The effects of food restriction on the perception of hunger, metabolic state and stress responsiveness in sheep

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Abstract

Sheep in temperate regions may be exposed to periods of limited food availability and a range of environmental conditions. Insufficient dietary intake may change live weight (LW) and body condition score (BCS) resulting in an increase in hunger and alterations in metabolic state, which may influence the welfare of sheep. In Chapter 2, a behavioural demand methodology to measure feeding motivation was refined and improved; five different walking distances (costs) were used and the number of rewards consumed, the total distance walked, P_{max} (maximum price) and O_{max} (maximum expenditure) were used as measures of motivation. Sixteen non-pregnant ewes were subjected to two feeding levels (24 h fasted and *ad libitum* fed control); fasted ewes consumed more rewards and walked a greater total distance compared to control ewes. The methodology provided good measures of feeding motivation and was a potential indicator of hunger. However, the range of costs used was too narrow for accurate assessment of P_{max} and O_{max}. In Chapter 3, the effects of body condition score (BCS) on feeding motivation and endocrine and metabolic responses in twin-bearing ewes were investigated to identify and quantify indicators of hunger and to assess the metabolic state of twin-bearing ewes with different BCS. Twenty-two ewes were divided into low BCS (LBC), medium BCS (MBC) and high BCS (HBC) treatments. Feeding motivation was assessed between day 91 and 105 of pregnancy and blood samples were collected once every 2 weeks between day 35 and 133 of pregnancy. The number of rewards consumed and O_{max} were significantly lower in the HBC ewes compared to LBC and MBC ewes, while the MBC ewes tended to show a lower motivation compared to the LBC ewes. LBC ewes were in a metabolically catabolic state and had low plasma concentrations of leptin, insulin and insulin-like growth factor-1. In Chapter 4, it was investigated whether a fast loss in BCS would increase hunger and alter the metabolic state more compared to a slow loss in BCS. Twenty-six twin-bearing ewes were assigned to Slow Loss (1 BCS loss in 13 weeks, SL), Fast loss (1 BCS loss in 10 weeks, FL) or Control (maintain BCS) treatments. Feeding motivation was assessed between day 42 and 67 of pregnancy and blood samples were collected once every 2 weeks between day 35 and 140 of pregnancy. SL and FL ewes had not lost much BCS at the start of the motivation period and no effects on feeding motivation were found. SL ewes were in a similar metabolic state as Control ewes. However, FL ewes were in a catabolic state in mid-pregnancy. In Chapter 5, it was investigated whether BCS affects the ability to maintain energy homeostasis and induce adaptive metabolic and stress responses to an environmental challenge. Eighteen shorn single-bearing ewes were divided into low, medium and high BCS treatments. Ewes were exposed to a 6 h acute cold challenge between days 85-87 of pregnancy. LBC ewes were able to maintain body temperature, but were slower to mobilise energy substrates and had a delayed and reduced cortisol response compared to MBC and HBC ewes. In conclusion, feeding motivation appeared to be an indicator of hunger in ewes; fasting and a loss in BCS markedly increased hunger. A loss in BCS induced a metabolically catabolic state. However, ewes were metabolically better able to adapt to a slow than a fast loss in BCS. Finally, low BCS ewes may have a reduced ability to respond to acute stressors.

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Abbreviations

°C	Degrees Celsius
ACTH	Adrenocorticotrophic hormone
AgRP	Agouti-related protein
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BAT	Brown adipose tissue
BCS	Body condition score
LW	Live weight
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
CV	Coefficients of variation
DM	Dry matter
EB	Energy balance
EG	Energy gain
FFA	Free fatty acids
g	Grams
GH	Growth hormone
GHS-R	Growth hormone secretagogue receptor
h	Hours
HPA-axis	Hypothalamo-pituitary-adrenal axis
ICV	Intracerebroventricular
IGFBP	Insulin-like growth factor binding protein
kg	Kilograms
l	Litre(s)
LPL	Lipoprotein lipase
m	Minutes
MJ	Mega Joules
ml	Millilitres
mmol	Millimoles
MR	Energy spent on maintain live weight
mRNA	messenger Ribonucleic acid
ng	Nanograms
NPY	Neuropeptide Y
O_{max}	Maximum expenditure
P_{max}	Maximum price
POA	Preoptic area
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
REML	Residual maximum likelihood

s	Seconds
SD	Standard deviation
SED	Standard error of the difference of the mean
SEM	Standard error of the mean
T3	3-5-3’-Triiodothyronine
T4	Thyroxine
T_{core}	Internal body temperature
TRH	Thyroid releasing hormone
TSH	Thyroid stimulating hormones
T_{skin}	Skin temperature
UCP1	Uncoupling protein 1
UCP2	Uncoupling protein 2
WAT	White adipose tissue
WR	Energy spent on walking
α-MSH	α-Melanocyte stimulating hormone
β-HBA	β-hydroxybutyrate

Chapter 1. General introduction

In pastoral farming systems, sheep are reared outdoors and may therefore be exposed to periods of limited food availability and a range of environmental conditions. In temperate regions, pasture growth and quality is limited during the winter months when sheep are pregnant (Kemp *et al.* 1992; McPhee *et al.* 1997), which may result in food restriction if supplementation is inadequate. Food restriction can be short-term (hours to days) or long-term (weeks to months). In this thesis, food restriction will be considered long-term when it leads to a loss in body reserves and weight. A measure for the amount of body reserves in sheep is the body condition score (BCS), which can be assessed by manual palpation of the fat cover over the backbone and spinal processes (Russel *et al.* 1969). According to the Russel system, body condition is scored on a six-point scale, with a score of 0 indicating an emaciated sheep and a score of 5 an extremely fat sheep. Therefore, BCS is a good reflection of long-term food restriction in sheep. Furthermore, there is evidence that changes in BCS lead to changes in metabolic and endocrine factors (Caldeira et al., 2007a; Caldeira et al., 2007b), which could make BCS a potential indicator of the metabolic status of ewes. However, very little is known about the welfare of food-restricted sheep with low BCS.

In recent years, it has been recognised that the mental states (also called affective states) and subjective experiences of animals are central to their welfare, in particular negative ones that may cause suffering (Dawkins 1988; Broom 1991; Dawkins 2008; Fraser 2009a). A major welfare concern in relation to food restriction and a loss in BCS is the resulting subjective feeling of hunger. In this thesis, hunger will be defined as the negative subjective state experienced by an animal that is chronically undernourished (D'Eath *et al.* 2009). However, the subjective experiences of animals are not directly measurable, and there is a need for methodologies that can obtain indicators of animals' subjective states. A second major aspect that is likely to influence the welfare of animals is the level of biological functioning (Mendl 2001). Biological functioning could imply the functioning of several different biological systems, from growth and reproduction to the immune system and the incidence of disease. The effects of nutrition on growth and reproduction have been studied extensively (Robinson 1996; Michels *et al.* 2000; Morrison *et al.* 2001; Smith and Dobson 2002; Chilliard *et al.* 2005; Abecia *et al.* 2006; Roche *et al.* 2009). However, the effect of food restriction on adaptive metabolic and endocrine responses in relation to the subjective experience of hunger has received relatively little attention. Therefore, the interpretation of biological functioning will be limited to measures that relate directly to the metabolic state of the animal. Therefore, the ability to maintain energy homeostasis and induce adaptive metabolic (and related endocrine) responses to food restriction is likely to reflect the level of metabolic functioning in ewes. The metabolic and endocrine responses to food restriction could potentially also be related to the subjective experience of hunger in sheep. Furthermore, the ability to induce adaptive metabolic and stress responses to additional environmental challenges (such as cold exposure) could provide a more in depth insight into the level of biological functioning in food restricted ewes. In this thesis, the focus will be on the consequences of food restriction on the subjective experience of hunger (including the development of a methodology to obtain indicators of hunger), metabolic and endocrine adaptations and stress responsiveness in sheep, and their implications for welfare.

1.1. Hunger and motivation for food

Most research on the topic of hunger and satiety has focused on the changes in endocrine signals in relation to changes in LW and/or food intake in order to understand how hunger is regulated (Friedman and Halaas 1998; Baskin *et al.* 1999; Blache *et al.* 2000; Schwartz *et al.* 2000; Tschop *et al.* 2000; Sugino *et al.* 2002b). Endocrine signals (e.g., insulin and leptin) provide a negative feedback to the hypothalamus and affect food intake and energy expenditure (Schwartz *et al.* 2000; Woods *et al.* 2000). However, hunger and satiety are not solely influenced by these endocrine signals. Many animals consume food without a metabolic need for energy or nutrients. For example, animals respond to external stimuli such as the taste and smell of food (Provenza *et al.* 1995; Ralphs *et al.* 1995) and experience and learned behaviour may influence diet selection and food intake (Kyriazakis *et al.* 1998; Villalba *et al.* 2008). In sheep, feeding behaviour is influenced by flock size (Penning *et al.* 1993), suggesting that hunger also has a social aspect. Therefore, hunger is a complex experience that is influenced by both internal and external factors.

1.1.1 Feeding motivation and behavioural demand

Subjective experiences of animals cannot be measured directly. However, animal welfare scientists have recognised the importance of subjective experiences and affective states for the well-being of animals, and many agree that subjective experiences directly affect welfare (Dawkins 1990, 2003, 2006; Duncan 2006; Fraser 2009a). This has resulted in the identification of indirect measures of subjective experiences (Dawkins 1983; Jensen and Pedersen 2008). In one of the first studies identifying important animal needs, hens with different degrees of food restriction were given a choice between a cage with litter and no food and a cage with food but no litter. It was found that with an increased duration of food restriction, animals preferred the cage with food and no litter over the cage with litter and no food, while non-food deprived hens would choose the cage with litter and no food (Dawkins 1983). The test clearly demonstrated that animals were able to make decisions based on their feeding level and that the subjective hunger state of animals can be assessed by offering them a choice between food and an alternative resource.

The idea of giving animals choices and asking what is important for their wellbeing has been further developed over the last few decades (Matthews 2008). An important step was the use of behavioural demand theory, an economic concept applied to animal behaviour (Dawkins 1990). This methodology requires the animal to “pay a price” for a certain resource, which is usually in the form of an activity an animal has to perform (e.g., work). The amount of work an animal is willing to do to obtain a resource is a measure of the motivation for that resource. Work has been measured in several different ways, for example by the number of panel or lever presses an animal performs (Matthews and Ladewig 1987; Lawrence and Illius 1989; Jackson *et al.* 1999; Savory and Lariviere 2000) or the distance that it is willing to walk (Schütz *et al.* 2006; Bokkers *et al.* 2007; Champion *et al.* 2007) and by the weight of a door that gives access to food (Jackson *et al.* 1999).

Generally, the strength of the motivation is measured using parameters derived from a demand curve, which describes the relationship between total consumption of the resource and its cost. A traditional demand curve is a straight line when plotted in log-log coordinates (Dawkins 1988; Jensen and Pedersen 2008). Its slope reflects proportional changes in consumption relative to price, and is used as a measure of motivational strength called “elasticity of demand” (Hursh and Winger 1995; Kirkden *et al.* 2003). If proportional changes in consumption are relatively small (the slope is less negative than -1) compared to proportional changes in cost, then demand is said to be inelastic. Demand is elastic when proportional reductions in consumption are large with a small increase in cost and the slope of the curve has a value more negative than -1. It is argued (Dawkins 1990; Kirkden *et al.* 2003) that the elasticity of demand assigns a single value to each resource so that different resources can be compared to determine which resources are the most important. Furthermore, the interpretation of an inelastic demand is that an animal is highly motivated to “defend” a certain level of consumption because it only reduces consumption slightly with an increased cost.

Elasticity of demand is the measure of motivational strength that has been traditionally used by several animal welfare scientists (Dawkins 1990; Jensen *et al.* 2004; Jensen and Pedersen 2008). However, the elasticity of demand as a measure of motivation strength has been criticised for a number of reasons (Kirkden *et al.* 2003). Kirkden et al. (2003) make the point, among others, that the use of elasticity of demand is only valid when elasticity is constant across a range of costs (and the demand curve is a true straight line on log-log coordinates). Several studies have shown that this is often not the case when complete demand curves are generated (i.e., demand curves obtain from a sufficiently large range of costs) (Greenwald and Hursh 2006; Hursh and Silberberg 2008). Usually, demand is inelastic at low cost and becomes more elastic when cost increases. Therefore, assigning a single value to a resource may not be valid. Furthermore, it may be incorrect to assume that an animal is defending a certain level of consumption, because an inelastic demand may also reflect a tendency to become easily satiated with the resource (see for explanation: Kirkden *et al.* 2003). Finally, the elasticity of demand does not take the amount paid (or expenditure) into account. Kirkden et al (2003) therefore suggest using alternative demand curve derived measures such as the consumer surplus and the reservation price. The consumer surplus is a measure of expenditure (price × consumption of the resource) and corresponds to an area under the demand curve. The reservation price is the highest price an animal would pay for the first unit of the resource. Kirkden et al (2003) suggest that these measures of motivation are more valid that the elasticity of demand, because they do not attempt to assign a single value to a resource (but rather evaluate specific quantities of a resource) and because they do not make any assumptions about the animal (such as that animals are defending certain levels of consumption) and that they take expenditure into account (rather than looking at the rate of change in consumption). The reservation price is a relatively new measure in animal welfare science, although there is some evidence that it is able to rank motivation for different resources (Cooper and Mason 2001; Mason *et al.* 2001; Asher *et al.* 2009). However, the methodology to determine the reservation price requires the animal to cease responding for the resource, or in behaviour analytic terms to undergo extinction. Because food is an essential resource, reducing consumption to zero also imposes ethical and practical issues.

Alternative measures of motivation could overcome the problems of elasticity of demand and the reservation price. These are O_{max} (maximum expenditure) and P_{max} (maximum price) and can be derived from the following generic demand function (Hursh and Winger 1995):

$$\ln(Q) = \ln(L) + b[\ln(P)] - a(P) \qquad [1]$$

where,

Q is the measured consumption of the resource,

P is the price for a unit of the resource, and

L, b and a are parameters characterising the initial level of the curve at ‘minimal cost or price’ (the minimal price is determined by the researcher, and usually means that the resource is freely available), the corresponding slope at minimal price, and the acceleration or increase in slope with increases in price, respectively.

The P_{max} is found on the point of the demand curve where elasticity of demand is exactly -1, and therefore indicates the point at which consumption reduces from proportionally less than the increase in cost to proportionally more than the increase in cost. O_{max} is the area under the curve bounded by the maximum price (on the x axis) and the level of demand (on the y axis), and is therefore a measure of expenditure. P_{max} and O_{max}, therefore, do not rely on the assumption that elasticity of the demand curve is constant across price and take expenditure into account. These measures also do not require consumption of the resource to reduce to 0, as they simply measure the point at which the animal is no longer willing to increase work to obtain a resource. The point elasticity of demand (η, required to calculate P_{max}) can be calculated as:

$$\eta = b - \alpha(P) \qquad [2]$$

P_{max} as:

$$P_{max} = (1 + b)/\alpha \qquad [3]$$

And O_{max} as:

$$O_{max} = P_{max} \text{ x } RW_{Pmax} \qquad [4]$$

where RW_{Pmax} is the number of rewards collected at P_{max}.

Unlike P_{max}, O_{max} accounts for differences in the quantity of a reward consumed and price paid (Hursh and Winger 1995; Madden *et al.* 2007a, b) and may therefore be a better measure for quantifying different motivational strengths. O_{max} has been validated as a good measure of need for alcohol (Murphy and MacKillop 2006), cigarettes (Johnson and Bickel 2006) and opioid drugs (Winger *et al.* 2002; Greenwald and Hursh 2006) in humans. Recent evidence presented by Madden et al. (2007a, b) suggests that O_{max} is also suitable for ranking the motivation for food (pellets), fat (oil) and water in rats. Therefore, there is substantial evidence that O_{max} is a suitable measure of motivational strength for several needs or resources in humans and some other animals.

P_{max} and O_{max} are relatively unused in animal welfare science. However, they could potentially be very useful indicators of motivation for different resources, including food. Furthermore, they could overcome the problems encountered by elasticity of demand (e.g., elasticity does not need to be constant across price) and the reservation price (e.g., demand does not need to be reduced to 0). If the level of food restriction affects P_{max} and O_{max} then this could be an indirect indication of hunger and could be a useful tool in assessing the welfare of ewes with different levels of food restriction.

1.1.2 Other behavioural measures of hunger

There are several alternative approaches to measuring hunger in animals. One common approach is to measure compensatory food intake after a period of food restriction. For example, Sibbald et al. (1997) have shown that the number of meals and intake rates increase in 18 h restricted sheep compared to non-restriction sheep. Furthermore, ewes with a BCS of 2.5 spend significantly more time eating compared to sheep with a BCS of 3.5, leading to an increase in food consumption (Sibbald 1997). Also Newman et al. (1994) have shown that a 24 h fast leads to increased food intake in sheep, which was mainly due to increases in bite mass and increased time spend eating. Interestingly, fasting also affected diet preferences with 24 h fasted sheep proportionally grazing less clover (and more grass) compared to non-restricted sheep (Newman *et al.* 1994). Another study showed that lambs offered a diet high in energy but low in protein have a preference for food that is relatively low in energy but high in protein when offered a choice (Villalba and Provenza 1999). Therefore, the nutritional state of sheep not only influences the amount of food eaten, but also the preferences for different types of food. Compensatory increases in food intake may be suitable indicators of feeding motivation in sheep.

In species other than sheep, compensatory food intake has also been used as an indicator of feeding motivation. Dairy cows on a restricted feeding level spend more time grazing compared to cows on an unrestricted feeding level (O'Driscoll *et al.* 2010), and restricted fed broilers also show an increased compensatory food intake (de Jong *et al.* 2003). Rats that have been trained to expect a period of fasting learn to eat more in the 3 h preceding the fast, and this anticipatory increase in food intake is more pronounced with a longer fasting time (Jarvandi *et* *al.* 2009). The level of frustration behaviour has also been used as an indicator of feeding motivation. For example, pigs fed a high fibre diet showed less stereotypic behaviour (licking, biting and chewing the empty trough or a chain, or chewing without any substrate in the mouth) compared to pigs fed a concentrate diet (Robert *et al.* 2002). Similar results were found in a different study in pigs (Zonderland *et al.* 2004). The level of activity could also be an indicator of a motivation for food; broilers on a restricted diet show higher activity levels compared to non-restricted broilers (Savory and Lariviere 2000). In addition, restricted fed broilers peck objects more frequently compared to broilers that received appetite suppressants and oat hulls with the same restricted diet (Sandilands *et al.* 2005). Therefore, activity level and frustrated behaviour may potentially be indicators of feeding motivation in sheep, although I am not aware of any studies assessing activity and frustrated behaviour after food restriction in sheep. However, activity and frustrated behaviours may be difficult to use as measures of motivation, because there may be other reasons for changes in these behaviours. For example, an increased weight due to higher food intake may reduce activity and decreased frustration behaviour with increased food intake may be due to an increased feeding time, rather than a reduction in hunger *per se* (D'Eath *et al.* 2009). Furthermore, the relationship between activity or frustration behaviour and hunger may not be linear and therefore difficult to interpret (de Jong *et al.* 2003). Therefore, obtaining measures of motivation strength (e.g., P_{max} and O_{max}) in combination with amount of food consumed (compensatory food intake) may provide better measures of the motivation for food.

1.2. Metabolic and endocrine signals and hunger

Metabolic and endocrine signals play important roles in maintaining energy homeostasis. The metabolic and endocrine responses to food restriction are potential indirect indicators of the level of biological functioning. Furthermore, endocrine signals provide feedback to the hypothalamus that responds with signals affecting energy expenditure and food intake (Schwartz *et al.* 2000). The metabolic and endocrine signals that play a role in energy homeostasis, LW and food intake regulation in non-pregnant as well as pregnant animals will be discussed in the following paragraphs. The focus will be on evidence obtained from ruminants. However, evidence from different mammalian species will be used when information on ruminants is lacking.

1.2.1 Metabolic signals

1.2.1.1. Adipose tissue and fatty acids

The main storage site of body reserves is adipose tissue. During times of food abundance, nutrients such as carbohydrates and fatty acids are stored as lipids in the adipocytes. Sheep adipose tissue can synthesize lipids de novo from acetate and to a lesser extent from lactate. Adipose tissue lipids also originate from plasma triacylglycerols hydrolysed by lipoprotein lipase (LPL). When food is restricted, body lipids are mobilised from the adipocytes and used as energy to maintain important body functions. The degree of lipid mobilisation depends on the severity and duration of food restriction in ruminants (Chilliard et al., 2000). Lipolysis of the adipocyte triacylglycerols occurs under the control of hormone-sensitive lipase and results in the release of non-esterified fatty acids or free fatty acids (FFA) from the adipocytes into the circulation. FFA are a readily available energy source for several body tissues to support body functions when food is restricted.

Research has shown that non-pregnant/non-lactating sheep of BCS 1.25, 2 and 3 had higher serum FFA concentrations and lower serum triglycerides than sheep with a BCS of 4 (Caldeira *et al.* 2007b). Furthermore, an increase in BCS is accompanied by a reduction in FFA, while a decrease is accompanied by an increase in FFA (Caldeira *et al.* 2007a). Several studies have also shown that plasma FFA concentration increase when sheep are undernourished or fasted

(Ingle *et al.* 1973; Sano *et al.* 1999). The level of FFA in plasma of sheep serves therefore as a good indicator of the degree of body fat mobilisation.

Sufficient body reserves and lipid storage in adipose tissue may be of particular importance for pregnant sheep. In early pregnancy, lipid reserves accumulate in order to support high metabolic demands in late pregnancy and lactation (Vernon and Pond 1997). Mobilisation of lipids begins in late pregnancy to support the fast growing fetus and to prepare the ewe for lactation (Robinson 1986). A study by Vernon (1981) showed that serum FFA concentrations were significantly higher at 135 days of pregnancy compared to sheep at less than 100 days of pregnancy. This increase in FFA was accompanied by a significant decrease in the rate of fatty acid synthesis per cell and in the activity of LPL after 100 days of pregnancy. Furthermore, the mean volume of subcutaneous adipocytes increased significantly between 70 and 100 days of pregnancy and fell by 105 days of pregnancy. In a similar study by Vernon et al. (1985) it was found that lipids were deposited in the adipocytes during the first 55 days of pregnancy while deposition of lipids ceased around mid-pregnancy. After 125 days of pregnancy, fatty acid synthesis was decreased and serum FFA concentrations had increased. These studies indicate that sheep are in a catabolic state after day 100 of pregnancy. FFA released during this catabolic state can be used as energy for maternal body tissues, but plays no role as energy supplier to the gravid uterus (Pethick *et al.* 1983). As pregnancy progresses, the maternal liver relies more on the use of FFA as an energy substrate in order to spare other metabolites for different purposes such as the synthesis of glucose (Freetly and Ferrell 2000) which is the major energy substrate for the growing fetus (Bell and Ehrhardt 2002).

1.2.1.2. Glucose

Glucose is the major energy source for the brains and other tissues in most species, including sheep. In contrast to non-ruminants, sheep absorb little or no glucose from their diet and are dependent on gluconeogenesis in the liver for their glucose supply. The volatile fatty acid propionate is the main precursor for gluconeogenesis in sheep (Bergman and Wolff 1971). When food intake is reduced and supply of propionate is insufficient, the body relies on the use of other glycogenic substrates such as amino acids, lactate, acetate and fatty acids. Muscle and adipose tissue of ruminants have adapted to use these alternative substrates efficiently when glucose is limited. Storage and mobilisation of glucose is regulated by insulin and glucagon, respectively. Insulin is secreted by the pancreas and stimulates the storage of glucose in the liver and muscle as glycogen and in adipose tissue as fatty acids. However, around 60-70% of glucose utilisation is independent of insulin in sheep allowing gluconeogenesis to continue after feeding despite high glucose availability (Weekes *et al.* 2000).

Glucose requirements increase during pregnancy and are obtained from dietary precursors and body reserves. Furthermore, the uterus and placental tissues (Bell and Bauman 1997) and the fetus (Bell and Ehrhardt 2002) rely on glucose as their main fuel for metabolic processes. Sheep fetuses are not able to generate glucose by gluconeogenesis and are therefore entirely dependent on maternal glucose supply (Kalhan and Parimi 2000). In fully fed ewes, maternal plasma glucose levels do not significantly change over the different stages of pregnancy (Vernon et al., 1981). However, it has been shown that undernutrition (Luther *et al.* 2007) and a reduction in BCS (West 1996) lead to significantly lower maternal plasma glucose concentrations. Therefore, low BCS in pregnancy is likely to reduce glucose availability and affect maternal energy availability and fetal development. The development of maternal insulin resistance results in reduced sensitivity of maternal liver and peripheral tissues to insulin, decreased storage of glucose in maternal body tissues and therefore higher availability of glucose for the fetus (Prior and Christenson 1978; Leturque *et al.* 1984). Maternal insulin resistance does not affect glucose uptake by the uterus, because this is mainly independent of insulin (Hay *et al.* 1983). Therefore, insulin resistance favours glucose supply to the fetus. During times of food restriction, this adaptation may serve to protect the fetus from reductions in glucose. In contrast, the higher insulin resistance in moderate versus lean BCS ewes results in a higher glucose supply to the fetus leading to fatter offspring (McNeill *et al.* 1997b), which may improve the survival chances of the newborn lamb.

1.2.1.3. β-hydroxybutyrate

The digestion of food in ruminants depends largely on microbial fermentation in the rumen. In fully fed sheep, the end products of carbohydrate fermentation acetate and butyrate are converted into the ketone bodies acetoacetate and β-hydroxybutyrate (β-HBA) during absorption across the rumen epithelium (reviewed by Bergman and Wolff 1971). The liver also secretes ketone bodies, using FFA as the main precursor. In fasted sheep, secretion of ketone bodies from the rumen ceases completely due to lack of substrates and the liver becomes the main source of ketone bodies. Furthermore, the amount of ketone bodies secreted from the liver dramatically increases during fasting, and this is accompanied by an increase in hepatic uptake of FFA (Heitmann *et al.* 1986). This is an important adaptation during insufficient energy availability and provides several tissues with ketone bodies as an energy substrate, including the heart, kidney, skeletal muscle and mammary gland (reviewed by Heitmann *et al.* 1987). This saves glucose for important tissues that cannot use ketone bodies efficiently as energy substrates, such as the brain (Pell and Bergman 1983) and developing fetus (Bell and Ehrhardt 2002).

Because β-HBA is part of an important glucose sparing process during periods of undernutrition, it is a potential indicator of the metabolic status in sheep. It has been shown that plasma concentrations of β-HBA increase with long-term food restriction in pregnant ewes (Charismiadou *et al.* 2000; Banchero *et al.* 2006) and depend on the BCS of non-pregnant/non-lactating sheep (Caldeira *et al.* 2007a), although not all studies have found an effect of feeding level on β-HBA (Farrell *et al.* 1972b; Caldeira *et al.* 2007b). However, the ability to utilise β-HBA reduces in late pregnancy due to changes in metabolism that are not yet understood (Harmeyer and Schlumbohm 2006). This often leads to an increase in β-HBA in late pregnancy, which combined with a decreased glucose production rate (Schlumbohm and Harmeyer 2008), makes pregnant ewes susceptible to ketosis. Ketosis results from the ewe not being able to meet the nutritional demands of the growing fetus and is characterized by an increase in β-HBA and a decrease in glucose and insulin (Scott *et al.* 1995) and can lead to clinical symptoms of disease (Van Saun 2000). It has been shown that twin-bearing ewes are particularly at risk of developing ketosis (Schlumbohm and Harmeyer 2008).

1.2.1.4. Urea

Protein is an important macronutrient obtained from the diet. Bacteria ferment ingested protein in the rumen resulting in the production of ammonia (Annison 1956), which is absorbed through the intestinal wall and converted to urea in the liver. In ruminants, blood urea then either exits the body through urine or re-enters the digestive system via saliva or directly through the intestinal wall (reviewed by Huntington and Archibeque 2000). Therefore, urea is not just an end product of protein metabolism, but provides an important source of nitrogen (N) for bacterial protein synthesis. This urea recycling mechanism allows ruminants to keep N supply to the rumen relatively constant during periods of low dietary protein intake, without using body tissues as an N source (Whitelaw *et al.* 1990; Sarraseca *et al.* 1998). Furthermore, it has been shown that infusion with protein leads to a significant increase in plasma urea concentrations (Potter and Bergen 1974). The feeding level and the protein content of the diet also influence urea plasma concentrations (Preston *et al.* 1965; Torell *et al.* 1974; Amos *et al.* 1976; Hoaglund *et al.* 1992). In addition, there are some indications that BCS affects urea concentrations (Caldeira *et al.* 2007b). Therefore, urea provides a good indication of the protein status of the animal.

1.2.2 Endocrine signals

1.2.2.1. Leptin

Other than the metabolism and storage of body lipids, adipose tissue also has an endocrine function. Its main endocrine secretive product is leptin. Leptin is a peptide hormone, which plays a role in the regulation of energy homeostasis by acting on the hypothalamus (Henry 2003; Shimizu *et al.* 2007). Leptin suppresses appetite, decreases food intake and increases fat mobilisation and energy expenditure (Friedman and Halaas 1998; Wynne *et al.* 2005). Morrison et al. have shown that intracerebroventricular (ICV) administration (2001), but not peripheral administration (2002) of leptin decreases food intake in lambs. It is also involved in the control and regulation of reproduction and immune functions (Houseknecht *et al.* 1998). Adipose tissue is the main site of leptin production, but small quantities of leptin are produced in the stomach of rodents (Bado *et al.* 1998) and placenta of humans (Masuzaki *et al.* 1997). However, studies in sheep found evidence of only very low levels of leptin expression in the ovine placenta (Thomas *et al.* 2001; Bispham *et al.* 2003). Leptin secretion is episodic and does not appear to follow a diurnal rhythm in sheep, in contrast to other species (Blache *et al.* 2000; Daniel *et al.* 2002; Delavaud *et al.* 2007).

Leptin levels can be influenced by short-term changes in food intake (Delavaud *et al.* 2007) and are a good reflection of body fat mass in sheep (Delavaud *et al.* 2000; Daniel *et al.* 2002), rodents (Frederich *et al.* 1995) and humans (Maffei *et al.* 1995). Blanche et al. (2000) subjected ewes and rams with an average BCS of 1.75 to a high, medium and low feeding level for a period of 2 months. At the end of the 2 months, the sheep had reached BCS of 2.54, 2.00 and 1.21, respectively. The plasma leptin concentrations increased according to BCS in the medium and high feeding levels, while no changes in leptin in animals on the low feeding level were observed. Miller et al. (2007) fed rams with a BCS of 2.1 *ad libitum* to increase BCS, rams with a BCS of 2.8 at maintenance to maintain BCS and rams with a BCS of 3.2 restricted to decrease BCS for a period of 6 weeks. At the end of the 6 weeks, the rams had similar BCS while plasma leptin concentrations had increased in the increasing BCS but remained unchanged in the stable and decreasing BSC groups. Therefore, changes in leptin concentrations are a reflection of changes in BCS in sheep, and seem to respond in particular to increased BCS.

Recent studies have found that leptin concentrations vary according to pregnancy stage. In a study by Erhardt et al. (2001) ewes were fed at maintenance throughout pregnancy and insulin, glucose and NEFA concentrations did not vary according to stage of pregnancy, indicating that ewes were in equilibrium energy balance and did not enter a catabolic state in late-pregnancy. Despite the stable diet and stable maternal LW, leptin was found to increase twofold during mid-pregnancy and decrease through late-pregnancy and lactation. The increased leptin levels in mid-pregnancy were associated with a 2.3 fold increase in maternal white adipose tissue leptin gene expression. Kulcsár et al. (2006) also found a leptin peak in mid-pregnancy in ewes. These patterns of leptin gene expression and secretion also correspond to findings in goats (Bonnet *et al.* 2005), rats (Chien *et al.* 1997), mice (Gavrilova *et al.* 1997) and humans (Masuzaki *et al.* 1997; Highman *et al.* 1998; Lage *et al.* 1999). In contrast, it has also been shown that leptin is a good reflection of body fatness in pregnant ewes with high fed ewes having significantly higher plasma leptin concentrations compared to restricted or moderately fed ewes (Thomas *et al.* 2001; Bispham *et al.* 2003). These findings imply that feed intake and body fat mass may not the only factors regulating the expression of leptin during pregnancy. It has not yet been established what these other factors are. Placental leptin is a potential candidate based on studies in rodents and humans (Masuzaki *et al.* 1997). However, sheep express negligible amounts of leptin in the placenta and therefore this source is unlikely to play a role in the pregnancy-related increase in leptin (Thomas *et al.* 2001; Bispham *et al.* 2003). Insulin has been shown to be an important regulator of leptin expression and secretion in non-pregnant/non-lactating rats (Barr *et al.* 1997), humans (Lee *et al.* 2007) and sheep (Asakuma *et al.* 2003) and may therefore also be involved in the regulation of leptin in pregnancy, although the role of insulin in leptin regulation in sheep has been questioned (Kauter *et al.* 2000).

There are some indications that leptin does not suppress food intake during pregnancy, despite the increase in mid-pregnancy. It has been found that rats accumulate large quantities of body fat in early pregnancy and increase their food intake dramatically (Naismith *et al.* 1982). This is accompanied by a rise of plasma leptin levels according to the increase in body fat (Chien *et al.* 1997). Despite the increased leptin levels, there is no evidence of a decreased food intake during pregnancy as would normally occur in non-pregnant/non-lactating rats (Mounzih *et al.* 1998). Therefore, it appears that animals are in a leptin resistant state during pregnancy. The study by Thomas et al. (2001) that showed overfed sheep became fat despite high levels of leptin also suggests leptin resistance in pregnant sheep. Leptin resistance during pregnancy allows an increase in maternal food intake despite large stores of body reserves and may serve to prepare the mother for the large metabolic demands of lactation. The exact mechanisms of leptin resistance are not known but pregnancy-related hormonal changes, changes in leptin transport into the brain, alterations in leptin signal transduction (Ladyman and Grattan 2005; Grattan *et al.* 2007) and binding to a soluble receptor (Gavrilova *et al.* 1997) have been proposed.

1.2.2.2. Insulin

Like leptin, insulin is also an adiposity signal that reflects the body fat stores and long-term feeding levels of sheep (Bergman *et al.* 1989; Rhind *et al.* 2000; Caldeira *et al.* 2007b, a; Miller *et al.* 2007) as well as short-term changes in food intake (Sano *et al.* 1995; Henry *et al.* 2004). Insulin secretion patterns are diurnal in most species (Kalsbeek *et al.* 2010). Insulin is transported into the brain by a receptor mediated mechanism. Upon entering the brain, it acts as an anorexigenic signal and decreases food intake and LW (Baskin *et al.* 1999; Wynne *et al.* 2005). In sheep it has been shown that a six day intercerebral infusion with insulin decreases food intake by 40% and also decreases LW compared to control sheep (Foster *et al.* 1991). The majority of insulin receptors in the brain are found in the olfactory bulb and the hypothalamus (Marks *et al.* 1990).

Insulin is also an indicator of body fatness in sheep, with insulin concentrations generally lower in lean compared to fat sheep (Bergman *et al.* 1989; Caldeira *et al.* 2007b, a). It has been shown that the feeding level can change insulin sensitivity and responsiveness. For example, the insulin response after an injection with glucose was larger in obese sheep than in lean sheep (McCann *et al.* 1986; McNeill *et al.* 1997a). Similar results were found by Bergman et al. (1989) who showed reduced insulin sensitivity in severely obese sheep compared to lean sheep. These findings suggest that the obese condition leads to a state of insulin resistance. Bergman et al. (1989) suggested that insulin resistance has two components: reduced insulin sensitivity due to reduced insulin receptors in peripheral tissues and reduced tissue responsiveness to insulin caused by changes in post receptor signals. In food restricted sheep, enhanced tissue responsiveness to insulin, but not sensitivity, was found compared to maintenance fed sheep (Sano *et al.* 1999). This indicates that body tissues adapt to undernutrition by enhancing the response to insulin.

As discussed previously, the development of maternal insulin resistance ensures sufficient glucose availability for the uterus and the fetus. Maternal plasma insulin concentrations generally peak between days 70 and 100 of pregnancy in fully fed ewes and a decrease after day 105 (Vernon *et al.* 1981). However, there is some evidence that an undernutrition period around the time of conception impairs this normal development of maternal insulin resistance (Jaquiery *et al.* 2009), which may have implications for fetal growth and development.

1.2.2.3. Ghrelin

Ghrelin is a peptide hormone secreted from the oxyntic cells in the stomach and is released into the bloodstream (Kojima *et al.* 1999; Date *et al.* 2000a). Ghrelin binds to the growth hormone secretagogue receptor (GHS-R), which is found in the pituitary and hypothalamus (Howard *et al.* 1996; McKee *et al.* 1997). One of its functions is the stimulation of growth hormone (GH) secretion from the pituitary (Date *et al.* 2000b), along with GH-releasing hormone and somatostatin. Another function of ghrelin is its involvement in the regulation of energy homeostasis and food intake. In rodents, Tschöp et al. (2000) found that levels of ghrelin increased during fasting and decreased after refeeding. They also showed that daily peripheral administration of ghrelin resulted in increased weight gain. Furthermore, intracerebroventricular administration of ghrelin leads to a dose-dependent increase in LW and food intake, resulting from decreased fat utilisation without changing energy expenditure or locomotor activity. Similar results were found by others in rats (Wren *et al.* 2000; Nakazato *et al.* 2001; Murakami *et al.* 2002; Olszewski *et al.* 2003). In addition, ghrelin increases the time spent eating in pigs (Salfen *et al.* 2004) and cows (Wertz-Lutz *et al.* 2006). Therefore, ghrelin has an orexigenic effect by stimulating food intake and body fatness. In sheep, it has been shown that the expression of GHS-R in the pituitary gland is higher in lean than in fat animals (Kurose *et al.* 2005; French *et al.* 2006), indicating that ghrelin might also be involved in the regulation of body fatness in sheep. However, peripheral and intracerebroventricular administration of ghrelin in sheep led to increased GH secretion but did not result in an increase in food intake in sheep (Iqbal *et al.* 2006), which suggests that the effects of ghrelin on food intake are different between species. However, it has been shown that mice genetically deficient in ghrelin (ghrelin-null mice) had normal responses to starvation and diet-induced obesity compared to wild-type mice, suggesting that ghrelin may not be an essential component to the regulation of food intake (Sun *et al.* 2003).

Circulating ghrelin concentrations may be a good indicator of the level of food intake in sheep. Sugino et al. (2002a; 2002b) observed a transient surge in plasma ghrelin concentrations shortly before a scheduled meal in sheep. Within 1 h after feeding, plasma ghrelin dropped to minimal values and slowly rose again to peak before the next scheduled meal. Pseudo-fed (i.e., sheep trained to expect food, but without actually being fed) sheep also had a peak in ghrelin concentrations shortly before pseudo-feeding, and levels slightly declined after pseudo-feeding but rose again and remained higher compared to sheep that had been fed. No significant peaks in ghrelin secretion were found in *ad libitum* fed sheep. This secretion pattern suggests that ghrelin is secreted in anticipation of a meal and not upon contact with ingesta or nutrients. Ghrelin may therefore be an anticipatory factor that could play a role in meal initiation. In humans, Cummings et al. (2001) showed that ghrelin secretion peaked shortly before a meal and reduced within 1 h after a meal. This pattern was reciprocal to insulin secretion. Furthermore, intermeal ghrelin levels showed a diurnal pattern that was in phase with leptin secretion. It is therefore possible that insulin, leptin and ghrelin are coordinately regulated, or that they influence each others' secretion, which has been suggested by several authors (Toshinai *et al.* 2001; McCowen *et al.* 2002; Takahashi *et al.* 2006; Takahashi *et al.* 2007) but questioned by others (Broglio *et al.* 2002). Another possibility is that ghrelin secretion is regulated by the autonomic nervous system through the vagus nerve, which projects to the gastro-intestinal tract and interacts with the oxyntic glands (Berthoud 2008). Sugino et al. (2003) found that administration of a cholinergic antagonist in sheep increased ghrelin secretion while a cholinergic agonist decreased ghrelin secretion, suggesting that cholinergic activity affects ghrelin secretion. Such an effect has also been demonstrated for the vagal control of gastrin release in the stomach (Andersson 1973; Walsh 1975). However, Broglio et al. (2003) found no evidence for cholinergic regulation of ghrelin secretion in humans.

