic variability amongst the alpacas used in the studies for this thesis was much broader than that of the sheep that they were compared to. For future research with alpacas, it would be beneficial to have animals of close genetic relationship and subjected to more selection so they are aligned with the population used for breeding. It could be considered a limitation of the work in this thesis that we did not know to what extent the animals' genes were influencing the results and how selection for fibre attributes influenced the attributes examined in this thesis.

Considering the results from this thesis and the alpaca's ability to survive in a harsh environment, it would be interesting to gain a more detailed understanding of their nitrogen and energy metabolism when they are faced with extreme environmental conditions and how they cope with distinct changes in feed availability and quality.

Future experiments could investigate the nitrogen and energy balance of alpacas fed below maintenance and their response to different levels of protein and energy in their diet. It would also be beneficial to use cannulated alpacas so that *in vitro* and *in situ* studies could be conducted. Such information may allow us to manipulate factors such as the efficiency of microbial protein synthesis, and ultimately provide a better understanding of other interrelated nutritional factors, such as mineral absorption and metabolism, in order to help optimise alpaca production in Australia.

From the results presented in this thesis it can be concluded that alpacas obtain glucose for energy predominantly from the protein component of their diet as part of an adaptation to the harsh conditions of their native environment. Therefore producers need to consider the quality, as well as the quantity, of the protein that they offer to their animals. With the knowledge that the amount and type of protein is of prime importance for optimum nutrition of alpacas, producers should use this knowledge to feed diets that better match the animal's requirements with its physiological state. Ensuring that alpacas receive a diet suited to their physiological state will help prevent nutritional diseases such as obesity that can impact on productive processes, especially reproductive performance.

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Appendices

The following appendices contain work from this thesis in its published form.

Appendix 1.

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Undegradable dietary protein in alpaca diets affects fibre diameter and time spent urinating

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Abstract. There is evidence that alpacas derive most of their glucose for energy from the deamination of amino acids. Consequently, they may have an insufficient supply of amino acids to meet their requirements for fibre growth. To optimise fibre production, it may be necessary to supply alpacas with supplemental protein to meet their requirement for extra amino acids. In this study, we examined if the proportion of rumen-degradable dietary protein (RDP) to undegradable dietary protein (UDP) from canola meal influenced the fibre growth of alpacas. We hypothesised that alpacas fed at maintenance a diet containing canola meal protein high in UDP would produce more fibre and spend less time urinating than peers fed a similar amount of canola meal protein with a low proportion of UDP. Four groups of eight alpacas were fed diets with the following ratios of UDP : RDP: 0 : 100, 30 : 70, 60 : 40 or 100 : 0 from canola meal protein. The fibre growth of the animals was measured over 2 months and the behaviour of the animals in the two extreme groups (0 and 100% UDP) was measured over 5 days. The alpacas fed the 0% UDP diet produced fibre of finer diameter than the alpacas fed diets containing higher levels of UDP ($P = 0.039$) and the 0% UDP group also spent more time urinating ($P = 0.027$). This result suggests that alpacas may have a limited ability to recycle nitrogen to the fermentative chambers of their stomach when fed a diet low in UDP. Consequently, microbial protein synthesis in the fermentative chambers may have limited the supply of amino acids available to the alpacas.

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Introduction

Alpacas were introduced to Australia to establish a new fibre industry with a focus to produce large quantities of quality fibre (Fysh 2003; McGregor 2006). In sheep, most of the glucose requirement is derived from propionate and fibre production is markedly influenced by the supply of dietary protein (Black and Reis 1979; McDonald *et al*. 2002). There is evidence that alpacas obtain most of their glucose from the deamination of dietary amino acids rather than from propionate (Van Saun 2006). Consequently, when alpacas are fed to meet their energy requirements for maintenance they may utilise most of the amino acids absorbed from the small intestine to meet their needs for glucose, especially since they maintain a blood glucose level higher than sheep (Kaneko *et al*. 2008). Therefore, under these conditions, alpacas may have an inadequate supply of amino acids to meet their requirements for fibre growth and it may be necessary to supply them with supplemental protein to optimise fibre production.

In ruminants, the extent to which ingested protein is degraded depends upon its solubility and the time it is retained in the rumen. The protein that enters the rumen is either degradable (rumen degradable protein, RDP) or undegradable (undegradable dietary protein, UDP) (Bach *et al*. 2005). In the rumen, RDP can be degraded to varying degrees from peptides through to ammonia and these products can all be utilised for the synthesis of microbial protein (Bach *et al*. 2005). Ammonia that is not captured for microbial protein synthesis passes from the rumen and is converted to urea by the liver. This urea can be recycled back to the rumen, either via saliva or across the rumen wall, or excreted in urine (McIntyre 1970; McDonald *et al*. 2002). Alpacas are thought to be particularly efficient at recycling nitrogen (N), which would be advantageous in environments where the feed that is available is of low N content (Genin *et al*. 1994; Genin and Tichit 1997). If there is adequate fermentable carbohydrate in the diet, the N recycled by alpacas, in the form of urea, may be the main source of N for microbial protein synthesis occurring in the first and second chambers of the alpaca stomach (fermentative chambers). Excess N, for example from RDP when there is inadequate fermentable carbohydrate for microbial protein synthesis, will

be liberated as ammonia, absorbed from the fermentative chambers and converted to urea in the liver. Urea is known to be a diuretic agent (Owen *et al.* 1943) and when the level of plasma urea is high diuresis will occur. When alpacas are fed protein high in RDP this is likely to lead to diuresis and the frequency of urination may be considerably higher than usual.

Undegradable dietary protein that passes from the rumen is digested in the abomasum and small intestine to amino acids, that are then absorbed and used for synthetic processes, including fibre growth, or can be converted to glucose. Undegradable dietary protein can be produced from protein meals by heating the meal in the presence of reducing sugars to promote a mild Maillard reaction, without overheating to render the protein insoluble in the lower digestive tract. Masters *et al.* (1999) demonstrated that the wool growth of sheep supplemented with UDP as expeller canola meal (~50% UDP) was 11% higher than that of sheep fed a similar level of protein as lupins (~25% UDP). Based on these results, canola meal protein was considered an appropriate supplement to investigate the responses in fibre growth with alpacas.