Because ghrelin stimulates food intake and body fatness in non-pregnant/non-lactating animals, it is likely that is also affects food intake and body fatness in pregnant animals. Apart from secretion from the stomach, ghrelin and its messenger ribonucleic acid (mRNA) have also been found in the ovaries of rats (Caminos *et al.* 2003) and sheep (Miller *et al.* 2005) and placenta of rats and humans (Gualillo *et al.* 2001). Ghrelin appears to peak during pregnancy, but the timing of the peak may differ between tissues and species; ghrelin peaks in early pregnancy in the rat ovaries (Caminos *et al.* 2003), in early pregnancy in the pig (Govoni *et al.* 2007), in early-pregnancy in the human placenta and mid-pregnancy in rat placenta (Gualillo *et al.* 2001), and fasting levels of serum ghrelin are highest around mid-pregnancy in humans (Fuglsang *et al.* 2005; Palik *et al.* 2007). In sheep, ghrelin is present in the ovary throughout pregnancy, with the highest concentrations in the first half of pregnancy (Du *et al.* 2009). However, it is unclear if ghrelin corresponds to maternal body fatness levels in mid-pregnancy in humans, with some finding no evidence (Fuglsang *et al.* 2005) and others finding a negative correlation with maternal body fatness (Palik *et al.* 2007). The involvement of ghrelin in the regulation of LW, BCS and food intake associated with pregnancy is therefore uncertain. Only one study, to my knowledge, has measured the effect of intramuscular ghrelin administration on food intake in pregnant ewes. Negligible effects of food intake were found, but ghrelin decreased insulin and FFA and increased GH concentrations (Melendez *et al.* 2006). The role of ghrelin during pregnancy and patterns of secretion in pregnant sheep remain to be investigated.

1.2.2.4. Insulin-like growth factor-1

Insulin-like growth factor-1 (IGF-1) is one of the components of the somatotropic axis, which consists of growth hormone (GH), IGFs (several types exist) and their associated receptors and binding proteins. The majority of circulating IGF-1 is bound to binding proteins (IGFBP) from which six different types have been identified. These IGFBP play a major role in the coordination and regulation of the biological actions of IGFs (reviewed by Jones and Clemmons 1995). IGF-1 is secreted into the circulation from the liver in response to GH and has an endocrine function. Many tissues and bone mass also synthesise IGF-1, where it promotes cell differentiation and proliferation when stimulated by GH. Therefore, IGF-1 also acts in an autocrine and paracrine manner (reviewed by Breier 1999). IGF-1 also functions independently of GH. IGF-1 administration induces a state of hypoglycemia by increasing peripheral glucose uptake in rats (Jacob *et al.* 1989). Furthermore, IGF-1 administration has anabolic effects on protein synthesis and nitrogen balance and reduces protein breakdown in sheep (Jacob *et al.* 1989; Douglas *et al.* 1991; Koea *et al.* 1992; Tomas *et al.* 1992). Therefore, IGF-1 has both GH-like and insulin-like actions.

Circulating IGF-1 concentrations are under nutritional control. In both sheep and cows it has been shown that long-term undernutrition results in significantly lower concentrations of IGF-1 (Breier *et al.* 1986; Bass *et al.* 1991; Osgerby *et al.* 2003b). Furthermore, ewes with a BCS of 3.6 have higher levels of IGF-1 compared to ewes with a BCS of 2.2 (Meza-Herrera *et al.* 2007). The main dietary factor controlling IGF-1 concentrations appears to be the protein content of the diet. Protein supplementation in pregnant dairy cows has been shown to increase IGF-1 concentrations (Perry *et al.* 2002). However, a short-term increase in dietary protein did not affect serum IGF-1 concentrations in ewes (Meza-Herrera *et al.* 2007). Another study in lambs showed that energy and protein restriction abolish the IGF-1 increase in response to GH, while energy restriction with protein supplementation restores the IGF-1 response to GH (Kriel *et al.* 1992).

The reduction in IGF-1 response to GH may be an important mechanism to maintain energy homeostasis rather than growth and development during protein restriction.

Recently, an important role of IGF-1 in fetal and placental growth has been identified. The sheep placenta (Lacroix *et al.* 1995; Reynolds *et al.* 1997) and fetus (Butler and Gluckman 1986; Owens *et al.* 1994) both produce IGF-1 and associated binding proteins. The importance of IGF-1 during fetal development has become evident from studies in IGF-1 knock-out mice. These mice are born at only 60% of LW of wild type mice and over 95% of them die shortly after birth (Powell-Braxton *et al.* 1993), suggesting a crucial role for IGF-1 in fetal development. The nutrition of the ewe also has an important influence on fetal development through IGF-1. It has been shown that maternal nutrient restriction significantly reduces hepatic fetal IGF-1 (Bauer *et al.* 1995; Brameld *et al.* 2000) and placental IGFBPs (Osgerby *et al.* 2003b, a) in sheep. This could have consequences for the growth and development of the fetus because IGF-1 has anabolic effects on fetal protein and carbohydrate metabolism (Harding *et al.* 1994; Liu *et al.* 1994).

1.2.2.5. Cortisol

Cortisol is one of the output factors of the hypothalamo-pituitary-adrenal (HPA) axis. Two main neuropeptides called corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) are released from the hypothalamus upon activation of the HPA-axis. These peptides act on the pituitary to release adrenocorticotrophin (ACTH), which stimulates the adrenals to produce glucocorticoids, including cortisol. Cortisol, in turn, gives a negative feedback signal to the hypothalamus, which results in the HPA-axis returning to baseline values after stimulation. The HPA-axis plays a role in the classical stress response to environmental challenges. Cortisol has many metabolic effects in the body, including mostly catabolic effects (proteolytic and lipolytic) in peripheral tissues and anabolic effects in the liver, including gluconeogenesis and protein synthesis (Sapolsky *et al.* 2000). Cortisol also reduces the amount of glucose entering cells, and therefore increases plasma glucose and insulin concentrations (McMahon *et al.* 1988). Plasma concentrations of cortisol vary throughout the day, with highest concentrations measured around mid-night and lowest around mid-day in sheep (Fulkerson and Tang 1979).

Cortisol may also play a role in the regulation of food intake. For example, cortisol administration for 21 d has been shown to increase food intake in sheep (Bassett 1963) and corticosterone implants in the hypothalamus stimulate carbohydrate intake in adrenalectomised rats (Tempel and Leibowitz 1989). Furthermore, adrenalectomy decreases feeding and food seeking behaviour while dexamethosone restores food intake (Santana *et al.* 1995). In sheep, it has been shown that the effects of cortisol on food intake depend on the time of the year, with a larger increase in food intake observed in August compared to January (southern hemisphere), which most likely relates to fluctuations in cortisol and food intake depending on circannual rhythms (Henry *et al.* 2010). However, acute stressors have also been shown to reduce food intake (Shibasaki *et al.* 1988; Shimizu *et al.* 1989). The exact mechanisms of how cortisol and other HPA-axis components regulate feeding behaviour are not yet completely understood, but a link between chronically increased cortisol, increased food intake and a resulting increase in body fat has been suggested in rats (la Fleur 2006).

1.2.3 Effects of endocrine signals on food intake

The effects of ghrelin, insulin and leptin on feeding motivation have not been entirely unravelled, but it has been established that the hypothalamus plays a major role in modulating their effects. The neurons of the arcuate nucleus in the hypothalamus contain several subpopulations of neurons that produce neuropeptides with either anabolic or catabolic effects. One subpopulation expresses neuropeptide Y (NPY) and agouti-related protein (AgRP), which have mostly anabolic effects. In rats, fasting results in an increased mRNA expression of both peptides, and they are thought to have important stimulatory effects on food intake (Hahn *et al.* 1998). Furthermore, injection of NPY in the paraventricular nucleus results in a dramatic increase in food intake and LW gain in rodents (Stanley *et al.* 1986). In sheep, there is some evidence that hypothalamic AgRP expression is decreased in *ad libitum* fed wethers compared to food restricted wethers (Relling *et al.* 2010).

A different subpopulation of neurons in the arcuate nucleus expresses a neuropeptide called Pro-opiomelanocortin (POMC), which has mostly catabolic effects. Activation of POMC neurons results in expression of α-melanocyte stimulating hormone (α-MSH), which has inhibitory effects on food intake (Cone 1999). It has been shown that a melanocortin receptor agonist inhibits feeding while a receptor antagonist increases feeding in rats (Fan *et al.* 1997). Low insulin and leptin plasma concentrations as a result of a reduction in body fat are associated with an activation of NPY/AGRP expression and an inhibition of POMC expression, while an increase in insulin and leptin as a result of body fat gain has the opposite effect (reviewed by Schwartz *et al.* 2000). Therefore, the effects of insulin and leptin on food intake is likely to be mediated by hypothalamic neuropeptides that can have either inhibiting or stimulating effects on food intake depending on the concentrations of insulin and leptin. However, recent evidence questions the role of leptin in activating neuropeptides in the arcuate nucleus in sheep. It has been found that leptin does not have a primary action on the arcuate nucleus, but may exert direct effects on the dorsal medial, paraventricular and ventromedial nuclei of the hypothalamus (Qi *et al.* 2010). Other structures in the hypothalamus may also be involved in regulation of feeding. The perifornical and lateral hypothalamus receive input from both AgRP/NPY and α-MSH containing neurons and express the neuropeptides melanin-concentrating hormone (MHC) and orexin (Elias *et al.* 1998). In sheep, it has been shown that orexin and MHC are inversely correlated with adiposity, being high in genetically selected lean sheep and low in genetically selected fat sheep (Anukulkitch *et al.* 2010). The effects of endocrine signals on food intake are therefore complex, and a number of different neuropeptides and brain structures may be involved in the regulation.

1.2.4 Short-term and long-term regulation of feeding motivation

The short-term (hours) regulation may be different from long-term (weeks-months) regulation of food intake and motivation. Short-term regulation of food intake is partly determined by gut fill and it has been shown that there is a negative relationship between the amount of digesta in the rumen and voluntary food intake in sheep (Church *et al.* 1980; Allen 1996). Upon contact with digesta, distension of the reticulo-rumen activates mechano-receptors and specific nutrients (i.e., glucose, FFA, amino acids) stimulate chemo-receptors in the rumen wall that provide afferent information to the hindbrain via the vagus nerve (Forbes 1996; Havel 2001). Furthermore, several peptides (i.e, gastrin, cholesystokinin (CCK), pepsin and ghrelin) are secreted in response to the presence of digesta (Huntington and Eisemann 1988; Hayashida *et al.* 2001). Together, these signals result in the regulation of satiety and the end of a meal.

As discussed in above, endocrine signals such as insulin, leptin and ghrelin directly stimulate food intake in sheep. Therefore, it is likely that long-term regulation of feeding motivation is driven by endocrine signals that are a reflection of body fatness and nutritional status. In contrast, short-term feeding motivation may be more driven by rumen fill. However, long-term signals cannot be entirely separated from short-term regulatory signals because both signals will need to be integrated in order to maintain energy homeostasis. Furthermore, the effects of long-term food restriction may affect short-term food intake. Sibbald and Rhind (1997) have shown that voluntary food intake is increased in sheep that had a previous low BCS (2.15) compared to sheep with a previous high BCS (3.15), even after weeks of *ad libitum* feeding when BCS were no longer different. In addition, the insulin response to a meal and a glucose injection was decreased in previously low BCS, suggesting that long-term changes in endocrine signals had occurred as a consequence of low BCS. Therefore, short-term signals can be overridden by long-term regulatory signals. It is likely that food restricted animals will have altered short-term and long-term signals that may affect the motivation for food.

1.3. Other indicators of biological functioning

1.3.1 Wool growth

Changes in nutrient availability may affect wool growth, and poor wool production may be an indicator of metabolic status in sheep with a low BCS. Research has shown that restricted feeding can reduce wool growth in pre-weaned lambs (Allden 1968a, b). However, when food intake was increased, lambs were able to recover from the undernutrition period and had normal wool production at 23 months after weaning. Similar results have been found in weaner lambs (Olivier and Olivier 2005). Furthermore, a relationship has been found between LW, wool growth and staple strength. Weaner wethers with a low LW produced significantly less wool and with a lower staple strength than heavier weaners (Masters *et al.* 1998). In adult sheep, undernutrition also significantly reduced wool growth (Adams *et al.* 2002; Li *et al.* 2006). Therefore, poor metabolic status and low BCS may affect wool production negatively.

1.3.2 Effects of maternal food restriction on the weight of the lambs

Food restricted ewes with little substrate availability may potentially give birth to smaller and lighter lambs. Undernutrition in well-fed ewes decreased fetal growth rate by 40%, and the loss in growth could not be compensated for by refeeding of the ewes (reviewed by Mellor 1987). Others have found a reduction in birth weight in lambs born to light ewes compared to heavy ewes (Clarke *et al.* 1997) and a tendency for lower fetal weights at day 135 of pregnancy in ewes fed 70% of maintenance requirements after day 22 of pregnancy (Osgerby *et al.* 2002). Other than feed restriction during pregnancy, LW and BCS at mating may be an important determinant of fetal growth and lamb birth weight (Greenwood *et al.* 2000). However, other studies showed that maternal feed restriction of singleton-bearing ewes for 4 weeks during mid-pregnancy did not affect the birth weight of the lambs (Oddy and Holst 1991; Holst *et al.* 1992). Also, a 5 day fast in mid-pregnancy did not affect lamb birth weight (McMullen *et al.* 2005). This suggests that the sensitivity of fetal and lamb birth weight to feed restriction may depend on the stage of pregnancy and that the impact of food restriction during pregnancy may be related to the BCS at mating or LW gain during pregnancy.

The impact of food restriction on fetal growth and development may be mediated by the placenta. The placenta is directly involved in the partitioning of nutrients to the fetus and therefore has a large impact on growth and development (reviewed by Wallace *et al.* 2001; Bell and Ehrhardt 2002). Within the placenta, the placentomes are the site of fetal and maternal circulation exchange, and consists of a fetal part (cotyledons) and a maternal part (caruncle). Fetal growth and the capacity of the placenta to supply nutrients to the fetus are highly dependent on the number of placentomes in the placenta (Bell and Ehrhardt 2002). It has been shown that a low BCS in mid-gestation reduces the number of placentomes and fetal size (Osgerby *et al.* 2003b, a) and that feed restriction in early to mid-gestation reduces placental weight (Heasman *et al.* 1999; Osgerby *et al.* 2002; McMullen *et al.* 2005).

The alteration of the placental mass resulting from a restricted diet may be an adaptive mechanism to maternal feed restriction and low BCS (Symonds *et al.* 2001). Placental weight is highly correlated to lamb birth weight (Mellor and Murray 1981; Dwyer *et al.* 2005), and it is therefore possible that maternal nutrition and BCS influence fetal growth and birth weight through modulation of the placenta. Several experiments have shown that growth restriction of the placenta lead to growth restriction of the fetus (Boyle *et al.* 1996; Murotsuki *et al.* 1997). Growth restricted fetuses have lower birth weights than non-growth restricted fetuses (Wallace *et al.* 2005; De Blasio *et al.* 2007) and low birth weight has been associated with lower survival rates (Clarke *et al.* 1997; De Blasio *et al.* 2007) and poorer health in later life (Barker 1997; Godfrey and Robinson 1998). Low BCS, therefore, may have consequences for the productivity of ewes, as it may reduce birth weight, lamb survival and reduce the health of the lambs.

1.3.3 The importance of adequate body condition score for twin-bearing ewes

Most of the previously mentioned studies have focused on singleton-bearing ewes. However, many lambs are born from multiple pregnancies. The average lambing percentage in New Zealand was 122.1% according to Meat and Wool New Zealand (2007) and 128% according to the New Zealand government statistics (2006). The nutritional demands of twin-bearing ewes may be higher than singleton-bearing ewes. Ewes need to supply both fetuses with sufficient nutrients and therefore require a larger and more efficient placenta. The larger placenta and fetal number increases demands for uterine blood flow and the transport of oxygen and nutrients. Research has shown that the placenta of twin-bearing ewes is more efficient, heavier and with more placentomes than that of single-bearing ewes, but has a reduced placental mass and number of placentomes per individual fetus (Greenwood *et al.* 2000; Dwyer and Lawrence 2005; Grazul-Bilska *et al.* 2006). This has consequences for fetal growth and generally results in a lower birth weights of twin lambs compared to singleton lambs (Gootwine and Rozov 2006). Furthermore, the mortality rate of newborn twin lambs is higher than that of singletons, which most likely relates to prematurity and lower birth weight due to nutritional constraints (Davies 2005; Luke 2005; Rosello-Soberon *et al.* 2005). It has been suggested that healthy and fully fed twin-bearing sheep may not be able to meet their fetuses' nutritional demands (Greenwood *et al.* 2000; Gootwine *et al.* 2007). Therefore, twin-bearing ewes have high metabolic requirement and their welfare may be more affected by food restriction compared to dry or single-bearing ewes. In the experiments conducted in this thesis a main focus will be on the effects of food restriction on the welfare of twin-bearing ewes.

1.4. Cold exposure and adaptation

As previously mentioned, the level of biological functioning is likely to have a major impact on ewe welfare. Of particular interested is the metabolic aspect of the biological functioning in food restricted ewes. In the previous sections, some of the metabolic and endocrine signals that may be altered when ewes are food restricted and subjected to BCS loss have been discussed. Therefore, the measurement of metabolic and endocrine responses to a change in BCS could provide indicators of the metabolic functioning of the ewes. However, food restricted ewes are likely to encounter additional environmental challenges in a farming environment, such as cold exposure. Therefore, the ability to maintain energy homeostasis and induce appropriate adaptive responses during environmental challenges may provide a deeper insight into the level of biological functioning in food restricted ewes.

Extensively reared sheep are outdoors on pasture all year around, and require sufficient physiological and behavioural adaptations to cold conditions. A first barrier that protects sheep from cold (and heat) is the insulating fleece that grows on its body surface. However, it is common practice to shear sheep in mid-pregnancy to improve lamb growth and survival. Lamb survival is improved by shearing because ewes are more willing to look for shelter when exposed to cold thereby protecting newborn lambs from harsh conditions (Lynch and Alexander 1977; Lynch *et al.* 1980). Furthermore, mid-pregnancy shearing has been shown to result in higher lamb birth weights (Revell *et al.* 2000; Corner *et al.* 2006; Kenyon *et al.* 2006). Shearing during winter months, however, could make ewes more susceptible to cold stress. It has been shown that mid-pregnancy shearing can lead to higher ewe mortality rates due to cold stress (Dabiri *et al.* 1995). High mortality rates have also been observed when shorn sheep were undernourished and subjected to cold stress (Panaretto 1968). In addition, sheep that lost LW prior to shearing were more likely to die during winter, while LW at shearing had no effect on mortality. Deaths after shearing could not be prevented by hay supplementation (Hutchinson and McRae 1969). Therefore, shearing and cold exposure impose an additional challenge to food restricted ewes.

1.4.1 Thermoregulation

Healthy mammals keep their internal body temperature constant even though the environmental temperature is much lower or higher than their body temperature. Within the environmental thermoneutral zone, the animal is not required to invest energy in thermoregulation. The heat production from sustaining vital functions is sufficient for maintenance of body temperature and this is called obligatory thermogenesis (Silva *et al.* 2006). However, a mechanism that prevents the body temperature from decreasing is needed when the environmental temperature drops below the lower critical temperature. Thermal sensory receptors located in the skin measure the skin temperature (T_{skin}) and thermal receptors located in the preoptic area (POA) of the hypothalamus, anterior hypothalamus, brain stem and spinal cord measure the core temperature (T_{core}) (Bicego *et al.* 2007). These temperature signals are integrated by the central nervous system (CNS) with information on heat exchange with the environment, diet and LW (Young 1981;

Bachman *et al.* 2002; Lowell and Bachman 2003; Bicego *et al.* 2007). When the T_{core} deviates from a reference, or set-point temperature, thermoregulatory reflexes result in increased heat conservation and heat production. The main thermo-integrative and thermoregulatory area in the brain is the POA, which contains warm-sensitive neurons, cold-sensitive neurons and temperature insensitive neurons (Boulant 1998; Griffin *et al.* 2001). The warm-sensitive neurons are more abundant than cold-sensitive neurons (Boulant 1998) and inhibitory effects of warm-sensitive neurons contribute more to heat production then cold-sensitive neurons (Kanosue *et al.* 1999).

The first response to cold exposure is vasoconstriction of the blood vessels in the skin. The cutaneous circulation is a major effector of thermoregulation and the skin contains both adrenergic sympathetic vasoconstrictor nerves and cholinergic sympathetic vasodilator nerves that regulate skin blood flow (Kellogg 2006). Furthermore, a reduction in respiratory rate has also been observed in sheep in order to prevent heat loss (Phillips and Raghavan 1970; Schaefer *et al.* 1982). This is followed by an increase in the so-called facultative or adaptive thermogenesis (Silva 2006). There are two different forms of adaptive thermogenesis: shivering thermogenesis and non-shivering thermogenesis. Shivering thermogenesis is defined as the involuntary shaking of the body due to muscle contractions resulting in heat production (Blood and Studdert 1998). The shivering response is mainly controlled by efferent inhibitory signals running from the POA to the posterior hypothalamus in response to reductions in T_{skin} and T_{core} (Nagashima *et al.* 2000). Non-shivering thermogenesis results from an enhancement of normal calorigenic processes and leads to increased heat production (Blood and Studdert 1998). The exact mechanisms of non-shivering thermogenesis in non-rodent mammals are not yet understood. However, it is clear that non-shivering thermogenesis is partly a result of an increased metabolic rate and increased heat production in muscle (Block 1994; Silva 2006; Bicego *et al.* 2007). In sheep, it has been demonstrated that short-term cold exposure results an increased heart rate (Slee 1972) and long-term cold exposure to reductions in LW and increased food intake (Sano *et al.* 1995; Moibi and Okine 2000). Recent research has also demonstrated a clear effect of food intake on thermogenesis (postprandial thermogenesis) in muscle and fat (Henry *et al.* 2008), indicating that non-shivering thermogenesis also exists in non-rodent mammals. However, the exact mechanisms of non-shivering thermogenesis during cold exposure in ruminants are still unclear.

The ability to increase metabolic rate is largely dependent on food intake and body fatness. Epke and Christopherson (2000) subjected 6 month old lambs to an *ad libitum* or a restricted diet (135% of maintenance requirements) and placed them in either a warm (23°C) or a cold (0°C) environment. Lambs in the cold environment on an *ad libitum* diet had the highest feed intake and an increased metabolic rate. Heat production tended to be greater in the *ad libitum* fed lambs in the cold environment compared to the *ad libitum* fed lambs in the warm environment, but heat production was reduced in feed restricted lambs in both environments. Li et al. (2000) also found an increased heat production in cold exposed sheep. In addition, they found that fasting decreased heat production in sheep kept in a warm environment but this effect was less obvious in the cold exposed sheep. Bennett (1972) showed a linear relationship between LW and metabolic rate when sheep were exposed to cold (between –0.5°C and +0.5°C). They also found that metabolism significantly reduced when sheep were fasted during cold exposure. Therefore, the increased demand for energy due to increased metabolic rate triggered by cold exposure requires a sufficient amount of body reserves and food intake. To my knowledge, no studies on the effects of different BCS on metabolic and endocrine responses to cold have been conducted in pregnant ewes.

1.4.2 Substrates for shivering thermogenesis

The adenosine triphosphate (ATP) required for heat production in muscle fibres is obtained from the oxidation of carbohydrates, lipids and protein (Haman 2006). In humans, the main substrate for heat production is glycogen, which is stored in the muscles. However, the percentage of glycogen contribution to shivering thermogenesis depends on the amount of glycogen reserves in the muscle and on the intensity of shivering (Haman *et al.* 2004a). Muscle protein content and plasma glucose (about 15%) contribute little to thermogenesis (Haman *et al.* 2004a; Haman *et al.* 2005). Haman et al. (2004a; 2004b) found that humans with few glycogen stores shifted their dependence on glycogen for thermogenesis to lipids when exposed to cold. The shift of substrate use did not affect shivering thermogenesis indicating that humans can use different substrate types without compromising heat production and shivering intensity. In rats it has been shown that glycogen is used a main substrate for shivering thermogenesis initially, but the dependence on glycogen is rapidly shifted to lipids during prolonged cold exposure, while protein only has a minor contribution (Vaillancourt *et al.* 2009). These results suggest that non-shivering thermogenesis is uncompromised as long as body reserves of either glycogen or lipids are abundant. To my knowledge, no studies have investigated the main substrates used for thermogenesis in sheep.

1.4.3 Non-shivering thermogenesis and brown adipose tissue

The body tissue that has had most attention with regards to non-shivering thermogenesis is brown adipose tissue (BAT). Small mammals (e.g., rats and mice) have substantial amounts of BAT, which is the major source of heat production in these animals (Rothwell and Stock 1985). Lambs also have substantial reserves of BAT that supplies them with heat from non-shivering thermogenesis. However, the ability of non-shivering thermogenesis in BAT in lambs declines rapidly within weeks after birth (Thompson *et al.* 1989; Lomax *et al.* 2007) and BAT is replaced by white adipose tissue (Gemmell *et al.* 1972). In humans, it was believed for a long time that adults did not possess any BAT. However, recent discovery of small amounts of functional BAT in humans and its activation during cold exposure have changed this view (Cypess *et al.* 2009; Saito *et al.* 2009; Van Marken Lichtenbelt *et al.* 2009; Virtanen *et al.* 2009). No evidence of BAT in adult sheep has been detected so far. However, the techniques used to detect BAT in the human studies could potentially also be used to study whether adult sheep possess active BAT. An alternative source of non-shivering thermogenesis in adult mammals and possibly adult sheep is muscle tissue (Block 1994). In sheep, acute and chronic cold exposure (3°C) lead to increased blood flow to skeletal muscle and also to the thyroid glands (Schaefer *et al.* 1982). However, the exact mechanisms of non-shivering thermogenesis are not known. It has been suggested that thermogenesis is under control of the central nervous system and that thyroid hormones and possibly others such as leptin and insulin are involved in its regulation.

1.4.4 Glucose metabolism during cold exposure

It has been shown that blood plasma levels of glucose are increased during cold exposure in sheep (Sano *et al.* 1995; Sano *et al.* 1999), indicating that glucose is a substrate for thermogenesis. The availability of glucose is dependent on insulin and glucagon secretion from the pancreas and cold exposure has been associated with higher glucagon (Sano *et al.* 1995) and insulin (Ekpe and Christopherson 2000) concentrations and an increased basal glucose metabolism (Takebayashi *et al.* 2003). Furthermore, the insulin response to an injection of glucose was reduced at a temperature of 0°C (Sasaki and Takahashi 1980). There are also indications that cold exposure increases tissue responsiveness to insulin (Weekes *et al.* 1983) and insulin sensitivity (Sano *et al.* 1999), which suggest that insulin binding to the receptor (insulin sensitivity) and post-receptor events in response to insulin action (tissue responsiveness) are improved during cold exposure. These adaptations may stimulate glucose availability in thermogenic body tissues in order to increase heat production. This mechanism is possibly mediated by sympatho-adrenomedullary activity (Sasaki and Takahashi 1980). The body tissues involved in thermogenesis have not been studied in sheep, but like in human thermoregulation, muscle tissue may play a role.

1.4.5 Hormonal control of thermogenesis

1.4.5.1. Thyroid hormones

Thyroid hormones play several roles in energy homeostasis in animals. Two types of thyroid hormones exist: 3-5-3'-triiodothyronine (T_3) and thyroxin (T_4). T_3 is the active form of the thyroid hormones and originates from the thyroid gland or from de-ionidisation of T_4 in peripheral tissues (Chopra *et al.* 1975; Chopra 1978). The thyroid gland releases thyroid hormones in response to thyroid stimulating hormones (TSH) from the pituitary, which in turn is stimulated by thyroid releasing hormone (TRH) from the hypothalamus. Thyroid hormones directly affect energy expenditure in animals (Dauncey 1990) by increasing ATP-turnover via stimulation of its utilisation and by reducing the efficiency of ATP synthesis; both these pathways result in increased heat production (Silva 2006). In addition, thyroid hormones have an orexigenic effect and stimulate food intake by acting on the ventromedial nucleus in the hypothalamus in rats (Kong *et al.* 2004). Furthermore, thyroid hormones are an indication of metabolic state and body fatness. In sheep, thyroid hormones are decreased during food restriction (Wester *et al.* 1995; Rhind *et al.* 2000), increased with increased LW (McCann *et al.* 1992) and vary with different BCS (Caldeira *et al.* 2007b). Thyroid hormone secretion depends on the season with higher plasma concentrations measured in periods with short day length (winter) compared to periods with longer day length (summer) in sheep (Henneman *et al.* 1955; Rhind *et al.* 2000) and goats (Todini *et al.* 2007). Increased thyroid levels in winter may serve to increase heat production and to stimulate food intake during cold exposure. However, Rhind et al. (2000) did not find a correlation between plasma thyroid levels and level of food intake throughout the year.

Because thyroid hormones affect energy expenditure and food intake, they may play a role in increased heat production with cold temperatures. Schaefer et al. (1982) exposed sheep to cold and found that blood flow to the thyroid gland was increased, indicating a higher activity. Ekpe and Christopherson (2000) kept lambs in a cold or warm environment and fed them a restricted or an *ad libitum* diet. In the first five weeks of cold exposure, they found a significant temperature by food intake interaction with food restriction in both environments reducing T_3 concentrations while *ad libitum* food intake and cold exposure increased T_3 concentrations. There was also a significant temperature by food intake interaction on T_4, but this effect was much smaller. However, a different study found no interactions between temperature and food intake in pigs and thyroid hormone levels were only influenced by level of food intake but not temperature (Dauncey 1990). Kennedy et al. (1977) also found increased T_3 and T_4 plasma levels in cold exposed sheep. These results suggest that thyroid hormones may play an important role in energy homeostasis during food restriction and cold exposure.

1.4.5.2. Leptin

As discussed previously, leptin is secreted in response to body fatness and food intake. However, temperature may also effect leptin secretion. In mice, a four week period of exposure to 4°C resulted in a significant reduction in plasma leptin levels, similar to levels observed during fasting (Korhonen and Saarela 2005). Zeyl et al. (2004) exposed healthy human male subjects to acute cold and measured plasma leptin levels. They also measured the effect of local temperature on leptin secretion in subcutaneous adipose tissue in vitro. It was found that acute cold exposure reduced plasma leptin levels in the subjects and that the local effect of a reduced temperature in subcutaneous adipose tissue was large enough to account for the reduced plasma leptin levels. This suggests that leptin secretion can be directly affected by subcutaneous adipose tissue temperature. Because leptin has a food intake and LW reducing effect and is thought to play a role in energy expenditure (heat production), leptin is a potential candidate for regulation of the increase in food intake and heat production during cold exposure.

Most of the research on the role of leptin in thermoregulation has focused on rodents that rely on BAT for thermogenesis. BAT can produce heat by means of uncoupling protein 1 (UCP1) located in the mitochondria membrane (Himms-Hagen 1985) and is also found in small quantities in white adipose tissue (WAT) and muscle in mice (Nagase *et al.* 1996). Commins et al. (1999b) showed that genetically leptin deficient obese *ob/ob* mice expressed less UCP1 mRNA and proteins in both BAT and WAT compared to normal lean mice. Treatment with leptin reduced LW and also resulted in a 4-5 fold increase in UCP1 mRNA expression and protein in BAT and WAT, indicating that leptin stimulates weight loss by increasing heat production. Rouru et al. (1999) found that leptin infusion favoured UCP1 BAT mRNA expression in lean rats. This effect of leptin on BAT UCP1 is most likely mediated by the actions of noradrenaline (Commins *et al.* 1999a). Leptin stimulates sympathetic nerve activity and noradrenaline secretion (Collins *et al.* 1996) and noradrenaline has been shown to activate UCP1 in BAT (Bouillaud *et al.* 1984).

Recent evidence of an effect of leptin on non-shivering thermogenesis in sheep has been presented by Henry et al. (2008), who showed that postprandial thermogenesis in muscle is enhanced by leptin. Also in lambs there is evidence of an effect of leptin on thermogenesis. Birth is associated with a peak in UCP1 in adipose tissue, but UCP1 is lost shortly after birth and has disappeared completely after 30 days (Mostyn *et al.* 2003). UCP1 has not been detected in body tissues other than BAT in lambs (Thompson *et al.* 1989). It has been shown that leptin administration improved thermoregulation in the first 2 days after birth in lambs. However, this was not associated with an increase in thermogenic potential of UCP1 in BAT. Furthermore, chronic leptin administration resulted in an accelerated reduction of UCP1 in BAT after birth (Mostyn *et al.* 2002). Leptin may also be required for thermogenesis that is independent of UCP1. Ukropec et al. (2006) found that leptin administration increased heat production in leptin and UCP1 deficient mice by novel thermogenic mechanisms that possibly involve the thyroid axis. They also suggested that BAT is not the target of enhanced fat oxidation by leptin during cold exposure. Also Van Marken Lichtenbelt and Daanen (2003) suggest the existence of thermoregulatory mechanisms independent of UCP1. Leptin may therefore be involved in thermoregulation in several ways with both central and local effects. The targets of leptin may include BAT. However, the existence of BAT in adults mammals is still unclear, although there is evidence of active BAT in humans (Heath 2009). Possible other targets of leptin are muscle tissue and WAT. In sheep, other mitochondrial proteins and uncoupling protein 2 (UCP2) which are present in several body tissues may play a role in thermoregulation and have been proposed as targets for leptin (Gnanalingham *et al.* 2005). Research on these subjects is limited and the involvement of leptin in thermoregulation during cold exposure in adult sheep has to my knowledge not been investigated.

1.4.6 Stress responses and cortisol

Cold exposure, or any other metabolically demanding environmental challenges, may compromise the animal’s energy homeostasis. Stress is defined as a state in which homeostasis is actually threatened or perceived to be so (Chrousos 2009). The stress response has many consequences, including increased energy mobilisation and decreased energy storage, gluconeogenesis, enhanced cardiovascular tone, stimulation of immune functions, inhibition of reproduction, decreased appetite and a heightened state of alertness and cognition (Sapolsky *et al.* 2000; Chrousos 2009). These responses allow the animal to give behaviourally and physiologically appropriate reactions to the challenge and increase survival chances. The stress response is characterised by the activation of the HPA-axis and results in the release of cortisol. As discussed previously, cortisol facilitates the availability of metabolic substrates that can be used for energy during a challenge. Therefore, the ability to mount an appropriate stress response may be of crucial importance to successfully cope with a challenge. Even though cortisol release is often interpreted as an indication of poor welfare in animal welfare literature (because many painful procedures result in the release of cortisol, see for examples: Apple *et al.* 1993; Dwyer and Bornett 2004; Fitzpatrick *et al.* 2006), I will use cortisol as a measure of the ability to mount an appropriate response to a challenge in this thesis, because of its important function in energy substrate mobilisation. Changes in cortisol, together with other metabolic alterations, could be an indirect indicator of the level of biological functioning of food restricted ewes and their ability to cope with environmental challenges.

1.5. Aims of the thesis

In this thesis, the focus will be on the welfare of food restricted ewes. One welfare aspect is the subjective experience of hunger, which could potentially be measured by assessing the strength of the motivation for food. However, a suitable methodology for the measurement of feeding motivation in sheep needs to be developed. The second welfare aspect is the level of biological functioning, which will be limited to those functions/signals that are relevant for metabolic functioning during food restriction. The metabolic functioning of the ewes will be measured by assessing the ability to maintain energy homeostasis and induce adaptive metabolic and endocrine responses during food restriction and an environmental cold challenge. Furthermore, the combination of feeding motivation with metabolic and endocrine responses to food restriction could provide useful indicators of hunger in sheep.

The aim of this thesis is to evaluate the effects of food restriction on feeding motivation, metabolic and endocrine responses and responses to a cold challenge in sheep, in order to obtain objective indicators of hunger and metabolic functioning in food restricted ewes.

1.6. Objectives

I. *To develop objective quantitative behavioural measurements to evaluate motivation for food in sheep and to measure the effects of short-term food restriction on feeding motivation.*

Objective and quantitative measures for hunger are required in order to study the effect of food restriction on welfare. As discussed in the review above, the strength of the motivation for food can provide insight in the subjective experience of hunger. Therefore, the aim was to improve and refine a methodology for the assessment of feeding motivation suitable for ewes and to apply this methodology in assessing the effects of short-term food restriction in non-pregnant/non-lactating ewes.

II. *To study the effects of body condition score on feeding motivation and metabolic and endocrine responses in pregnant ewes.*

Measures of feeding motivation were obtained as indicators of hunger in order to gain insight in the subjective experiences of ewes with different BCS and to assess their welfare. Furthermore, metabolic and endocrine responses to changes in BCS were assessed, which could be indicative of metabolic functioning of the ewes. In addition, metabolic and endocrine signals may provide feedback to the hypothalamus and affect energy expenditure and feeding motivation and could be indicators of hunger.

II. *To assess the effects of different rates of BCS loss on feeding motivation and endocrine responses in twin-bearing ewes.*

Food restriction over several weeks or months during pregnancy leads to a loss in BCS and LW. However, the rate at which this loss occurs may have an impact on the perception of hunger and how well ewes are able to mobilise sufficient energy substrates to adapt to the loss. Data on different amounts and rates of BCS loss and its effect on health and welfare are very limited in pregnant sheep. Therefore, the aim of the study was to determine the effects of two different rates of BCS loss on feeding motivation and metabolic and endocrine responses in twin-bearing ewes.

IV. *To study the effects of body condition score on metabolic and stress responses to an acute cold challenge in pregnant ewes.*

The ability to maintain energy homeostasis and to cope with environmental challenges are main determinants for the health and welfare of animals. Food restricted ewes may be exposed to additional environmental challenges, such as cold exposure. In order to successfully cope with a cold challenge, ewes have to activate appropriate metabolic and stress responses resulting in the mobilisation of sufficient energy substrates. Therefore, food restricted ewes may have more difficulties adapting to metabolically demanding challenges. These metabolic and stress responses may be indirect indicators of the level of biological functioning of food restricted ewes. Therefore, the aim was to investigate the effects of different BCS on the metabolic and endocrine responses during BCS change and to assess the metabolic, endocrine and stress responses to an acute cold challenge in mid-pregnancy.

Chapter 2. Measurement of feeding motivation in sheep and the effects of food restriction

2.1. Abstract

The availability of food is a crucial factor determining the health and growth of animals. Under pasture grazing conditions, food availability fluctuates throughout the year with certain times not providing sufficient quantities to maintain optimum levels of feed intake. The main welfare concern in regards of food restriction is the subjective experience of hunger. It is essential to develop objective measures of hunger to understand the animal's perception of it. A behavioural economic methodology for the measurement of feeding motivation was refined and the effects of food restriction on feeding motivation were evaluated. Sixteen ewes were exposed to five different cost levels (i.e., 2, 6.6, 12.1, 22, or 40m walking distances per reward) and two feeding levels (24 h restriction and un-restricted control). Twenty-four h restricted ewes worked for a higher number of rewards, walked a greater total distance and approached the feeding station faster on the first visit. The methodology, therefore, provides suitable and sensitive measures of feeding motivation and is a potential indirect indicator of hunger in sheep. A generic demand function fitted to the data showed that economic measures of motivation (i.e., the elasticity of demand, the maximum price paid (P_{max}) and maximum expenditure (O_{max})) could not detect differences between restricted and control ewes. The methodology could be used to quantify feeding motivation in grazing animals by detecting significant changes in their willingness to work depending on their level of food deprivation, which would be useful in establishing acceptable feeding levels from an animals' perspective.

2.2. Introduction

Grazing ruminants are highly dependent on pasture growth and availability to maintain body reserves and biological functions like reproduction. Feed intake may be decreased when food is limited during, for example, droughts or cold and/or wet winters, and may trigger hunger. Understanding the animal's perception of hunger is relevant because animals' experiences are an important determinant for their welfare (Rooijen 1984; Dawkins 1988, 1990). Even though it is currently impossible to measure animal experiences directly, it is possible to measure external indicators of motivations underlying animal behaviour. Previous studies have shown that determining the strength of the motivation to obtain a resource may provide insight into the perception and value of that resource (Dawkins 1983, 1990; Matthews and Ladewig 1994; Jensen and Pedersen 2008). It is assumed that the stronger the animal's motivation for a resource, the more important that particular resource is for the animal. A generally accepted principle is that if an animal is highly motivated to obtain a resource such as pasture, but the husbandry environment does not allow them to access it, welfare is likely to be compromised (Dawkins 1990). However, the welfare status of a food deprived animal may depend on the severity of food restriction and resulting hunger. It is likely that a period of food restriction increases feeding motivation, but a slight increase in feeding motivation does not necessarily indicate hunger or poor welfare. An important question therefore is: what is the level of feeding motivation at which the welfare of ewes is significantly compromised? The development of sensitive methods that can quantify different levels of feeding motivation are a crucial first step in answering this question.

One methodology used to measure perceptions of animals involves assessing motivation with behavioural economic principles (Dawkins 1983; Kirkden *et al.* 2003; Kirkden and Pajor 2006). Motivation tests often use operant conditioning methodologies to impose a "cost or price" on the acquisition of a certain resource, such as food (Dawkins 1983; Mason and Garner 1998; Kirkden and Pajor 2006;

Matthews 2008). The relationship between the price an animal is willing to pay and consumption of a resource is described by a demand function. Demand functions could be used, for example, to detect important shifts in an animal's willingness to work depending on the level of food deprivation experienced. Several measures of motivational strength can be obtained from the demand curve: one of them is the elasticity of demand (which uses the slope of straight-line curve fitted to the data points as a measure of motivation) that has been used frequently in previous studies. However, this approach is only valid when elasticity is constant across price (Jensen and Pedersen 2008), which is often not the case for demand curves derived from a wide range of cost, termed here "complete demand curves" (Kirkden *et al.* 2003; Greenwald and Hursh 2006; Hursh and Silberberg 2008). Two alternative measures, called P_{max} and O_{max} (Hursh and Winger 1995), are derived from complete demand curves and have been developed in the field of psychology. P_{max} and O_{max} have been validated as suitable measures of motivational strength for several needs or resources (including food) in humans and other animals (Winger *et al.* 2002; Greenwald and Hursh 2006; Johnson and Bickel 2006; Murphy and MacKillop 2006; 2007b, a). P_{max} is the maximum price paid for a resource (and corresponds to the point on the demand curve where elasticity is exactly -1) and O_{max} is the maximal expenditure at P_{max}. O_{max} is equivalent to the area under the demand curve bounded by P_{max} and the corresponding level of consumption (Hursh and Winger 1995) and is the main measure of interest because it accounts for differences in the quantity of a reward consumed (Hursh and Winger 1995; Madden *et al.* 2007a).

The method proposed in this study utilises a number of unique features, and novel combinations of them, to measure motivation and assess animal welfare. These include the measurement of feeding motivation over an extended period of time taking into account diurnal variation of food intake, the use of a "cost" that is related to a natural grazing situation (i.e., walking), the use of a demand curve to calculate the strength of the motivation for food and the use of measures (P_{max} and

O_{max}) that have been validated in experimental contexts, but not previously used in animal welfare studies. This integrated approach may overcome many of the criticisms levelled at previously used methods, even though some of the elements of the methodology have been used previously. Criticism of previous methods include the short testing sessions to assess demand for food in sheep (Jackson *et al.* 1999), dairy cows (Schütz *et al.* 2006), broilers (Bokkers *et al.* 2004; Bokkers *et al.* 2007) and pigs (Matthews and Ladewig 1994). Short testing sessions do not take diurnal feeding patterns into account and animals may reach satiety quickly, which may mask motivation over a long period of time. Furthermore, to my knowledge there is only one published study using a demand curve methodology to assess feeding motivation (Matthews and Ladewig 1994), in which constant rather than varying elasticity of demand across cost was assumed. Studies by Schütz (2006) and Bokkers (2004; 2007) calculated a non demand-curve derived measure of maximum price paid (also called break point) for food with the use of progressive ratio schedules (i.e., if the cost of the first reward is one key peck, the cost of the second reward is 2 key pecks etc.) in which cost is increased in a single session until animals stop responding. However, this approach imposes a series of prices in one session and animals may reach satiety during these short sessions. It has been suggested that the break point is arbitrary and may differ between individuals and resources (Kirkden *et al.* 2003). The use of demand curves for assessing the subjective experience of hunger is relatively unexplored.

If ewes show different motivational strengths to obtain food with different levels of food restriction, then measures obtained from the demand curve should be related to the level of hunger experienced by the ewes. This study aims to validate the demand function methodology and associated measures using different durations of short-term food restriction. The advantage of using short-term restriction is that it is likely to have a less severe impact on the animals and it allows the use of the animals as their own controls in comparing motivation for food at both restricted and unrestricted feeding levels. Therefore, feeding motivation was measured with walking as cost to obtain a food reward and this methodology was applied to evaluate the effects of 24 h and 0 h food restriction on feeding motivation in ewes. This study provides a novel insight into the animals' experience of hunger by providing quantitative measurements of the strength of feeding motivation in sheep.

2.3. Methods

2.3.1 Animals and housing

Sixteen non-pregnant/non-lactating Romney x Dorset ewes between 19 and 31 months of age (average age 28 ± 4 (standard deviation) months) were used. The ewes weighed an average of 87 ± 7 kg and had an average body condition score of 5 ± 0.1 (Russel *et al.* 1969; Russel 1984) at the start of the experiment. Sheep were kept in small paddocks with limited grass and were offered a mixture of two different food pellets (Table 2.1), one with high fibre content (9.8 MJ/kg, CamTech, Hamilton, New Zealand) and a second custom made pellet with a smaller diameter (4 mm) and a slightly lower fibre content (12.4 MJ/kg, Seales, Morrinsville, New Zealand). Equal weights of the pellets were offered on non-test days (see below) such that the total offered equalled 120% of theoretical maintenance requirements for a 90 kg sheep (NRC 2007).

Table 2.1. Nutritional values of the daily diet.

	CamTech pellet	Seales pellet
Dry matter (%)	84.3	84.3
Metabolisable energy (MJ/kg DM)	9.8	12.4
Crude protein (% DM)	14.3	9.7
Lipid (% DM)	1.4	5.4
Ash (% DM)	3.2	2.3
ADF (% DM)	17	8.7
NDF (% DM)	47.4	23.3

2.3.2 Testing facilities

The facility for the feeding motivation tests consisted of two test races, each housing one individual test sheep (Fig. 2.1.). Each race was 34 x 3 m and constructed of steel posts and 1.3 m high standard farm type sheep netting covered with weed matting and standard farm gates at the entrance/exit of the races. Ewes could not see outside the race environment. The race floor was covered with a layer of sawdust on top of a layer of post peelings. A single companion ewe was placed in a pen (3 x 3 m) at each end of the race to minimize isolation stress of the test ewe. Shelters (1.0 x 2.0 x 1.2 m high) were provided for test and companion ewes, with water freely available and food for companions only. Direct physical contact between the test and companion ewes was not possible because they were always separated by 2.5 m. The race consisted of a “reward end” and a “home end” and was traversed by an automatic movable steel mesh gate (3 x 1 m) that could be programmed to give the ewe access to the feeding station at the reward end (Fig. 2.1). The gate was supported on rails at 2.25 m above ground level and driven by a motor. When the gate was at the fully retracted at the reward end, an ultrasound sensor placed approximately 50 cm in front of the feeding station at a height of 2.1 m detected the presence of the ewe. After detecting the animal’s presence, a 2 s tone was sounded, followed by delivery of 5 g food reward by an automatic feed delivery system. The food reward was the previously mentioned custom-made sheep pellet (Seales 4 mm diameter pellet). Thirty-five s after reward delivery, a second tone was sounded and the gate was programmed to move the ewe slowly (about 0.26 m/s) to a pre-programmed distance along the race, and then the gate returned after 2 s at the same speed to the feeder position. The ewe could repeat this process an unlimited number of times during the test period (22 h and 50 min). As a safety measure the gate was programmed to time-out when it was unable to move for more than 3 s, in order to prevent injury to a sheep obstructing the race. A software program controlled the speed of the gate movements, the travelling distance, tones, time and amount of food delivery and also recorded all tasks during the feeding motivation test and the total number of gate movements. Each test was recorded by video cameras (black and white, infra-red camera model AVC 307R, Jaycar Electronics, Sydney, Australia), painted in camouflage colours and adapted to field conditions by combining the power and video cabling into one waterproof plug.

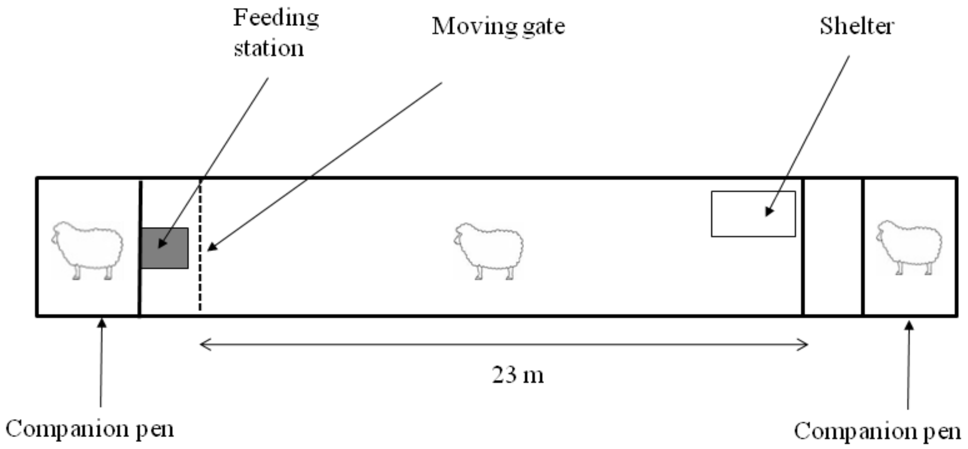


Figure 2.1. Test race with walking space for test ewe, companion pens, feeding station and moving gate.

The reward size was calculated so that the test animals should have been able to obtain at least their maintenance food allowance at the highest cost (40 m). Sheep walk between 3 and 17 km /day under grazing conditions (Squires and Wilson 1971; Squires 1976; Brand 2000) and it was assumed that ewes would walk up to 12 km/day on a testing day. A total walking distance of 12 km/day is equivalent to 300 visits to the feeding station at 40 m per visit. The theoretical maintenance requirements of non-pregnant/non-lactating ewes with a 90 kg live weight (LW) are 11.37 MJ per day (NRC 2007). The energy requirements for walking horizontally are 2.7 J/kg LW/m (averaged from Clapperton (1964) and Farrell (1972a)), which means that a 90 kg ewe making 300 visits of 40 m needs 2.9 MJ per day for walking. The total energy requirements are then 14.3 MJ per day. In order to provide energy requirements for 300 rewards, an average reward size of 48 kJ was used, or around 4-5 g, for a diet with an energy content of 12.4 MJ/kg. The reward size was calibrated at 5 g before the start of the experiment. After each test, the food remaining in the feeder and troughs was collected and weighed.

2.3.3 Training

The sheep were given a total of seven weeks training before the start of the feeding motivation test. Initially, 26 sheep were selected at the start of the training (i.e., ten more than the required sample size). The purpose of training was to gradually familiarise the sheep to the apparatus and the testing procedure (including gate movements and tones). Training consisted of nine steps, which are outlined in Table 2.2. Each step was repeated until ewes were calm and eating the rewards. The number of sheep in each race was gradually reduced as sheep became familiar with the procedure and environment. From the ten excluded ewes, eight were randomly selected as companions.

Table 2.2 Nine training steps, with duration of each step, number of sheep per race and reward sizes (g).

Training step	Duration (min)	Number of sheep per race	Reward size (g)	Tasks
1	n/a	n/a	n/a	Familiarization with handlers, food and general farm environment
2	20	3	300	Introduction to the race, no gate movements or tones
3	20	3	300	In race, no gate movements or tones
4	20	2	15	Introduction to the moving gate, manual gate movements
5	20	2	15	Feeding motivation test, without tones
6	20	2	15	Feeding motivation test, introduction to tones
7	20	1	15	Individual feeding motivation test
8	120	1	15	Individual feeding motivation test
9	600-840	1	5	Individual feeding motivation test

n/a = not applicable

2.3.4 Treatments and design

Different levels of hunger were created by two different feeding levels: 24 h food restriction and a control feeding level (no restriction). Twenty-four h before the start of a test session, ewes were placed in individual holding pens bedded with sawdust and water freely available and either fasted for 24 h (restricted) before the test or offered 750 g of both types of pellet around 1000 h and again at 1600 h (total 3 kg of pellets per sheep on a pre-testing day, control). Individual residual feed was weighed to measure feed intake the day before the motivation test. All ewes were exposed to the five different cost treatments (walking distances). Seven days of rest were given between each motivation test. The tests lasted for 22 h and 50 min on average for each cost treatment. Cost levels were 2, 6.6, 12.1, 22, and 40 m walking per reward and each ewe was tested once on each cost. The cost levels were chosen to be on a logarithmic scale, to make them more compatible with calculations for the demand curve and to ensure that cost levels were evenly spaced on a log-log scale. Each ewe was exposed to both the restricted and control feeding levels: for eight sheep, three of the five costs were undertaken under feeding restriction (and the remaining two costs were under control conditions). For the remaining eight ewes, three of the five costs were undertaken under control feeding levels (and the remaining two costs were under feed restriction). Pairs of restricted and control ewes were formed and were always tested on the same day and cost level. Run order (chronological order in which the test were conducted) for the pairs over the five tests was randomized using 5X5 Latin square layouts with pairs as rows and run order as columns. Three kg of pellets were placed into the feeder before each feeding motivation test. After the test, all the remaining food in the feeders and feeding troughs was weighed so that individual intake and average reward size for each day could be calculated. The reward size was kept consistent at 5 g for all distance and food restriction treatments. The speed at which animals approached the feeding station on their first visit was calculated from the computer outputs. Approaching speed was calculated as the cost level (m) on the testing day divided by the latency to access the first food reward.

2.3.5 Ethical note

All experimental manipulations were approved by the Ruakura and the University of Waikato Animal Ethics Committees. All the ewes in this experiment were exposed to a 24 h fast. However, a food restriction time of 24 h in ruminants can be considered mild to moderate for well-fed ewes. A fasting period of 24 h or longer is often used in scientific studies and no negative effects on sheep welfare have been reported in these studies (McCann *et al.* 1986; Newman *et al.* 1994; Daniel *et al.* 2002; Henry *et al.* 2004). Furthermore, the ewes were offered food above theoretical maintenance requirements on non-testing days. The general health of the ewes and the condition of the hoofs and legs were monitored regularly throughout the trial. LW and body condition were measured weekly. Ewes did not show signs of health problems at any time and did not lose LW or condition. An emergency time out was programmed to avoid injuries to ewes by the automatic gate, which resulted in the moving gate stopping after it had been unable to move for 3 s. No ewes were found to display injuries or illness during the trial.

2.3.6 Statistical analysis

In total there were 62 tests included in the analysis (33 control and 29 restricted tests). Tests in which ewes did not complete at least 21 h and 20 min of testing gave results that deviated from a normal food consumption level in the race (over 22 h and 50 min) and were excluded from the statistical analysis. On some testing days, technical malfunctions resulted in problems with gate movements or an incorrect delivery of the reward size (less than 3.0 g or more than 6.5 g per reward). All these data were excluded from analysis.

Data are presented as means ± standard error of the mean (SEM), unless stated otherwise. The effects of feed restriction, cost and their interaction on the number of rewards the sheep collected were tested by analysis of the data as an incomplete blocks design using the Residual Maximum Likelihood (REML) procedure in GenStat 11 (VSN International Ltd., Hemel Hempstead, United Kingdom), first regarding cost levels as a five level treatment factor. An additional term in the REML random model was incorporated to reflect greater error variation in the data from the control ewes. Despite this, the residuals were found to be normally distributed. Effect of race used and run order were also checked.

The residuals of the data on approaching speed did not pass the Shapiro-Wilk test for normality and were therefore transformed by 1/ (0.4-speed), with 0.4 being the maximum speed of the gate (m/s). These residuals passed the Shapiro-Wilk test of normality and significance levels were obtained by analysing the transformed data by the REML procedure. Also, residuals from actual food consumption did not pass the Shapiro-Wilk test of normality and therefore data were log transformed. These data were analysed by the REML procedure to test for significant differences in food consumption between restricted and control ewes, and are presented as a percentage of maintenance requirements to give an indication of the level of food intake in the race. The energy balance (EB) of the ewes was calculated as $EB = EG - (MR + WR)$ with EG as energy gain from food consumption in the race (in MJ), MR as energy expended on maintaining LW (theoretical maintenance requirements of 11.37 MJ) and WR as energy spend on walking in the race (2.7 J × kg LW × m).

In order to obtain the demand curve, the estimated treatment by cost means from a REML analysis were log-transformed before fitting the generic demand function. The model of Hursh and Winger (1995) was used to calculate the demand curve (Formula 1, Chapter 1), P_{max} (Formula 3, Chapter 1) and O_{max} (Formula 4, Chapter 1). In addition, a constant elasticity model was investigated by regressing the log-transformed means against log cost within treatment group. This constant elasticity model fits a straight-line curve to the data points. Significance levels of elasticity of demand, O_{max} and P_{max} were tested with a two-tailed Student's t-test (GenStat 11).

2.4. Results

All 16 test ewes learned to perform the motivation test within 7 weeks. The average reward size was 4.97 ± 0.14 g over the course of the experiment. The restricted ewes overall consumed more rewards than the control ewes (P < 0.001, Fig. 2.2). There was also an effect of cost on rewards obtained, with the mean number of rewards obtained reducing with increasing cost for the restricted and the control ewes (P < 0.001, Fig. 2.2). There was no interaction between restriction level and cost on the number of rewards. However, there was a tendency for a run (chronological order in which test were conducted) effect (P < 0.1), with ewes increasing the number of rewards consumed with increasing run number. No significant effect of race on number of rewards obtained was found.

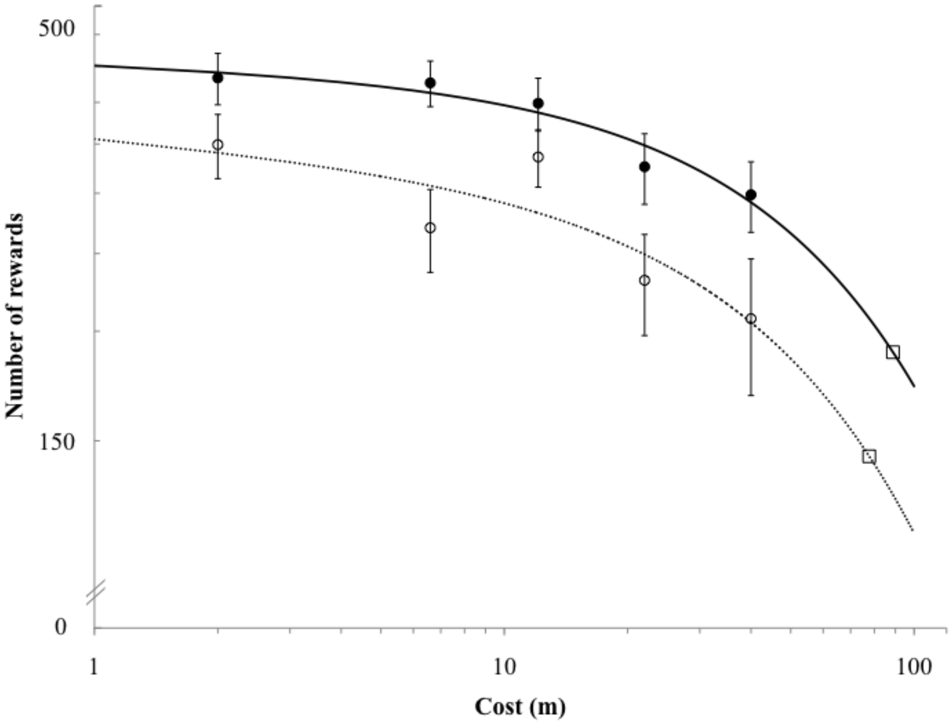


Figure 2.2. Demand curve showing the mean ± standard error of the difference (SED) number of rewards during the feeding motivation test for control ewes (open line and circles) and restricted ewes (closed line and circles). P_{max} values are indicated by the open squares. Upper half and lower half of the restricted and control SED bars, respectively, indicate within restricted group variation. Lower half and upper half of the restricted and control SED bars, respectively, indicate between restricted/control group variation.

The total distance walked by the restricted ewes was higher compared to the total distance walked by the control ewes ($P < 0.001$, Fig. 2.3). At the highest cost (40 m), the restricted ewes walked a total distance of 9.9 km compared to 6.3 km for control ewes. There was also an effect of cost on total distance walked, with both restricted and control ewes walking a higher total distance with an increasing cost ($P < 0.001$, Fig. 2.3). There was an interaction between restriction and cost in relation to total distance walked ($P < 0.01$), with the restricted ewes increasing the total distance walked more compared to control ewes.

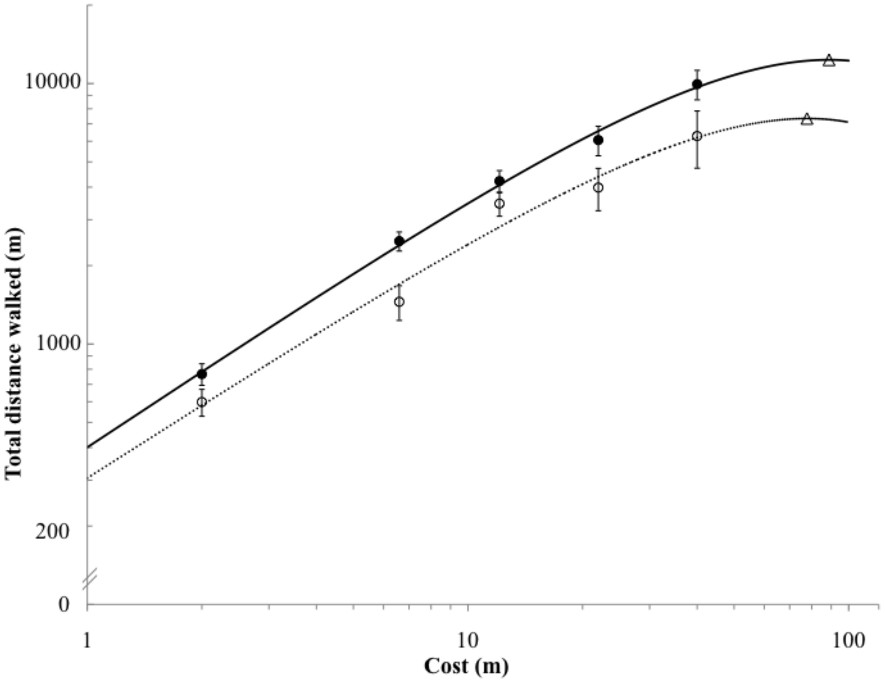


Figure 2.3. Mean ± SED total distance walked (m) during the feeding motivation test for control ewes (open line and circles) and restricted ewes (closed line and circles). O_{max} values are indicated by the open triangles. Upper half and lower half of the restricted and control SED bars, respectively, indicate within restricted group variation. Lower half and upper half of the restricted and control SED bars, respectively, indicate between restricted/control group variation.

The elasticities, obtained from the constant elasticity model, for restricted and control ewes were -0.15 ± 0.06 and -0.20 ± 0.06, respectively. The difference was not significant. The P_{max} values, obtained from the non-constant elasticity model, were outside the range of the imposed costs for both the restricted and control ewes, and were estimated by extrapolation of the fitted curves. The P_{max} for the restricted and control ewes were estimated to be 88.8 ± 84.9 m and 77.7 ± 65.9 m, respectively (Fig. 2.2). The total distance walked (expenditure) O_{max} was 7,338 ± 3,520 m and 12,327 ± 7,333 m for the control and restricted ewes, respectively (Fig. 2.3). There were no significant differences in the P_{max} and O_{max} values between the restricted and control ewes. However, numerically the O_{max} of the restricted ewes was almost twice as high as the O_{max} of the control ewes. The non-constant elasticity model gave a R^2 of 0.81 compared to 0.73 for the constant elasticity model; the difference was not significant.

The level of feeding and cost also affected the speed at which the animals approached the feeding station on the first visit (Table 2.3). The restricted ewes approached the feeding station significantly faster compared to control ewes ($P < 0.01$). Furthermore, speed at first approach increased for both treatments when cost was increased ($P < 0.001$).

Table 2.3. Mean walking speed ± SEM (m/s) when approaching the feeding station on first visit for control and 24 h restricted ewes.

		Cost (m)				
Treatment		2.0	6.6	12.1	22.0	40.0
Control	Mean	0.05	0.18	0.22	0.21	0.24
	SEM	0.02	0.03	0.03	0.04	0.05
Restricted	Mean	0.14	0.25	0.24	0.30	0.31
	SEM	0.03	0.03	0.03	0.00	0.00

At the lowest cost (2 m), all ewes consumed quantities of food above calculated maintenance requirements with food consumption at 114 ± 16.1% of maintenance for the control ewes and 173 ± 33% for the restricted ewes (Fig. 2.4). At 22 m cost, food consumption of the control ewes dropped under theoretical maintenance requirements (65 ± 12% of maintenance). The restricted ewes continued to consume food at or above maintenance at all cost levels (97 ± 7% of maintenance at 40 m). The differences in feed intake between control and restricted ewes were significant ($P < 0.001$). As the energy consumption of the restricted ewes was equal to or greater than that spent on walking at all costs, they remained in positive energy balance (Fig. 2.4). The control ewes did not work hard enough and were, therefore, in negative energy balance on the two highest costs. The day before the test, mean food consumption of the control ewes was 1,678.6 ± 89.6 g (125% of maintenance requirements for a 90 kg ewe).

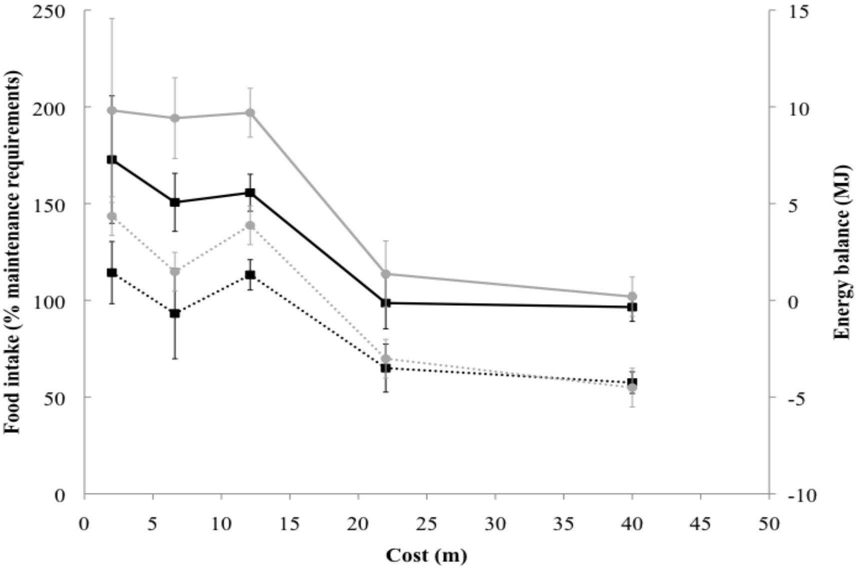


Figure 2.4. Percentage of theoretical maintenance requirements consumed at each cost levels for control (closed square and dashed line) and restricted ewes (closed square and closed line), and energy balance (MJ) for control (closed circle and dashed line) and restricted (closed circle and closed line) ewes.

2.5. Discussion

The effects of 0 and 24 h food restriction on feeding motivation were evaluated in ewes, using a novel behavioural demand methodology with walking distance as the cost to obtain a food reward. The ewes that were fasted for 24 h consumed significantly more rewards and walked longer total distances compared to the control ewes. This is a clear indication that 24 h food restriction increased feeding motivation in ewes. This is further supported by the higher approach speeds on the first visit to the feeding station of the restricted ewes compared to control ewes. No interaction was found between cost and the number of rewards consumed, indicating that the reduction in food consumption with increasing cost occurred at a similar rate for control and restricted ewes. However, the 24 h restricted ewes always consumed more rewards than control ewes, independent of cost. At the lowest cost, both restricted and control ewes consumed food at or above the theoretical maintenance requirements. When costs became relatively high, the control ewes reduced their food below maintenance requirements, while restricted ewes always consumed food above theoretical maintenance level. Furthermore, data on energy balance showed that the control ewes were in negative energy balance at the higher cost levels, meaning that they spent more energy walking and maintaining their own LW than they gained from food consumption. However, the calculations of energy balance only accounted for the energy gained and expended during a test session (and ewes were therefore assumed to be having 0 MJ energy available at the start of each session), because no quantitative measures of energy balance were available before the start of the test.

The elasticity of demand was numerically higher for the control ewes compared to the restricted ewes, although not significantly. An elasticity of more than -1 is generally considered to be an indication of “inelastic demand” suggesting a highly essential resource (Dawkins 1990; Hursh and Winger 1995; Jensen and Pedersen 2008). The P_{max} was also similar between the restricted and control ewes. Therefore, both the elasticity of demand and the P_{max} suggest that the value of the food reward was not perceived differently between restricted and control ewes, which contradicts the observation that a significantly higher number of rewards were obtained by the restricted ewes. The higher numerical value of O_{max} is suggestive of an increased level of feeding motivation of the restricted ewes compared to the control ewes, although this did not reach statistical significance. The economic measures were therefore unable to statistically distinguish the strength of the motivation between the restricted and control ewes. However, a complete demand curve was not obtained in this study because the range of costs used was not wide enough. The lack of a complete demand curve could have led to the inability to detect significant differences in P_{max} and O_{max} between restricted and control groups because they were outside the range of assessed cost and had to be estimated. The proportion of the total variation explained by the model (R^2) was slightly higher for the non-constant elasticity model compared to the constant elasticity model, suggesting that the elasticity was not constant across costs. In the literature, the demand for food and other resources typically show non-constant elasticity across cost when complete demand curves have been obtained (Greenwald and Hursh 2006; Madden *et al.* 2007a, b; Hursh and Silberberg 2008). Demand curves with constant elasticity are most likely incomplete demand curves resulting from a too narrow range of cost, which should be taken into consideration when interpreting results.

It is possible that the control ewes ingested sufficient energy the day before the motivation test to compensate for a reduction in food intake, meaning that it could have been less important for them to maintain a high food intake with an increased cost. Foraging animals have to make decisions continuously on where to graze and on what type of forage, and how much energy to spend on walking in search of food. Foraging theory predicts that animals maximize foraging efficiency, which is the rate of energy intake in relation to energy expenditure (Ydenberg and Hurd 1998; Bergman *et al.* 2001). This implies that there is a theoretical optimum between energy expended on walking in search of food and the energy obtained from the diet. However, there is a lack of knowledge in what determines the choice of the animal between continuing to spend energy on searching for food or to use energy from other sources, such as body fat reserves. One of the key pieces of information required is whether there is a large energetic cost involved in walking. Only a few studies have assessed the metabolic energy required for walking, and it has been found that the energetic cost is relatively small compared to theoretical maintenance requirements (Clapperton 1964; Farrell *et al.* 1972a). Furthermore, the energetic cost of walking was taken into account when calculating the reward size and ewes should have been able to obtain energy in excess of that expended for every walk to the feeder. However, in a review by Lachica and Aguilera (2005) it was concluded that the energy spent on locomotion depends on the efficiency of locomotion as well as the distance walked. They also highlighted the lack of studies on the subject and the need for more accurate measurements of energy expenditure on locomotion in grazing animals. Therefore, it is possible that walking imposed a greater metabolic cost than calculated, and that the control ewes may have compensated by reducing the number of food rewards obtained at the higher costs. However, energy spent per metre on walking would have been similar for the two groups (because they had similar LWs). Therefore, this does not explain why restricted ewes continued to obtain at least maintenance requirements (by walking up to 10 km), whilst the control ewes did not.

It is possible that the effects of varying the distance walked influenced not only the energetic cost of obtaining food but also some other perceived cost dimension such as time (Johnson and Collier 1991; Collier *et al.* 2002). In my study, the time between the consumption of each reward increased with cost, because walking a longer distance takes more time to complete. Furthermore, ewes increased the approaching speed to the feeding station as the distance increased, perhaps to compensate for time taken. Dumont et al. (1998) studied the value of a good quality hay compared to poor quality hay with ewes walking across an indoor test arena and varying the distance walked (23 or 46 m). They found that when the walking distance (or cost) was increased the ewes significantly increased the walking speed (from 1.11 to 1.26 m/s). One reason why time could be perceived as a cost is that when animals spend large amounts of time on one activity, they will have less time available to spend on other important activities. One of these behaviours could be resting. Resting times reported in the literature range from around 13% (Fierro and Bryant 1990) in grazing sheep to around 60-65% for penned sheep (Bøe *et al.* 2006; Jørgensen *et al.* 2009). These differences are most likely due to food availability, with penned animals having sufficient food available freeing plenty of resting time, while grazing animals spent a high proportion of their time walking in search of food. When rats were tested in an operant setting with time interval as the only cost to obtain a food reward (without the requirement of physical effort), they reduced the number of meals consumed with increasing time intervals (Mathis *et al.* 1995). Results from other rat studies also suggest that time is perceived as a cost, but that effort required (for example, wheel running or lever presses) may also add to the cost (Johnson and Collier 1991; Collier *et al.* 2002). Unfortunately, time spent on other behaviours were not measured in this study, but there may have been a trade-off between time spent on feeding behaviour and other activities. The actual perceived cost by animals is most likely a combination of different factors that may include physical effort, metabolic energy and time. It would be useful to further investigate what drives animals to make a decision between walking for food and using different energy conserving strategies.

There is no general consensus on what hunger is or how it can be measured because it consists of internal as well as external components. Many studies have used operant conditioning procedures to measure hunger in sheep (Jackson *et al.* 1999), dairy cows (Schütz *et al.* 2006), broilers (Bokkers *et al.* 2004; Bokkers *et al.* 2007) and pigs (Matthews and Ladewig 1994). However, as mentioned in the introduction of this chapter, there are some methodological issues that should be taken into consideration when interpreting the results. Other studies have quantified hunger by depriving animals of food and measuring compensatory food intake or time spend eating after food has been made available (1994; Sibbald 1997; Cockram *et al.* 1999). Researchers have also used the expression of appetitive behaviour (Douglas *et al.* 1998), activity (Douglas *et al.* 1998; Savory and Lariviere 2000; Toscano *et al.* 2007), or feeding-related stereotypies (Robert *et al.* 2002) as indicators of hunger. The latter behavioural indicators may be a sign of the existence of a motivation, but are difficult to interpret because they may be masked by motivation for other resources or they may be indicators of other types of frustration. These measures are therefore not ideal as the sole measure of motivation. The advantage of using behavioural demand functions is that these provide a more direct measure of the strength of the motivation. Animals may consume large quantities of food when cost is low or non-existent. Yet this does not directly mean that these animals are hungry or highly motivated to obtain food. Imposing a cost on a resource forces the animals to weigh the cost against the benefits of food consumption, so that only highly motivated animals will maintain food consumption with increasing cost. Furthermore, when food intake is the only measure of motivation it is possible that differences in motivation are masked by satiety effects due to high caloric intake or limited capacity of the gastro-intestinal tract, in particular when measurements are taken over short periods of time. Demand curves, however, are only valid when a sufficiently wide range of cost is used so that the shape of the curve can be accurately determined (i.e., constant elasticity or varying elasticity across price), and economic measures precisely estimated.

It is important to develop quantitative and sensitive measurements of hunger, because food deprivation is a potential welfare problem for many animals (Dawkins 1988). It is clear that when an animal is severely food deprived so that its survival is threatened, its welfare is compromised. However, when food availability is at a level that survival and reproduction are not directly threatened, does that mean that an animal has good welfare? In the recent decades it has become acceptable to discuss animal welfare in terms of the animal's subjective experiences and it has been recognised that these are the main determinants of animal welfare (Dawkins 1983, 1988, 1990). Therefore, an important question that should be asked is: how is the animal's state perceived by the animal itself? With regards to the welfare issue of hunger, the method currently available to provide the best answer to this question is to measure the strength of the motivation for food, such as the one reported in this study. In this study, it has clearly been shown that 24 h food restriction increases the subjective experience of hunger in non-pregnant/non-lactating ewes.

It needs to be kept in mind that it will be extremely difficult to find a "golden standard" of animal welfare in terms of optimum feeding motivational strength. It would be very useful to investigate feeding motivation of animals in different physiological states (i.e., pregnant and lactating animals) because metabolic demand varies highly between physiological states. Furthermore, there may be different levels of "severity" of food restriction. For example, it has been suggested that long-term food restriction is more severe compared to short-term restriction in broilers (Savory *et al.* 1993). Different lengths of short-term and long-term food restriction, as well as different levels of feeding (i.e., different percentages of theoretical maintenance requirements) and their interaction could be investigated and compared to a control that is in energy balance (e.g., by measuring plasma glucose and free fatty acids). This would provide a whole range of feeding motivational strengths that could provide insight into the relationship between food restriction, feeding motivation and welfare.

In this study, different levels of feeding motivation with different short-term restriction levels were successfully quantified in sheep, using a behavioural demand approach. The methodology was able to provide insight in the strength of the motivation for food by increasing the cost ewes had to pay for a food reward. Even though the energetic cost of walking most likely would have been small and similar for restricted and control ewes, the imposed cost had a major effect on willingness to work for food. The behavioural demand approach is therefore a suitable and sensitive measure of feeding motivation, at least in sheep. The methodology, using the number of rewards consumed combined with economic measures, can potentially differentiate feeding motivation of animals with different deprivation levels that are more or less severe than the ones in the current study. However, this study also highlights the importance of obtaining complete demand curves in order to accurately assess economic measures of demand (P_{max} and O_{max}). In future experiments, the methodology could be used to find the range of feeding levels between which animal welfare is optimal.

Chapter 3. The perception of hunger and metabolic and endocrine responses to changes in body condition score in pregnant sheep

3.1. Abstract

Food restriction in pregnant ewes is likely to result in adaptive metabolic and endocrine responses and in the subjective experience of hunger, which could potentially affect the welfare of ewes. The relationships between body condition score (BCS), feeding motivation and endocrine and metabolic responses in twin-bearing ewes were investigated in order to (1) identify and quantify indicators of hunger and (2) to assess the metabolic status of twin-bearing ewes with different BCS. Ewes were divided into three groups at day 37 of pregnancy, creating a low BCS (LBC, n=8), a medium BCS (MBC, n=8) and a high BCS (HBC, n=6) treatment. Blood samples were collected every 2 weeks from day 37 until the end of pregnancy. Feeding motivation was assessed between day 91 and 105 of pregnancy and the number of rewards, maximum price paid (P_{max}) and expenditure (O_{max}) were measured using a behavioural demand methodology. LBC ewes consumed more rewards and had an increased O_{max} compared to HBC ewes, while the MBC ewes were intermediate. No differences in P_{max} were found. LBC ewes were in a catabolic state reflected by low glucose and high free fatty acid (FFA) concentrations, while MBC and HBC ewes had sufficient energy substrates available. Leptin, insulin and insulin-like growth factor 1 (IGF-1) were also lower in LBC compared MBC and HBC ewes. In conclusion, feeding motivation was altered by a change in BCS, suggesting that feeding motivation was an indicator of hunger. Metabolic and endocrine plasma concentrations were good indicators of the metabolic state of ewes with different BCS.

3.2. Introduction

Pregnant animals have high metabolic requirements in order to support the growth and development of the placenta, fetus(es) and mammary gland. Ewes in extensive farming systems depend mainly on pasture availability to optimise food intake, which can fluctuate throughout the year. In temperate regions, pasture growth is generally lowest in winter (Kemp *et al.* 1992; McPhee *et al.* 1997) when ewes are pregnant. Ewes may not be able to obtain sufficient energy substrates to maintain live weight (LW) when dietary intake is inadequate; this is especially relevant for ewes bearing multiple lambs. As a result, food restricted ewes may experience changes in (LW) and body condition score (BCS, scored on a scale between 1 and 5: Russel *et al.* 1969), which could lead to hunger. Hunger is defined as a negative subjective state when animals are chronically undernourished (D'Eath *et al.* 2009).

It is important to obtain insights in the subjective state of hunger, because subjective animal experiences are one of the main determinants of their welfare (Dawkins 1983, 2006; Jensen and Pedersen 2008; Matthews 2008; Fraser 2009a). In Chapter 2, it was shown that measuring the motivation for food (an indirect measure of hunger) can provide insight in the subjective experience of hunger. With this methodology, ewes are given the opportunity to repeatedly walk a set distance for a small food reward, and by varying the distance per reward (i.e., changing the “cost” of the reward) the demand for food can be measured. The relation between total consumption of the food reward and its cost is described by a demand curve, and measures such as the level of reward consumption, the maximum price (P_{max}) and expenditure (O_{max}) indicate the strength of the motivation (Hursh and Winger 1995; Hursh and Silberberg 2008). Of these, O_{max} has been shown to be the most useful indicator of demand for resources in animals (Madden *et al.* 2007a, b), because it takes into the account the level of consumption as well as the total amount paid for the resource. The feeding motivation methodology, therefore, has the potential to provide insight into the subjective hunger state of animals. However, P_{max} and O_{max} were unable to detect differences in motivation between ewes with different levels of short-term food restriction in Chapter 2, because no complete demand curves were obtained. In order to detect differences in motivation between ewes with different BCS it is important to use a wider range of cost than used in Chapter 2, so that a complete demand curve can be generated and P_{max} and O_{max} accurately assessed.