In this study, we fed alpacas a maintenance diet and tested whether the proportion of RDP to UDP from canola meal in the diet influenced fibre growth. We hypothesised that alpacas fed at maintenance a diet containing canola meal high in UDP would produce more fibre and spend less time urinating than peers fed a similar amount of canola meal with a low proportion as UDP.

Materials and methods

Alpacas were fed diets of similar metabolisable energy (ME) content at a level calculated to maintain bodyweight (Van Saun 2006) with the following ratios of UDP : RDP; 0 : 100 (0% UDP), 30 : 70 (30% UDP), 60 : 40 (60% UDP) or 100 : 0 (100% UDP) from canola meal protein. The behaviour of the alpacas in the 100 and 0% UDP protein groups was monitored using a video recorder. The fibre characteristics of the alpacas were analysed to determine whether fibre production was affected by the different proportions of UDP in the diet.

Animals

Castrated male Huacaya alpacas (n = 32) aged 24–35 months were transported to Shenton Park Research Station at the University of Western Australia and housed randomly in bare, outdoor individual pens, ~3 by 10 m. The alpacas were allowed to become acclimatised to their new environment, feeding routine and handlers for ~2 months before the commencement of a 3-month feeding period during June–August (winter). Each alpaca had *ad libitum* access to fresh water, a feed shelter and a shaded area. They were fed once daily at ~0800 hours and their refusals from the previous day were recorded. All animals were weighed to the nearest 0.5 kg and their body condition was scored at the same time each week. The amount of feed offered to each animal was adjusted over the 3-month period as necessary to ensure that all animals were fed to maintain bodyweight and condition.

Feed treatments and analyses

The 32 alpacas were randomly allocated to four dietary treatments each with eight animals of similar mean liveweight (48.0 ± 0.22 kg, range of 37–62 kg) and body condition score (2.3 ± 0.05 units, range of 2.0–3.5 units, scale 1 = emaciated to 5 = obese; Fysh 2003). The weight of each feedstuff fed to the individual alpacas was calculated based on the individual's metabolic weight. The alpacas received a basal diet of milled barley straw at 22 g/kg$^{0.75}$, a roughage-based pellet at 9.7 g/kg$^{0.75}$ (Macco 101 pellet, Macco Feeds Australia, Williams, Western Australia), 25 g/head.day of a complete mineral mix and 25 g/head.day of dried sugarcane molasses (Palabind, Laverton North, Vic., Australia). A supplement of 4.6 g/kg$^{0.75}$ of canola meal was added to the basal diet as either untreated, flaky cold-pressed canola meal (0% UDP), or the same canola meal finely milled and mixed with a water solution to provide 2.5% dextrose and 0.5% sodium hydroxide (by weight) before being heated to ~85°C in a paddle mixer fitted with a thermostatically controlled heating belt that renders it undigestible at rumen pH (100% UDP). Two treatment groups were fed mixes of the treated and untreated canola meal to give two treatments of 30 and 60% UDP. The untreated and treated canola meal were analysed for acid detergent insoluble N to determine the extent of non-enzymatic browning due to overheating and buffer soluble N to determine the overall solubility of the canola meal protein (Licitra *et al.* 1996). The canola meal in all treatments provided ~50% of the total protein in each diet.

Sampling and analyses of fibre

Each animal had a mid-side patch ~10 by 10 cm clipped to skin level before the start of the dietary treatments using a Laube clipper with a size 40 blade (Kim Laube and Co., Oxnard, CA, USA). At the end of the 3-month feeding period the same mid-side area was clipped and the fibre was removed. The fibre that was removed at the start and the end of the treatment period was dried and weighed. At both time points, the dimensions of the mid-side patch were measured to accurately determine the area from which the fibre was removed in order to calculate dry fibre growth per square centimetre of the area sampled. The diameter of a representative sample of the fibre clipped from the mid-side of each animal was measured by an accredited commercial company (MicronMan, Bibra Lake, Western Australia) using an Optical Fibre Diameter Analyser 2000 (BSC Electronics, Perth, Australia; number of counts ranged from 800 to 1250 per sample).

Blood collection and analyses

Blood samples were taken via jugular venipuncture into vacuette EDTA tubes before feeding on 1 day each week. After centrifugation at 3000g at 4°C for 10 min, the plasma was removed and frozen at −20°C for subsequent analysis. Plasma urea N was measured in plasma samples taken the week before the start of the experiment and in Weeks 2, 8 and 9 of the feeding period using a Kinetic UV test with an Olympus test kit OSR6134 on an Olympus AU400 analyser (Olympus Diagnostics, Tokyo, Japan). These four time points coincided with the weeks when it was necessary to adjust food

intake to ensure the animals were always fed to maintain liveweight and body condition.

Behavioural observations

The behaviour of each of the eight alpacas fed the 0 and 100% UDP treatments was recorded using four closed-circuit television cameras and digital surveillance system software (Kguard DVR7134 version 1.1, Kguard Security, Taipei, Taiwan). Each animal was recorded for 8 h over 5 days commencing at the start of feeding each morning. The video footage was analysed using Interact software (Interact, version 8, Mangold International GmbH, Arnstorf, Germany) to determine the total time each animal spent performing each activity. Behaviour was classified into eight categories (eating, lying, standing, walking, grooming, defecating, drinking, and urinating).

Statistical analyses

The mean fibre diameter and the dry fibre growth of the eight animals in each treatment group were compared using an ANOVA (GENSTAT, 11th edition, VSN International Ltd, Hemel Hempstead, UK). The fibre diameter value at the beginning of the treatment was used as a covariate. The mean change in liveweight, body condition and plasma urea N concentration in the blood of the eight animals in each treatment were compared using an ANOVA (GENSTAT 2008). The liveweight and body condition score from the first week of the treatment period were used as a covariate. Pairwise comparisons between the four treatment groups were also conducted with Dunnett's test using the statistical program R (R, version 2.14.0, The R Foundation for Statistical Computing: http://www.r-project.org/, verified 5 June 2012). The mean time spent in each behaviour for the eight animals in the two extreme treatment groups was arcsine transformed. A proportion of video footage was used for analysis due to several video recordings stopping before the 8-h period had concluded. For most animals, ~90% or more of the video footage was used, except for one animal in the 100% UDP group where only 66% of the footage was suitable for analysis. The transformed data were analysed using ANOVA with repeated-measures (GENSTAT 2008).