Another aspect of animal welfare is the level of biological functioning (Mendl 2001). Biological functioning comprises a range of different biological systems and functions (i.e., growth, health and reproduction). However, the focus will be mainly on measures that are most relevant for metabolic functioning of food restricted ewes, such as plasma concentrations of metabolic and endocrine signals. Metabolic signals such as glucose and free fatty acids (FFA) are good reflections of the metabolic state of ewes (Caldeira *et al.* 2007b, a). Endocrine signals such as insulin, leptin and ghrelin may directly affect food intake and appetite in mammals (Schwartz *et al.* 2000; Woods 2005). Furthermore, plasma concentrations of endocrine signals also reflect changes in body fat and energy reserves. Research has shown that a reduction in body fat, BCS or plane of nutrition results in a decrease in leptin, IGF-1, insulin and an increase in ghrelin in non-pregnant/non-lactating ewes (Bergman *et al.* 1989; Friedman 1998; Blache *et al.* 2000; Daniel *et al.* 2002; Kurose *et al.* 2005; Caldeira *et al.* 2007b, a) as well as in pregnant ewes (Bispham *et al.* 2003; Osgerby *et al.* 2003b; Banchero *et al.* 2004). Because of their involvement of in the regulation of LW and BCS, endocrine signals are potential indicators of hunger in sheep. In addition, wool growth and the weight of the lambs could be potential indicators of the biological function of food restricted pregnant ewes.

Very little is known about the relationship between metabolic and endocrine responses to food restriction and the perception of hunger in sheep. Measures of feeding motivation are likely to be a reflection of the subjective hunger state of ewes. However, it would be valuable to investigate whether metabolic and endocrine responses are also indicators of hunger, because such indicators could be used as a hunger assessment tool in order to determine the welfare of food restricted ewes. Metabolic and endocrine indicators of hunger could possibly be identified by combining measures of feeding motivation with metabolic and endocrine responses to food restriction. Therefore, the aim was to investigate the relationships between BCS, feeding motivation, and endocrine and metabolic responses in twin-bearing ewes in order to identify and quantify indicators of hunger and to assess the metabolic status of food restricted ewes.

3.3. Methods

3.3.1 Animals, housing, diets and management

Animals were selected from a base flock of 100 4-5 year old Coopworth x Texel ewes. All ewes were kept on pasture from the start of the study (60 days prior to mating) until day 70 of pregnancy. Ewes were also supplemented with a mixture of two different pelleted rations (see Table 2.1, Chapter 2), with each ration providing half of the daily energetic requirements (NRC 2007). All 100 ewes were synchronised for mating using Eazi-breed^{TM} CIDRS$^{®}$ (intravaginal controlled internal drug release devices containing 0.3 g progesterone, Pfizer Animal Health, Auckland, New Zealand) and mated in two different groups (50 ewes per group; randomly allocated, balanced for weight and BCS) on pasture. Mean weights and BCS for Groups 1 and 2 were 56.4 ± 0.7 kg and 3.1 ± 0.07 BCS, and 58.0 ± 0.9 kg and 3.1 ± 0.08 BCS, respectively. CIDRs were removed on the day the rams were introduced. Ewes normally cycle 48 hours after CIDR removal (Wheaton *et al.* 1993) and, therefore, the second day after CIDR removal was taken as day 1 of pregnancy (day 1 of pregnancy on 9/04/2008 and 30/04/2008 for group 1 and group 2, respectively). Rams equipped with harnesses and crayons were present 48 h after CIDR removal and allowed to mate for three consecutive days. Pregnancy status and the number of fetuses were assessed on day 41 of pregnancy by ultrasound scanning by a commercial scanner. The number of fetuses was reassessed on day 62 and 70 of pregnancy. The aim was to select twin-bearing ewes only. However, two ewes gave birth to singletons (both HBC treatment, see below) and two gave birth to triplets (both MBC treatments, see below); all other ewes gave birth to twins.

From day 70 of pregnancy until 1 week after lambing, the ewes were housed indoors in group pens. All animals within a pen were allocated to the same treatment (BCS level, see below). A total of seven group pens were used (between 24 and 120 m^2 per pen), each housing between 2 and 15 ewes (depending on stocking density and diets). All pens were bedded with sawdust and water was freely available. Sheep were offered the same two rations used at pasture and received additional hay daily (around 50 g per sheep). Ewes and lambs were returned to pasture one week after lambing during which time they received the same diet as in the pens for 7 days. After that, all ewes were grazed (but not supplemented) together as one group until weaning of the lambs at 10 weeks of age.

3.3.2 Body condition score treatments

All 100 ewes were fed to achieve BCS 3 starting at 60 days prior to mating. Within each mating group, ewes with a BCS between 2.5 and 3.5 were randomly allocated to three different BCS treatments balanced for LW and BCS. The three BCS treatments were low BCS (LBC; targeted at BCS 2), medium BCS (MBC; targeted at BCS 3) and high BCS (HBC; targeted at BCS 4). The feeding level for each treatment was designed to achieve the target BCS score by day 76 of pregnancy. The nutritional manipulations to attain the appropriate BCS started on day 37 of pregnancy at which time the LBC, MBC and HBC ewes were offered

65%, 130% and 200% of theoretical maintenance requirements, respectively (NRC 2007). BCS was monitored weekly and feeding levels adjusted accordingly. Feeding levels were different for each paddock (before day 70 of pregnancy) and pen (after day 70 of pregnancy) and individual ewes were changed, if necessary, to a higher/lower feeding level depending on amount of BCS loss/gain required. Therefore, animals within the same treatment may have been on a different feeding level between day 35 and 80 of pregnancy. Ewes from the same mating group and treatment were housed together once the target BCS was reached and fed the same diet at least one week before the start of the feeding motivation test. Target BCS were maintained until lambing.

3.3.3 Body condition score, weight, body fat and muscle measures

LW and BCS were measured weekly from 60 days before mating until the end of pregnancy and then monitored fortnightly until weaning of the lambs. BCS was assessed by manual palpation of the backbone and spinal processes by a trained assessor. A second highly experienced assessor scored the ewes independently every 3 weeks and the scores between the two assessors were compared. In case of any discrepancies between scorers, the BCS were reassessed and both scorers agreed upon a final score. There was a high agreement in BCS scoring between the two assessors, which ensured the accuracy of BCS scoring. The width (A-measure) and depth (B-measure) of the eye muscle (*m. longissimus dorsi*) and thickness of the fat covering the eye muscle (C-measure) and the thickness of the tissue 110 mm lateral to the mid-line over the 12th rib (GR-measure) were measured by ultrasound scanning using the procedure of Fernandez et al. (1997; 1998) on day -36, 1, 35 (baseline), 57 (mid-way through the BCS change), 76 (targeted end of BCS change), 119 of pregnancy (after feeding motivation testing; see below) and at 157 days after mating (end of experiment).

3.3.4 Feeding motivation facility

The facility used for motivation testing consisted of four identical races in which ewes could walk for access to food. These races were identical to the two races in Chapter 2 (Fig. 2.1), except that the walking space was increased from 23 to 28 m, so that a maximum cost was increased to 50 m. The total length and width of the races was 39 x 3 m, and included two companion pens, shelters for the test and companion ewes, water for all ewes, a feeding trough for the companion ewes and the feeding station for the test ewe.

The reward size was calculated so that the test animals could obtain at least their maintenance food allowance under the most stringent cost conditions (50 m). Based on a previous experiment (Chapter 2) it was assumed that ewes with a low BCS would walk up to 20 km on a testing day. A total walking distance of 20 km a day is equivalent to 400 visits to the feeding station of 50 m each. The maintenance requirement of twin-bearing ewes with a 70 kg LW is 18.3 MJ per day. Additional energy at 2.7 MJ/kg/LW (averaged from Clapperton 1964; Farrell *et al.* 1972a) is required for walking, making the total daily energy requirements 21.0 MJ. The energy requirements were equally divided over 400 rewards, giving an average reward size of 4.2 g per visit (or 53 kJ per reward) from pellets with an energetic value of 12.4 MJ/kg. Therefore, the automatic feeders were calibrated to deliver between 4.0 and 4.5 g per reward. Each testing day, the food placed into the feeder and the food collected after completion of the test were weighed. The difference was used to calculate the mean reward size (amount of food consumed/number of rewards) for each test session for each ewe.

3.3.5 Training

Sheep were handled daily in groups of 6 for 20 min with food available in troughs in standard sheep yards (before mating). Group size was slowly reduced until animals remained calm when placed individually in the yards. This process took 2 months. After that, ewes were trained in the motivation races for 5 weeks. Groups 1 and 2 were trained at the same time (group 1 between day 55 and 85 of pregnancy; group 2 between days 40 and 69 of pregnancy). Only pregnant ewes were selected for training in the races. The purpose of training was to gradually familiarise the sheep to the apparatus and the testing procedure (including gate movements and tones; see Chapter 2 for details). Training consisted of 9 steps, which are outlined in Table 2.2 (Chapter 2). Each step was repeated until ewes were calm and eating the rewards. The number of sheep in each race was gradually reduced as sheep became familiar with the procedure and environment. Sixteen ewes were selected as companions (not used for feeding motivation assessment), based on calmness in the companion pens and ease of handling.

3.3.6 Feeding motivation assessment

Feeding motivation was assessed between days 90 and 105 of pregnancy. From the original ewes allocated to each BCS treatment, 11 ewes from the LBC, 12 from the MBC and eight ewes from the HBC treatment across both mating groups were scanned pregnant with twins at the third ultrasound scan. Of these, three LBC, four MBC and two HBC ewes failed to attain the target BCS score. The final numbers available for each treatment were eight LBC, eight MBC and six HBC ewes. Each ewe was tested at five different cost levels in random order (2 m, 7.2 m, 13.8 m, 44 m and 50 m) for 23 h on average per test. The reward size was kept constant for all cost levels and BCS treatments. Between 1100 h and 1300 h on the day before each feeding motivation test, ewes were placed in individual pens and were fed their daily ration (as calculated to maintain the appropriate BCS score). Residual food was weighed out the next day so that the individual ewe intake could be calculated. After each testing session, each ewe was returned to the appropriate group pen for at least 2 days before the next test. All individual tests were recorded on videotape and later viewed in order to detect any technical issues that may have occurred.

3.3.7 Metabolic and endocrine measurements

Blood samples were taken by venipuncture of the jugular vein on day 0, 35, 36, 37, 49, 63, 77, 91, 105, 119 and 133 of pregnancy. Blood was collected in one 15 ml EDTA vacutainer for leptin and ghrelin and in one 15 ml heparinised vacutainer for glucose, FFA, β-hydroxybutyrate (β-HBA), urea, insulin, cortisol and IGF-1 per sheep. Food was removed at 17:00 h the day before the blood samples were taken in order to reduce the short-term effects of food intake on metabolic and endocrine plasma concentrations. For periods when the sheep were maintained at pasture, the animals were brought indoors the evening before sampling (samples taken at day 0, 35, 36, 37, 49, and 63 of pregnancy). All blood samples were taken between 8:00 and 11:00 h, before feeding and were stored on ice immediately and centrifuged within 1 h of collection. Plasma was stored at -20°C until analysis.

3.3.8 Blood sample analyses

Specialised laboratories analysed blood plasma for concentrations of FFA, cortisol, insulin, glucose, IGF-1, β-HBA and urea (The Liggins Institute, University of Auckland, New Zealand) and leptin and ghrelin (School of Animal Biology, University of Western Australia, Australia).

Glucose was analysed by enzymatic colorimetric assay (Roche, Mannheim, Germany), urea by kinetic UV assay (Roche), FFA by enzymatic colorimetric assay (Randox Laboratories Ltd, Ardmore, Crumlin, UK) and β-HBA by kinetic UV assay (Randox Laboratories Ltd). The average intra-assay coefficients of variation (CV) were 2.2, 1.7, 2.7, and 3.2 % for glucose, urea, free fatty acids, and β-HBA, respectively.

Plasma hormone concentrations were measured by specific radioimmunoassay (RIA) established and validated for sheep plasma. Plasma insulin was measured according to previously published methods (Oliver *et al.* 1993) except that ovine insulin was used as the standard (Sigma Chemical, St. Louis, MO, batch # I9254). The minimal detectable concentration was 0.03 ng/ml plasma and the inter- and intra-assay CVs were 9.3% and 12.4%, respectively. Plasma IGF-I was measured using an IGFBP-blocked RIA (Blum and Breier 1994; Vickers *et al.* 1999). The detection limit was 0.7 ng/ml and the inter- and intra-assay coefficients of variation were 9.5% and 10.0%, respectively. Cortisol was measured using mass spectrometry using a previously published method (Rumball *et al.* 2008). Mean inter- and intra-assay CV values were 11.2% and 7.1%. Estradiol and Progesterone were also measured using mass spectrometry and intra-assay CV values were 20.4% and 9.6%, respectively.

Plasma leptin concentrations were measured in duplicate by double-antibody radio-immuno-assay (RIA) method (Blache *et al.* 2000). Bovine recombinant leptin (b/o-leptin) was kindly donated by Dr. Ross L. Tellam (CSIRO Livestock industries, Queensland Biosciences Precinct, St Lucia, Australia). The minimum detection limit was 0.05 ng/ml. The intra-assay and inter-assay CVs were 4.2% and 8.3%, respectively. Plasma ghrelin was measured in duplicate by a double-antibody RIA method based on the Linco Total Ghrelin RIA Kit (cat no. GHRT-89HK). It was modified by according to the method described by Miller (2009). The minimum detection limit was 25 pg/ml. The intra-assay and inter-assay CVs were 4.2% and 4.4%, respectively.

3.3.9 Wool measurements

Wool growth (fibre length and diameter) was determined by clipping a 30 mm^2 patch on the mid-side of the sheep (Morley *et al.* 1955) on day -7 (baseline) and 100 (after achieving target BCS) of pregnancy. After collection, wool characteristics were determined with image analysis using OFDA 2000 (BSC electronics, Ardross, Australia) by a specialised laboratory (SGS wool testing services, Timaru, New Zealand).

3.3.10 Pregnancy length and lamb birth and weaning weights

At the end of pregnancy, ewes gave birth in the indoor group pens equipped with video cameras to record the exact lambing time. Lambs were weighed between 6 and 18 h after birth, ear-tagged and tail docked (with rubber docking rings). Lambs were weaned at 10 weeks of age and their weaning weights were recorded.

3.3.11 Ethical note

All experimental procedures were approved by the Ruakura Animal Ethics Committee and the University of Waikato Animal Ethics Committee. All ewes were closely monitored throughout the experiment, in particular the leg and hoof health as well as general health and behaviour. Ewes were foot bathed in a zinc sulphate monohydrate solution 1-2 times weekly for 10 m as a preventive measure for lameness. From day 100 of pregnancy, urine samples were collected (urine release was induced by manually obstructing the airflow through the nose for a maximum duration of 30 s) every 10 days from LBC ewes and measured ketone levels by urine dip sticks (Multistix, Siemens health care diagnostics, Deerfield, USA). Ketone levels were normal at all times and intervention was not needed. The nutritional treatment and feeding motivation procedure did not lead to any animal health or welfare problems at any time. During the BCS change phase, individual ewes were moved to a different pen depending on their BCS loss/gain. However, all ewes were grazed together as one group before the start of the experiment and were therefore relatively familiar with each other. No behavioural problems were observed at any time as a result of changing pens.

3.3.12 Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Because not all ewes carried twins, the number of lambs (i.e., triplets, twins and singletons) was used as a covariate in the statistical analysis. No effects of number of lambs were found on any of the parameters and, therefore, all 22 ewes were included in the analysis (except for analysis of pregnancy lengths, birth and weaning weights which were for twin lambs only). The effects of BCS, cost level and their interaction on the number of rewards the sheep collected in the motivation races were tested by Residual Maximum Likelihood (REML) procedure in GenStat 10, with BCS treatment, cost levels, run and race as fixed effects and ewe as a random effect. Some test sessions were shorter than 20 h and 45 min and in some sessions the reward size was lower than 3.5 g or higher than 6 g, due to equipment malfunctioning or ewes interfering with the moving gate; these were all excluded from the analysis. A total of 85 tests were included in the analysis (28 LBC, 36 MBC and 21 HBC tests). In order to calculate the demand curve, the number of trips to the feeding station was log-transformed before analysis. The demand curve (Formula 1, Chapter 1), P_{max} (Formula 3, Chapter 1) and O_{max} (Formula 4, Chapter 1) were calculated according to the model by Hursh and Winger (1995).

The effects of BCS treatments on fat and muscle stores and endocrine and metabolic responses were analysed over time by REML procedure in GenStat 10 using the appropriate ante dependence covariance model, with mating group and

BCS treatment as fixed effects and ewe as a random effect. On day 105, all eight ewes that had been in the motivational races the previous day were excluded. Insulin, cortisol, estradiol, progesterone, FFA, urea and β-HBA data required log-transformation for normalization and are shown as observed means ± SEM but statistical significance levels were obtained from log-transformed data. Effects of BCS treatment on pregnancy lengths, lamb birth and weaning weight were assessed by REML with BCS and treatment as fixed effect and ewe as a random effect. Finally, a two-way analysis of variance (ANOVA) in GenStat 10 was used to test for significant differences in body reserves and metabolic and endocrine plasma concentrations between treatments on a particular day of pregnancy. Correlations (R) between BCS and endocrine, muscle and fat reserves were also calculated in GenStat and tested for significance at the 1% and 5% level, using individual animal data. Differences in wool growth and LW at mating and day 140 of pregnancy were assessed with a two-way ANOVA in GenStat 10.

3.4. Results

3.4.1 Live weights, body condition score, fat and muscle

LW, BCS (Fig. 3.1), fat and muscle reserves (Fig. 3.2) were not different between BCS treatments at day 35 of pregnancy (baseline).

The mean ± SEM BCS of the LBC, MBC and HBC ewes were 2.0 ± 0.0, 2.9 ± 0.1 and 3.7 ± 0.1, respectively, before the start of the motivation test at day 91 of pregnancy (Fig. 3.1). At this time, the BCS and LW were lower in LBC ewes than MBC ewes ($P < 0.001$ and $P < 0.05$, respectively) and HBC ewes ($P < 0.001$ and $P < 0.001$, respectively) and the BCS and LW were lower in the MBC ewes than the HBC ewes ($P < 0.001$ and $P < 0.05$, respectively).

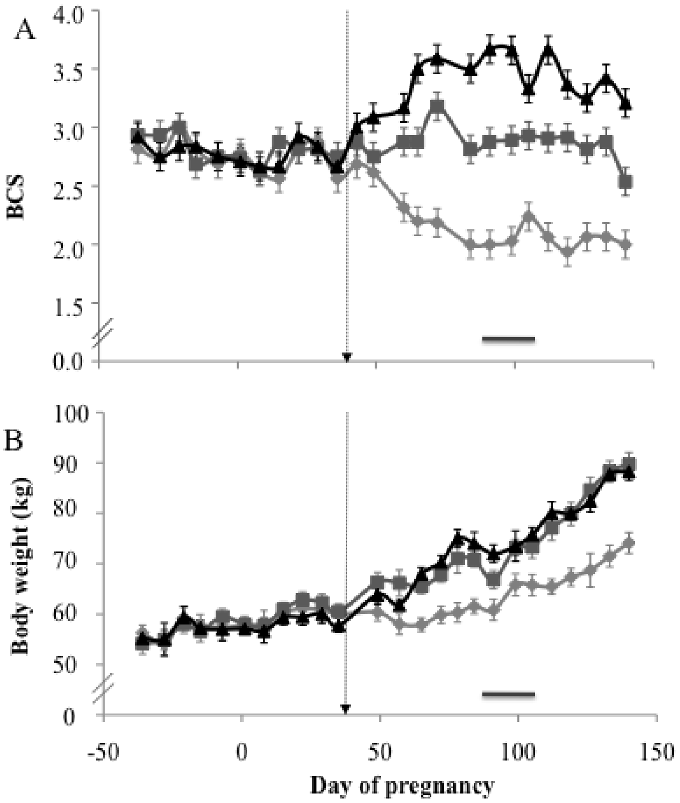


Figure 3.1. The effect of BCS treatment on mean ± SEM (A) BCS and (B) LW for LBC (rhombus), MBC (square) and HBC (triangle) from day -40 to 140 of pregnancy. The arrows indicate the start of the treatments (day 37 of pregnancy). The grey lines indicate the period of feeding motivation testing (day 91 to 105 of pregnancy).

Eye muscle width was affected by BCS treatments ($P < 0.001$, Fig. 3.2A). The LBC ewes had a significantly lower eye muscle width compared to HBC ($P < 0.01$) and MBC ($P < 0.05$) ewes. The HBC ewes had a greater eye muscle width compared to MBC ewes ($P < 0.01$). Eye muscle depth was also influenced by BCS ($P < 0.01$, Figure 3.2B). The LBC had a decreased muscle depth compared to HBC ewes ($P < 0.01$) and tended to have a smaller muscle depth than MBC ewes ($P < 0.1$). The muscle depth did not significantly differ between MBC and HBC ewes. A time by BCS treatment interaction was evident for muscle width and depth (both $P < 0.001$), with the MBC and HBC ewes increasing and LBC slightly reducing muscle dimensions.

The fat covering the eye muscle was different between the BCS treatments ($P < 0.01$, Figure 3.2C). This difference was mainly due to the LBC having significantly less fat covering the eye muscle compared to HBC ewes ($P < 0.01$). GR depth was influenced by BCS ($P < 0.001$, Figure 3.2D), with LBC ewes having significantly lower GR depth compared to HBC ewes ($P < 0.01$) and MBC ewes ($P < 0.01$). GR depth and eye muscle fat did not differ between MBC and HBC ewes. Time by BCS treatment interactions for the GR and eye muscle fat measures were significant (both $P < 0.001$), due to the larger increases in MBC and HBC ewes over time compared to LBC ewes.

3.4.2 Correlations between body condition score, muscle dimensions, fat and GR depth

The correlations between BCS, muscle dimensions, fat and GR depth varied at different time points when BCS was changing (day 37 to 84, Table 3.1). In general, there were few significant correlations early in the experiment (except at day 1, when BCS was significantly correlated with both muscle width and depth, both $P < 0.05$). Correlations between the BCS and eye muscle dimensions, fat and GR depth became significant after day 76 of pregnancy. Throughout the experiment, eye muscle depth and width were always highly correlated (average $R = 0.933$, $P < 0.001$).

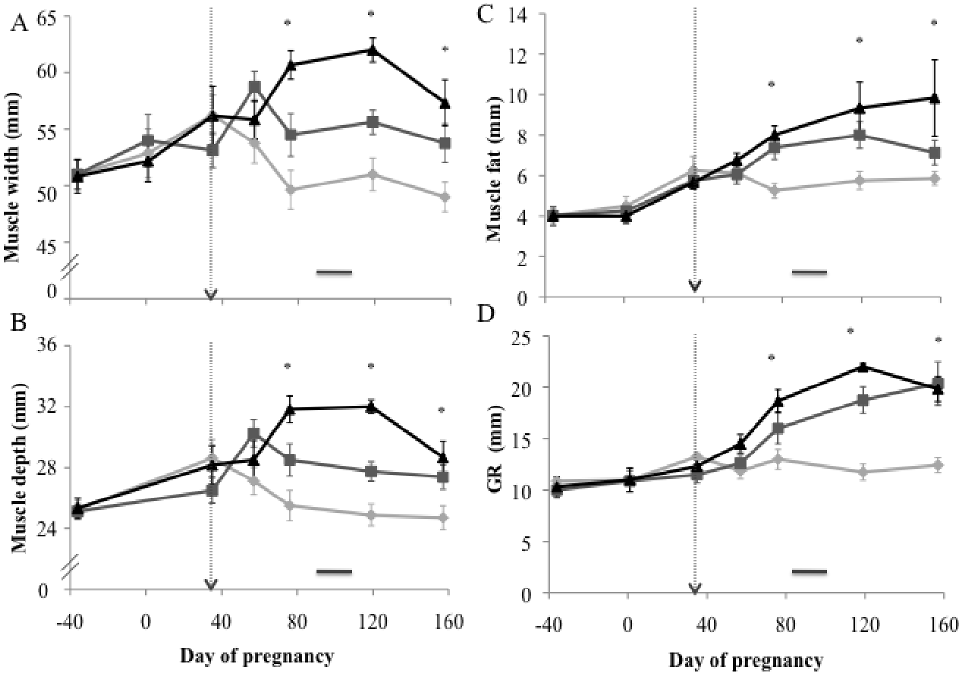


Figure 3.2 The effects of BCS treatment on mean ± SEM muscle and fat reserves for LBC (rhombus), MBC (square) and HBC (triangle) ewes between day -40 and 157 of pregnancy: (A) eye muscle width, (B) eye muscle depth, (C) eye muscle fat and (D) GR depth. The arrows indicate the start of the treatments (day 37 of pregnancy). The grey line indicates the period of feeding motivation testing (day 91 to 105 of pregnancy).* $P < 0.05$ for differences at individual time points.

Table 3.1 Correlation coefficients (R) between BCS, eye muscle width, depth, fat and GR depth at each sampling point.

Day of pregnancy	Measure	BCS	Width	Depth	Fat	GR
Day -36	BCS	1				
	Width	0.373	1			
	Depth	0.409	0.929***	1		
	Fat	0.429	0.424	0.409	1	
	GR	0.447	0.389	0.413	0.286	1
Day 1	BCS	1				
	Width	0.67*	1			
	Depth	0.692*	0.96***	1		
	Fat	0.386	0.173	0.177	1	
	GR	0.128	0.255	0.276	0.344	1
Day 35	BCS	1				
	Width	0.162	1			
	Depth	0.228	0.969***	1		
	Fat	-0.238	0.086	0.039	1	
	GR	0.229	0.623**	0.601**	0.065	1
Day 57	BCS	1				
	Width	0.313	1			
	Depth	0.286	0.968***	1		
	Fat	0.265	0.209	0.245	1	
	GR	0.318	0.196	0.271	0.453	1
Day 76	BCS	1				
	Width	0.53*	1			
	Depth	0.569*	0.849***	1		
	Fat	0.536*	0.502	0.454	1	
	GR	0.568*	0.479	0.315	0.603*	1
Day 119	BCS	1				
	Width	0.683***	1			
	Depth	0.795***	0.935***	1		
	Fat	0.787***	0.552*	0.661**	1	
	GR	0.888***	0.753***	0.791***	0.732***	1
Day 157	BCS	1				
	Width	0.877***	1			
	Depth	0.859***	0.921***	1		
	Fat	0.728***	0.648**	0.623**	1	
	GR	0.684**	0.681***	0.653**	0.604**	1

***P < 0.001, **P < 0.01, *P < 0.05

3.4.3 Feeding motivation and food consumption

The demand curve (Fig. 3.3) shows that the HBC ewes consumed fewer rewards at all costs compared to LBC and MBC ewes ($P < 0.001$). The LBC ewes tended to consume more rewards than the MBC ewes ($P < 0.1$). The mean number of rewards consumed decreased when cost was increased for all treatments ($P < 0.001$). The maximum expenditure (O_{max}) values were affected by BCS and were 13,347 m, 9,186 m and 1,522 m for LBC, MBC and HBC ewes ($P < 0.05$), respectively. The maximum prices paid (P_{max}) were 49 m for LBC, 35 m for HBC and 12 m for HBC ewes (Fig. 3.3), respectively. However, the differences were not significant.

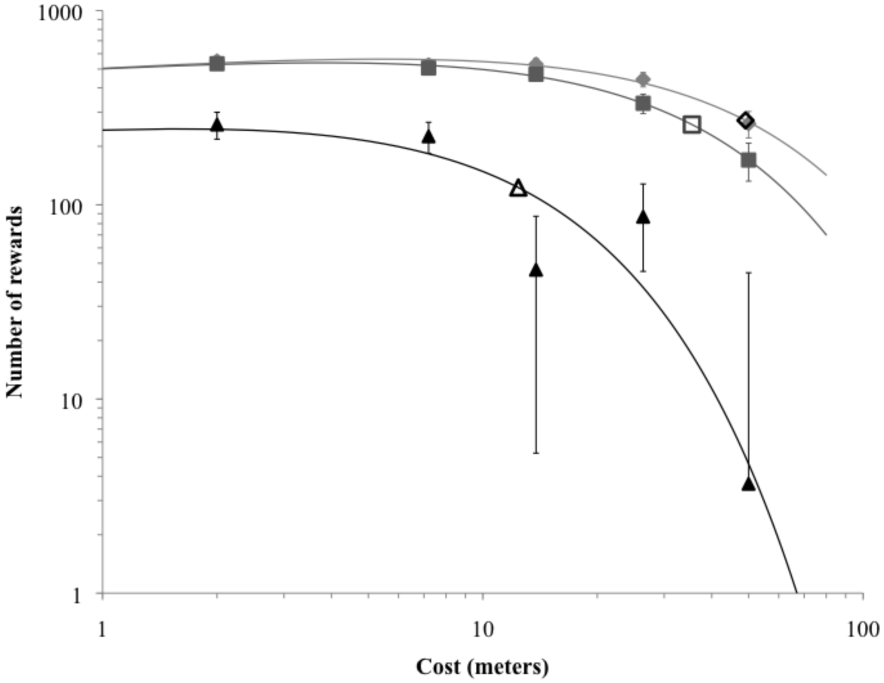


Figure 3.3. The effect of BCS treatment on the mean ± SEM number of rewards consumed in the feeding motivation test conducted between day 90 and 105 of pregnancy by LBC (closed rhombus), MBC (closed square) and HBC (closed triangle) ewes. P_{max} values are indicated by the open rhombus for LBC ewes, open square for MBC ewes and open triangle for HBC ewes.

The LBC and MBC ewes were fed restrictedly according to their BCS treatment and the HBC ewes were fed *ad libitum* on days when feeding motivation was not assessed. Average food consumption measured on the day before each test session was 0.9 kg (64% of theoretical maintenance requirements) for LBC ewes, 1.9 kg (130% of theoretical maintenance requirements) for MBC ewes and 2.3 kg (160% of theoretical maintenance requirements) for HBC ewes. The amount of food consumed during the motivation test sessions, expressed as a percentage of the theoretical maintenance requirements, was more than 100% when LBC and MBC ewes were exposed to costs between 2 m and 44 m (Figure 3.4). Only at the highest cost of 50 m did food consumption drop below 100% of maintenance requirements (83% and 64% for LBC and MBC ewes, respectively). The HBC ewes consumed less than maintenance requirements at all costs, reducing to only 10% of maintenance requirements at the highest cost of 50 m.

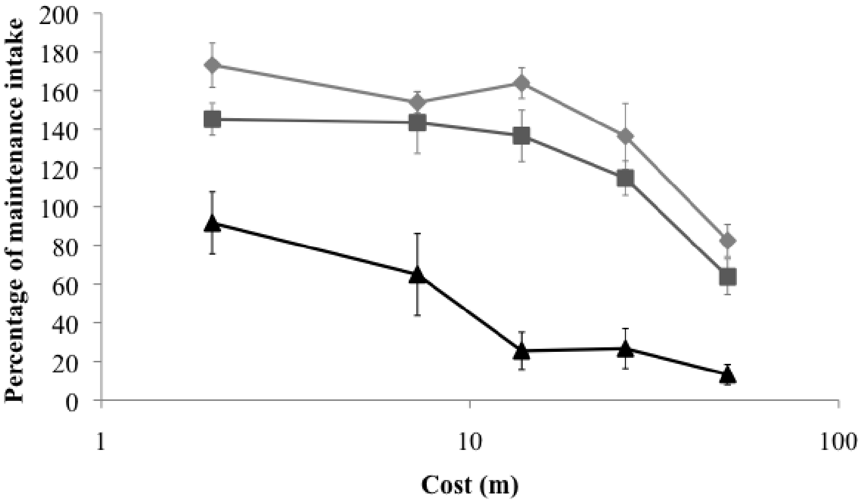


Figure 3.4. The effect of BCS treatment on mean ± SEM food intake as a percentage of theoretical maintenance requirements during the feeding motivation test conducted between day 90 and 105 of pregnancy by the LBC (rhombus), MBC (square) and HBC (triangle) ewes.

3.4.4 Metabolic and endocrine baselines

Metabolic (Fig. 3.5) and endocrine (Fig. 3.6) plasma concentrations did not differ between BCS treatments at day 35 of pregnancy.

3.4.5 Metabolic responses

Plasma glucose ($P < 0.001$, Fig. 3.5A), FFA ($P < 0.001$, Fig. 3.5B), urea ($P < 0.01$, Fig 3.5.C) and β-HBA ($P < 0.01$, Fig 3.5D) concentrations were influenced by BCS treatment. Glucose concentrations were higher in HBC ewes compared LBC ($P < 0.01$) and MBC ($P < 0.05$) ewes. FFA concentrations were higher in LBC ewes compared to MBC ($P < 0.01$) and HBC ($P < 0.001$) ewes. β-HBA concentrations were lower in MBC ewes than LBC ($P < 0.05$) and HBC ewes ($P < 0.01$). Urea concentrations were higher in HBC ewes than LBC ewes ($P < 0.001$) and tended to be higher than MBC ewes ($P < 0.1$). There was a time by BCS treatment interaction for urea concentrations ($P < 0.01$) with LBC ewes remaining relatively stable while MBC and HBC showed an increase in mid-pregnancy followed by a decrease in urea towards the end of pregnancy.

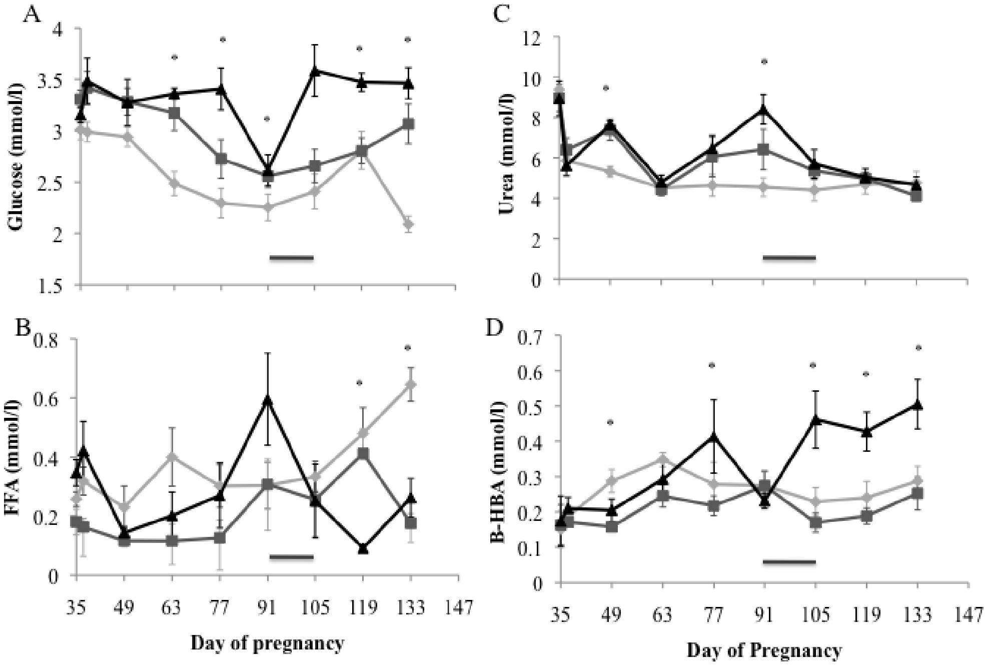


Figure 3.5. The effect of BCS treatment on mean ± SEM metabolic responses between day 37 and 133 of pregnancy for LBC (rhombus), MBC (square) and HBC (triangle): (A) glucose, (B) FFA, (C) urea and (D) β-HBA plasma concentrations. The grey line indicates the feeding motivation period (day 91-105 of pregnancy). * $P < 0.05$ for differences at individual time points.

3.4.6 Endocrine responses

Plasma insulin ($P < 0.05$, Fig. 3.6A), leptin ($P < 0.01$, Fig. 3.6B), cortisol ($P < 0.001$, Fig. 3.6C) and IGF-1 ($P < 0.05$, Fig. 3.6D) concentrations were affected by BCS, while plasma ghrelin, estradiol and progesterone (Fig. 3.7) concentrations were not influenced by BCS. Insulin concentrations were higher in HBC ewes compared to LBC ($P < 0.05$) and MBC ($P < 0.05$) ewes. There was a time by BCS treatment interaction for insulin ($P < 0.001$), with HBC ewes showing a larger increase towards the end of pregnancy compared to LBC and HBC ewes. Plasma leptin concentration was lower in LBC compared to MBC and HBC ewes (both $P < 0.01$). A time by BCS treatment interaction was also found for leptin (P <

0.001), with MBC and HBC ewes showing an increase in leptin while the LBC ewes showed a decrease with advancing pregnancy. Plasma IGF-1 was lower in LBC ewes compared to HBC ewes (P < 0.05) and tended to be lower than MBC ewes (P < 0.1). There was an evident time by treatment interaction for IGF-1 (P < 0.001), with the HBC ewes showing a larger increase in IGF-1 when pregnancy advanced compared to MBC ewes, while IGF -1 concentrations of the LBC ewes increased the least. Cortisol was higher in HBC compared to LBC and MBC ewes (both P < 0.01). There was a time by BCS treatment interaction for cortisol (P < 0.05) mostly due to the large reduction in late pregnancy observed in the HBC ewes.

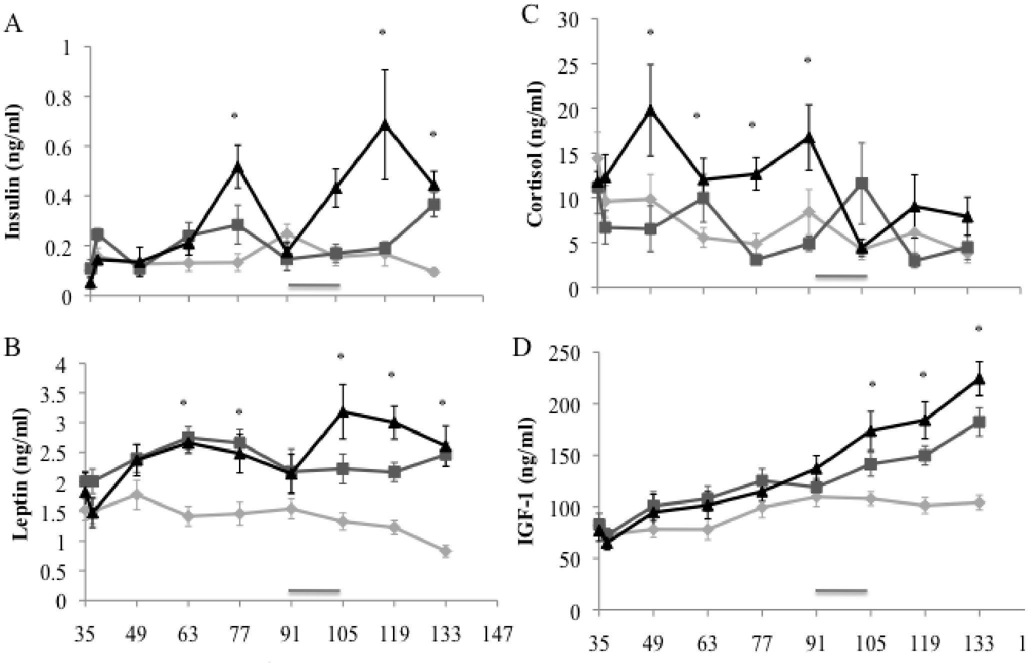


Figure 3.6. The effect of BCS treatment on mean ± SEM endocrine responses between day 37 and 133 of pregnancy for LBC (rhombus), MBC (square) and HBC (triangle) ewes: (A) insulin, (B) leptin, (C) cortisol and (D) IGF-1 plasma concentrations. The grey line indicates the period of feeding motivation testing (day 91 -105 of pregnancy). *P < 0.05 for differences at individual time points.

3.4.7 Correlations between body condition score and plasma endocrine concentrations

BCS was significantly correlated with leptin at day 49, 63, 119 and day 133 of pregnancy (see Table 3.2 for significance levels). The correlations between BCS and IGF-1 and insulin were not significant early in the experiment, but BCS was significantly correlated with IGF-1 at day 119 and insulin at day 119 and 133 (Table 3.2). Leptin concentrations were significantly correlated with insulin concentrations at most time points.