Results

All alpacas consumed the pellets and canola meal offered each day over the 3-month feeding period. On a few occasions some animals did not eat a small amount of the straw offered. The mean ME intake did not differ between groups ($P = 0.985$). The change in liveweight ($P = 0.662$) and body condition ($P = 0.278$) did not differ between groups (Table 1).

The weight of fibre grown per unit area over the 14-week feeding period was similar between all treatment groups ($P = 0.313$). The mean diameter of the fibre grown by the animals in each group was thinner than the mean diameter of their fibre at the start of the 14-week experiment. The alpacas fed the diet with 0% UDP grew fibre of finer diameter than the alpacas fed the three diets with higher levels of UDP ($P = 0.039$; Table 1).

There was no effect of treatment on plasma urea N ($P = 0.530$), however the group that received 0% UDP had the lowest plasma urea N concentration with a mean of 4.2 ± 0.19 mmol/L compared with the 30, 60 and 100% UDP treatment groups with mean concentrations of 4.8 ± 0.25, 4.7 ± 0.12 and 4.6 ± 0.16. The mean plasma urea N concentration for all animals in the week before the start of the experiment was significantly higher than the mean values for Weeks 2, 8 and 9 of the experiment ($P = 0.004$).

There were no differences between the 0 and 100% UDP treatment groups for any of the observed behaviours ($P > 0.05$; Table 2), except that the alpacas fed the diet containing 0% UDP spent a significantly greater proportion of time urinating compared with alpacas fed the 100% UDP diet ($P = 0.027$). However, the frequency of urination was not different between the two groups (0% diet: 2.0 ± 0.5 urinations/day; 100% diet: 2.5 ± 0.9 urinations/day; $P > 0.05$).

Discussion

The hypothesis that alpacas fed canola meal with a high proportion of UDP would produce more fibre and spend less time urinating than peers fed a similar amount of canola meal with a low proportion of UDP was partially supported. The fibre from the alpacas fed 0% UDP was finer than that of the alpacas in the other groups. This result is consistent with those of Masters *et al.* (1999) where sheep supplemented with UDP in the form of canola meal grew more wool of greater diameter than those supplemented with a lower proportion of UDP as lupins. While the fibre diameter was finer in the alpacas fed only RDP (0% UDP), the alpacas fed higher proportions of UDP did not produce more fibre. This result suggests that if fibre production is N limited, that the availability of N was lower in the alpacas fed

Table 1. Mean (±s.e.) metabolisable energy (ME) intake, change in liveweight, body condition, fibre growth and fibre diameter of alpacas fed diets containing different proportions of undegradable dietary protein (UDP) over 14 weeks

Within rows, values followed by different letters are significantly different at $P = 0.05$

Measurement	Proportion of UDP from canola meal in diet			
	0%	30%	60%	100%
ME intake (MJ/kg$^{0.75}$/day)	4.2 ± 0.22	4.3 ± 0.21	4.2 ± 0.27	4.2 ± 0.26
Change in liveweight (kg)	1.7 ± 0.28	1.5 ± 0.85	2.9 ± 1.11	1.5 ± 1.03
Change in condition score (1–5)	–0.6 ± 0.15	–0.2 ± 0.16	0.0 ± 0.19	–0.2 ± 0.16
Fibre growth (mg/cm^2)	33.8 ± 2.42	39.6 ± 3.29	42.2 ± 3.97	37.7 ± 3.10
Fibre diameter (μm)	18.1 ± 0.50a	20.4 ± 0.93b	21.4 ± 0.63b	20.4 ± 0.82b

Table 2. Time budget of alpacas fed a diet containing either 0% undegradable dietary protein (UDP) or 100% UDP for each behaviour category observed

Times for each behaviour category are expressed as percentage of total time. Within rows, values followed by different letters are significantly different at $P = 0.05$

Behaviour	Proportion of UDP from canola meal in diet	
	0%	100%
Eating	27.1 ± 4.68	32.6 ± 3.71
Lying	20.2 ± 4.27	17.1 ± 1.65
Standing	75.5 ± 3.91	76.4 ± 2.26
Walking	4.1 ± 0.77	6.4 ± 1.79
Grooming	0.8 ± 0.10	1.6 ± 0.57
Defecating	0.4 ± 0.13	0.2 ± 0.06
Drinking	0.4 ± 0.13	0.2 ± 0.11
Urinating	0.4 ± 0.13a	0.1 ± 0.04b

0% UDP. While fibre growth appeared to be limited by N availability in the 0% UDP group, the alpacas in all treatment groups maintained their liveweight throughout the experiment, suggesting that protein was probably not limiting and that the mechanisms for retention and utilisation of N for fibre growth may differ between alpacas and sheep. The apparent difference in the fate of N between alpacas fed 0% UDP and those fed higher levels of UDP could be partly explained by the behavioural observations.

The general characteristics of the behavioural attributes measured for the two extreme groups were similar, except that the alpacas in the 0% UDP group spent four times as much time urinating as those in the 100% UDP group. One interpretation of these results is that the latter group retained more N. However, as urea is a diuretic agent it is possible that the alpacas in the 0% UDP group excreted more urea in their urine with less being recycled to the fermentative chambers. Under these conditions, N may have limited microbial protein synthesis in the fermentative chambers with the result that less amino acids were available to be absorbed and used for fibre production.

Llamas fed a low protein diet were found to efficiently recycle urea and utilised ~85% of the urea recycled for microbial protein synthesis (von Engelhardt and Schneider 1977). Although similar conclusions have been drawn for alpacas, they appear to be less efficient at utilising high levels of dietary N than llamas (Davies *et al.* 2007). In our experiment, it is possible that the canola meal low in UDP was mostly degraded in the fermentative chambers and ultimately excreted as urinary urea. Nevertheless, the increase in plasma urea N concentration observed 2 weeks after the alpacas began to receive the experimental diets, and that still persisted in Weeks 8 and 9, may indicate that the microbes in the fermentative chambers adapted to the experimental diets by increasing protein degradation. Although not significantly different, the mean plasma urea N concentration of the alpacas fed 0% UDP tended to be lower than in the alpacas fed higher proportions of UDP. Taken together, our results lend support to the view that N metabolism in alpacas may differ from that of true ruminants such as sheep and that alpacas might have a limited ability to recycle N to their fermentative chambers.

To help elucidate these possible differences, it would be useful to compare the proportion of N apparently absorbed that is excreted in the urine of alpacas and sheep fed 0 and 100% UDP from canola meal. As part of this experiment to gain a better understanding of the ability of alpacas to recycle N to the fermentative chambers, blood samples for measurement of plasma urea N should be taken at an appropriate interval after, rather than before, feeding.

Conclusions

Alpacas fed 0% UDP produced fibre of finer diameter than those fed the higher proportions of UDP. Although there was no difference in the amount of fibre produced between the treatment groups, the alpacas fed diets containing UDP tended to produce more fibre of greater diameter. Alpacas fed 0% UDP spent more time urinating than those fed 100% UDP probably as a result of them excreting more urea in their urine and possibly indicating less recycling of urea to the fermentative chambers. Under the conditions of this experiment, it is possible that N may have limited microbial protein synthesis in the fermentative chambers resulting in less amino acids being available for absorption and ultimately fibre production.

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Appendix 2.

Gradual Training of Alpacas to the Confinement of Metabolism Pens Reduces Stress When Normal Excretion Behavior Is Accommodated

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Abstract

Confinement in metabolism pens may provoke a stress response in alpacas that will reduce the welfare of the animal and jeopardize the validity of scientific results obtained in such pens. In this study, we tested a protocol designed to successfully train alpacas to be held in a specially designed metabolism pen so that the animals' confinement would not jeopardize their welfare. We hypothesized that the alpacas would show fewer behaviors associated with a response to stress as training gradually progressed, and that they would adapt to being in the confinement of the metabolism pen. The training protocol was successful at introducing alpacas to the metabolism pens, and it did reduce the incidence of behavioral responses to stress as the training progressed. The success of the training protocol may be attributed to the progressive nature of the training, the tailoring of the protocol to suit alpacas, and the use of positive reinforcement. This study demonstrated that both animal welfare and the validity of the scientific outcomes could be maximized by the gradual training of experimental animals, thereby minimizing the stress imposed on the animals during experimental procedures.

Key words: animal welfare; behavior; habituation; stress

Introduction

The confinement of an animal is known to alter its normal behaviour, and can be a significant stressor (Bowers et al. 1993; Fraser 2008). The use of metabolism crates in research, where the animal is confined for an extended period of time, is a controversial practice because both the welfare of the animal and the validity of scientific results can be compromised (Fraser 2008). Little has been reported with respect to the reaction to stress of alpacas in metabolism crates. Although some reports on confinement in other species appear in the literature, it is important to bear in mind that large interspecies variability can occur in animals' capacity to tolerate and adjust to confinement (Bowers et al. 1993; Fraser 2008).

The effect of confinement has been studied in other farm animals such as sheep, pigs, and horses (Bowers et al. 1993; Jaskulke and Manteuffel 2011; Mal et al. 1991). Acclimatization periods and habituation training can assist sheep in adapting to confinement and restraint during handling (Grandin 1989). Sheep that are acclimatized to a procedure or routine generally display reduced physiological and behavioral responses associated with the stress response compared with sheep that experience the same procedure for the first time (Grandin 1997). Alpacas are social animals with a strong group hierarchy (Fowler 1998). Therefore the act of placing them in confinement where they are exposed to a novel environment and have limited social contact is likely to induce changes in behavior and physiology associated with the "stress response" that is seen in other social species such as sheep (Bowers et al. 1993; Done-Currie et al. 1984).

The main impetus that led to our study was the need to conduct nutrition experiments, which require alpacas to spend approximately 7 days in a metabolism pen so that energy and nitrogen balance can be determined. The aim of the study was to design a protocol to train alpacas successfully to remain in a specially designed metabolism pen that would be used for future nutritional studies (Figure 1). The training protocol and metabolism pens were developed to decrease the welfare compromise associated with the transition from a paddock and group situation to the semi-isolation of the metabolism pen (the pen is not total isolation because an alpaca in the pen can see and communicate with other alpacas). For ethical reasons and safety concerns, we did not compare the behavioral stress response of trained and untrained

alpacas, but instead compared the same animals when they were naive to following training. It was expected that the alpacas would gradually show fewer stress-associated behaviors as training progressed, and that they would adapt to the confinement of the metabolism pen.

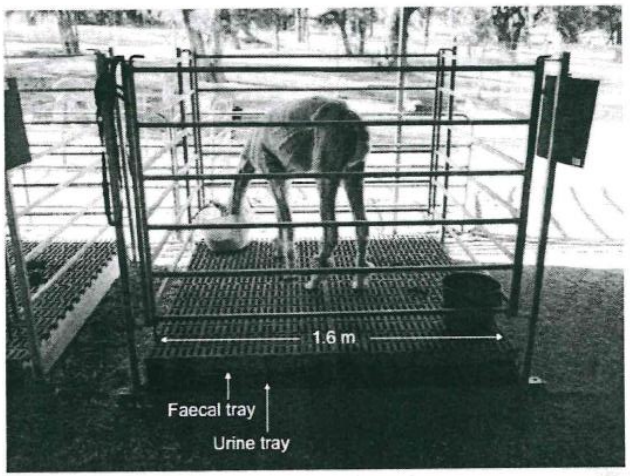

Figure 1. The metabolism pen designed for alpacas with sufficient floor space and high sides.