3.4.8 Wool growth responses to changes in body condition score

Fibre volume (fibre diameter ^{2} x staple length: LBC 95 ± 7, MBC 128 ± 4, and HBC 121 ± 10 m^3) was affected by BCS ($P < 0.05$) and staple length tended to be affected by BCS (LBC 89 ± 4, MBC 102 ± 6 and HBC 98 ± 5 mm; $P < 0.1$). Fibre diameter was not different between BCS treatments (LBC 33 ± 1, MBC 36 ± 1 and HBC 35 ±1 mm).

3.4.9 Pregnancy lengths, lamb birth and weaning weights

Pregnancy length was affected by BCS treatment ($P < 0.05$), with HBC ewes having the shortest (143 ± 1 days), the MBC ewes intermediate (145 ± 0 days) and the LBC the longest pregnancy length (146 ± 1 days). Lamb birth weights were not different between BCS treatments (LBC females 4.4 ± 0.2 and males 4.2 ± 0.2 kg, MBC females 4.8 ± 0.3 and males 4.8 ± 0.3 kg and HBC females 4.4 ± 0.2 and males 4.8 ± 0.2 kg). The weaning weights of lambs tended to differ between treatment groups (LBC females 21 ± 0 and males 22 ± 1 kg, MBC females 23 ± 2 and males 25 ± 1 kg and HBC females 25 ± 1 and males 24 ± 1 kg, $P < 0.1$), with the lambs born to LBC ewes tending to be slightly lighter.

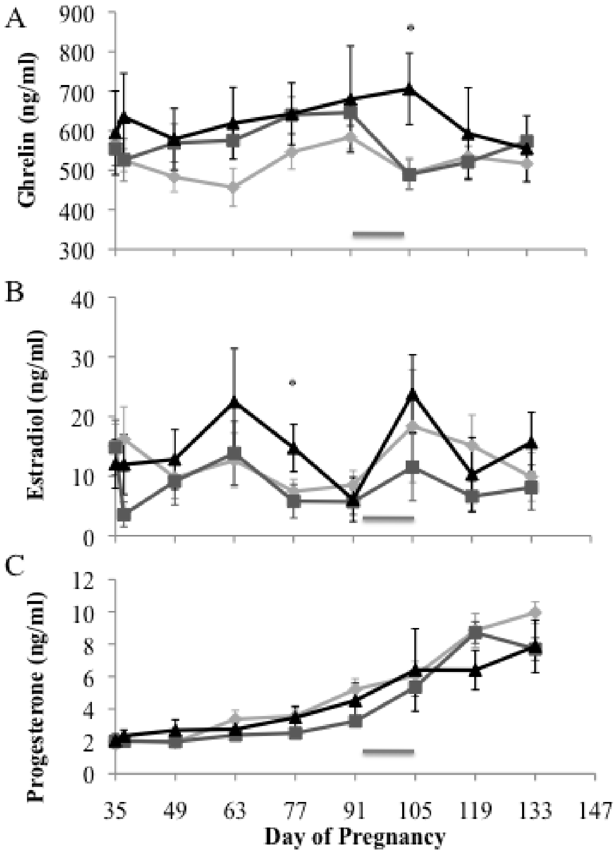


Figure 3.7. The effect of BCS on mean ± SEM endocrine responses between day 37 and 133 of pregnancy for LBC (rhombus), MBC (square) and HBC (triangle): (A) ghrelin, (B) estradiol and (C) progesterone plasma concentrations. Grey line indicates period of feeding motivation testing (day 90-105 of pregnancy). *P < 0.05 for differences at individual time points.

Table 3.2. Correlation coefficients (R) between BCS, IGF-1, insulin, and leptin at each sampling day.

Day of pregnancy	Measures	BCS	IGF-1	Insulin	Leptin
Day 37	BCS	1			
	IGF-1	-0.224	1		
	Insulin	0.475	0.084	1	
	Leptin	0.314	0.065	0.53	1
Day 49	BCS	1			
	IGF-1	0.225	1		
	Insulin	0.301	0.297	1	
	Leptin	0.593**	0.543*	0.463	1
Day 63	BCS	1			
	IGF-1	0.318	1		
	Insulin	0.276	0.315	1	
	Leptin	0.644**	0.367	0.652**	1
Day 77	BCS	1			
	IGF-1	0.314	1		
	Insulin	0.53	0.198	1	
	Leptin	0.452	0.367	0.272	1
Day 91	BCS	1			
	IGF-1	0.303	1		
	Insulin	-0.151	0.096	1	
	Leptin	0.431	0.269	0.5*	1
Day 105	BCS	1			
	IGF-1	0.352	1		
	Insulin	0.203	0.362	1	
	Leptin	0.451	0.386	0.659**	1
Day 119	BCS	1			
	IGF-1	0.725***	1		
	Insulin	0.451	0.415	1	
	Leptin	0.864***	0.757***	0.532*	1
Day 133	BCS	1			
	IGF-1	0.633**	1		
	Insulin	0.742***	0.673**	1	
	Leptin	0.824***	0.535*	0.863***	1

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

3.5. Discussion

The aim of this study was to identify indicators of hunger in ewes with different BCS, because subjective experiences are central to animal welfare. This is the first study that has successfully shown that BCS affects feeding motivation in sheep. Using a behavioural demand methodology, it was found that BCS significantly affected the maximum expenditure (O_{max}) as well as the total number of rewards consumed, with LBC ewes expressing the highest, MBC ewes intermediate and HBC ewes the lowest level of feeding motivation. In Chapter 2, it was found that a 24 h fast in non-pregnant/non-lactating ewes with a BCS of 5 significantly increased the number of rewards obtained during a feeding motivation test, although no differences in O_{max} were found because no complete demand curves were obtained. In this study, complete demand curves were obtained (due to an increase in the maximum cost from 40 to 50 m), which enabled accurate assessment of P_{max} and O_{max}. However, P_{max} was not able to detect differences in feeding motivation between ewes with different BCS, most likely because P_{max} does not account for differences in the level of food consumption and total amount paid. Together, the previous and current studies provide evidence that both long-term food restriction (affecting the amount of body reserves) and short-term food restriction (fasting without significantly affecting body reserves) increase the motivation for food in sheep, suggesting that food restricted ewes experience hunger.

In pigs, short-term food deprivation increases the number of button pushes in order to obtain food (Farmer *et al.* 2001). Short-term food restriction also increases activity and standing behaviour in pigs, which could be indicative of an increased sensation of hunger (Toscano *et al.* 2007). Long-term food restriction resulting in changes in LW has been shown to increase the number of key pecks to get access to food in broilers (Bokkers *et al.* 2004). In sheep, studies have shown that fasted (Newman *et al.* 1994) and low BCS (Sibbald 1997) ewes consume large quantities of food when offered, which could also be an indication of an increased motivation for food. However, the advantage of using a behavioural demand approach is that a more direct measure of the strength of the motivation can be obtained (as discussed in Chapter 2; Mason and Garner 1998; Jensen and Pedersen 2008). Furthermore, animals may show high levels of food intake when food is freely available, but may not necessarily show high levels of intake when a cost is imposed. For example, the HBC ewes consumed food at 160% of theoretical maintenance requirements (NRC 2001) when food was freely available, but were never willing to consume food at maintenance requirements during the feeding motivation test, not even at lowest cost. Similar results were found in Chapter 2: the control ewes consumed food at 125% of theoretical maintenance when freely available, but food intake was below maintenance levels at a cost of 22 m and higher. Therefore, high intake levels when food is freely available may indicate that a feeding motivation exists, but could also simply be a response to having free access to food.

A second aim of the study was to assess the metabolic state of the animal as an indicator of the level of biological functioning. Furthermore, combining measures of feeding motivation with metabolic and endocrine responses to changes in BCS could provide a deeper insight in the experience of hunger. Leptin was considerably lower in LBC ewes compared to MBC and HBC ewes, and was highly correlated to BCS at several points in pregnancy. Leptin is a signal of adiposity and affects food intake and LW via the hypothalamus (Schwartz *et al.* 2000; Woods and Seeley 2000) and my results are in line with other studies that found that pregnant ewes on a low or moderate feeding level had lower levels of leptin compared to ewes on a high feeding level (Thomas *et al.* 2001; Bispham *et al.* 2003). There is also evidence that leptin is directly involved in the regulation of food intake. Central (intracerebroventricular) infusion with leptin has been shown to decrease food intake in fully fed lambs (Morrison *et al.* 2001) and ewes (Henry *et al.* 2001). However, intravenous infusion of leptin did not have an effect on food intake in lambs (Morrison *et al.* 2002). Interestingly, leptin levels were not different between the BCS treatments at the start the feeding motivation test (day 91 of pregnancy), but leptin was significantly different between BCS treatments by the end of the motivation test period (day 105 of pregnancy). At day 105 of pregnancy, leptin levels were markedly different between LBC and MBC ewes, but the difference in feeding motivation was relatively small between LBC and MBC ewes. This suggests that leptin may only play a minor role in the regulation of feeding motivation around this time of pregnancy.

Insulin was also considerably lower in LBC and MBC ewes compared to HBC ewes. This is in agreement with other studies in sheep that also showed that maternal insulin concentrations depend on body fatness and nutrition during pregnancy (Wallace *et al.* 1999; Luther *et al.* 2007). Insulin could also play a role in feeding motivation; it has been shown to decrease food intake in sheep (Foster *et al.* 1991) and rats (Air *et al.* 2002) and increase food intake and consequently weight gain in rats (Dryden *et al.* 1998). Although not completely conclusive, it is unlikely that insulin influenced feeding motivation in the current study because significant treatment differences around the time of feeding motivation were not observed. One possible reason for the lack of a similar pattern in insulin concentrations and feeding motivation could be the development of maternal insulin resistance, a common adaptation to pregnancy in ewes (Jaquiery *et al.* 2009). There are some indications that peripheral insulin resistance could also affect the appetite controlling centres in the hypothalamus of humans, which could reduce the food intake inhibiting effects of insulin (Anthony *et al.* 2006). The development of maternal insulin resistance could therefore explain the apparent lack of relationship between feeding motivation and insulin. It was also found that insulin and leptin were highly correlated at most measuring points, and there could possibly be a combined or additive effect on feeding motivation. There is evidence that insulin increases leptin secretion in rats (Barr *et al.* 1997; Mueller *et al.* 1998) but this effect has not been observed in sheep (Kauter *et al.* 2000; Asakuma *et al.* 2003). The mechanisms by which plasma concentrations of insulin and leptin influence feeding motivation in pregnant sheep, or whether they influence feeding motivation at all require further investigation.

However, blood samples were only collected once every 2 weeks. It was expected that the protocol of blood sampling would be sufficient to detect metabolic and endocrine responses to changes in BCS and that these could be used to detect relationships between metabolic and endocrine responses and the motivation for food. The main problem when linking the measures of feeding motivation to endocrine responses is the lack of a single accurate measure of feeding motivation; an O_{max} value for each individual animal is needed in order to calculate a correlation, which is problematic due to the large losses of data. In hindsight, the blood sampling schedule may not have been ideal, because most endocrine signals respond to both long-term (BCS) and to short-term changes in feeding level (within hours, see also Chapter 5) and blood samples should have been collected just before (or during) each motivation test in order to get accurate baseline values. In addition, there was a large reduction in glucose and insulin in the HBC ewes at day 91 of pregnancy (sample taken before the motivation test period). The reason for this is unclear, but is most likely due to an inexplicable reduction in food intake, which questions the suitability of endocrine concentrations at day 91 of pregnancy as indicators of BCS and hunger, at least in HBC ewes. Endocrine concentrations at day 105 of pregnancy may be more suitable for calculating a correlation with feeding motivation. However, eight ewes (four from each mating group) had just completed a motivation test session on that day and had to be excluded from the analysis because of the freely available food in the motivational races, reducing the statistical power of the test. Therefore, the relationship between feeding motivation and concentrations of plasma endocrine signals has been described but no formal statistical analysis has been applied.

Cortisol was lower in MBC and LBC than HBC ewes, in particular during early pregnancy. Previously it has been shown that cortisol levels increase during pregnancy, and that this increase results from a change in the negative feedback action of cortisol on the hypothalamic-pituitary-adrenal (HPA) axis (Keller-Wood 1998; Keller-Wood and Wood 2001). However, cortisol concentrations are suppressed when ewes are exposed to several weeks-months of undernutrition (Bispham *et al.* 2003; Jaquiery *et al.* 2006). In this study, the increased cortisol concentrations in the HBC ewes could simply be due to the increased food intake or body fatness (Knoll *et al.* 1984; Bjorntorp and Rosmond 2000; Rask *et al.* 2001). There is some evidence that cortisol, together with other components of the HPA axis, could be involved in mediating the motivation for food. Administration of cortisol stimulates food intake in sheep (Bassett 1963) and in humans and rats (reviewed by Dallman *et al.* 2004; Dallman *et al.* 2006). In this study, cortisol concentrations were significantly different at day 91 of pregnancy with HBC ewes having higher concentrations than LBC and MBC ewes (before motivation testing), but the difference between treatments had disappeared after motivation testing (day 105 of pregnancy). Cortisol may also influence food intake in concert with leptin, as research has shown that leptin and cortisol mutually regulate each other and affect energy balance (reviewed by Leal-Cerro *et al.* 2001).

Ghrelin was not affected by BCS treatment. However, the fortnightly blood sampling protocol may not have been optimal to detect changes in ghrelin. Ghrelin is mainly secreted in anticipation of a meal (Sugino et al. 2002; Sugino et al. 2004) and may not be directly related to body fat mass in rats (Qi *et al.* 2008), although other studies have suggested that ghrelin is related to body fat mass in humans (Shiiya *et al.* 2002). No data on body fat mass and ghrelin are available in sheep. The fortnightly blood sample collection schedule may not have been sufficiently frequent to detect changes in ghrelin with more short-term changes in food intake. It has been suggested that ghrelin is an endocrine link between the digestive system and the higher brain centres (Tschop *et al.* 2000). Potentially, ghrelin could mediate feeding motivation through its involvement in short-term regulation of food intake and through anticipatory regulation of feeding (Sugino *et al.* 2002a; Sugino *et al.* 2004; Drazen *et al.* 2006). However, the relationship between ghrelin and feeding motivation in sheep needs more investigation.

Plasma IGF-1 concentrations reflected the differences in metabolic status of ewes with different BCS. These results are in agreement with previous studies that have shown that IGF-1 concentrations are significantly higher in ewes on a high diet compared to ewes fed to maintain BCS (Wallace *et al.* 1999; Wallace *et al.* 2000) and significantly lower in undernourished ewes compared to ewes fed at maintenance requirements (McMullen *et al.* 2005). In this study, the first BCS treatment differences were observed at day 105 of pregnancy (after motivation testing) and the difference between the treatments became larger as pregnancy progressed. However, there is no evidence that IGF-1 plays a role in food intake regulation in sheep (Foster *et al.* 1991). Rather, IGF-1 plays an important role in carbohydrate and protein metabolism, as well as cell replication and differentiation (see reviews by Gluckman *et al.* 1987; Jones and Clemmons 1995). Furthermore, IGF-1 is essential in the regulation of growth of the placenta and fetus (see reviews by Wathes *et al.* 1998; Fowden 2003). Recent evidence suggests that maternal nutrition may influence fetal development through IGF-1 (Bassett *et al.* 1990; Brameld *et al.* 2000; Kenyon *et al.* 2009b; Sullivan *et al.* 2009), either directly or through partitioning of nutrients between maternal and fetal tissues (Fowden 2003).

There are several other factors involved in the regulation of feeding motivation that were not measured. Recently, it has been suggested that oxytocin may play a role in the increased food intake observed in pregnant animals (Douglas *et al.* 2007). It has been shown that central oxytocin administration inhibits food intake in non-pregnant rats (Verbalis *et al.* 1986; Arletti *et al.* 1989). Furthermore, oxytocin neurons become activated during feeding in non-pregnant rats, suggesting a role in meal termination (Mitra *et al.* 2010). However, the excitability of oxytocin neurons as well as oxytocin secretion are decreased in mid-pregnancy, and it has been demonstrated that administration of an oxytocin antagonist increases feeding in non-pregnant rats but has no effect on pregnant rats (Douglas *et al.* 2007). Therefore, oxytocin could be mediating feeding motivation in pregnant animals due to a reduction in its inhibiting effect on food intake.

There may by other central and peripheral factors involved in the regulation of feeding motivation. For example, cholecystokinin (CCK) is released from the gastro-intestinal tract upon contact with digesta and circulating CCK levels may induce satiety (Della-Fera *et al.* 1981; Della-Fera and Baile 1985; Gibbs and Smith 1986). Neurons located in the hypothalamus also produce peptides that could affect feeding motivation. One set of arcuate nucleus neurons produces proopiomelanocortin (POMC), and uses a-melanocyte stimulating hormone (a-MSH) as its transmitter (Cone 1999). A different set of arcuate nucleus hormones produces neuropeptide Y (NPY) and agouti-related protein (AgRP) (Woods *et al.* 2000; Woods 2005). Neuronal synthesis of these peptides are altered with changes in adiposity (Schwartz *et al.* 2000). Furthermore, high levels of leptin and insulin are thought to activate POMC neurons and inhibit NPY and AgRP resulting in a decrease in food intake, while low levels of leptin and insulin have the opposite effect (reviewed by Schwartz *et al.* 2000). Therefore, several brain peptides in the arcuate nucleus may need to be studied in order to determine how feeding motivation is regulated.

The metabolic state of food restricted ewes was assessed in order to obtain relevant measures of their biological functioning. Plasma metabolites are a good reflection of the metabolic status and energy balance of animals. The LBC ewes were in a catabolic state in mid- and late-pregnancy, which was reflected by low glucose combined with high FFA plasma concentrations. It is perhaps surprising that urea concentrations were low in LBC ewes because high urea concentrations could be a reflection of high protein catabolism. However, the concentrate diet offered to the ewes was relatively high in protein and intake may have been sufficient to prevent internal protein catabolism. Furthermore, ruminants are able to use body fat as a buffer to prevent protein catabolism during energy restriction (Chowdhury and Orskov 1997). In contrast, there were high concentrations of glucose and urea and low concentrations of FFA observed in HBC ewes, which implied they had sufficient energy substrates available. However, HBC ewes also showed a large increase in β-HBA in late pregnancy, which is an indication of an increased catabolic state. This most likely reflects the loss of BCS experienced by the HBC ewes in late pregnancy. MBC ewes were generally in a relatively good metabolic state, with sufficient glucose and moderate FFA concentrations. Unlike the HBC ewes, the MBC ewes did not show an increase in β-HBA in late pregnancy. The only metabolite that was significantly different at day 105 of pregnancy (sample taken after feeding motivation testing) was glucose. LBC and MBC ewes had relatively low levels of glucose, while HBC ewes had high levels. These low glucose concentrations could have been a metabolic signal to increase food intake in low and medium BCS ewes.

With respect to indicators of body composition, BCS was highly correlated with ultrasound measures of GR depth, subcutaneous fat over the eye muscle and eye muscle dimensions, particularly in late pregnancy. This aligns with the results from other studies in which high correlations between BCS and subcutaneous fat measures were found in non-lactating/non-pregnant ewes after slaughter (Delfa *et al.* 1989; Sanson *et al.* 1993). Muscle and fat measures changed significantly during pregnancy with changes in BCS. The MBC and HBC ewes gained significant amounts of subcutaneous fat and muscle mass in early pregnancy. However, some fat and muscle was lost towards the end of pregnancy, even though food consumption did not decrease. It has been well documented that there is a change from net fat accumulation to net mobilisation when ewes enter the third trimester of pregnancy (Vernon *et al.* 1981; Vernon *et al.* 1985; Robinson 1986). A different pattern was observed in the LBC ewes, which lost muscle mass after the start of the treatments, and showed a slight increase in GR depth towards the end of pregnancy. This could be an indication that the LBC ewes partitioned any spare energy towards subcutaneous fat stores. Others have also reported changes in body reserve partitioning with undernutrition, either towards body fat (McNeill *et al.* 1998; Wallace *et al.* 1999) or muscle (McCrabb *et al.* 1992). Therefore, these studies suggest that a change in feeding level during pregnancy affects the pattern and site of fat and muscle accumulation and mobilisation in pregnant ewes.

A low BCS may also have consequences for lamb survival, in particular because the lambs of the LBC ewes tended to be lighter at weaning. Low birth weight is associated with reduced lamb survival (Clarke *et al.* 1997; De Blasio *et al.* 2007) and may affect the health of lambs in later life (Godfrey and Robinson 1998; Barker 2001; Bloomfield *et al.* 2003a; Wu *et al.* 2004). A study by Kenyon et al. (2009a) found that low LW ewes had lighter lambs than high LW ewes at 100 days of lactation. There is some evidence that nutrition during pregnancy can affect birth weight of the lambs, although this effect depends on the timing and lengths of the under- or over-nutrition period (Redmer *et al.* 2004; Kenyon *et al.* 2009a). Pregnancy lengths were shorter in HBC compared to LBC ewes, with MBC ewes having intermediate pregnancy lengths. Others have found that undernutrition around conception can lead to preterm birth (West 1996; Bloomfield *et al.* 2003b). However, ewes were allowed to mate for three days in this study and the exact day at which ewes became pregnant cannot be determined. A single mating day or artificial insemination should be used in order to accurately assess the effects of BCS in pregnancy length. Wool growth was reduced in LBC ewes, which tended to have a shortened staple length and a reduced fibre volume. Other studies have also shown that wool growth is affected by nutrition (Adams *et al.* 2002; Adams *et al.* 2007).

A main concern for welfare arises when ewes are food restricted and experiencing hunger. In this study, measures of feeding motivation were used as an indicator of hunger. It is a generally accepted principle that welfare is likely to be compromised when an animal is unable to obtain a resource for which it shows a high motivation (Dawkins 1990). Therefore, the increase in feeding motivation observed in the LBC ewes, and to a lesser extent in MBC ewes, may have compromised welfare. However, the interpretation of the effects of feeding motivation on welfare may be more complex than that. For example, it is well documented that nutrient intake in sheep and other mammals is season dependant and the majority of body fat stored in the time prior to mating is lost in late pregnancy and lactation (reviewed by Robinson 1986). Therefore, the problem when assessing welfare in terms of feed restriction and BCS change in pregnant ewes is not that changes may occur, but what is an acceptable level of change.

Measures of the metabolic state of food restricted ewes were obtained as an indicator of the level of biological functioning, and could be an important welfare indicator. The combined measures of feeding motivation and metabolic state could provide a more accurate assessment of welfare. Unfortunately, no gold standard for an appropriate level of feeding motivation or metabolic state of ewes exists. However, it is likely that the risk of compromised welfare increases when ewes show a very high motivation to feed combined with a poor metabolic state. The LBC ewes showed low levels of body reserves and low metabolic status. These observations combined with the high feeding motivation strongly suggest that LBC ewes were hungry. The low concentrations of endocrine signals that theoretically provide a feedback to the brain to increase food intake (e.g., leptin and insulin) could also be an indication of hunger. However, as discussed previously, plasma concentrations of endocrine signals did not seem to be related to feeding motivation. The MBC ewes also showed a relatively high feeding motivation, but had intermediate body reserves, intermediate metabolic status and intermediate plasma concentrations of endocrine signals (i.e., MBC values were in between those of LBC and HBC ewes). Therefore, they may have been less hungry than the LBC ewes. The HBC ewes, in contrast, showed very low levels of feeding motivation and had plenty of body reserves, a relatively high metabolic status combined with high endocrine signal concentrations. Therefore, these ewes did not appear to experience hunger. However, high body fatness does not necessarily imply good welfare. The high concentrations of β-HBA observed in HBC ewes could have negative health implications because β-HBA is involved in the development of metabolic diseases such as ketosis (Harmeyer and Schlumbohm 2006; Schlumbohm and Harmeyer 2008). Furthermore, fat or obese animals are at an increased risk of developing health related conditions such as insulin resistance, hypertension and cardio-respiratory disease (Jellinger 2009). Because health is an aspect of the biological function of the animals, overly fat animals may not be in an optimal welfare state. The MBC ewes had moderate glucose and urea concentrations combined with low FFA and β-HBA concentrations indicating that sufficient substrates for metabolic processes were available, without the risk of developing obesity related conditions. Therefore, pregnant ewes with a moderate BCS may have better welfare than low and high BCS ewes.

In conclusion, feeding motivation was considerably increased in LBC ewes compared to HBC ewes, and slightly increased compared to MBC ewes, suggesting that feeding motivation was an indicator of hunger. The metabolic state was also reduced in LBC ewes compared to MBC and HBC ewes, as shown by the reduced glucose and increased FFA concentrations. The high feeding motivation combined with the poor metabolic state strongly suggests that LBC ewes experienced hunger. No clear descriptive relationship between plasma concentrations of endocrine signals such as insulin and leptin with feeding motivation were observed, suggesting that these were not suitable indicators of hunger. Other aspects of biological functioning, such as lamb weaning weight and wool growth were only mildly affected by food restriction. Therefore, the main welfare implication for food restricted ewes is the subjective experience of hunger. Other aspects of biological functioning, such as productivity and immune function remain to be investigated.

Chapter 4. The effects of different rates of body condition score loss on feeding motivation and metabolic and endocrine responses in twin-bearing ewes

4.1. Abstract

Food restriction leading to a loss in body condition score (BCS) increases feeding motivation and leads to a metabolically catabolic state in twin-bearing ewes, which could be interpreted as hunger. However, a fast loss in BCS may increase hunger more than a slow loss in BCS. The aim was to study the effects of two different rates of BCS loss on feeding motivation and metabolic and endocrine responses in twin-bearing ewes, in order to assess hunger and the metabolic state. Ewes were assigned to treatments at day 37 of pregnancy: Slow Loss (SL) that lost 1 BCS over 13 weeks (n=8); Fast Loss (FL) that lost 1 BCS over 10 weeks (n=8) and Control with a stable BCS (n=10). Feeding motivation was assessed between day 42 and 67 of pregnancy (using a behavioural demand methodology, with maximum price P_{max} and expenditure O_{max} as measures of motivation). Blood samples were collected at day 35, 37, 42, 70, 84, 98, 112, 126 and 140 of pregnancy. SL and FL ewes had not lost any BCS at the time of the motivation test period and no effects on feeding motivation were found. FL ewes were in a catabolic state in mid-pregnancy as indicated by the increased plasma β-hydroxybutyrate and decreased glucose and leptin concentrations, while SL ewes were in a similar metabolic state as Control ewes. In conclusion, food restriction that has not resulted in significant BCS loss does not increase feeding motivation in early-pregnancy. Therefore, it appears that feeding motivation is only increased when animals are either fasted or when a substantial amount of BCS has been lost. The metabolic states of the ewes in mid-pregnancy suggests that they are better able to adapt to a slow than a fast loss in BCS.

4.2. Introduction

Ewes carrying twins have high metabolic requirements in order to provide sufficient energy and nutrients to the developing fetus and mammary gland. Food restriction over several weeks or months during pregnancy leads to a loss in BCS and live weight (LW). BCS is a measure of the amount of body reserves in sheep (Russel *et al.* 1969) and is highly correlated to the size of the eye muscle (*m. longissimus dorsi*) and subcutaneous fat depth (Chapter 3; Delfa *et al.* 1989; Sanson *et al.* 1993). Furthermore, a loss in LW and/or BCS results in reductions in plasma concentrations of glucose, insulin, leptin, insulin-like growth factor 1 (IGF-1), and cortisol (Chapter 3; Thomas *et al.* 2001; Bispham *et al.* 2003; McMullen *et al.* 2005; Jaquiery *et al.* 2006; Luther *et al.* 2007) and to a significant increase in feeding motivation in pregnant ewes (Chapter 3). These observed changes in metabolic and endocrine signals and motivation for food in response to changes in BCS may affect the metabolic state and the subjective experience of hunger in ewes.

The subjective experiences of animals have received considerable attention in the last few years because they are considered to be an important determinant of animal welfare (Desire *et al.* 2002; Dawkins 2006; Boissy *et al.* 2007; Dawkins 2008; Yeates and Main 2008). An increase in motivation for food combined with changes in endocrine signals that regulate this motivation could be interpreted as indicators of hunger. However, it is very difficult to assess the impact of food restriction on welfare, because no gold standard exists. In pregnant animals, a transition from body reserve accumulation to mobilisation occurs naturally towards the end of pregnancy (Robinson 1986) resulting in a loss of LW and reserves. The question, therefore, is not whether a loss in condition is acceptable or not, but how much BCS loss is acceptable and over what length of time.

In Chapter 3, it has already been shown that a loss of 1 BCS over a period of 7 weeks leads to a catabolic state and increased hunger. However, the time period over which the BCS loss occurs may influence the metabolic and hunger state of ewes. In dairy cattle, it has been shown that a marked loss in BCS from the dry to near calving period results in a significant increase in metabolic and reproductive diseases around calving compared to a moderate loss (Kim and Suh 2003). It is likely that ewes are better able to adapt to a slow loss (months) compared to a fast loss (weeks) in BCS, because a fast loss is more likely to induce adaptive metabolic and endocrine responses leading to a catabolic state in order to free sufficient energy substrates than a slow loss. Furthermore, a fast BCS loss is likely to result in an increased feeding motivation compared to a slow BCS loss, and therefore increased hunger. If this is the case, then ewes losing BCS at a slow rate may have better welfare compared to ewes losing BCS at a fast rate. Data on different rates of BCS loss and its effect on feeding motivation, metabolic and endocrine status and welfare are to my knowledge non-existent in pregnant sheep. Therefore, the aim of the study was to investigate whether a slow loss in BCS would have less impact on hunger and lead to smaller metabolic and endocrine responses compared to a fast loss in twin-bearing ewes. This information is important when determining minimum feeding levels from both a metabolic and subjective hunger perspective.

4.3. Methods

4.3.1 Animals, housing, diets and management

Animals were selected from a base flock of 200 Coopworth x Texel ewes (4-5 year old). All ewes were kept on pasture from 60 days prior to mating until day 30 of pregnancy. Ewes were fed two different lucerne forages: FiberPro, produced by Fiber Fresh Ltd, Reporoa, New Zealand and lucerne chaff produced by lucerne Products Ltd, Putaruru, New Zealand (Table 4.1), with each ration providing half of the daily energetic requirements (NRC 2007). Ewes were synchronised for mating using Eazi-breed^{TM} CIDRs$^{®}$ (Controlled Internal Drug Release intravaginal devices containing 0.3 g progesterone, Pfizer Animal Health, Auckland, New Zealand) and mated in two different groups (100 ewes per group) during outdoor housing on pasture. Mean weights and BCS ± SEM for Groups 1 and 2 were 67 ± 1 kg and 2.9 ± 0.1 BCS, and 67 ± 1 kg and 2.8 ± 0.0 BCS, respectively. CIDRs were removed on the day the rams were introduced. Ewes normally cycle 48 h after CIDR removal (Wheaton *et al.* 1993) and, therefore, the second day after CIDR removal was taken as day 1 of pregnancy (day 1 of pregnancy on 1/04/2009 and 29/04/2008 for group 1 and group 2, respectively). Rams equipped with harnesses and crayons were present 48 h after CIDR removal and allowed to mate for three consecutive days. Pregnancy status and the number of fetuses were assessed on day 38 of pregnancy by ultrasound scanning by an experienced scanner.

Table 4.1. Nutritional values of FiberPro and chaff.

	FiberPro	Chaff
DM (%)	44	87
Organic matter (% DM)	92	89
Crude protein (% DM)	18	22
Acid detergent fibre (% DM)	37	27
Neutral detergent fibre (% DM)	52	33
Digestibility (% DM)	56	66
Metabolisable energy (MJ/kg DM	9.0	10.6

MJ = Mega Joules; DM = Dry matter

From day 30 until day 70 of pregnancy, the ewes were housed in indoor group pens. All animals within a pen were allocated to the same BCS loss treatment (see below). There were a total of seven pens (between 24 and 120 m^2), each housing between 2 and 15 ewes (depending on stocking density and diets). All pens were bedded with sawdust and water was freely available. Sheep were offered the same types of forages as provided at pasture.

4.3.2 Body condition score treatments

All 200 ewes were fed to maintain/achieve BCS 3 starting at 60 days prior to mating. Within each mating group, ewes were randomly allocated to one of three BCS loss treatments balanced for LW and BCS: Slow loss (SL) aimed at 1 BCS loss over a 12 week period, Fast Loss (FL) aimed at 1 BCS loss over a 6 week period and Control, aimed to maintain BCS 3. The nutritional manipulations to achieve the desired BCS loss started on day 37 of pregnancy at which time the Control ewes were maintained on their previous feeding level, the SL ewes were reduced to 70% of their previous feeding level and the FL ewes were reduced to 50% of their previous feeding level. The previous feeding levels were either 135% (5 SL, 5 FL and 7 Control ewes) or 175% (3 SL, 3 FL and 3 Control ewes) of maintenance requirements, depending on the BCS of the ewes. Once the treatments had started, the daily ration was adjusted on a weekly basis in order to achieve the desired BCS loss rate. Once SL and FL ewes had lost 1 BCS, they were maintained at that BCS until the last blood sample at day 140 of pregnancy had been obtained. After that, food intake was increased to more than maintenance requirements for all ewes by allowing access to paddocks with plenty of grass. Forage supplementation was gradually reduced over a 2-week period after lambing and BCS and LW were monitored once every 2 weeks until weaning of the lambs.

4.3.3 Feeding motivation methodology

Feeding motivation testing facilities were described in Chapter 3. Ewes were trained in the race according to the previously described method in Chapter 2 (Table 2.2).

4.3.4 Feeding motivation assessment

Feeding motivation was measured between day 42 and 67 of pregnancy, in order to assess feeding motivation during the period when the largest BCS loss of the FL ewes should occur. Feeding motivation was assessed with 12 ewes in the Control group, 12 in the SL group and 12 in the FL group. However, at lambing some ewes gave birth to triplets, quadruples or single lambs and these ewes were excluded from data analysis, leaving 10 twin-bearing ewes in the Control group, 8 in the SL group and 8 in the FL group. Each ewe was tested at five different cost levels (2 m, 7.2 m, 13.8 m, 44 m and 50 m) for 23 h on average per test session in random order. The reward was the lucerne chaff (Table 4.1) and the reward size was kept constant for all cost levels and treatments. The day before each feeding motivation session the ewes were placed in individual pens and were fed half their daily ration (as calculated to attain the appropriate BCS loss rate) in the morning (between 1100 and 1300 h) and half in the afternoon (between 1630 and 1700 h). Refusals were weighed out the next day so that individual ewe intake could be calculated. After each testing session, ewes were returned to the appropriate group treatment pen for 4 days.

4.3.5 Body condition score and physiological measurements

LW and BCS were measured weekly from 2 months before mating until the end of pregnancy. The width (A-measure) and depth (B-measure) of the eye muscle (*m. longissimus dorsi*), the thickness of the fat covering the eye muscle (C-measure) and the thickness of the tissue 110 mm lateral to the mid-line over the 12th rib (GR-measure) were measured by ultrasound scanning to get an accurate assessment of the body reserves (Fernandez et al. 1997; Fernandez et al. 1998). Measures were taken on day -6, 35 (baseline), 55 (day at which FL ewes should have lost 0.5 BCS), 76 (day at which FL ewes should have lost 1 BCS and SL ewes should have lost 0.5 BCS), 98, 119 (day at which SL ewes should have lost 1 BCS) and 140 (end of experiment) of pregnancy.

Metabolic and endocrine responses to loss in BCS were measured by obtaining blood samples by venipuncture of the jugular vein taken at day 35, 37, 42, 70, 84, 98, 112, 126 and 140 of pregnancy. For each sheep, blood was collected in one 15 ml heparinised vacutainer for glucose, free fatty acids (FFA), urea, β-hydroxybutyrate (β-HBA), IGF-1 and insulin analysis, and in one 15 ml EDTA vacutainer for leptin and ghrelin analysis. Food was removed around 1700 h the day before samples were taken. All blood samples were taken between 0800 and 1100 h, before feeding and were stored on ice immediately after collection. After centrifugation, plasma was stored at -20 °C until analysis.

4.3.6 Blood sample analysis

Specialist laboratories analysed blood plasma for concentrations of FFA, insulin, glucose, IGF-1, β-HBA and urea (The Liggins Institute, University of Auckland, New Zealand) and leptin and ghrelin (School of Animal Biology, University of Western Australia, Perth, Australia).

Plasma metabolite concentrations were measured on a Hitachi 902 autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan), glucose by enzymatic colorimetric assay (Roche, Mannheim, Germany), urea by kinetic UV assay

(Roche), FFA by enzymatic colorimetric assay (Randox Laboratories Ltd, Ardmore, Crumlin, UK) and β-HBA by kinetic UV assay (Randox). The average intra-assay coefficients of variation (CV) were 1.5, 1.4, 2.9 and 5.3 % for glucose, urea, FFA, and β-HBA, respectively.

Plasma hormone concentrations were measured by specific radioimmunoassay (RIA) established and validated for sheep plasma. Plasma insulin was measured according to previously published methods (Oliver et al. 1993) except that ovine insulin was used as the standard (Sigma Chemical, St. Louis, MO). The minimal detectable concentration was 0.03 ng/ml plasma and the intra- and inter-assay CVs were 11.2% and 5.6%, respectively. Plasma IGF-I was measured using an insulin-like growth factor binding protein (IGFBP)-blocked RIA (Blum & Breier 1994; Vickers et al. 1999). The detection limit was 0.7 ng/ml and the intra- and inter-assay CV were 10.8% and 12.7%, respectively.

Leptin in plasma was measured in duplicate by double-antibody RIA method (Blache *et al.* 2000). Bovine recombinant leptin (b/o-leptin), was kindly donated by Dr. Ross L. Tellam (CSIRO Tropical Agriculture, Indooroopilly, Queensland, Australia). The minimum detection limit was 0.06 ng/ml. The intra-assay and inter-assay CV were 4.2% and 8.3%, respectively. Plasma ghrelin was measured in duplicate by a double-antibody RIA method based on the Linco Total Ghrelin RIA kit. It was modified according to the method described by Miller et al. (2009). The minimum detection limit was 25 pg/ml. The mean intra-assay and inter-assay CV were 4.4 and 4.2%, respectively.

4.3.7 Pregnancy length, lamb birth and weaning weights

Lambs were weighed between 6 and 18 h after birth and ear-tagged and tail docked (with docking rings) at the same time. Lambs were weaned at 11 weeks of age and the their weaning weights were recorded.

4.3.8 Ethical note

All experimental procedures were approved by the Ruakura Animal Ethics Committee and the University of Waikato Animal Ethics Committee. All ewes were closely monitored throughout the experiment, in particular for general health and behaviour and leg and hoof health. Ewes were foot bathed in a zinc sulphate monohydrate solution 1-2 times weekly for 10 min as a preventive measure for lameness. After day 100 of pregnancy urine samples were collected weekly from the SL and FL ewes and ketone levels measured (Multistix, Siemens health care diagnostics, Deerfield, USA). Three ewes in the FL treatment showed high ketone levels (no clinical symptoms) at day 118 of pregnancy, and were treated with acetol (Ethical agents Ltd, Auckland, New Zealand, containing 85.86 g/100 ml 1-2 Propanediol, 60 ml administered twice daily) and the feed allowance was increased substantially until ketone levels returned to normal. The ewes previously showing high ketones gave birth to triplets. These ewes were therefore excluded from all data analysis. LW and BCS were measured weekly in order to monitor the health of the animals. The nutritional treatments and feeding motivation procedure did not lead to any welfare problems at any time.

4.3.9 Statistical analysis

Data are presented as means ± standard error of the mean (SEM). The effects of BCS loss rate, cost and their interaction on the number of rewards the sheep collected in the motivation races were tested by the Residual Maximum Likelihood (REML) procedure in GenStat 11, with BCS loss treatment, cost levels, run and race as fixed effects and ewe as a random effect. Some testing sessions were shorter than 20 h and 45 min and in some the reward size was lower than 3.0 g or higher than 6.5 g due to equipment malfunctioning or ewes interfering with the moving gate. Data from these sessions were excluded from analysis. In addition, all data from the ewes showing high ketones were excluded from analysis. A total of 118 testing sessions were included in the analysis (46 of Control, 39 of SL and 33 of FL ewes). In order to obtain the demand curve, the number of trips were log-transformed before analysis. The demand curve was calculated according to the model by Hursh and Winger (1995) described in Chapter 1 (Formula 1). The maximum price (P_{max}) and maximum expenditure (O_{max}) were derived from the demand curve and were the main measures of motivation (Formula 3 and 4 in Chapter 1).