Material and Methods

The University of Western Australia Animal Ethics Committee approved all of the methods described in this article (RA/3/100/877). In the study, the alpacas were gradually trained to the confinement of a metabolism pen. During the training, the alpacas' behavioral response to this stress was monitored and used as a measure of the amount of strain the animal was experiencing.

Preliminary Halter and Lead Training

Ten alpacas underwent halter training and were used in this study. They were sourced from local breeding facilities where they had been housed in a paddock environment and were rarely yarded. The alpacas had received little to no training before being used in the experiment. Upon arrival at Shenton Park Research Station at the University of Western Australia, we allowed the alpacas 1 week to become accustomed to their new environment. We kept them in a paddock with an open-sided shelter. This paddock was the location for the training periods, and when they were not in the metabolism pens, we permitted the alpacas to roam freely in the paddock. Before beginning the metabolism pen training protocols, the alpacas were familiarized with the handlers and underwent halter and lead training. The same method of halter training was used for all of the alpacas (Table 1). Each training session lasted approximately 15 to 20 minutes although the time taken for each step of the protocol varied according to the progress made by each individual alpaca. Generally, we introduced a new step every 2 days, and we modified some sessions according to the progress made by the individual alpaca. Within 10 days, the majority of the alpacas were accustomed to the halter and would walk on the lead without pulling. We offered food rewards when a training step was successfully completed, and we used this method of positive reinforcement throughout the entire training process.

Table 1. General method of halter and lead training for alpacas

Training step	Description of method
1	Alpaca caught and held by handler. Gently rubbed over neck and shoulders.
2	Alpaca introduced to the halter; allowed to sniff, halter gently placed in front of nose, nosepiece gradually eased into place and removed.
3	Once nosepiece was in place, strap behind ears was fastened. Alpaca taught to stand quietly and allow halter to be removed.
4	Alpaca was led in straight line, encouraged not to pull. Introduced to "walk on" and "stand" commands.
5	Alpaca was led in a more complicated trajectory (e.g., weaving, over obstacles).

Metabolism Pen Training

Monitoring During Training

The level of stress that an animal perceives can be assessed using either physiological or behavioral indicators, or using both (Baldock and Sibly 1990; Grandin 1997). For the present study, we chose behavioral indicators to assess the response to stress because they are noninvasive and/or impose no additional stress on the animal. They also provide an immediate measure of the response to stress and therefore constitute a more useful and practical assessment during this experiment. Alpacas display a number of behavioral reactions to stressful situations such as holding their tail above their back, spitting, rolling their head, making loud screaming vocalizations, and flaring their nostrils (McGee-Bennett 2001). During the introduction of an alpaca to a metabolism pen, we defined a stressed alpaca by the following possible behavioral indicators of stress: repetitively lying down and getting up over short time intervals, attempts to escape, kicking toward the pen, restlessness or pacing around the pen, loud vocalizations, excessive alertness defined by the ears pricked forward and flared nostrils, or pushing against the sides of the pen. We quantified the incidence of these behavioral responses during the training of the alpacas and used the data as a guide to the degree of success and subsequent progression through the training. During constant monitoring, we recorded the alpaca's behavior every 5 minutes or when the alpaca showed signs of distress. If an alpaca showed three or more of the stress signals over two time monitoring periods (every 5 minutes to every 2 hours depending on the training step), we removed the animal from the metabolism pen and retrained from the previous training step. Throughout the study, whenever one alpaca was in the metabolism pen, we kept its companions in a small yard that was constructed around the pen, or in other metabolism pens placed close by, so that all of the animals remained in contact with each other.

Training Protocols

The study consisted of two parts. First, we conducted a preliminary study to assess a training protocol designed from knowledge of the social behavior of alpacas. We used four alpaca wethers (2-2.5 years old) who were successfully accustomed to the metabolism pens using the training protocol (see Protocol 1 below) within 11 days. During step 6, we arranged for the construction of the metabolism pen under the open-sided shelter so that the alpacas had sufficient cover while in the pen. We left in the pen the alpaca's water bucket and daily food ration of milled barley straw, along with a roughage-based pellet (Macco 101 pellet, Macco Feeds Australia, Williams, Western Australia) and dry, granulated sugar cane molasses (Palabind, Probiotec). We used dry molasses as a food reward during the other steps of the protocol.

Protocol 1

1. We marked out an area of ground with the same dimensions as the metabolism pen (1.6 × 1.6 meters) using metal pickets ~ 1.5 meters high and rope. The alpacas were individually confined in this makeshift pen for 30 minutes while a handler standing several meters away constantly monitored the animals. We repeated this process two or three times until the animals settled and showed no stress-associated behaviors.
2. We left the alpacas in the makeshift pen for 2 hours under constant supervision.
3. We placed the flooring of the metabolism pen with the alpacas in a small yard where they spent half a day exploring and become familiar with it.
4. We restricted the alpacas to the flooring for 2 hours with constant monitoring. For practical reasons, we modified this step during the training so that the handler led the alpacas over the flooring and left them standing on the floor for progressively longer periods of time (10, 30, and 60 seconds).
5. We gradually added the sides of the metabolism pen, and we left the alpacas in the constructed pen for 30 minutes.
6. We gradually increased the time the alpacas spent in the metabolism pen from 2 to 4, 8, and 24 hours.

The behavior of the four alpacas indicated that the gradual construction of the metabolism pen sufficiently desensitized the alpacas to confinement. This desensitization of the animals enabled them to become familiar with the pen and to remain in the pen with apparent lack of concern for long periods of time (Table 2).