Data on fat and muscle reserves, endocrine and metabolic responses were analysed to compare the three BCS loss treatments over time by REML procedure in GenStat 11 using the appropriate ante dependence covariance model, with mating group and BCS loss treatment as fixed effects and ewe as a random effect. Some of the physiological data required a log-transformation for normalization. This data is shown as observed means ± SEM but statistical significance levels were obtained from the log-transformed data. The effects of BCS loss treatment on lamb birth and weaning weight were assessed by REML with BCS loss treatment as a fixed effect and ewe as a random effect. Finally, a two-way analysis of variance (ANOVA) in GenStat 11 was used to test for significant differences between BCS loss treatments on a particular day of pregnancy.

4.4. Results

4.4.1 Body condition score, muscle and fat reserves

No differences in LW, BCS (Fig. 4.1), fat and muscle reserves (Fig. 4.2) were found between Control, SL and FL treatments at day 35 of pregnancy (baseline).

BCS (Fig. 4.1A) and LW (Fig. 4.1B) did not differ between treatments at the start of the feeding motivation test period (day 42 of pregnancy), but were different by the end of the motivation test period (day 62 of pregnancy, both $P < 0.001$), with the Control ewes having a higher BCS and LW compared to SL ewes ($P < 0.05$ and $P < 0.01$, respectively) and FL ewes ($P < 0.01$ and $P < 0.001$, respectively). The FL ewes had a lower BCS than SL ewes by day 84 of pregnancy ($P < 0.05$) and a lower LW by day 71 of pregnancy ($P < 0.01$). The FL ewes lost 1 BCS over a period of 10 week and the SL ewes lost a similar amount over 13 weeks (Fig. 4.1A).

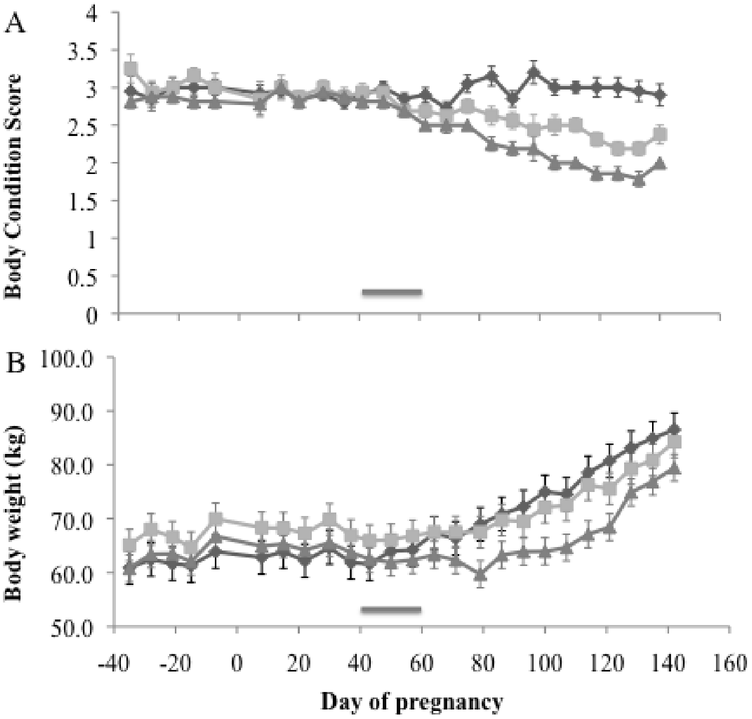


Figure 4.1. The effect of BCS loss treatment on mean ± SEM (A) BCS and (B) LW for Control (rhombus), SL (square) and FL (triangle) ewes. BCS loss period for FL treatment was between days 37 and 105 of pregnancy, and for SL treatment between days 37 and 126 of pregnancy. The grey horizontal line indicates the period of feeding motivation testing (between days 42 and 62 of pregnancy).

Between days 37 and 126 of pregnancy, the muscle width (Fig. 4.2A) and depth (Fig. 4.2B) were not different between treatments. However, the FL ewes had lower muscle width and depth compared to the Control ewes at day 140 of pregnancy (both $P < 0.05$). Fat reserves were affected by BCS loss treatment during pregnancy (eye muscle fat, $P < 0.001$, Fig. 4.2C; and GR depth, $P < 0.001$, Fig. 4.2D), with Control ewes having more fat reserves compared to SL and FL ewes (eye muscle fat and GR depth $P < 0.01$). There were time by treatment interactions for fat reserves (eye muscle fat, P < 0.01 and GR depth, P < 0.001) with the FL ewes increasing eye muscle fat less and decreasing GR depth compared to the Control ewes, while the SL ewes were intermediate.

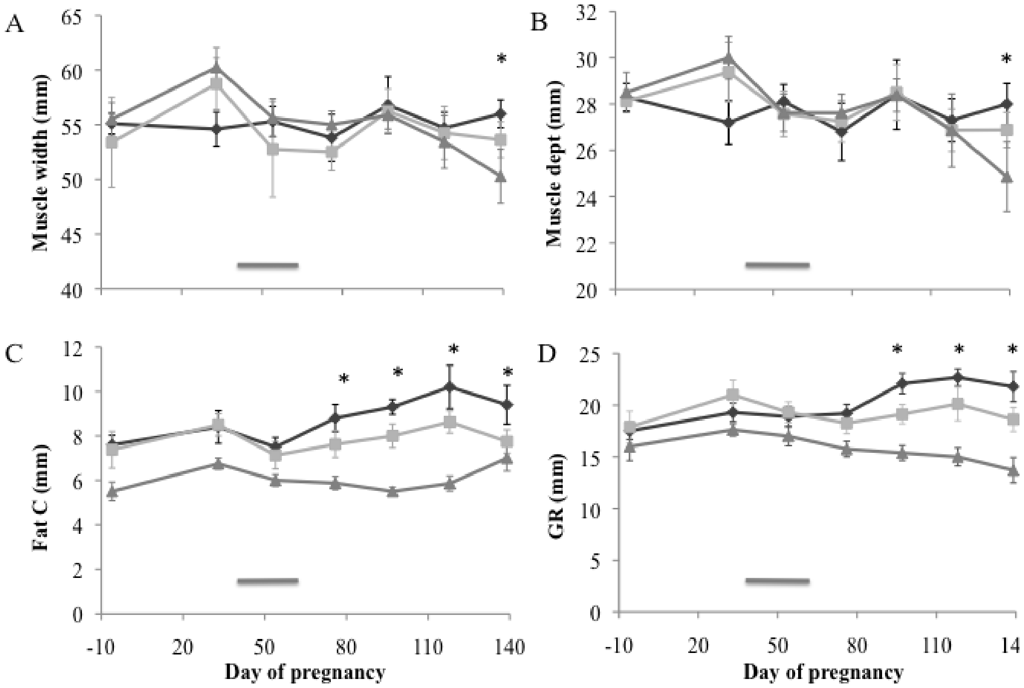


Figure 4.2. The effect of BCS loss treatment on mean ± SEM body reserves for Control (rhombus), SL (square) and FL (triangle) ewes: (A) muscle width, (B) muscle depth, (C) eye muscle fat and (D) GR depth. BCS loss period for FL treatment was between days 37 and 105 of pregnancy, and for SL treatment between days 37 and 126 of pregnancy. The grey horizontal line indicates the period of feeding motivation testing (days 42 and 62 of pregnancy). *P < 0.05 for differences at individual time points.

4.4.2 Feeding motivation and feeding levels

Feeding levels were adjusted weekly according to BCS loss and pregnancy stage. The FL ewes were maintained at their feeding level (reduction to 50% of previous feeding level, adjusted for pregnancy stage) until day 120 of pregnancy when the feeding level was slowly increased to 100% of maintenance requirements (due to some ewes showing high ketone levels, see section 4.3.8). The SL ewes were maintained at their feeding level (reduction to 70% of previous feeding level, adjusted for pregnancy stage) throughout pregnancy while the Control ewes were offered *ad libitum* food after day 120 of pregnancy, which was necessary to maintain a BCS of 3. The number of rewards consumed was not affected by the BCS loss treatment (Fig. 4.3). P_{max} values were 69 ± 19 m for Control ewes, 47 ± 7 m for SL ewes and 54 ± 10 m for the FL ewes; the difference was not significant. O_{max} values were 12,132 ± 1,916 m for Control ewes, 10,320 ± 1,108 m for SL ewes and 11,459 ± 1,366 for the FL ewes; the differences did not reach statistical significance.

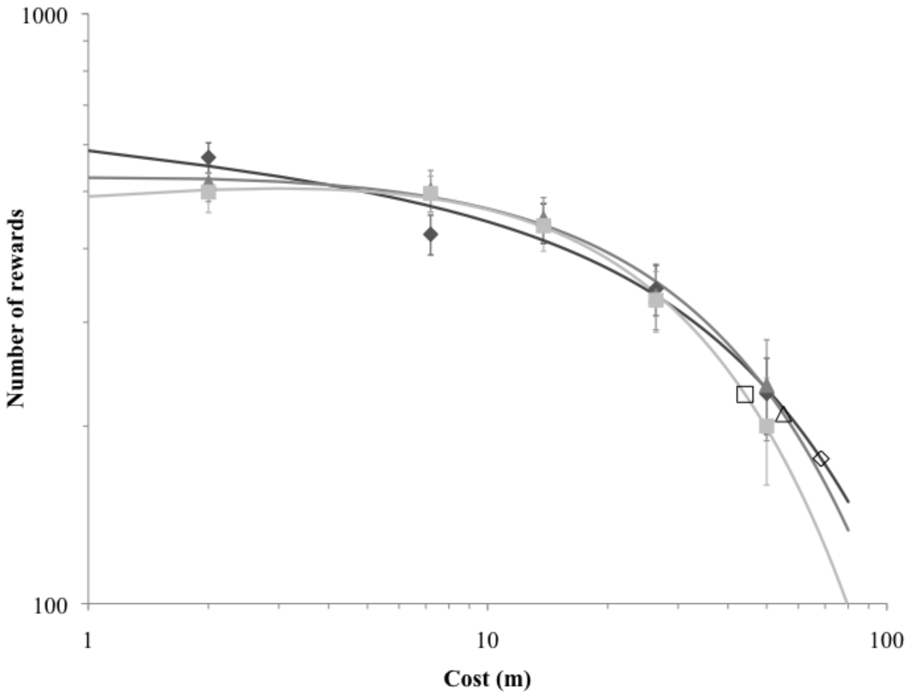


Figure 4.3. The effect of BCS loss treatment on mean ± SEM number of rewards consumed during the feeding motivation test (between days 42 and 62 of pregnancy) for Control (rhombus), SL (square) and FL (triangle) ewes. P_{max} values are indicated by the open markers for Control (open rhombus), SL (open square) and FL (open triangle).

4.4.3 Metabolic and endocrine baselines

Metabolic (Fig. 4.4) and endocrine plasma concentrations (Fig. 4.5) were not different between Control, SL and FL treatments at day 35 of pregnancy.

4.4.4 Metabolic responses

Plasma glucose concentrations were influenced by BCS loss treatment ($P < 0.05$, Fig. 4.4A), with the FL ewes having lower glucose concentrations compared to Control ewes ($P < 0.05$), while the SL ewes were not different from FL or Control ewes. There was also a time by BCS loss treatment interaction on glucose ($P < 0.05$), with FL ewes showing a decrease in mid-pregnancy and an increase in late-pregnancy while the SL and Control ewes showed a small but steady decrease. β-HBA was affected by BCS ($P < 0.01$, Fig. 4.4B), with FL ewes having higher concentrations compared to SL ewes ($P < 0.01$). β-HBA concentrations in Control ewes were not different from FL or SL ewes. Time by BCS loss interactions were also found for β-HBA ($P < 0.001$), with SL ewes increasing less than Control and FL ewes. FFA was not different between treatments overall (day 37–140 of pregnancy). However, there was a strong time by BCS loss treatment effect on FFA ($P < 0.001$, Fig. 4.4C), mainly due to the large increase in FFA in the Control ewes compared to FL and SL ewes. Urea concentrations were not different between treatments (Fig. 4.4D).

4.4.5 Endocrine responses

Plasma leptin concentrations were different between BCS loss treatments ($P < 0.001$, Fig. 4.5A); this difference was mainly due to the lower leptin concentrations of the FL ewes compared to SL ewes ($P < 0.001$) and Control ewes ($P < 0.001$). There were no differences between Control and SL ewes. The time by BCS loss treatment interaction for leptin was also significant ($P < 0.001$), with the FL ewes having lower leptin but decreasing less over pregnancy compared to Control and SL ewes. Ghrelin tended to be lower in FL ewes than SL and control ewes ($P < 0.1$, Fig. 4.5B); the time by BCS loss treatment interaction for ghrelin was not significant. Insulin concentrations were not different between BCS loss treatments (Fig. 4.5C). However, there was a tendency for a time by BCS loss treatment interaction (P < 0.1) with Control ewes showing a peak in insulin at day 112 of pregnancy while SL and FL ewes peaked at day 126 of pregnancy. While overall IGF-1 concentrations were not different between BCS loss treatments (Fig. 4.5D), there was a strong time by BCS loss treatment (P < 0.01) with Control ewes increasing more compared to SL and FL ewes.

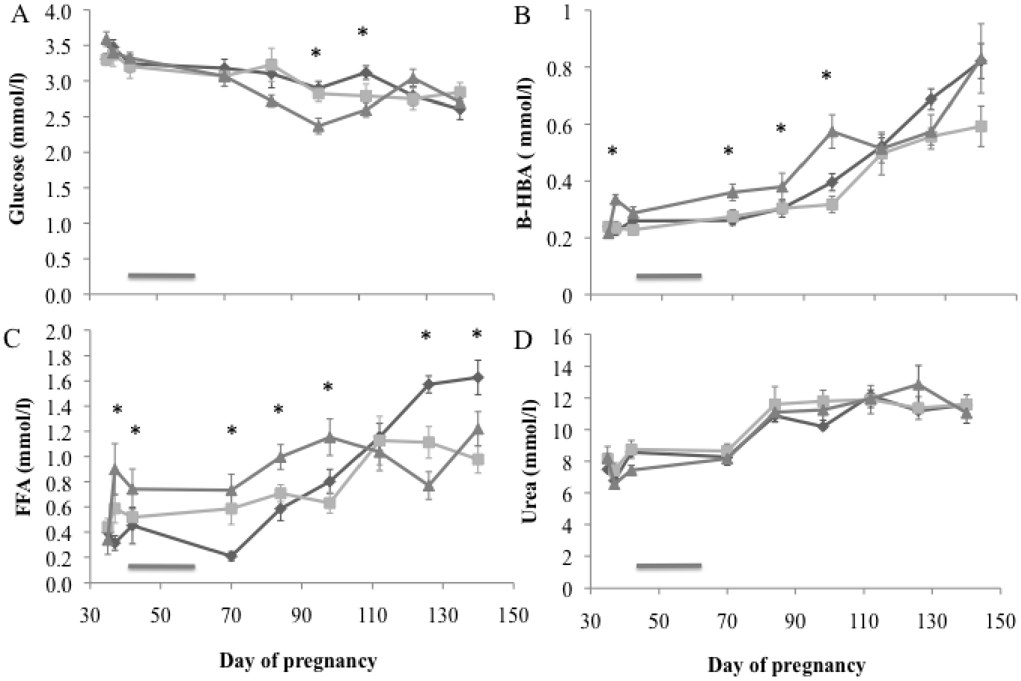


Figure 4.4. Effects of BCS loss treatments on mean ± SEM metabolic responses for Control (rhombus), SL (square) and FL (triangle) ewes: (A) Glucose, (B) β-HBA, (C) FFA and (D) Urea concentrations. BCS loss period for FL treatment was between days 37 and 105 of pregnancy, and for SL treatment between days 37 and 126 of pregnancy. The grey horizontal line indicates the period of feeding motivation testing (days 42 and 62 of pregnancy). *P < 0.05 for differences at individual time points.

4.4.6 Lamb weights

Lamb birth weights tended to be affected by BCS (Control females 4.7 ± 0.2 kg and males 4.1 ± 0.3, SL females 4.4 ± 0.2 and males 4.7 ± 0.2 kg and FL females 4.8 ± 0.2 and males 5.2 ± 0.4 kg, $P < 0.1$). There was also a tendency for sex by treatments interaction for birth weight ($P < 0.1$), with Control female lambs heavier than males, but SL and FL male lambs heavier than females. Weaning weights were unaffected by BCS loss (Control female lambs 27 ± 1 kg and males 28 ± 1, SL female lambs 26 ± 2 and males 27 ± 2 kg and FL female lambs 27 ± 1 and males 29 ± 2 kg). Pregnancy lengths were 146 ± 0 days for Control ewes, 147 ± 0 days for SL ewes and 147 ± 0 days for FL ewes; the difference was not significant.

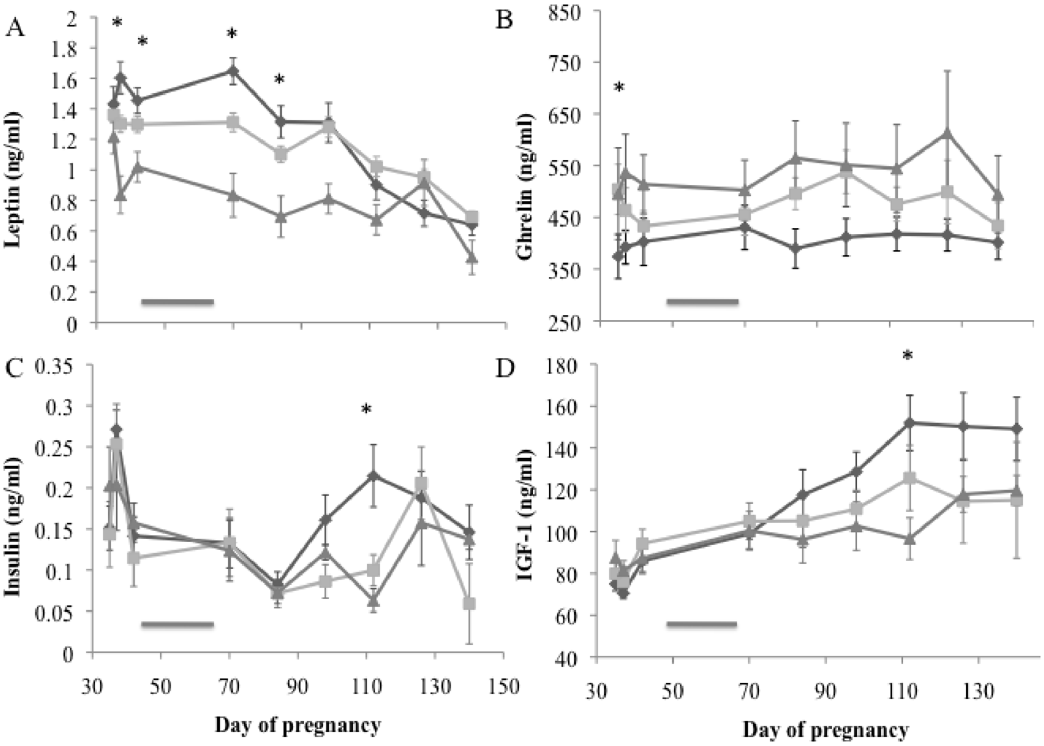


Figure 4.5. Effects of BCS loss treatments on mean ± SEM endocrine responses for Control (rhombus), SL (square) and FL (triangle) ewes: (A) Ghrelin, (B) leptin, (C) insulin and (D) IGF-1 plasma concentrations. BCS loss period for FL treatment was between days 37 and 105 of pregnancy, and for SL treatment between days 37 and 126 of pregnancy. The grey horizontal line indicates the period of feeding motivation testing (between days 42 and 62 of pregnancy). *P < 0.05 for differences at individual time points.

4.5. Discussion

The aim of the study was to determine whether a slow loss in BCS would have less impact on hunger and result in smaller metabolic and endocrine responses compared to a fast BCS loss in twin-bearing ewes. Both the subjective experience of hunger and the metabolic state may affect the welfare of pregnant ewes (for more information, see Chapter 1). Therefore, determining the level of food restriction at which welfare is compromised is important so that minimum feeding levels from both a metabolic and subjective hunger perspective can be established. No effects of BCS loss rate on feeding motivation were found in this study. Motivation was tested relatively early in the experiment (only 5 days after the start of the nutritional treatments to manipulate different BCS loss rates) and, therefore, ewes on the FL and SL treatments had not lost significant amounts of LW or BCS. This may explain the lack of BCS loss treatment affect on feeding motivation. Differences in LW, BCS and eye muscle fat between BCS loss treatments started to appear towards the end of the motivation-testing period; however, no change in motivation over time could be detected. The reason for choosing to assess motivation so early in the experiment was based on previous experience that ewes could lose 1 BCS in 7 weeks on a moderate deprivation diet (60-80% maintenance requirements, Chapter 3). In this study, feeding motivation was assessed in the period that the largest loss in BCS would occur, rather than at the end of the loss period (otherwise it would simply be a repetition of Chapter 3). Because it takes around 4 weeks to test one group, testing had to commence shortly after the start of the nutritional treatments. However, the FL ewes in the current experiment took around 10 weeks to lose 1 BCS after a reduction to 50% of the previous diet. A different type of food was used in this study compared to Chapter 3 (lucerne forage instead of a pelleted ration). Studies have reported that the digestibility of a lucerne pellet is 10% lower than a lucerne forage (Woodford and Murphy 1988; de Vega *et al.* 2000). Therefore, the use of lucerne chaff instead of a concentrate pellet could explain the higher resistance to BCS loss in this study. However, other studies comparing grass-based diets with lucerne concentrate diets found little difference in LW and BCS (Dawson *et al.* 2005) and energy availability (Goetsch *et al.* 1997) in sheep.

It has to be emphasised, however, that the amount of food consumed on a daily basis was very different between BCS loss treatments during the feeding motivation period, even though it had not yet resulted in large losses in LW and BCS. In previous studies presented in this thesis, it was shown that both a 24 h fast and a low BCS of 2 lead to considerable increases in feeding motivation (Chapter 2 and 3). It has also been shown that an increased fasting time (i.e., 0, 3, 6 and 9 h) in lactating dairy cows leads to an increase in the distance walked for food (Schütz *et al.* 2006). Therefore, there is sufficient evidence implying that food restriction leads to an increase in feeding motivation. However, the results from the current study suggest that even when food intake is restricted to 50% of previous feeding level there is no increase in feeding motivation, provided that no significant losses in BCS have occurred. It appears that feeding motivation is only increased when animals are either fasted or when a substantial amount of LW and BCS has been lost.

Plasma concentrations of metabolites are generally good indicators of the metabolic state of ewes. In early pregnancy, glucose concentrations were similar for all BCS loss treatments and did not significantly change between day 42 and 70 of pregnancy (sample taken before and after the motivation period, respectively). However, slightly higher concentrations of FFA and β-HBA were observed in the FL ewes compared to SL and Control ewes between day 42 and 70 of pregnancy. This, therefore, suggests that the FL ewes may have been in a mildly catabolic state during the feeding motivation period. However, this was not sufficient to result in an increase in feeding motivation. At day 84 of pregnancy, the FL ewes experienced a reduction in plasma glucose levels accompanied by an increase in FFA and β-HBA. However, glucose concentrations returned to values close to the SL and Control ewes by day 126 of pregnancy and FFA values also decreased, most likely due to an increase in food intake. Three FL ewes were showing high ketone levels in urine at day 118 of pregnancy and feeding levels were increased for all FL ewes as a precaution. Interestingly, the SL ewes did not show major reductions in glucose and were not significantly different from the Control ewes at any time. SL ewes also did not require an increase in feeding level towards the end of pregnancy. The Control ewes showed a major increase in FFA towards the end of pregnancy, which could be due to the higher availability of body reserves that were mobilised to support the development of the mammary glands and meet the demands of the growing fetus. The results therefore showed that the SL ewes were not in a more catabolic state compared to Control ewes that were fed *ad libitum* in late pregnancy. This suggests that ewes were able to metabolically cope with mild food restriction in late pregnancy. However, the FL ewes were in a catabolic state in mid-pregnancy. The catabolic state may have consequences for their welfare, as it has been shown in Chapter 3 that a catabolic state combined with a low BCS in mid- to late-pregnancy is associated with an increase in feeding motivation.

Endocrine markers such as insulin and leptin provide negative feedback to the hypothalamus and are involved in the regulation of LW, food intake and energy expenditure (Schwartz *et al.* 1992; Schwartz *et al.* 2000; Woods and Seeley 2000; Woods 2005). There were large differences between BCS loss treatments in leptin concentrations, even before any losses in LW and BCS had occurred. Similar results were found in a study by Delavaud (2007), in which sheep were subjected to several weeks of underfeeding resulting in a major decrease in leptin after 14 days, before any changes in LW/BCS. Other studies have also shown effects of BCS on leptin concentrations and a strong correlation between leptin and BCS (Chapter 3; Thomas *et al.* 2001) and between eye muscle fat and leptin (Blache *et* *al.* 2000) in sheep. Interestingly, even though leptin concentrations were different between BCS loss treatments around the feeding motivation period (samples taken at day 42 and 70 of pregnancy), the measures of motivation could not detect any differences between BCS loss treatments. Plasma leptin concentrations were also not a good reflection of feeding motivation measures in Chapter 3. However, other studies have shown that intracerebroventricular leptin administration reduces food intake in sheep (Henry *et al.* 2001; Morrison *et al.* 2001), suggesting that leptin is involved in the regulation of feeding motivation. The reason why leptin was not related to feeding motivation in this study is not entirely clear. It is possible that interactions between leptin and other hormones (perhaps insulin and ghrelin) at the level of the hypothalamus modulate feeding motivation in concert with each other, and interactions between them may be complex. Therefore, leptin is an important adiposity signal and a good reflection of feeding level and body reserves, but the plasma leptin concentration may not be a good indicator of feeding motivation.

Insulin is also an important adiposity signal that is involved in the regulation of LW and energy homeostasis (Woods *et al.* 2000; Woods and Seeley 2000). However, no major effects of BCS loss treatment on plasma insulin concentrations were found. Insulin, therefore, was not a good indicator of BCS loss. However, other studies reported that insulin was a good reflection of BCS in sheep (Chapter 3; Caldeira *et al.* 2007b, a), and LW in sheep (Miller *et al.* 2007). There is some evidence that insulin may affect food intake, because intracerebroventricular (ICV) insulin infusion decreases food intake in sheep (Foster *et al.* 1991). No differences in feeding motivation and plasma insulin concentrations around the feeding motivation period were found between the BCS loss treatments. In Chapter 3, feeding motivation was very different between BCS treatments and this pattern was not reflected in insulin concentrations, suggesting that insulin may not affect feeding motivation. However, in order to investigate the effects of insulin (and also leptin) on feeding motivation in more detail, more frequent sampling before or during the feeding motivation sessions may be needed in order to track short-term changes in insulin (and leptin) concentrations in relation to hunger and satiety.

IGF-1 is an important mediator of energy homeostasis and plays a key role in protein and carbohydrate metabolism (see reviews by Gluckman *et al.* 1987; Jones and Clemmons 1995). Another important role of IGF-1 is its involvement in the regulation of placental and fetal growth (see reviews by Wathes *et al.* 1998; Fowden 2003). In this study, plasma IGF-1 concentrations increased more towards the end of pregnancy in the Control ewes than in the FL and SL ewes. The FL ewes showed a small drop in IGF-1 concentrations at day 112 of pregnancy, but values were similar to the SL ewes by day 126 of pregnancy. This was most likely due to the increase in food intake by the FL ewes. IGF-1 is generally a good indication of the metabolic state in pregnant sheep, but does take several months to reflect changes in BCS (Chapter 3). No differences in IGF-1 around the feeding motivation period were observed, which is not surprising because nutritional treatments had only just started. Furthermore, there is no evidence that IGF-1 influences food intake in sheep (Foster *et al.* 1991). Therefore, based on the current and the Chapter 3 study, IGF-1 is a good indicator of long-term changes in BCS but not of feeding motivation.

Another endocrine signal that may be involved in the regulation of LW and feeding motivation is ghrelin. A tendency for lower ghrelin concentrations was found in FL ewes. However, increased adiposity per se may not influence plasma ghrelin concentrations (Qi *et al.* 2008). Rather, ghrelin may be a short-term regulator of food intake. For example, it has been shown that fasting increases ghrelin concentrations compared to well fed rats (Tschop *et al.* 2000). In sheep, it has been shown that ghrelin is secreted in anticipation of a meal and may play a role in meal initiation (Sugino *et al.* 2002a; Sugino *et al.* 2004). Furthermore, administration of ghrelin stimulates food intake in cattle (Wertz-Lutz *et al.* 2006) and food intake and LW gain in rats (Tschop *et al.* 2000). However, ghrelin administration does not lead to a significant increase in food intake in sheep (Iqbal *et al.* 2006; Melendez *et al.* 2006). No differences in plasma ghrelin concentrations between BCS loss treatments were observed during the feeding motivation period. However, more frequent sampling during the feeding motivation sessions would be useful in investigating the role of ghrelin on feeding motivation. Furthermore, total ghrelin (sum of des-acyl and acyl ghrelin) was measured in this study, and there are indications that the acylated form of ghrelin has a biologically different action than the desacylated form (Castañeda *et al.* 2010). The acylated form most likely plays a more important role in the regulation of appetite than the desacylated form (Castañeda *et al.* 2010). It would be valuable to measure acyl-ghrelin separately in relation to feeding motivation in order to detect any regulatory effects. In summary, ghrelin may not be a good indication of BCS in sheep. More research is required in order to find any regulatory effects of ghrelin on feeding motivation.

Subjective experiences of animals have received considerable attention in the past decade and are considered key determinants of animal welfare (Desire *et al.* 2002; Dawkins 2006; Boissy *et al.* 2007; Dawkins 2008; Yeates and Main 2008). In this study, the hunger experienced by ewes losing BCS at different rates was of particular interest and feeding motivation was used as an indicator of hunger. The motivation for a resource is a good indicator of the importance of that resource to the animal (Dawkins 1990; Jensen and Toates 1993; Jensen and Pedersen 2008), and the welfare of animals is considered to be poor when they have a high motivation for the resource, but are unable to get it (Dawkins 1990). In this thesis it was found that feeding motivation was increased with short-term (Chapter 2) and long-term hunger (Chapter 3), suggesting that feeding motivation provides insight in the subjective hunger state of the animal. The lack of a difference in feeding motivation in the current study indicated that a dietary restriction to 50% of previous feeding level not leading to significant losses in LW and BCS does not increase hunger compared to ewes fed to maintain BCS. It appears that ewes have a good ability to cope with food restriction in early pregnancy and hunger does not increase until a significant amount of BCS has been lost.

The biological functioning (i.e., growth, health and reproduction) of the animal is generally also considered to be a key determinant of animal welfare (Mendl 2001). In this study, measures of biological functioning were limited to those that are directly related to the metabolic state of the animal. FL ewes had lower glucose and higher β-HBA in mid-pregnancy, which indicates they were in a metabolically catabolic state. A catabolic state with high levels of β-HBA could potentially lead to the development of metabolic diseases such as ketosis (Scott *et al.* 1995; Van Saun 2000), which could compromise the health and welfare of ewes. There was a tendency of a BCS loss effect on lamb birth weights. There are several studies that have shown that lambs born to undernourished ewes may be smaller, have reduced growth, survival and health in later life (Clarke *et al.* 1997; Barker 2001; Bloomfield *et al.* 2003b; De Blasio *et al.* 2007). Interestingly, the lambs born to the FL ewes tended to be heavier than the SL and Control ewes; the reason for this is not clear. In addition, there was a tendency for a sex by BCS loss interaction, with male lambs born to the FL and SL ewes heavier than females, but female lambs born to Control ewes heavier than males. The reason for this is not entirely clear, because in most circumstances male lambs are heavier than females (Miller *et al.* 2010).

No additional measures of biological functioning were obtained in this study. Other studies have used immunological markers as indicators of health and welfare (Douglas *et al.* 1998; Tuchscherer *et al.* 2004; Boissy *et al.* 2007). It has been shown that there is a strong link between nutrition and immunity against parasites in sheep (Van Houtert and Sykes 1996) and the immune response to a parasite infection is enhanced in sheep fed at a high feeding level compared to a 70% of maintenance restriction level in early pregnancy (Valderrabano *et al.* 2006). Furthermore, a link between body fat and immunity has been established in rodents (De Rosa *et al.* 2007; Desruisseaux *et al.* 2007). The function of the immune system in ewes with different BCS could be further explored in order to obtain immunological measures as welfare indicators.

In conclusion, the different rates of BCS loss did not affect feeding motivation. These results suggest that twin-bearing ewes subjected to food restriction to 50% of previous feeding levels between day 42 and 67 of pregnancy were not hungrier than ewes fed to maintain BCS, provided no major losses in BCS had occurred. Therefore, it appears that feeding motivation is only increased when BCS is low or when ewes have been fasted. SL ewes were in a similar metabolic state compared to Control ewes, indicating that pregnant ewes are metabolically well able to adapt to mild food restriction in pregnancy. FL ewes experienced a catabolic state in mid-pregnancy, which may have implications for their welfare as this is likely to result in an increased feeding motivation. Several aspects of biological functioning of ewes losing BCS at different rates remain to be investigated.

Chapter 5. The effects of body condition score on metabolic and stress responses to an acute cold challenge in pregnant ewes

5.1. Abstract

The dietary intake of grazing sheep may not be constant due to alterations in pasture growth, which may lead to changes in body condition score (BCS) and the metabolic state of sheep. Additional environmental challenges such as cold exposure may compromise the ability to maintain energy homeostasis, in particular when ewes are food restricted. The aim was to expose pregnant ewes with different BCS to an acute cold challenge in order to assess whether BCS affects the metabolic and stress responses. Ewes were divided into three BCS treatments at day 35 of pregnancy (low BCS, LBC; medium BCS, MBC; and high BCS, HBC; with six ewes per treatment) and shorn 2 weeks before the acute cold challenge (day 71 of pregnancy). Ewes were exposed to a 6-hour acute cold challenge between days 85-87 of pregnancy in a climate-controlled room (4.5ºC) equipped with water sprinklers and wind fans. Fourteen blood samples were collected via jugular catheters at regular intervals. Plasma insulin and cortisol responses to the acute cold challenge were lower in LBC ewes compared to MBC and HBC ewes. LBC ewes also took longer to increase cortisol and thyroxine (T4) concentrations. Furthermore, LBC and MBC ewes were slower to mobilise free fatty acids (FFA) than HBC ewes. All ewes were able to increase plasma glucose concentrations and internal body temperature in response to the cold challenge. In conclusion, BCS considerably affected the cortisol and metabolic responses to a cold challenge. The decreased and delayed stress response observed in LBC ewes implied that undernutrition is a chronic stressor in pregnant ewes. Therefore, pregnant low BCS ewes may have a reduced ability to respond to acute challenges.

5.2. Introduction

Grazing sheep in temperate regions may be exposed to chronic environmental challenges, such as long-term undernutrition leading to a loss of body reserves due to inadequate pasture growth and nutritional supplementation. Grazing sheep may also be exposed to acute challenges at the same time, such as low temperatures, wind and rain during winter. Furthermore, it is common practice in New Zealand to shear ewes in mid-pregnancy to improve lamb birth weights (Kenyon *et al.* 2003), which leads to acute cold exposure. The ability to induce adaptive metabolic and endocrine responses in order to maintain energy homeostasis is likely to be a main determinant of the health and welfare of animals during environmental challenges (Chapter 1).

Exposure to stressful challenges, such as cold, will normally lead to activation of the hypothalamo-pituitary-adrenal (HPA) axis resulting in the release of cortisol. The latter facilitates the mobilisation of energy substrates and supports energy homeostasis. Therefore, it is essential that an appropriate HPA-axis stress response is mounted during metabolically demanding challenges. In addition, an increase in food intake (Ekpe and Christopherson 2000) and resting metabolic rate (Slee 1972; Ekpe and Christopherson 2000) have been observed in cold exposed sheep. However, undernourished ewes with low body fat reserves that are unable to increase food intake may have difficulties obtaining sufficient substrates to conserve or generate heat (thermogenesis). In previous research it has been shown that pregnant ewes with low body condition score (BCS) have lower levels of plasma insulin, leptin, cortisol, glucose and insulin like growth factor 1 (IGF-1) and increased FFA and β-hydroxybutyrate (β-HBA) compared to ewes with a moderate or high BCS (Chapter 3 and 4), raising the question of whether low BCS ewes are able to maintain energy homeostasis during acute challenges, such as sudden reductions in temperature.

The adaptive metabolic, endocrine and stress responses to an acute challenge could provide a deeper insight into the level of biological functioning of ewes with different BCS. A reduced level of biological functioning could be an indication of reduced welfare (Mendl 2001). Therefore, the aim of the study was to investigate the effects of different BCS on the metabolic and stress responses to an acute cold challenge in mid-pregnancy. Furthermore, the metabolic and endocrine responses during BCS change were also studied in order to assess the metabolic state of ewes with different BCS.

5.3. Methods

5.3.1 Animals and management

Animals were selected from a base flock of 100 (4-5 year old) Coopworth x Texel ewes. Ewes initially maintained on pasture were synchronised for mating using Eazi-breed^{TM} CIDRS$^{®}$ (intravaginal controlled internal drug release devices containing 0.3 g progesterone, Pfizer Animal Health, Auckland, New Zealand) and mated in two different groups (50 ewes per group). Mean weights and BCS for Groups 1 and 2 were 56.4 ± 0.7 kg and 3.1 ± 0.07 BCS, and 58.0 ± 0.9 kg and 3.1 ± 0.08 BCS, respectively. Ewes should have been cycling 48 h after CIDR removal (Wheaton *et al.* 1993) and this day was taken as day 1 of pregnancy (day 1 of pregnancy on 9/04/2008 and 30/04/2008 for group 1 and group 2, respectively). Rams equipped with harnesses and crayons were present at CIDR removal and allowed to mate for three consecutive days. Pregnancy and the number of fetuses were confirmed on day 41 and 62 of pregnancy by ultrasound scanning. All ewes were kept on pasture from the start of the study (60 days prior to mating) until day 60 of pregnancy and were then housed in individual pens (1.3 x 1.0 x 1.0 m high pens constructed of steel posts and mesh with water available *ad libitum*). Ewes were grazed together as one group after completion of the cold challenge (day 90 of pregnancy until weaning of the lambs). Lamb birth weights were measured between 6 h and 16 h after birth, weaning weights were measured at 10 weeks of age.

5.3.2 Body condition score treatments

All 100 ewes were fed to attain BCS 3 starting at 60 days prior to mating. Within each mating group, ewes were randomly allocated to three different BCS treatments balanced for live weight (LW) and BCS. The three BCS treatments were low BCS (LBC; aimed at BCS 2), medium BCS (MBC; aimed at BCS 3) and high BCS (HBC; aimed at BCS 4). Ewes were fasted for 24 h on day 36 of pregnancy in order to obtain an accurate estimation of LW and measure metabolic and endocrine responses to fasting. The nutritional manipulations to change BCS started on day 37 of pregnancy at which time the LBC, MBC and HBC ewes were offered a pelleted ration (UniC pellet, Table 2.1 in Chapter 2) at 65%, 130% and 200% of theoretical maintenance requirements, respectively (NRC 2007). The daily ration was adjusted weekly depending on BCS gain/loss in order to reach the target BCS at day 83 of pregnancy. Ewes were fed in single pens from day 60 of pregnancy onwards according to individual needs and were offered the same sheep pellets and an additional handful of hay per day.

5.3.3 Body condition score change phase

LW and BCS were measured weekly until the end of pregnancy. The width (A-measure) and depth (B-measure) of the eye muscle (*m. longissimus dorsi*), the thickness of the fat covering the eye muscle (C-measure) and the thickness of the tissue 110 mm lateral to the mid-line over the 12th rib (GR-measure) were measured by ultrasound scanning on day 35 and 76 of pregnancy to obtain an accurate assessment of the body reserves (Fernandez *et al.* 1997; Fernandez *et al.* 1998).

Ewes were shorn at day 71 of pregnancy and blood samples on the pre-shearing day (day 70) and post-shearing day (day 72) were collected by venipuncture of the jugular vein (experimental timeline shown in Fig. 5.1). Internal body temperature was measured every 10 min using a modified CIDR® fitted with a microprocessor-controlled MinilogTX data logger (Vemco Ltd., Shad Bay, Nova Scotia, Canada). Additional blood samples were taken at day 0, 35, 36, 37, 49, 79 and 87 or 89 of pregnancy (Fig. 5.1). Food was removed around 1700 h the day before each sample was taken. For periods when the sheep were kept at pasture, the animals were held indoors (in familiar group pens) the night before samples were obtained (samples taken at day 0, 35, 36, 37, and 49 of pregnancy). All blood samples were taken between 0800 and 1100 h, before feeding and were stored on ice immediately after collection. After centrifugation, plasma was stored at -20°C until analysis. The environmental temperature in the indoor housing facility was recorded every 10 min by HOBO Pro Dataloggers (Onset Computer Corporation, Bourne, MA, USA) and the average temperature was 10.8 ± 2.7 °C.

5.3.4 Acute cold challenge

Six ewes per treatment (14 single and 4 twin-bearing ewes) were exposed to a 6 h acute cold challenge at day 85-87 of pregnancy (Fig. 5.1). The acute cold challenge took place in an environmental chamber that could be controlled for temperature and humidity and contained wind fans and mist sprinklers. Ewes were divided into four subgroups and were tested on separate days (between 3 and 5 ewes per testing day), with ewes from different BCS treatments as balanced as possible on each day. Intracath blood sampling devices consisting of 12G needles and 040 PVC tubing (Biocorp, Huntingdale, Australia) were inserted into the jugular vein 2 days before the cold challenge under local anaesthesia. Ewes were transported to the environmental chamber in a standard sheep trailer (approximately 2 min drive) and kept in metabolic crates the day before the acute cold challenge for 2 h without being exposed to cold for acclimatization and were then returned to their home pens. Six calibrated I-buttons (Kooltrak Standard Temperature Logger Unmounted, Kooltrak GmbH, Geisenheim, Germany) were attached to electrocardiograph (ECG) patches and glued to 2-3 cm^2 areas of closely clipped skin (one on the base of each ear, one on each side of the mid-trunk and one on each hind leg) after the acclimatization period. On the morning of the cold challenge, ewes were transported to the environmental chamber and placed in metabolic crates at ambient temperature (11.8–14.7 °C). A baseline blood sample was taken 1 h after arrival. The acute cold challenge started exactly 2 h after the baseline blood sample had been taken by reducing the chamber temperature to 5°C, activating the mist sprinklers and wind fans and by wetting the sheep with approximately 10 L of water from a standard plastic bucket. Temperature and wind data were recorded with a Kestrel® 3000 pocket wind meter (Nielsen-Kellerman, Boothwyn, USA). The fans produced wind for the full 6 h (average 0.5 m/s, minimum 0.2 m/s and maximum 3.2 m/s) and mist sprinklers were activated every 30 min for 5 min producing 15.4 mm/s of water to keep ewes moist. Blood samples were collected at 1, 10, 20, 30, 40, 50, 60, 90, 120, 150, 180, 240, 300 and 360 min. After the last blood sample had been collected the chamber temperature was increased to 18 ºC and ewes were towel dried and transported back to their home pens within 30 min. Recovery blood samples were taken 2 h post-challenge and the following morning around 0900 h (day 86-88 of pregnancy). Ewes were fed immediately after collecting the 2 h post-challenge sample.

Figure 5.1. Experimental timeline

5.3.5 Blood sample analysis

Specialised laboratories were employed to analyse blood plasma for concentrations of FFA, cortisol, insulin, glucose, IGF-1, β-HBA and urea (The Liggins Institute, University of Auckland, New Zealand) and leptin and ghrelin (School of Animal Biology, University of Western Australia, Perth).

Glucose was analysed by enzymatic colorimetric assay (Roche, Mannheim, Germany), urea by kinetic UV assay (Roche), FFA by enzymatic colorimetric assay (Randox Laboratories Ltd, Ardmore, Crumlin, UK) and β-HBA by kinetic UV assay (Randox). The average intra-assay coefficients of variation (CV) were 2.2, 1.7, 2.7, and 3.2 % for glucose, urea, free fatty acids, and β-HBA, respectively.

Plasma hormone concentrations were measured by specific radioimmunoassay (RIA). Plasma insulin was measured according to previously published methods (Oliver *et al.* 1993) except that ovine insulin was used as the standard (Sigma Chemical, St. Louis, MO, batch # I9254). The minimal detectable concentration was 0.03 ng/ml plasma and the inter- and intra-assay CV values were 9.3% and 12.4%, respectively. Plasma IGF-I was measured using an insulin-like growth factor binding protein (IGFBP)-blocked RIA (Blum and Breier 1994; Vickers *et al.* 1999). The detection limit was 0.7 ng/ml and the inter- and intra-assay CV were 9.5% and 10.0%, respectively. Cortisol was measured using mass spectrometry according to previously published methods (Rumball *et al.* 2008); mean inter- and intra-assay CV values were 11.2% and 7.1%.

Plasma leptin concentrations were measured in duplicate by double-antibody RIA method (Blache *et al.* 2000). Bovine recombinant leptin (b/o-leptin) was kindly donated by Dr. Ross L. Tellam (CSIRO Tropical Agriculture, Indooroopilly, Queensland, Australia). The minimum detection limit was 0.05 ng/ml. The intra-assay and inter-assay CV were 4.2% and 8.3%, respectively. Plasma ghrelin was measured in duplicate by a double-antibody RIA method based on the Linco Total Ghrelin RIA Kit. The method was modified according to the method described by Miller et al. (2009). The minimal detection limit was 25 pg/ml. The intra-assay and inter-assay CV were 4.2% and 4.4%, respectively.

5.3.6 Ethical note

All experimental procedures were approved by the Ruakura Animal Ethics Committee and the University of Waikato Animal Ethics Committee. All ewes were closely monitored for body posture, alertness, and food intake throughout the experiment. After catheter insertion ewes were also monitored for swelling or inflammation at the catheter site and internal body temperature twice a day. No adverse signs were observed at any time. During the acute cold challenge, ewes were continuously and closely monitored for behavioural signs of inability to cope (i.e., body posture and alertness); no negative signs were observed at any time. Ewes recovered quickly and ceased shivering within 30 min after the completion of the challenge. Ewes were fed immediately after the 2 h post-challenge blood sample was collected and body posture and alertness, food intake and internal body temperature were also checked.

5.3.7 Statistical analysis

Data are presented as means ± standard error of the mean (SEM), unless stated otherwise. Data on metabolic and endocrine responses to fasting and shearing were analysed by a repeated measures analysis in GenStat 11 on day 36 and 37 with day 35 as a covariate and on pre- and post-shearing days, with day 35 as a covariate. In addition, a two-way analysis of variance (ANOVA) in GenStat 11 was done for each individual day. Data on metabolic and endocrine responses to changes in BCS over time were analysed with the Residual Maximum Likelihood (REML) procedure in GenStat 11 using the appropriate ante dependence covariance model, with BCS treatment as a fixed effect and ewe as a random effect. Data were log-transformed when residuals did not pass the Shapiro-Wilk test for normality. Fat and muscle data on day 76 of pregnancy were analysed by general ANOVA in GenStat 11 with day 35 as a covariate. Effects of four different factors - BCS treatment, actual BCS (individual animal BCS measured at day 83 of pregnancy), actual LW (individual animal LW measured at day 83 of pregnancy) and the change in LW from the start of the treatments (day 35 of pregnancy) - on the area under the curve (AUC), the highest plasma concentration (peak) and the rate at which this value was reached (rate to peak) were tested by linear regression in GenStat 11, with the baseline value taken 2 h before the start of the challenge as covariate. The cold challenge was divided into a 0-60 min period and a 60-360 min period for the statistical analysis because of the biphasic nature of most of the responses. Exceptions were leptin and glucose plasma concentrations; leptin reduced during the acute cold challenge and was therefore analysed with a repeated measurements analysis. Glucose did not peak in the first 60 min and a regression analysis was therefore used to analyse the full 360 min. The changes in skin temperature (T_{skin}) were analysed by repeated measurement analysis. Data on internal body temperature (T_{core}) were analysed by linear regression of BCS treatment on peak values. Data on lamb birth and weaning weight were analysed with REML in GenStat 11, with BCS treatment and lamb sex as fixed effects and ewe as a random effect (singleton lambs only).

5.4. Results

5.4.1 Baselines

LW, BCS (Table 5.1), muscle, fat and GR depth (Table 5.2) were not different between BCS treatments at day 35 of pregnancy. Endocrine and metabolic plasma concentrations did not differ between BCS treatments at day 35 of pregnancy (Table 5.3).

5.4.2 Body condition score change phase

The BCS and LW were lower in LBC ewes compared to MBC (both $P < 0.01$) and HBC (both $P < 0.01$) ewes at the time of the acute cold challenge (day 83 of pregnancy, Table 5.1). Eye muscle width ($P < 0.05$), eye muscle depth ($P < 0.01$), eye muscle fat cover ($P < 0.01$) and GR depth ($P < 0.001$) were influenced by BCS treatments at day 76 of pregnancy, with LBC ewes having lower muscle and fat reserves compared to MBC and HBC ewes (Table 5.2).

Table 5.1. Mean ± SEM body condition score (BCS), live weight (LW) and change in weight (between day 35 and 83 of pregnancy, WC) of LBC, MBC and HBC ewes at day 35 (baseline) and 83 of pregnancy.

	Day of pregnancy	35		83	
Factor	Treatment	*Means	SEM	*Means	SEM
BCS	LBC	2.7	0.1	2.4^{A}	0.15
	MBC	2.7	0.1	3.2^{B}	0.11
	HBC	2.7	0.1	3.6^{B}	0.16
LW	LBC	54.4	2.0	52.3^{A}	1.7
(kg)	MBC	57.1	2.2	64.3^{B}	1.6
	HBC	54.0	0.8	65.3^{B}	1.6
WC	LBC			-2.1	1.8
(kg)	MBC			7.2	0.7
	HBC			11.3	1.1

^{*A,B}Within a factor within a column mean values with a different superscript letters are significantly different ($P < 0.01$).

5.4.2.1. Fasting

Plasma FFA ($P < 0.001$) and β-HBA ($P < 0.001$) concentrations increased and plasma insulin ($P < 0.001$), IGF-1 ($P < 0.01$), and leptin ($P < 0.001$) concentrations decreased in response to the fast at day 36 of pregnancy (Table 5.3). Leptin was the only hormone that was affected by BCS at day 37 of pregnancy, and was lower in LBC compared to MBC ewes ($P < 0.05$).

Table 5.2. Mean ± SEM eye muscle width, depth, fat and GR depth for LBC, MBC and HBC ewes at day 35 and 76 of pregnancy.

	Day of pregnancy	35		76	
Factor	Treatment	*Mean	SEM	*Mean	SEM
Muscle width	LBC	55.3	2.7	53.2^{a}	1.8
(mm)	MBC	53.8	1.2	58.6^{b}	1.2
	HBC	52.7	0.4	$57.3^{a,b}$	1.4
Muscle depth	LBC	28.2	1.6	26.7^{A}	1
(mm)	MBC	27.2	0.5	31.0^{B}	0.5
	HBC	26	0.3	$29.0^{A,B}$	0.9
Muscle fat	LBC	6	0.7	5.0^{A}	0.5
(mm)	MBC	5.5	0.4	$6.3^{A,B}$	0.7
	HBC	5.8	0.8	8.0^{B}	0.7
GR depth	LBC	11.5	1.1	12.5^{A}	1.4
(mm)	MBC	9.8	0.9	17.8^{B}	0.6
	HBC	12.2	0.7	17.5^{B}	0.6

[*a,b]Within factor within a column, mean values with different superscript letters are significantly different, lower case letters indicating significance of $P < 0.05$ and capitals of $P < 0.01$.

Table 5.3. The effects of BCS treatment on mean ± SEM metabolic and endocrine baseline values (day 35), fasting (day 36) and start of nutritional treatments (day 37) for LBC, MBC and HBC ewes.

Day of pregnancy	BCS	Glucose (mmol/l)		FFA (mmol/ml)		β-HBA (mmol/l)		Leptin (ng/ml)		Cortisol (ng/ml)		IGF-1 (ng/ml)		Insulin (ng/ml)	
		*Means	SEM	*Means	SEM	*Means	SEM	*Means	SEM	*Means	SEM	*Means	SEM	*Means	SEM
Day 35	LBC	3.28	0.13	0.26	0.04	0.15	0.02	1.84	0.25	15.20	3.08	54.80	3.90	0.13	0.03
	MBC	3.13	0.13	0.33	0.06	0.15	0.02	1.71	0.29	12.96	2.80	55.73	5.54	0.06	0.01
	HBC	3.48	0.18	0.22	0.04	0.17	0.02	2.14	0.28	14.80	2.43	50.53	3.75	0.18	0.06
Day 36	LBC	3.07	0.23	0.93	0.09	0.31	0.02	0.71	0.10	18.77	3.00	39.85^{a}	3.73	0.10	0.04
	MBC	3.08	0.19	1.02	0.14	0.35	0.03	0.72	0.04	10.90	2.94	52.62^{b}	3.73	0.07	0.02
	HBC	3.00	0.20	0.82	0.09	0.28	0.03	0.73	0.06	12.94	2.41	$41.00^{a,b}$	3.43	0.08	0.03
Day 37	LBC	3.28	0.11	0.41	0.04	0.19	0.02	1.362^{a}	0.23	12.70	4.22	50.58	3.44	0.18	0.03
	MBC	3.57	0.17	0.38	0.14	0.22	0.06	1.868^{b}	0.25	11.43	4.84	54.57	3.29	0.19	0.05
	HBC	3.48	0.16	0.22	0.03	0.14	0.03	$2.072^{a,b}$	0.24	10.99	3.00	48.12	3.90	0.26	0.06
BCS effect		ns		0.026		0.044		ns		ns		0.021		ns	
Time effect		ns		<0.001		< 0.001		< 0.001		0.072		0.007		< 0.001	
Interaction		ns		0.02		ns		ns		ns		ns		0.088	

[*a,b] Within a day within a column, mean values with different superscript letters are significantly different (P < 0.05).Values in rows “BCS effect”,” Time effect” and “Interaction” are P values indicating the level of significance across the three days, ns indicates a non-significant effect.

Plasma glucose, FFA and leptin concentrations were lower in LBC ewes compared to MBC ($P < 0.01$, $P < 0.05$ and $P < 0.01$, respectively) and HBC ewes ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively) during the BCS change phase (Fig. 5.2); the MBC and HBC ewes were not different. A time by BCS treatment interaction was found for FFA ($P < 0.05$) and leptin ($P < 0.01$), with FFA increasing and leptin decreasing more in LBC ewes compared to MBC and HBC ewes. β-HBA, insulin, IGF-1, ghrelin and cortisol plasma concentrations were not influenced by BCS treatment during the BCS change phase (data not shown).

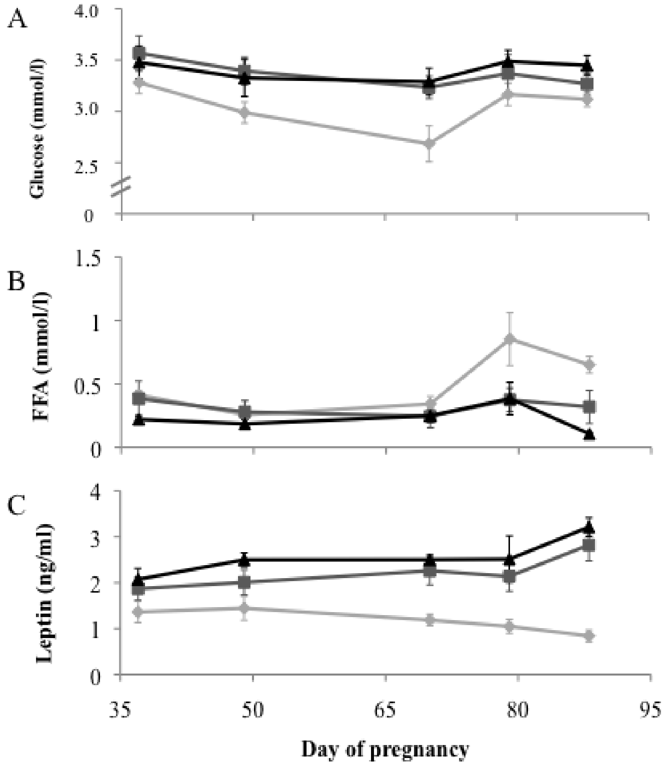


Figure 5.2. The effect of BCS treatment on mean ± SEM metabolic and endocrine responses for LBC (rhombus), MBC (square) and HBC (triangle) ewes between day 37 and 87 of pregnancy: (A) glucose, (B) FFA and (C) leptin plasma concentrations.

5.4.3 Shearing

Plasma glucose and FFA concentrations (both $P < 0.001$) increased and cortisol tended to increase ($P < 0.1$) in response to shearing; the time by BCS treatment interactions were not significant (Table 5.4). Leptin and ghrelin decreased in response to shearing (both $P < 0.01$, Table 5.5). There tended to be an interaction between shearing and BCS for leptin ($P < 0.1$), with leptin decreasing more in MBC and HBC ewes compared to LBC ewes in response to shearing. T4 also increased in response to shearing ($P < 0.001$). No responses to shearing in β-HBA, insulin and IGF-1 were found (Table 5.4 and 5.5).

Table 5.4. The effects of shearing (day 71 of pregnancy) and BCS treatment on mean ± SEM metabolic plasma concentrations of LBC, MBC and HBC ewes measured on pre-shearing (day 70 of pregnancy) and post-shearing (day 72 of pregnancy) days.

Day	BCS	Glucose		FFA		β-HBA	
		*Means	SEM	*Means	SEM	*Means	SEM
Pre-shearing	LBC	2.68^{a}	0.17	0.34	0.06	0.28	0.04
	MBC	3.23^{b}	0.11	0.25	0.09	0.25	0.04
	HBC	3.29^{b}	0.13	0.25	0.05	0.23	0.03
Post-shearing	LBC	3.43	0.10	0.83	0.17	0.30	0.04
	MBC	3.73	0.18	0.51	0.14	0.26	0.04
	HBC	3.61	0.17	0.67	0.10	0.27	0.04
BCS treatment effect		0.005		ns		ns	
Time effect		< 0.001		< 0.001		ns	
Interaction		ns		ns		ns	

^{*a,b}Within a day and within a column, mean values with different superscript letters are significantly different ($P < 0.05$). Values in rows “BCS effect”,” Time effect” and “Interaction” are P values indicating the level of significance across the three days, ns indicates a non-significant effect.

Table 5.5. The effects of shearing (day 71 of pregnancy) and BCS treatment on mean ± SEM endocrine plasma concentrations of LBC, MBC and HBC ewes measured on pre-shearing (day 70 of pregnancy) and post-shearing (day 72 of pregnancy) days.

Day	BCS	leptin		cortisol		IGF-1		T4		Insulin	
		*Means	SEM	*Means	SEM	*Means	SEM	*Means	SEM	*Means	SEM
Pre-shearing	LBC	1.19^{a}	0.13	6.01	0.443	55.05	2.70	76.95^{a}	4.75	0.1332	0.03078
	MBC	2.26^{b}	0.31	11.50	3.942	81.7	10.55	89.99^{a,b}	5.73	0.2055	0.04541
	HBC	2.50^{b}	0.10	9.94	1.038	71.52	3.89	98.52^{b}	7.15	0.2002	0.05407
Post-shearing	LBC	1.17^{a}	0.09	11.07	2.912	60.83	6.64	106.20	7.47	0.2488	0.06837
	MBC	1.84^{b}	0.25	12.75	2.702	75.45	6.32	109.60	6.24	0.2177	0.10196
	HBC	1.83^{a,b}	0.28	17.47	3.677	64	7.45	121.30	8.80	0.222	0.05121
BCS treatment effect		< 0.001		0.074		0.008		ns		ns	
Time effect		0.006		0.094		ns		< 0.001		ns	
Interaction		0.069		ns		ns		ns		ns	

^{*a,b} Within a day and within a column, mean values with different superscript letters are significantly different ($P < 0.05$). Values in rows “BCS effect”,” Time effect” and “Interaction” are P values indicating the level of significance across the three days, ns indicates a non-significant effect.

5.4.4 Acute cold challenge

5.4.4.1. Temperatures

The average temperature during the acute cold challenge was 4.43 ± 0.058 ºC. T_{core} of all ewes increased during the acute cold challenge ($P < 0.001$, Fig. 5.3); there was no time by BCS treatment interaction. There was a tendency for a BCS treatment effect on the peak T_{core}, with MBC having a lower peak T_{core} than HBC ewes ($P < 0.1$); this effect had disappeared after completion of the challenge. T_{skin} was also reduced during the acute cold challenge compared to before and after the challenge ($P < 0.001$ for ears, trunk and legs, Fig. 5.4). There was a time by treatment interaction for the ear T_{skin} ($P < 0.05$), due to a large increase in ear T_{skin} in LBC ewes during the recovery phase.

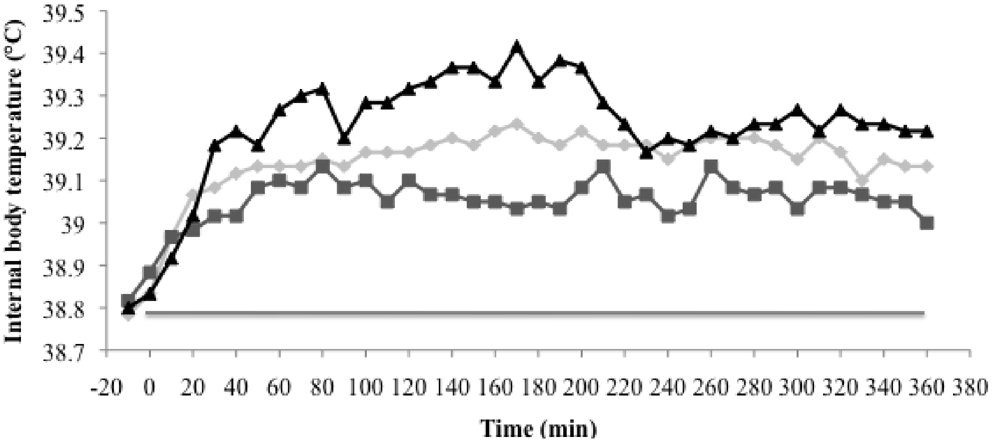


Figure 5.3. The effect of BCS treatment on mean internal body temperatures (ºC) for LBC (rhombus), MBC, (square) and HBC (triangle) ewes during the acute cold challenge at day 85-87 of pregnancy. The grey horizontal line indicates the period of the cold challenge (0-360 min).

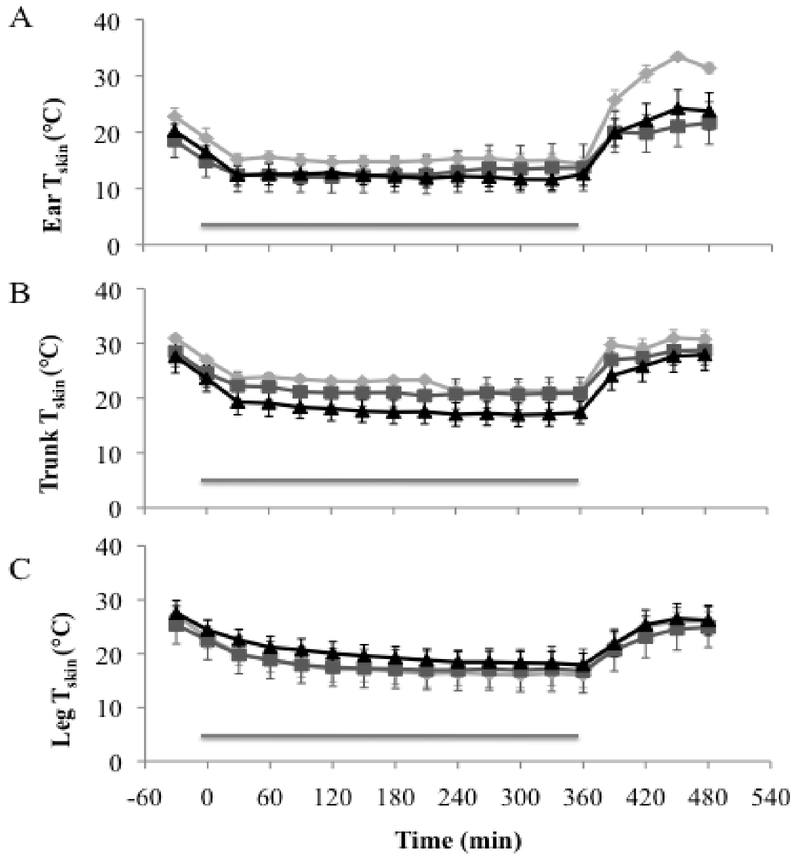


Figure 5.4. The effects of BCS treatment on mean ± SEM skin temperatures (ºC) during the acute cold challenge for LBC (rhombus), MBC (square) and HBC (triangle) ewes at day 85-87 of pregnancy: (A) ear skin temperature, (B) trunk skin temperature and (C) leg skin temperature. The grey horizontal line indicates the period of the cold challenge (0-360 min).

5.4.4.2. Metabolic and endocrine responses in the first 60 min

AUC, peak and rate to peak responses are presented for the factors with the highest significance level only (i.e., BCS treatment, actual BCS, actual LW or change in LW). The significance levels of all other factors are shown in the corresponding tables (see below). The plasma glucose (Fig. 5.5A, Table 5.5) and β-HBA (Fig. 5.5B) responses to the acute cold challenge were not affected by any of the factors. FFA (Fig. 5.5C) AUC was mostly affected by the change in LW (P

< 0.001); peak height was not influenced by any of the factors and rate to peak was mostly influenced by actual BCS (P < 0.01, Table 5.5).

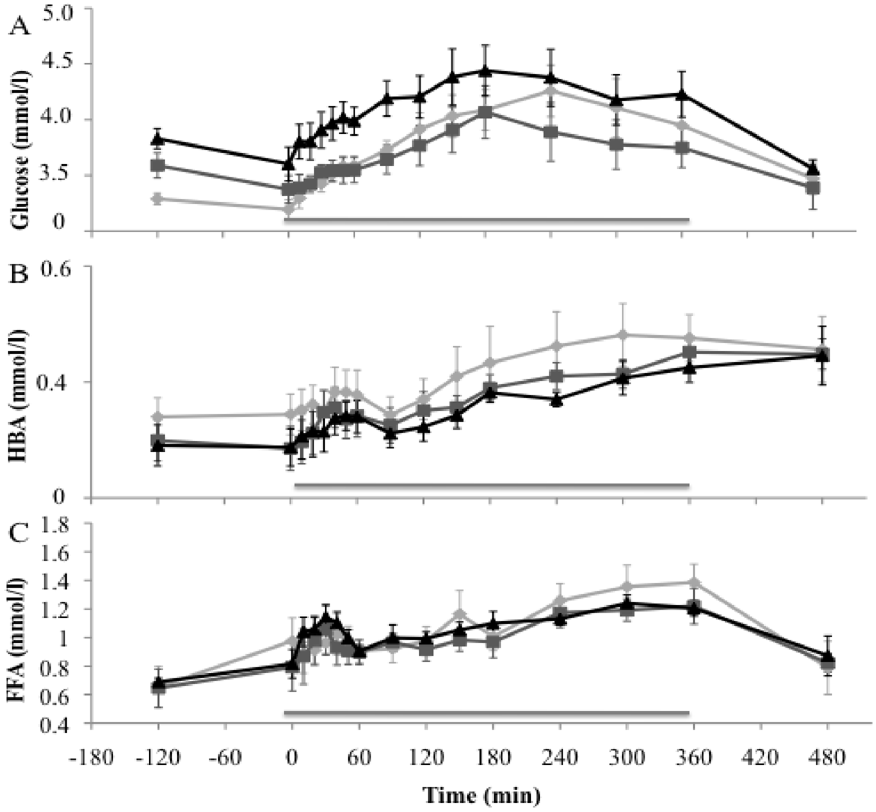


Figure 5.5. The effects of BCS treatment on mean ± SEM metabolic responses to the acute cold challenge for LBC (rhombus), MBC (square) and HBC (triangle) ewes at day 85-87 of pregnancy: (A) Glucose, (B) β-HBA, and (C) FFA plasma responses to the acute cold challenge. The grey horizontal line indicates the period of the cold challenge (0-360 min).

Table 5.6. The effect of BCS treatment, actual BCS, actual live weight (LW) and change in LW (WC) on area under the curve (AUC, mmol/l × min), peak values (mmol/l) and rate to reach the peak (mmol/l/min), with confidence intervals (CI) and P-values for glucose and FFA plasma concentrations during the acute cold stress challenge at day 85-87 of pregnancy.

Metabolite	Parameter	AUC			Peak			Rate to Peak		
		Slope	CI	P-value	Slope	CI	P-value	Slope	CI	P-value
Glucose (mmol/)	BCS linear	2.18	7.15	ns	0.17	0.68	ns	0.00	0.00	ns
	BCS actual	2.96	6.00	ns	-0.22	0.50	ns	0.00	0.00	ns
	LW	0.06	0.49	ns	-0.02	0.04	ns	0.00	0.00	ns
	WC	0.11	0.55	ns	-0.01	0.05	ns	0.00	0.00	ns
FFA (mmol/l)	BCS linear	10.11	6.34	0.05	0.04	0.37	ns	0.01	0.01	0.01
	BCS actual	7.84	5.06	0.01	0.07	0.21	ns	0.01	0.01	0.01
	LW	0.51	0.45	0.05	0.00	0.02	ns	0.00	0.00	ns
	WC	0.74	0.36	0.001	0.00	0.02	ns	0.00	0.00	0.05

Cortisol (Fig. 5.6A) AUC and peak height were mostly affected by the actual BCS ($P < 0.05$ and $P < 0.001$, respectively; Table 5.6) and the rate to reach the peak was mainly influenced by BCS treatment ($P < 0.01$), with LBC ewes taking longer to reach the peak compared to MBC and HBC ewes ($P < 0.05$). Insulin (Fig. 5.6B) AUC and rate to peak were not affected by any of the factors. Insulin peak height was equally determined by actual LW and change in LW (both $P < 0.001$, Table 5.6). Responses to leptin were analysed over the full 360 min (Fig. 5.7A). Leptin was different between BCS treatments ($P < 0.05$) and there was a significant time by treatment interaction for leptin ($P < 0.01$), with the MBC and HBC ewes reducing leptin concentrations while the LBC remained relatively unchanged. T4 (Fig. 5.7B) AUC and peak height were not influenced by any of the factors (Table 5.6), and rate to reach the peak plasma T4 concentrations was mostly affected by actual BCS ($P < 0.05$).

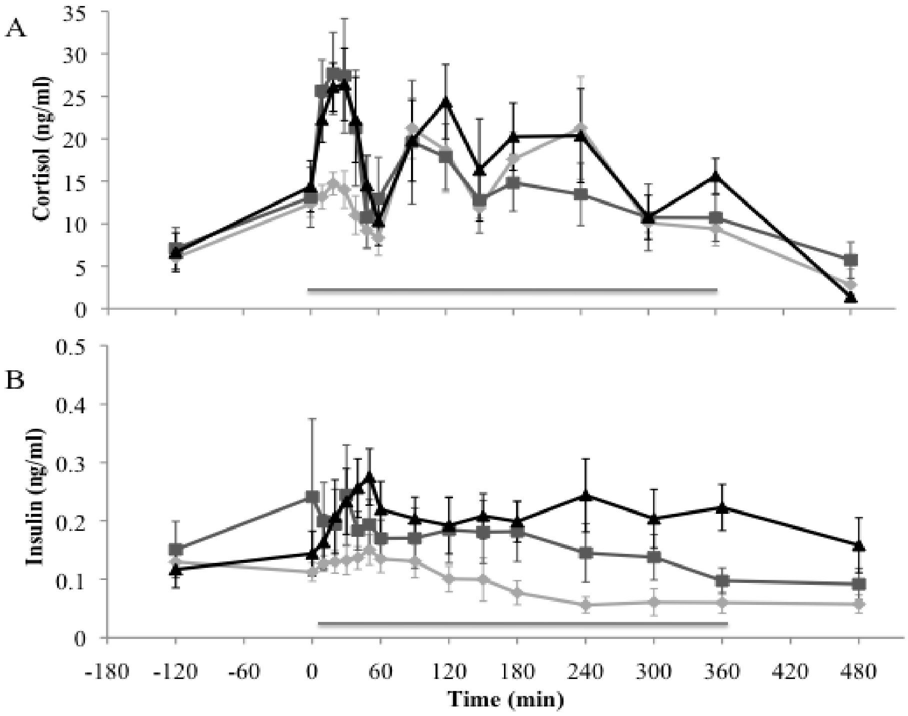


Figure 5.6. The effects of BCS treatment on mean ± SEM endocrine responses during the acute cold challenge for LBC (rhombus), MBC (square) and HBC (triangle) ewes at day 85-87 of pregnancy: (A) cortisol and (B) insulin plasma responses to the acute cold challenge. The grey horizontal line indicates the period of the cold challenge (0-360 min).

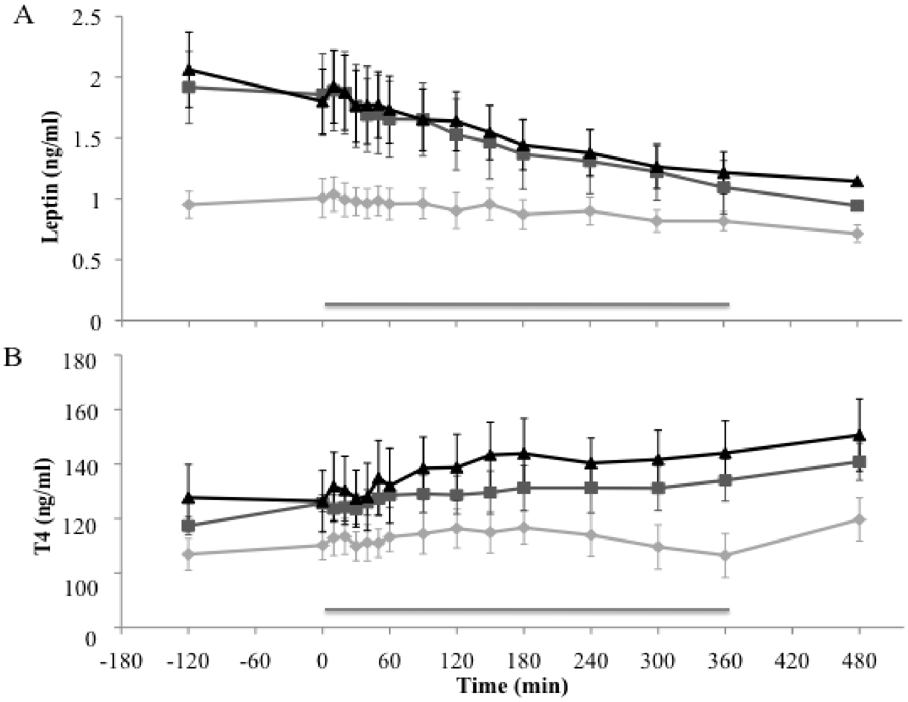


Figure 5.7. The effects of BCS treatment on mean ± SEM endocrine responses during the acute cold challenge for LBC (rhombus), MBC (square) and HBC (triangle) ewes at day 85-87 of pregnancy: (A) Leptin and (B) T4 plasma responses to the acute cold challenge. The grey horizontal line indicates the period of the cold challenge (0-360 min).

5.4.4.3. Metabolic and endocrine responses between 60 and 360 min

Insulin peak height was mostly affected by actual BCS ($P < 0.01$), while AUC and rate to peak were not influenced by any of the factors. Glucose, FFA, β-HBA, T4 and cortisol responses were not affected by any of the factors.

Table 5.7. The effect of BCS treatment, actual BCS, actual live weight (LW) and change in LW (WC) on area under the curve (AUC, ng/ml × min), peak values (ng/ml) and rate to reach the peak (ng/ml/min), with confidence intervals (CI) and P-values for plasma cortisol, insulin and T4 concentrations during the acute cold stress challenge at day 85-87 of pregnancy.

		AUC			Peak			Rate to Peak		
		Slope	CI	P-value	Slope	CI	P-value	Slope	CI	P-value
Cortisol (ng/ml)	BCS treat	338.40	416.20	ns	14.47	10.83	0.05	0.49	0.32	0.05
	BCS actual	380.50	313.20	0.05	15.97	7.86	0.001	0.37	0.28	0.05
	LW	26.68	25.56	0.05	1.20	0.65	0.01	0.03	0.03	0.05
	WC	19.19	27.85	ns	0.90	0.79	0.05	0.03	0.02	0.05
Insulin (ng/ml)	BCS treat	1.96	7.10	ns	0.22	0.29	0.01	0.00	0.00	ns
	BCS actual	2.49	5.52	ns	0.24	0.11	0.001	0.00	0.00	ns
	LW	0.15	0.45	ns	0.02	0.01	0.001	0.00	0.00	ns
	WC	0.08	0.47	ns	0.02	0.01	ns	0.00	0.00	ns
T4 (ng/ml)	BCS treat	62.83	205.20	ns	19.32	27.18	ns	-0.22	0.36	0.1
	BCS actual	-2.40	189.40	ns	0.40	8.66	ns	-0.29	0.26	0.05
	LW	-0.31	17.77	ns	0.47	0.77	ns	-0.02	0.03	ns
	WC	0.31	16.06	ns	0.18	0.73	ns	-0.02	0.02	0.05

5.4.4.4. Recovery phase

The recovery phase included the samples taken 2 h after completion of the cold challenge and the following morning at 0900 h. Plasma insulin concentrations increased after the cold challenge ($P < 0.001$) and were lower in LBC compared to MBC and HBC ewes ($P < 0.05$). Leptin was different between BCS treatments during the recovery period ($P < 0.01$), and there was a time by treatment interaction ($P < 0.01$) with MBC and HBC increasing leptin and LBC remaining relatively stable. T4 was lower in LBC ewes compared to MBC and HBC ewes during the recovery period ($P < 0.05$), and a time by treatment interaction ($P < 0.01$) was observed with MBC and HBC ewes decreasing T4 concentrations and LBC ewes remaining relatively stable. FFA, glucose, β-HBA and cortisol were not influenced by BCS during the recovery phase.

5.4.4.5. Lamb birth and weaning weight

Lamb birth weights (LBC female lambs 6.7 ± 0.7 and males 6.2 ± 0.5 kg; MBC females 5.7 ± 0.6 and males 5.3 ± 0.2; HBC females 5.6 ± 0.7 and males 6.4 ± 0.3 kg) and weaning weight (LBC female lambs 28 ± 3 and males 28 ± 2 kg; MBC females 27 ± 2 and males 28 ± 1 kg, HBC females 26 ± 4 and males 28 ± 3 kg) were not different between BCS treatments. However, there was a significant BCS treatment by sex interaction for lamb birth weight ($P < 0.05$) with male lambs born to HBC ewes being heavier than females, but female lambs born to LBC and MBC ewes being heavier than males.

5.5. Discussion

In order to assess whether BCS affects adaptive metabolic and stress responses in pregnant ewes, ewes with different BCS were exposed to an acute cold challenge in mid-pregnancy. The LBC ewes showed decreased metabolic and endocrine responses to the challenge and generally took longer to induce responses, but were able to release sufficient energy substrates as indicated by the responses in glucose and FFA concentrations and by the increase in T_{core}. The LBC ewes also had a delayed and reduced cortisol response to the challenge. In addition, the metabolic state of the ewes during the BCS change phase showed that low BCS ewes had markedly decreased plasma glucose and leptin concentrations and increased FFA concentrations. Therefore, low BCS ewes were in a metabolically catabolic state and had a reduced ability to respond to an acute challenge compared to moderate and high BCS ewes.

5.5.1 Body reserves during body condition score change phase

LBC ewes lost LW, BCS and eye muscle reserves as well as some of the fat covering the eye muscle during the BCS change phase, in contrast to MBC and HBC ewes that gained BCS, LW and both muscle and fat stores. However, the LBC ewes showed a slight increase in GR depth. A similar increase in GR depth in pregnant ewes with low BCS was found in a previous study that was undertaken on a different cohort of ewes from the same main flock (Chapter 3). Others have showed that undernutrition may favour partitioning of energy towards storage of body fat (McNeill *et al.* 1998; Wallace *et al.* 1999). However, McCrabb et al (1992) found that undernourished ewes partitioned energy towards muscle mass while McNeill et al (1997b) found no change in muscle and fat stores when pre-mated thin and fat ewes maintained their body fat levels throughout pregnancy. The discrepancy between these results may stem from the different timing and length of the undernutrition period, the methods and site of measurements and possibly the breed of sheep used in the different studies. However, these studies suggest that a change in feeding level during pregnancy affects the pattern and site of body reserve accumulation and mobilisation in pregnant ewes. In this study, however, other important internal fat depots were not measured, such as omental, mesenteric and perirenal fat, which account for a substantial amount of total body fat in sheep (Delfa *et al.* 1989; Arana *et al.* 2005). Low BCS ewes mainly store fat in the intermuscular and mesenteric fat depots, and fat is deposited in the perirenal depot followed by the omental and subcutaneous depots with increasing BCS (Teixeira *et al.* 1989). However, the lipogenic activity (i.e., the ability to incorporate fatty acids) in these tissues varies considerably with perirenal and omental fat having the highest activity and subcutaneous fat having a relatively low activity (Ingle *et al.* 1972; Faulconnier *et al.* 2001). Therefore, the amount of fat stored in the different depots may have varied considerably between the LBC, MBC and HBC ewes. It is likely that LBC ewes have mobilised substantial amounts of fat from the omental and perirenal fat depots during BCS loss, which would explain the small reduction in eye muscle fat.