Table 2 Assessment of training success achieved with each step of Protocol 1*

Training step	Degree of success and observations
1	All of the alpacas stayed in pen. Animals had to remain haltered because the rope fence did not always prevent them from leaving the defined area. No signs of any perceived stress; escape initiated out of boredom rather than fear.
2	Alpacas remained haltered. No signs of any perceived stress.
3	Repeated over 3 days. All alpacas observed exploring the flooring; were content to lie down near it and eat food from it.
4	All alpacas were comfortable with being led on and off the flooring. No signs of any perceived stress.
5	Two opposing sides added; all alpacas led over with no signs of perceived stress. Third side added; alpacas accepted gradual confinement with no signs of perceived stress. Fourth side added; some signs of perceived stress were evident. One alpaca circled the pen, looking for a way out, and attempted to escape several times by pushing and banging on the sides of the pen. This animal was removed from the metabolism pen and calmed down before starting again from the beginning of the training step. Second attempt was more successful; alpaca discovered his food and ate, which resulted in less circling and no attempts at escape.
6	All alpacas appeared comfortable. As time increased, the alpacas were observed to refrain from urinating and defecating until they were released. When required to stay in pen for ≥ 8 hours, the alpacas urinated and defecated. Some animals appeared to experience some discomfort, evident by small humming noises and sniffing at the flooring. When the animals did defecate, their feces were solid and packed together unlike the animals' normally excreted individual pellets. Consequently, the feces did not fall through the flooring of the metabolism pen to be trampled and spread over the floor, making collection for nutritional studies difficult and inaccurate.

*See text for a description of Protocol 1.

After the preliminary study, we developed a more flexible, modified protocol (see Protocol 2 below) in which we altered some of the training steps originally used to suit the progress of an individual alpaca and the degree of success achieved with each step. We then conducted a second study using six different alpaca wethers of similar age to test Protocol 2. The alpacas completed Protocol 2 within 6 to 7 days.

Protocol 2

1. We placed the flooring of the metabolism pen in a small yard where the alpacas spent half a day exploring and becoming familiar with it.
2. We led the alpacas across the flooring until we observed no behavioral indications of stress.
3. Gradually, we added the sides of the metabolism pen, and we led the alpacas through the constructed pen.
4. The alpacas spent a full day in the metabolism pen, in sight of peers, under constant supervision.

Design of the Metabolism Pens

The metabolism pens that we used in this study were specially designed for alpacas but are suitable for other production animals such as sheep and goats. The welfare of each animal in the pen was a high priority, thus the metabolism pen had a larger floor space (1.6 × 1.6 meters) than most conventional metabolism crates. Each pen accommodated one alpaca lying down with its neck stretched out in front, as is their habit (Figure 2). The flooring was situated as low as possible to the ground so that the animal was required to take only one small step up to enter, but the flooring was high enough to accommodate urine and feces collection apparatus underneath. The flooring was made of nonslip plastic material that is commonly used in piggeries (Stepper flooring, MIK International, Germany). The sides of the crate were made high (1.2 meters) but still allowed the animal to see its peers.

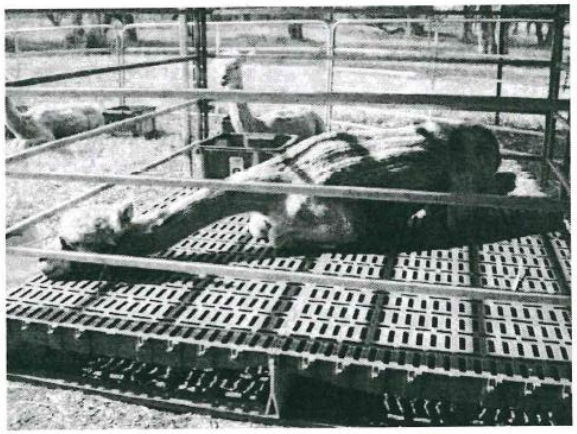

Figure 2. The metabolism pen has sufficient floor space to accommodate an alpaca lying down with its neck stretched out in front.

Results

Five of the six alpaca wethers were successfully trained to the metabolism pen using Protocol 2. One alpaca was excluded from the training phase because he showed no improvement during basic handling procedures such as being haltered and walked on the lead. We decided that any attempt to put him near the metabolism pen would impose a considerable stress and be dangerous to the animal and to the handler.

The alpacas showed no behavioral signs of stress during step 1 of the training protocol, when they explored the flooring of the metabolism pen. Initially, most of the animals sniffed at the floor and then ignored its presence. After approximately 1 hour, one alpaca walked over the floor.

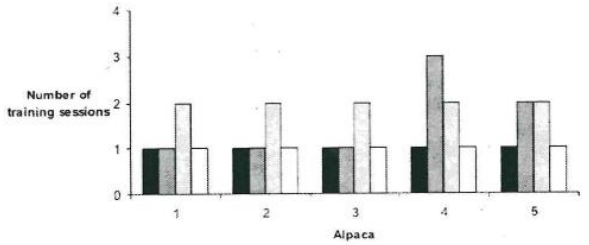

Figure 3. Number of training sessions required by individual alpacas for each training step. Black bars = training step 1 (half day); dark gray bars = training step 2 (sessions of 15-20 minutes in duration); light gray bars = training step 3 (15-20 minutes in duration); white bars = training step 4 (full day).

During step 2, we observed stress behaviors within the first 10 minutes of the training sessions. Three of five alpacas progressed to the next training step after one 20-minute session (Figure 3). The other two animals that repeated the training step showed five incidents, collectively, of excessive alertness in the first 10 minutes of the session. We also recorded restlessness in the form of pulling on the lead rope on three occasions (Table 3).

Table 3 Collective number of incidents of stress-associated behaviors expressed by all alpacas during each step of Protocol 2.* (Click anywhere on table for larger version.)

Step	Time (mins)	Stress indicators							Total no. of incidents
		Unsettled	Escape attempt	Kicking at pen	Pushing on pen	Loud vocals	Restlessness (pacing)	Excessive alertness	
1	Half day	-	-	-	-	-	-	-	0
2	5	1	-	-	-	-	1	3	5
	10	1	-	-	-	-	2	2	5
	15	-	-	-	-	-	-	1	1
	20	-	-	-	-	-	-	-	0
3	5	-	-	-	-	-	2	1	3
	10	-	-	-	-	-	2	-	2
	15	-	-	-	-	-	-	-	0
	20	-	-	-	-	-	-	-	0
4.	Whole day	2	2	-	1	-	7	-	12

*Dashes indicate that no behavioural response was observed.

During the first 10 minutes of step 3 of the training protocol, some alpacas demonstrated restlessness and excessive alertness. However, only two training sessions were needed for all of the animals to complete this step (Table 3).

Initially, the alpacas were restless when they were left in a metabolism pen for a day (step 4). One animal initially attempted to escape by pushing on the sides of the pen; however, all animals settled within 1 hour and ate their daily food ration. Throughout the day some animals made soft vocalizations, often in response to another animal. After 9 hours, most of the alpacas appeared to be unsettled and restless, and we observed repeated standing up, lying back down, pacing around their pen, and sniffing at the floor. It was evident that they were uncomfortable. Because alpacas typically defecate and urinate at communal dung heaps, their behavior suggested that they needed to defecate and urinate but were unwilling to do so. One animal attempted to escape by rearing. On the second attempt at escape, that alpaca appeared to urinate accidentally, which apparently prompted him to stand in the body position associated with excretion, after which he did urinate and defecate. After urinating and defecating, the alpaca reverted back to being calm and showed no further overt behaviors associated with perceived stress. The behavior of this animal suggested that the presence of feces in the pen could be used as a stimulus to trigger defecation. The feces from that animal's pen were transferred into the other pens in an attempt to trigger a response from the other animals (see Discussion). This method was successful in changing the alpaca's learned behavior of using communal dung heaps. After all of the animals had urinated and defecated, they showed no further behaviors associated with perceived stress, and they continued to urinate and defecate normally.

Discussion

The protocol developed in this study was successful at introducing alpacas to specially designed metabolism pens, and it led to a progressive decline in the occurrence of behavioral responses to stress that can be experienced by animals subjected to confinement. The alpacas adapted to the confinement of the metabolism pens and showed fewer behaviors associated with a response to stress as the training progressed. The success of the protocol may be attributed to the progressive nature of the training in which each alpaca was presented with one stressor at a time and given time to become comfortable with that stressor before proceeding to the next training step. Compared with a review of behavioral principles of sheep handling in which Hutson (2000) concluded that gradual training and handling can reduce the stress of handling in sheep, it is apparent from our results that his conclusions pertain also to alpacas. Gradual exposure to novelty can allow animals to become accustomed to stimuli that may otherwise prompt stress-associated behaviors (Grandin 1997). In this study, we exercised care to ensure that the alpacas appeared to be comfortable with each training step before we guided them to progress to the next step. For example, we did not introduce them to the flooring of the metabolism pen or lead them over it until they had walked calmly on the lead and had responded to verbal commands from their handler.

We also tailored the training protocol to the alpacas by using information and observations of alpaca behavior. These animals are naturally inquisitive and social, and they should find exploring novel objects a positive experience when kept in a stable group (Fowler 1998; Tennessen 1989). For that reason, we allowed the alpacas to explore the metabolism pen flooring in their own time when it was left in their paddock. By keeping them together as a group and letting them approach the flooring by themselves, it appeared that we effected a reduction in the stress of having a novel object in their yard. Similarly, because of their social nature, we had the metabolism pen designed to allow the alpacas to see each other, even when they were lying down as they do in the field. The freedom to express normal behavior is regarded as one of the "five freedoms" used to assess animal welfare (Farm Animal Welfare Council 1992). We designed the metabolism pen to be more accommodating of the normal behavior of alpacas than conventional metabolism crates, which tend to isolate the animal completely by blocking visual contact with conspecifics and restricting the amount of floor space to such a degree that the animal cannot easily turn around. It appears that in this study, the welfare of the animals was not compromised.

Although we developed the training protocol for alpacas, it was evident during our study that some behaviors that are characteristic of the species were more difficult to overcome. As mentioned above, alpacas defecate and urinate on communal dung heaps (Fysh 2003). When the alpacas were required to remain in the metabolism pens for extended periods, they initially displayed no signs of an agitated state but later became agitated and restless. Given the

subsequent behavior of the alpacas, we conclude that this restlessness was due to their resisting the micturition and defecation reflexes because they did not have access to a communal heap. It was possible, however, to modify this learned behavior and train the alpacas to defecate and urinate within a few hours of being in the pen by transferring fresh feces into the pen from a pile in the paddock or in another metabolism pen. This action appeared to act as a stimulus to release the inhibition of the excretion reflexes. After the initial excretion, the alpacas continued to defecate and urinate in the pens regardless of the amount of time they spent out of the metabolism pens between training sessions. This outcome was important because the objective of keeping animals in a metabolism pen is usually so that feces and/or urine can be collected. It also highlights the importance of the relationship that animals have between olfactory cues and behavior. It is clear from this experiment that rather that having clean and disinfected pens, the animals required the presence of some feces in the metabolism pens for them to perform natural functions. From a regulation and ethical view, keeping animal facilities very clean or sterile can likely affect the behavior of the animal and inadvertently cause stress.

The training protocol may also have been successful because we used positive reinforcement, mainly in the form of food. Sheep can be trained to accept restraint voluntarily with the assistance of grain as a reward (Grandin 1989). Similarly, Hutson (2000) has recommended that the aversive and stressful nature of handling could be reduced by using food rewards. In the present study, the alpacas appeared to remember that when they behaved in a particular way, they would be rewarded. The gradual absence of behavioral responses to stress suggested that the animals had a positive experience.

Although, according to our measures, the training protocol was successful in introducing alpacas to the metabolism pens and reducing the potential stress of being confined within the pen, it must be noted that we used only behavioral indicators to assess the response to stress. Other authors have suggested that it is best to use both behavioral and physiological indicators such as cortisol or heart rate (Arzamendia et al. 2010; Grandin 1997). However, behavioral indicators alone were a practical and immediate measure of the response to stress in this study. Because cortisol concentration was not immediately available, it was not an appropriate stress indicator in this study. Cortisol concentrations may have confirmed the response to stress by the animal, although there is evidence to suggest that cortisol may not always show a response during handling, particularly when the animals are habituated (Andrade et al. 2001; Lay et al. 1992). Likewise, heart rate measurements can be confounded by activity and may not provide an accurate measure of stress during the training. Moreover the training protocol was spread over a few weeks so that it was not practical to equip each alpaca daily with devices to measure heart rate or with indwelling cannulae to take serial blood samples. Both of these procedures can themselves act as stressors.

Changes in behavior are the first symptoms that can indicate the state of an animal's well-being (Dellmeier 1989). The nature of the training relied heavily on the handler recognizing when an alpaca showed reluctance to the task he was being asked to do and therefore being able to remove the animal from the stressful situation. Studies on the response to confinement in other species, such as horses, have also used behavior as the sole indicator of animal well-being (Mal et al. 1991). Likewise, a correlation between behavioral observations and physiological measures that reflect the physiological response to stress has been identified in cattle (Stockman et al. 2011).

Our study also highlights that although the training protocol worked for most of the alpacas, some animals may not become accustomed to handling or novel experiences regardless of the amount of time the handler spends in taming the animal. During this study, we excluded one alpaca from the training to the metabolism pen because he continued to show signs of stress during basic handling sessions. This animal's continued adverse reaction may be attributed to its previous life experiences, its genetic makeup, or an interaction between the two. Often an animal's previous experience can influence its response to stress (Grandin 1997). In addition, genetic factors such as temperament may influence an animal's resistance to stressors and their ability to be trained (Grandin 1997).

Conclusions

Overall, the training protocol that we developed to train alpacas to the confinement of metabolism pens was successful in reducing the incidence of the animals' stress-associated behaviors while in the pens. We largely

attribute this success to the gradual progression of the training steps and our tailoring of the protocol to suit alpacas by using positive reinforcement. The study demonstrates that when it is necessary to confine large experimental animals, such as during metabolic experimentation, it is clearly advantageous to develop adequate procedures for minimizing the animals' stress. By implementing such procedures, it is possible to maximize both animal welfare and the validity of scientific outcomes.

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Appendix 3.

278

Dietary levels of un-degradable dietary protein affects fibre diameter and nitrogen excretion in alpacas

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Introduction Alpacas can obtain most of their glucose from deamination of amino acids (AA) rather than from propionate (Van Saun, 2006). To reach their needs for glucose, alpacas, fed to meet their energy requirements for maintenance, may utilise most of the AAs absorbed from the small intestine. Consequently, the supply of AAs to meet their requirements for fibre growth might be compromised and it may be necessary to supply them with supplemental protein to optimise fibre production. Un-degradable dietary proteins (UDP) by-pass the rumen and are digested in the abomasum and small intestine where AAs are absorbed and used for synthetic processes including fibre growth or conversion to glucose. In sheep supplemented with UDP as expeller canola meal (about 50% UDP), wool growth increased by 11% compared to the wool growth of sheep fed lupins; a grain with a similar level of protein but only 25% UDP (Masters *et al.*, 1999). In this study, we tested if the proportion of UDP in the diet could influence fibre growth of alpacas. We hypothesised that alpacas, fed at maintenance a diet containing canola meal protein high in UDP, will produce more quality fibre and excrete less nitrogen than alpacas fed a similar amount of canola meal protein with a low proportion as UDP.

Materials and methods Four groups of 8 alpacas were fed diets of similar metabolisable energy (ME) content at a level calculated to maintain body weight with varying proportions of UDP; 0%, 30%, 60% and 100%, in the form of heat-treated canola meal. The fibre growth of the animals was measured over two months by clipping mid-side patches at the start and end of the treatment period. The fibre diameter was measured using a representative sample from the mid-side patch. The behaviour of the animals in the two extreme groups (0% and 100%) was measured over five days using CCTV cameras and digital surveillance software.

Statistical analyses The mean fibre diameter and the dry fibre growth (in g/cm2) was analysed using ANOVA. The fibre diameter value obtained at the beginning of the treatment was used as a covariate. Pairwise comparisons between the four treatment groups were also tested using the Student-Newman-Keuls test (GenStat®, 11th edition, VSN International Ltd., 2008). Behavioural data from the two extreme treatment groups were normalised using an arcsine transformation and then analysed using ANOVA with repeated measures.

Results All alpacas consumed most of the food on offer and only a small amount of straw was not eaten by some animals. All animals maintained live weight ($p = 0.662$) and body condition ($p = 0.278$; Table 1). Fibre grown was similar between all treatment groups ($p = 0.313$). The fibre diameter was smaller in alpacas fed 0% UDP than that of alpacas fed the higher levels of UDP ($p = 0.039$). There were no differences between the 0% and 100% treatment groups for any of the observed behaviours, but alpacas fed 0% UDP spent a more time urinating than alpacas fed 100% UDP ($p = 0.027$).

Table 1 Mean change (±SE) in live weight, body condition, fibre growth and fibre diameter of alpacas fed diets containing different % of UDP over 14 weeks and time spent urinating. [ab] values within a row with different superscripts are different ($p < 0.05$).

	Proportion of UDP from canola meal in diet			
	0%	30%	60%	100%
Change in live weight (kg)	1.7 ± 0.28	1.5 ± 0.85	2.9 ± 1.11	1.5 ± 1.03
Change in condition score (1-5)	-0.6 ± 0.15	-0.2 ± 0.16	0.0 ± 0.19	-0.2 ± 0.16
Fibre growth (mg/cm^2)	33.8 ± 2.42	39.6 ± 3.29	42.2 ± 3.97	37.7 ± 3.10
Fibre diameter (µm)	18.1 ± 0.50^a	20.4 ± 0.93^b	21.4 ± 0.63^b	20.4 ± 0.82^b
Urinating	0.4 ± 0.13^a			0.1 ± 0.04^b

Conclusions The increase in time urinating in alpacas fed 0% UDP suggested that they produce more urine and excreted more nitrogen, and consequently less urea would have being recycled to the fermentative organs. Therefore, nitrogen may have limited microbial protein synthesis, so less amino acids were available for fibre production, as suggested by the decrease in fibre diameter.

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