5.5.2 Metabolic and endocrine responses to body condition score change

Plasma glucose and leptin concentrations were significantly reduced, and FFA concentrations significantly increased in LBC compared to MBC and HBC ewes, with the differences becoming larger with progressing pregnancy. This indicated that LBC ewes were in a catabolic state. In Chapter 3, marked effects of BCS were found on plasma leptin, glucose and FFA as well as on cortisol, insulin and IGF-1 concentrations. Several other studies have shown effects of BCS, body fat or feeding level on leptin (Thomas *et al.* 2001; Bispham *et al.* 2003), insulin (Wallace *et al.* 1999; Luther *et al.* 2007), IGF-1 (Wallace *et al.* 2000; McMullen *et al.* 2005) and cortisol (Jaquiery *et al.* 2006; Rumball *et al.* 2008). In the current study, however, cortisol, insulin and IGF-1 plasma concentrations were not influenced by BCS. The differences in BCS between treatments were relatively small in the current study compared to the study outlined in Chapter 3 (i.e., LBC 2.4 vs. 2.0, MBC 3.2 vs. 2.9 and HBC 3.6 vs. 3.7 in the current and Chapter 3 studies, respectively). It is likely that the effect of BCS on metabolic and endocrine markers depends on the severity of the food restriction regime and the amount of BCS loss (Chapter 3 and 4; Blache *et al.* 2000; Thomas *et al.* 2001; Bispham *et al.* 2003; Caldeira *et al.* 2007b, a), and the changes in BCS in the current study may have been to small to affect plasma insulin, IGF-1 and cortisol concentrations. Another possible explanation is that differences in insulin and IGF-1 concentrations may not become apparent until after 100 days of pregnancy (Chapter 3). However, the current study was completed at day 90 of pregnancy. In summary, the studies presented in this thesis suggest that leptin is a suitable indicator for short-term (hours) changes in food intake as well as a medium-term indicator (weeks) of altered BCS. FFA is affected by both short-term (hours) changes in food intake and medium-term changes in BCS (weeks) and glucose is an indicator of medium-term (weeks) changes in food intake and BCS. Insulin and IGF-1 are suitable indicators of short-term changes in food intake (hours) as well as long-term (months) food restriction reflecting altered BCS.

5.5.3 Responses to shearing

Shearing generally leads to an increase in food intake (Dabiri *et al.* 1995), metabolic rate (Slee 1972; Ekpe and Christopherson 2000) and internal body temperature (Piccione *et al.* 2002), and therefore imposes a metabolic load on ewes. Shearing led to significant increases in plasma glucose, FFA and T4 and a reduction in leptin concentrations in this study. The increased glucose (Hargreaves and Hutson 1990a; Carcangiu *et al.* 2008) and T4 concentrations (Drews and Slebodzinski 1985; Symonds *et al.* 1989) after shearing are in agreement with other studies. However, no interactions between shearing and BCS were found for T4, in contrast to others who found a larger increase in undernourished ewes (Drews and Slebodzinski 1985; Symonds and Lomax 1989). Cortisol tended to increase in response to shearing. Hargreaves and Hutson (1990b, a) and Carcangiu et al. (2003) demonstrated that shearing increases plasma cortisol concentrations between the start of the procedure until 60 min after shearing. The long time period between shearing and blood sample collection (one day) in my study could have resulted in the low cortisol response to shearing compared to other studies (Hargreaves and Hutson 1990a, b; Carcangiu *et al.* 2008). MBC and HBC ewes showed a tendency for a larger decrease in leptin in response to shearing compared to LBC ewes. This decrease in leptin may be an important energy saving mechanism during cold exposure and may also stimulate food intake (Henry *et al.* 2001; Henry *et al.* 2008). However, LBC ewes were most likely already experiencing low levels of leptin, resulting in a smaller decrease. All other interactions between plasma metabolite/hormone responses to shearing and BCS were not significant. In summary, while shearing induced metabolic and endocrine responses there were no addition affects of BCS.

5.5.4 Acute cold challenge

When animals are exposed to cold, several mechanisms come into play that ensure that heat is conserved and/or generated. As a first response, vasoconstriction results in heat conservation and shivering leads to heat generating muscular activity (Silva 2006; Bicego *et al.* 2007). Second, adaptive thermogenesis (also called non-shivering thermogenesis) is activated and generates heat by mechanisms that are still largely unknown in (non-rodent) mammals (Dulloo and Samec 2001; Cannon and Nedergaard 2009). Adaptive thermogenesis is possibly mediated by endocrine factors such as thyroid hormones (Dauncey 1990; Nazifi *et al.* 2003; Todini 2007) and catecholamines (Bachman *et al.* 2002; Jansky and Jansky 2002), and indirectly by glucocorticoids (Silva 2006), insulin (Sano *et al.* 1992; Sano *et al.* 1995; Sano *et al.* 1999) and leptin (Henry *et al.* 2008) through the mobilisation of energy substrates and increases in energy expenditure.

The main result from this study was the significantly reduced stress (cortisol) response in the first 60 min of the cold challenge observed in the LBC ewes. Stress responses result from challenges that compromise energy homeostasis and lead to activation of the HPA-axis. Stress *per se* is not necessarily a problem because acute stress responses facilitate appropriate behavioural reactions

(Pijlman and van Ree 2002; Pijlman *et al.* 2003; Kanitz *et al.* 2009) and physiological responses (Sapolsky *et al.* 2000). These are required to maintain or restore energy homeostasis and increase the chances of survival. The ability to mount an appropriate stress response is therefore of crucial importance.

Interestingly, cortisol concentrations were not significantly different between ewes with different BCS before the start of the acute cold challenge. Furthermore, plasma cortisol concentrations were no longer different between ewes with different BCS after the first 60 min, indicating that low BCS were able to mount a stress response, but took longer to do so. The pattern of cortisol secretion was biphasic in all BCS treatments, with a peak in the first 60 min and a second peak within 120 min. Interestingly, the highest peak in the MBC and HBC ewes was the first peak, while the highest peak of the LBC ewes was the second peak. The activation of the HPA-axis and the release of cortisol most likely contributed to an increase in the availability of energy substrates. Cortisol stimulates gluconeogenesis and protein synthesis and indirectly increases plasma glucose and insulin concentrations due to its effects on the hypothalamus (McMahon *et al.* 1988; Mormède *et al.* 2007). Therefore, the reasons for the LBC ewes' reduced and delayed response in cortisol could be to conserve energy during environmental challenges.

An alternative explanation for the reduced cortisol response in LBC ewes is that undernutrition leading to a low BCS is a chronic stressor resulting in permanent changes in the HPA-axis. It is well documented that animals under chronic stress do not show elevated cortisol levels or even suppressed cortisol levels, which is an indication of HPA-axis down-regulation (Girotti *et al.* 2006; Dallman 2007). A possible function of down regulation of the HPA-axis to chronic or repeated stressors is to ensure that sensitivity to novel stressors can still be maintained. Several studies have shown that chronically stressed rats show facilitated HPA-axis responses to a novel stressor (Bhatnagar and Dallman 1998; Dallman *et al.*

2003; Ma *et al.* 2008), while others have showed a decrease in the HPA-axis response to acute stress in bulls (Ladewig and Smidt 1989). The HPA-axis response to a challenge may depend on the characteristics of the challenge (Retana-Márquez *et al.* 2003), which may explain the differences in results between the different studies. However, in undernourished sheep it has been shown that the cortisol response to an arginine vasopressin (AVP)−corticotrophin-releasing hormone (CRH) challenge was reduced after several weeks of food restriction (Jaquiery *et al.* 2006). Furthermore, lambs undernourished between 12 and 25 weeks of age showed a reduced adrenocorticotrophic hormone (ACTH) response to a corticotrophin-releasing factor (CRF)−AVP challenge at 1.5 years of age, while the cortisol response remained unchanged. Therefore, there is substantial evidence that undernutrition results in down-regulation of the HPA-axis.

During cold exposure, sufficient energy substrates need to be released in order to maintain internal body temperature. Other than activation of the HPA-axis, there could be a number of different hormones involved in freeing energy substrates. Thyroid hormones may be directly involved in thermoregulation (Silva 2005; Silva 2006). Furthermore, thyroid hormones play a major role in the increase of basal metabolic rate and increase glucose availability to cells, stimulate protein synthesis and lipid metabolism as well as cardiac and neural functions (Dauncey 1990; Todini 2007). T4 is also a good indication of the nutritional status of ruminants (Todini 2007) and a direct effect of triiodothyronine (T3) on the stimulation of food intake has been reported in rats (Kong *et al.* 2004). However, very little effect of BCS on the T4 response to cold exposure was found, except for an effect of actual BCS and change in LW on the rate at which peak levels of T4 were reached. Ekpe and Christopherson (2000) reported that food restriction in a warm (23°C) environment for 5 weeks reduced T4 plasma concentrations, while food restriction in a cold environment (0°C) did not change T4 concentrations compared to fully fed sheep. Therefore, there may be interactions between T4, BCS/feeding level and the environmental temperature.

Several other endocrine signals may affect thermoregulation directly or indirectly. Catecholamines may play an important role in thermoregulation, although these were not measured in this study. Infusion of adrenaline and noradrenaline stimulate energy expenditure in humans (Blaak *et al.* 1993) and in brown adipose tissue (BAT) in rats (Bouillaud *et al.* 1984). It has also been observed that cold exposure increases sympathetic activity in BAT in rats (Landsberg *et al.* 1984). Furthermore, noradrenaline regulates body temperature through the α-adrenergic receptor in the pre-optic area of the hypothalamus (Mallick *et al.* 2002; Jha and Mallick 2009), which is one of the main thermo-integrative structures in the brain.

Leptin is a potential endocrine signal facilitating the availability of energy substrates and the increase in expenditure during cold exposure. A steady reduction of leptin in the MBC and HBC ewes was found over the course of the cold challenge, while LBC concentrations remained relatively unchanged. Other studies have also shown a reduction in leptin during cold exposure in sheep (Asakuma *et al.* 2003) and rats (Bing *et al.* 1998; Korhonen and Saarela 2005). This reduction in leptin could contribute to an increased food intake during cold exposure (Henry *et al.* 2001; Morrison *et al.* 2001) and therefore increase the availability of energy substrates for thermogenesis. Some studies have pointed towards a more direct effect of leptin on energy expenditure. It has been observed that leptin administration leads to a loss of body fat that could not be accounted for by the reduction in food intake alone, suggesting a direct effect on thermogenesis in rodents (Halaas *et al.* 1997; Bowen *et al.* 2003). In sheep, it has been reported that intracerebroventricular administration of leptin increases postprandial thermogenesis in muscle, and to a lesser extent in fat (Henry *et al.* 2008). The reduction in leptin observed in this study could therefore have been an important energy saving mechanism during cold exposure.

Another candidate that may affect thermoregulation indirectly is insulin. Acute cold exposure increased insulin concentrations and the HBC ewes responded with a significantly larger increase in insulin compared to LBC ewes. Insulin does not appear to have a direct effect on thermoregulation, but may increase thermogenesis through its effects on the hypothalamus (Silva 2006). It has been demonstrated that cold exposure reduces insulin secretion (Sasaki and Takahashi 1980) and increases tissue responsiveness to insulin in sheep (Sano *et al.* 1999). These effects are most likely mediated by the sympathetic nervous-adrenomedullary systems and result in the facilitation of muscle glucose uptake (Vollenweider *et al.* 1993). Another potential way that insulin could influence thermoregulation is through its effect on leptin; depressed insulin action during cold exposure could result in reduced in leptin secretion (Asakuma *et al.* 2003; Korhonen and Saarela 2005). Therefore, responses in insulin observed in this study could have contributed to the increased availability of glucose and the maintenance of energy homeostasis and internal body temperature.

The T_{core} increased in response to the cold challenge in all ewes and remained elevated during the duration of the challenge. The HBC ewes showed the highest increase of 0.6 °C after 170 min. However, there was only a tendency for a BCS effect on T_{core}. It has been shown that sheep exposed to cold for four days have an increased rectal temperature (Li *et al.* 2000), but decreased rectal temperatures during five weeks of cold exposure have also been reported (Ekpe and Christopherson 2000). T_{skin} decreased considerably during the acute cold challenge in all ewes, most likely due to vasoconstriction of the blood vessels to conserve heat. This reduction in T_{skin} may lead to an increase the hypothalamic set-point of T_{core} (Hammel *et al.* 1963; Jessen 1996), suggesting that the observed fall in T_{skin} could have lead to an increase in the hypothalamic set-point temperature and therefore an increased T_{core}. However, because of the short duration of the acute cold challenge it is unlikely that there would have been any shifts in the set-point. Rather, the increased T_{core} could have been caused by an increase in metabolic heat production as a result of the reduced T_{skin} (Jessen

1996). The LBC ewes had a significantly higher ear T_{skin} than the MBC and HBC ewes 2 h post-challenge; the reason for this is not clear. It is possible that LBC ewes could have shivered more (shivering was not measured), or that there was a higher degree of vasodilatation after completion of the cold challenge leading to increased blood flow and therefore increased T_{skin}.

The endocrine and metabolic responses to the cold challenge were successful in releasing sufficient energy substrates and inducing thermoregulatory responses to maintain internal body temperature. Glucose levels were increased in all ewes throughout the cold challenge, indicating that all ewes were able to maintain energy balance. The low insulin response observed in the LBC ewes most likely facilitated the availability of glucose for thermoregulation. LBC ewes showed decreased responses in FFA compared to HBC ewes. However, at the end of the 6 h cold challenge, FFA levels appeared to be higher in LBC ewes compared to MBC and HBC ewes (although not significantly). Together these results suggest that LBC ewes were able to mobilise sufficient energy substrates to cope with the challenge, but they were slightly slower to do so.

More research would be needed to study the implications of the reduced cortisol response observed in the LBC ewes. Chronic stressors such as low BCS and undernutrition may have possible implications for the ability to cope with acute stressors. The acute cold challenge presented in this study was not severe enough to compromise the health and welfare of low BCS ewes. However, it would be valuable to investigate the potential impacts of exposing low BCS ewes to other stressors that occur regularly on farms (for example disease, lameness, social stress, or a more severe cold challenge) and assess any health and welfare impacts. It would also be valuable to investigate whether a combination of different stressors within a short time period affects the health and welfare of ewes. It is likely that additional challenges have an additive effect and low BCS ewes may eventually lack the energy substrates to adapt to the challenges, which may compromise energy homeostasis and may make ewes more vulnerable to developing metabolic diseases (for example ketosis). However, animal welfare is also largely determined by subjective experiences and the affective states (see Chapter 1). It is likely that a challenge could lead to a negative affective state when the ewes perceive the challenge to be aversive (Veissier and Boissy 2007), even if the health of the animal is not directly affected. To my knowledge, however, there are no methodologies that can provide direct insight into the affective states of animals. Such methodologies are necessary to determine the affective state of animals under a range of chronic and acute stressors.

5.5.5 Lamb birth weight

BCS did not affect lamb birth weights in this study. However, there was an interaction between lamb sex and BCS treatment with the male lambs born to the HBC ewes heavier than females, but the female lambs born to the LBC and MBC ewes heavier than males. Similar results were found in Chapter 3 (i.e, HBC male lambs heavier than females and LBC female lambs heavier than males, no difference between male and female lambs born to MBC ewes), although this was not significant. In Chapter 4, however, the Control female lambs were heavier than males, and SL and FL male lambs were heavier than females, which was close to being statistically significant. Generally, male lambs are heavier than female lambs (Miller *et al.* 2010). From the studies presented in this thesis, it appears that alterations in BCS may impact the growth of males and females differently, although the results of Chapter 4 are not consistent with the results of Chapter 3 and 5. Furthermore, relatively few lambs were used and higher numbers may be required to determine the effects of BCS on lamb birth weight. To my knowledge, no other studies have reported that BCS affects birth weight of males and female lambs differently. However, others have reported that nutrition during pregnancy affects birth weight, depending on the timing and length of the under- or over-nutrition period (reviewed by Redmer *et al.* 2004; Wallace *et al.* 2005; Wallace *et al.* 2006). Undernourished ewes could potentially give birth to low birth weight lambs, which consequently decreases lamb survival (Clarke *et al.*

1997; De Blasio *et al.* 2007) and may affect the health of lambs in later life (Godfrey and Robinson 1998; Bloomfield *et al.* 2003a; Wu *et al.* 2004; Oliver *et al.* 2007). Another possible way of affecting lamb birth weight is by shearing the ewes in mid-pregnancy, which has been shown to increase lamb birth weights (Kenyon *et al.* 2003; Corner *et al.* 2006). There is some evidence that the effects of shearing on birth weight depend on the nutrition of the ewe and on the number of lambs (Kenyon *et al.* 2002; Kenyon *et al.* 2007). In this study, it could not be establish whether there was an effect of shearing on lamb birth weighs, because all ewes were shorn.

In summary, BCS considerably affected the cortisol response to an acute cold challenge in mid-pregnancy. Ewes with a low BCS showed a reduced cortisol response in the first 60 min of the challenge, in contrast to ewes with moderate or high BCS. The reduced and delayed cortisol response is most likely indicative of an HPA-axis down regulation. Despite this, low BCS ewes were able to release glucose and FFA during the challenge and increase internal body temperature, indicating that energy homeostasis was maintained. The implications of the delayed cortisol response for ewe health and welfare remain to be further investigated, but may indicate that ewes with low BCS have a reduced ability to adapt to acute challenges. Ewes with low BCS also showed reduced plasma insulin and leptin responses and took longer to reach peak concentrations in insulin and T4 during the acute cold challenge. These endocrine signals are involved in energy balance and LW regulation, and may be directly or indirectly involved in thermoregulatory processes. This study has provided evidence for an interaction between these endocrine signals, BCS and environmental climatic conditions.

Chapter 6. General discussion

The main focus of this thesis has been on the effects of food restriction and changes in body condition score (BCS) on hunger and metabolic functioning in pregnant ewes. Measures of feeding motivation were obtained in order to assess the subjective experience of hunger, because subjective experiences are key determinants for the welfare state of animals (Dawkins 1988; Broom 1991; Dawkins 2008; Fraser 2009a). Another aspect of welfare is the level of biological functioning (Mendl 2001). Therefore, measures of metabolic and endocrine responses to changes in BCS were obtained in order to assess the level of metabolic functioning, which is one component of biological functioning. Finally, ewes with different BCS were exposed to an acute cold challenge in order to assess the ability to maintain energy homeostasis and induce adaptive metabolic and stress responses. Together, measures of feeding motivation and the relationship between feeding motivation and metabolic and endocrine signals have contributed to new knowledge of the perception of hunger in ewes. In addition, metabolic and endocrine responses to changes in BCS as well as to an acute cold challenge have provided a deeper understanding of the level of metabolic functioning in food restricted ewes.

6.1. Effects of food restriction on feeding motivation and hunger

The measures of feeding motivation taken in this study have clearly shown that short-term food restriction (24 h fast, Chapter 2) and long-term food restriction (loss of BCS, Chapter 3) lead to a marked increase in feeding motivation. However, an interesting finding was that the measures of motivation used in this study (i.e., the number of rewards consumed, total distance walked, O_{max} and P_{max}) could not detect differences in motivation between ewes restricted to 50-70% of previous feeding level and ewes fed to maintain BCS (Chapter 4). This suggests that feeding motivation may not be increased when feeding level is low (above 50% of maintenance requirements) compared to ewes fed to maintain BCS, provided that no major losses in live weight (LW) and BCS have occurred.

In other ruminants, it has also been shown that short-term food restriction affects the motivation for food. For example, a linear increase in the distance walked for food was found in lactating dairy cattle when food deprivation time was increased from 0 to 3, 6 and 9 h. However, dry cows did not increase distance walked with an increased food deprivation time (Schütz *et al.* 2006). The difference was most likely due to the higher metabolic demands of lactating dairy cows. However, a study investigating the effect of different durations of short-term (between 0 and 24 h) food restriction in sheep did not report any effects on feeding motivation (Jackson *et al.* 1999). The cost of the reward in that study was a door that sheep pushed through to get to the reward, and the cost per reward was increased by increasing the weight of the door; however, the cost may not have been high enough to reveal differences in motivation. Another study in sheep showed a large compensatory increase in food intake after a 24 h fast (Newman *et al.* 1994), which is also indicative of an increased feeding motivation after short-term food restriction. It has also been found that short-term food restriction increases feeding motivation measured by the number of button pushes and panel presses in pigs (Lawrence and Illius 1989; Farmer *et al.* 2001). However, it has not been shown previously that short-term food restriction increases feeding motivation with the use of a behavioural demand methodology in sheep. The study in Chapter 2 is the first study to successfully use a behavioural demand methodology to show that short-term food restriction increases the motivation for food in sheep.

Previous studies have provided evidence that long-term food restriction (i.e., leading to a loss in LW and BCS) increases feeding motivation in sheep, although this has not been assessed using a behavioural demand methodology. It has been found that sheep with a low BCS of 2.5 eat considerably more and spend a longer time eating compared to sheep with a high BCS of 3.5 (Sibbald 1997). Also in broilers it has been shown that compensatory food intake increases with increased food restriction levels (de Jong *et al.* 2003). A behavioural demand methodology has been used to assess feeding motivation in other species. For example, broilers weighing 50% of normal weight are more motivated to work for food compared to broilers weighing 75% of normal weight (Bokkers *et al.* 2004). However, Savory and Lariviere (2000) found no relation between food restriction level and feeding motivation, but rather a strong relation between LW and feeding motivation in broilers. Also in rats a strong relationship between LW and willingness to work (lever presses) for food has been found (Ferguson and Paule 1997). Therefore, the findings that long-term food restriction increases feeding motivation in pregnant ewes are robust, and well in agreement with previous findings in other (non-pregnant) animal species.

The methodologies to assess motivation used in the above studies have varied from simply measuring compensatory food intake to making animals work for their food. The work animals had to perform ranged from button pushes (Farmer *et al.* 2001), panel presses (Lawrence and Illius 1989), lever presses (Ferguson and Paule 1997), key pecks (Bokkers *et al.* 2004) or walking (Schütz *et al.* 2006; Bokkers *et al.* 2007), and all these methods were successfully used to demonstrate differences in feeding motivation to some degree. However, like in the study outlined in Chapter 4, it is not always possible to detect differences in feeding motivation between animals at different food restriction levels (Ferguson and Paule 1997; Jackson *et al.* 1999; Schütz *et al.* 2006). This suggests that detecting differences in motivation is possible when the differences in feeding levels are very large (e.g., comparing a food restricted animals with an *ad libitum* fed control), but may be less sensitive to detecting more subtle differences in feeding motivation (e.g., comparing a 50% with an 80% of maintenance requirements restriction level).

6.2. What does an increased feeding motivation mean for animal welfare?

To assess the welfare of sheep, it would be important to determine what the level of feeding motivation is at which ewes start experiencing hunger and whether this leads to a negative subjective state. Feeding motivation was increased after a 24 h fast (Chapter 2) and it is likely that feeding motivation would increase further with an increased fasting time (Schütz *et al.* 2006). In Chapter 4, however, food restriction was reduced to 50% of previous feeding level for around 4 weeks (before any changes in LW and BCS had occurred), and the Fast Loss (FL) and Slow Loss (SL) ewes were not willing to work harder for their food compared to Control ewes that were fed to maintain BCS. Because the low BCS (LBC) ewes in Chapter 3 tended to show an increased motivation for food compared to medium BCS (MBC) ewes, it appears that the long-term consequences of LW and BCS loss may result in a higher increase in feeding motivation compared to the short-term consequences of low feeding level. This idea is supported by a study by Savory et al. (1993) who showed that long-term restricted broilers had an increased motivation for food compared to 72 h fasted broilers. Possibly, food restriction levels of at least 50% of maintenance requirements are not experienced as hunger as long as sufficient body reserves are available (for mobilisation of energy substrates); however, this will need to be investigated.

In order to determine whether long-term restriction is more severe compared to short-term restriction in sheep, it would be important to investigate how short-term food restriction (i.e., several hours/days of fasting or low feeding levels) interacts with long-term food restriction (loss in LW and BCS) and affects feeding motivation. This could be done, for example, by creating six food restriction treatments with two different short-term restriction levels (24 h fast and *ad libitum*) and three different BCS (low, medium and high) in a crossover design. Furthermore, it would have been useful to compare all restrictedly fed ewes with an *ad libitum* fed control in the studies presented in this thesis (which was used in Chapter 2 and 3, but not in Chapter 4). It is possible that SL and FL ewes (food restricted sheep that had not lost any BCS, Chapter 4) would have had a higher motivation for food compared to *ad libitum* fed ewes (rather than the Control ewes fed to maintain BCS). In Chapter 3, it was found that the MBC ewes (fed close to 100% of maintenance requirements) had a relatively high motivation for food compared to the high BCS (HBC) ewes. This suggests that any restriction below *ad libitum* food intake would result in an increase in feeding motivation. Others have also suggested that animals experience hunger when they cannot choose their own level of intake (e.g., D'Eath *et al.* 2009).

However, this still does not answer the question whether an increased motivation for food equates to poor welfare. What can be said with certainty is that the increase in feeding motivation observed in LBC and fasted ewes indicate that they “wanted” food. The fact that ewes “wanted” more food implies that they perceived a lack of food. However, medium BCS ewes (i.e., ewes with a BCS of 3) were also willing to work hard for their food, suggesting that these ewes also “wanted” food. The medium BCS ewes received sufficient food to cover all metabolic needs and should not have experienced a metabolic drive to increase food intake (e.g. sufficient glucose combined with low FFA showed they were not in energy deficit, unlike the LBC ewes, Chapter 3). How can it then be explained that they also “wanted” food? And does this mean that ewes in positive or neutral energy balance can also experience hunger? And would this hunger be equally severe compared to ewes with a low BCS, or ewes that have been fasted?

In order to answer these questions, it has to be assumed that the small difference between LBC and MBC ewes in Chapter 3 and the lack of difference between BCS loss treatments in Chapter 4 was not due to lack of statistical power or other methodological issues (see below). According to Dawkins (1988), animal welfare is poor when an animal is motivated to perform a behaviour/obtain a resource, but is unable to do so. This implies that an increase in feeding motivation results in a decrease in welfare. However, this is a theoretical assumption rather than one based on empirical evidence. Furthermore, measuring the motivation for a resource only indicates whether the animal wants a resource or not, but does not necessarily provide insight in the overall affective state of the animal when it does not have access to the resource. The affective state of the animal is also considered to be a main determinant of welfare (Boissy *et al.* 2007; Fraser 2009a). Even though not obtaining a resource for which a high motivation exists is *likely* to induce a negative affective state, there are still no direct measures of these affective states (see below for more discussion). Until such measures are available, only assumptions can be made about the affects of having/not having a resource on the affective state and welfare of animals.

So how can assumptions be made about the welfare of animals with a high motivation for food? There is a possibility that a high motivation for food is not necessarily synonymous with poor welfare. During mammalian evolution, food availability has been variable and continued access to high quality food was rare (Stubbs and Tolkamp 2006). Furthermore, food availability is highly season dependent and animals have adapted to eat in excess of requirements in times of plenty in order to prepare for times of little in the future (Stubbs and Tolkamp 2006). Therefore, feeding motivation may be relatively high under most circumstances, which means that assessing welfare based on feeding motivation alone may be difficult. To demonstrate this point further, two hypothetical relationships between feeding motivation and food restriction and their impacts on welfare are indicated in Fig. 6.1. In the simplest case, the relationship between feeding motivation and food restriction is negative and linear, and every small increase in food restriction would lead to a corresponding increase in feeding motivation and decrease in welfare. In this case, feeding motivation would be a good and direct indication of welfare. However, a linear relationship between food restriction and motivation is not necessarily a correct assumption if animals have indeed evolved to express a high motivation for food. Alternatively, the relationship between feeding motivation and food restriction could be a hyperbolic one, and a relatively high feeding motivation does not imply bad welfare. Therefore, feeding motivation may not be a straightforward indicator of welfare. Of course, the relationships between food restriction and motivation and the welfare categories in Fig. 6.1 are only hypothetical, and empirical evidence is required to determine the relationship between food restriction, motivation and welfare (with a greater variety in short-term and long-term restriction levels than the ones used on this thesis).

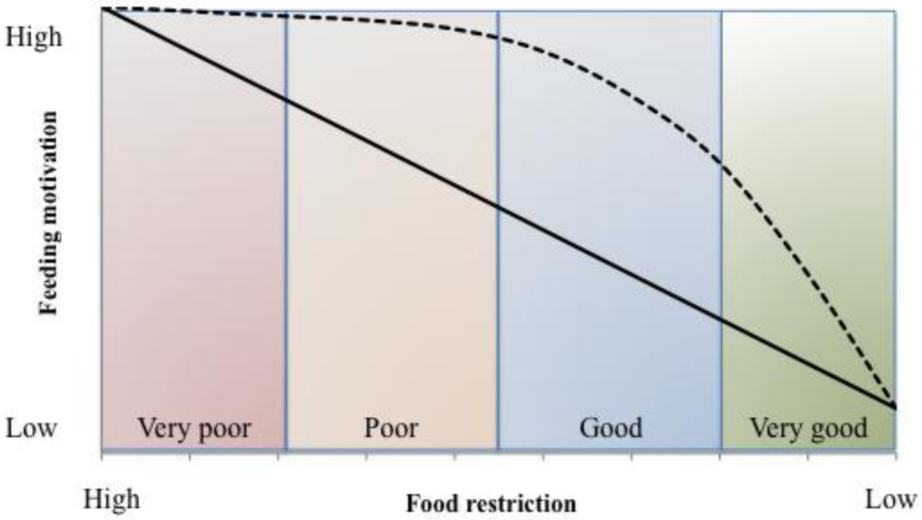


Figure 6.1. Possible relationships between feeding motivation and food restriction (scale 1-10), shown as linear (closed line) or hyperbolic (dashed line) relationships. See text for explanation.

There may be other methods that could provide insight in the subjective experiences of animals with a high motivation for food, in order to get a more accurate assessment of their welfare. There are suggestions that animals can experience similar basic emotions, also called affective states, as humans (Panksepp 2005) and animal welfare scientists have recently started using methods from human psychology to determine the affective states of animals. In human psychology, a strong link between cognition and affective states has been identified, and there is evidence that affective states result in alterations in cognitive function (Lazarus 1991). This provides a basis for exploration of whether such alterations in cognition depending on the underlying affective state also occur in animals (Paul *et al.* 2005). For example, it has been shown that rats interpret ambiguous cues as negative when they are chronically stressed, indicating a judgment bias (Harding *et al.* 2004). In sheep, it has been shown that release from restraint results in a positive judgment bias (Doyle *et al.* 2010). Therefore, it would be very valuable to investigate whether presumed negative states (such as hunger) would result in a cognitive bias. This may provide a more direct insight into the underlying affective state of the animal rather than simply asking what animals want. This does not undermine the importance of asking animals what they want, but adds to an understanding of how having or not having a resource influences affective states and animal welfare.

6.3. Limitations of feeding motivation methodology

In all feeding motivation studies conducted in this thesis (Chapter 2, 3 and 4), the measure of motivation most successful in detecting differences in motivation was the number of rewards consumed. P_{max} (maximum price) was not able to detect any differences in motivation in any of the studies, most likely because it does not account for differences in the level of food consumption. O_{max} (maximum expenditure) detected differences in feeding motivation between different BCS treatments in Chapter 3 only. The lack of differences in measures of motivation between treatments may reflect actual state of the animal or possibly limitations of experimental design. Aspects of experimental design that may have contributed include:

1. *Inability to obtain a full demand curve in Chapter 2 and 4.* In order to detect differences in motivational strength, a complete demand curve needs to be obtained with a wide range of costs so that economic measures O_{max} and P_{max} can be accurately assessed (see also Chapter 2). In Chapter 3, a full demand curve was obtained and O_{max} and P_{max} could be accurately determined. However, the range of costs was not wide enough to obtain a full demand curve in Chapters 2 and 4, resulting in O_{max} and P_{max} being outside the range of costs. It was surprising that no full demand curve was obtained in Chapter 4, because the same cost range as in Chapter 3 were used. Also, ewes in Chapter 4 were in an earlier stage of pregnancy and had not lost any LW/BCS

and were expected to be less motivated for food due to lower metabolic requirements.

2. *Lack of sensitivity of the methodology to detect motivational differences between BCS loss treatments in Chapter 4, and for detecting only small differences between LBC and MBC ewes in Chapter 3.* One reason for lack of sensitivity could be that there was insufficient time to obtain food at preferred levels (in particular at the highest cost); restricted ewes in both Chapter 3 and 4 may have spent the majority of the test sessions walking for food (although this was not recorded) which may have limited their maximum expenditure (O_{max}), and therefore a plateau could have been reached. Using a less time consuming cost, such as lever presses, could solve this problem. An alternative explanation is that the maximum price was not high enough; larger differences in motivation for food between BCS and BCS loss treatments may have been detected if the price of food would have been more expensive. However, it would be difficult to make the cost higher by increasing the distance to walk, because increased walking distance will also result in increased transit time. The reward size could be increased, but that would make the total cost less expensive. However, instead of increasing the distance, animals could be made to walk on a sloped surface instead of a flat surface, which requires more energy. Another option to detect more subtle differences in motivation would be by providing animals with a percentage of their maintenance requirements just before the start of the test, and they would have to work only for the remaining percentage (or more, if they chose to).
3. *Use of an artificial environment that did not provide any other resources (except visual contact with other sheep, shelter and water) or the opportunity to express other behaviours that may be important to ewes.* This may, for example, result in boredom and ewes may choose to walk for food because of a lack of anything else to do. However, the high BCS animals in Chapter 3 hardly walked for food at the highest cost, suggesting that boredom did not influence feeding motivation (at least at the highest cost). Another potential problem when making animals of different LW walk for food is that heavier animals may potentially have more trouble walking, or it may be more physically demanding for them. However, the animals used in Chapter 2 had a

BCS of 5 and differences in feeding motivation could still be detected, so this is unlikely to have influenced the results.

4. *Use of an open economy, meaning that the animals had access to food outside the testing environment (on off days).* Animals could have learned that they would always get food after the test session ended, which may have reduced their feeding motivation during the test. This problem could be solved by letting animals live in their testing environment (Mason and Garner 1998; Cooper and Mason 2001) where they would never have free access to food (i.e., closed economy). However, there could be significant increases in LW when animals are allowed to live in a testing environment and allowed to increase food intake to preferred levels, which could be a potential confounding factor in the interpretation of the results. An open economy, with testing sessions long enough to account for diurnal patterns of food intake (24 h), may be the best solution.

6.4. Alternative measures of motivation

Relating back to the literature in Chapter 1 and 2 on the measurement of feeding motivation, animal welfare scientists have traditionally used the elasticity of demand as an indicator of motivation (Dawkins 1990; Jensen and Pedersen 2008). Note that this elasticity of demand is assumed to be constant across cost. This is in contrast to the use of “point” elasticity to find P_{max} in this thesis. The advantage of using P_{max} and O_{max} measures as compared to elasticity of demand (constant elasticity model) is that they account for both the shape of the curve and the level of consumption, respectively. Animals in different hunger states could have similar elasticity of demand but consumption levels could be very different, which would not be taken into account by the constant elasticity of demand model. For future experiments, a wide range of costs should be used so that a full demand curve can be obtained. Appropriate cost ranges could perhaps be determined by a pilot study. A varying elasticity of demand model should then be applied to the data and both consumption levels and the shape of the curve should be taken into account, like the one in this thesis. Other possible measures of demand that could be obtained are the reservation price and consumer surplus (Kirkden *et al.* 2003).

However, these have some practical limitations because costs are required to increase until consumption falls to zero. This is unlikely to happen for a resource as essential as food. Therefore, the number of rewards collected combined with O_{max} is still the best measure of motivation available.

6.5. Comparisons of feeding motivation between the different studies

The P_{max} values were highest in the fasted ewes (Chapter 2) followed by the SL and FL ewes (Chapter 4) and finally the LBC ewes (Chapter 3, see Appendix A for the mean number of rewards consumed, distance walked, O_{max} and P_{max} across the different motivation studies). A higher P_{max} could imply a higher motivation for food. However, it is important to remember that P_{max} depends on the shape of the curve and is found at the point at which elasticity of demand (or slope of the curve) is exactly -1. In contrast, O_{max} (area under the curve bound by P_{max} and the level of consumption) is a measure of expenditure and depends on the level of consumption and the total distance walked. O_{max} is not necessarily higher with an increased P_{max} because consumption levels may be low. Therefore, the higher P_{max} values of the fasted ewes in Chapter 2 and the SL and FL ewes in Chapter 4 do not necessarily mean that their motivation was higher compared to LBC ewes in Chapter 3. Indeed, O_{max} values of SL and FL ewes (Chapter 4) and fasted ewes (Chapter 2) were lower than O_{max} of the LBC ewes (Chapter 3), suggesting that feeding motivation was also lower. It is interesting to note that the MBC ewes (Chapter 3) and Control ewes (Chapter 4) had a similar BCS of 3, however, O_{max} values were higher for the Control ewes. The reason for this is not entirely clear; feeding motivation was assessed relatively early in pregnancy in Chapter 4 and ewes were not expected to be as highly motivated as the ewes in Chapter 3 (feeding motivation assessed in mid-late pregnancy), due to the lower metabolic requirements. Perhaps it was easier to walk for ewes in early pregnancy due to the lower LW and smaller size of the mammary glands. However, both P_{max} and O_{max} values were outside the range of assessed cost in Chapter 2 and 4, which makes the values less reliable than the ones in Chapter 3.

6.6. Endocrine responses in relation to body condition score and feeding motivation

The findings from this thesis suggest that leptin and IGF-1 were endocrine indicators of BCS and feeding level, while insulin was more indicative of high BCS and did not respond much to reductions in BCS. In response to a change in feeding level, leptin was altered within a few days (Chapter 4) and after 3 to 4 weeks (Chapter 3 and 5). The difference between the studies was most likely due to the severity of food restriction (i.e., reduction to 50% of previous feeding levels in Chapter 4 vs 60-80% of maintenance requirements in Chapter 3 and 5). Leptin concentrations were also highly correlated to eye muscle fat and GR depth (Chapter 3). Furthermore, leptin responded to short-term changes in food intake and concentrations were reduced after a 24 h fast (Chapter 5). Others have also shown that 32 h fasting reduces leptin in fat but not lean ewes (Henry *et al.* 2004) and that 48 h fasting reduces leptin in both thin and fat ewes (Daniel *et al.* 2002). Therefore, leptin was a good indicator of both short-term (hours) and medium-term (weeks) changes in food intake and BCS. Insulin-like growth factor 1 (IGF-1) appeared to be a long-term indicator of body fatness and nutritional status, taking about 9 weeks to reflect changes in BCS (Chapter 3). A study by McCann et al. (1997) also showed that IGF-1 was profoundly higher in fat compared to thin sheep. In addition, IGF-1 was reduced by the 24 h fast (Chapter 5), a similar effect has also been found by others (Kosior-Korzecka *et al.* 2006). Therefore, it appears that IGF-1 is a good indicator of long-term changes in BCS and metabolic state in pregnant ewes.

Insulin increased in high BCS ewes after 10 weeks on the nutritional treatments, while the effects of food restriction on plasma insulin concentrations were relatively small (Chapter 3). In Chapter 4 and 5, similar results were found and BCS loss did not affect insulin concentrations. Therefore, insulin may be an indicator of high BCS, rather than low BCS. However, others have shown a reduction in insulin after a reduction in BCS in non-pregnant ewes (Caldeira *et al.* 2007b, a). Insulin was also considerably reduced after a fasting (Chapter 5; Henry *et al.* 2004) and, therefore, alters rapidly after a change in feeding level. In this thesis, the short-term effects of food restriction on insulin seem contradictory to the lack of effect of long-term food restriction. The reason for the low response in insulin to reductions in BCS is most likely due to its homeostatic glycaemic control. Insulin activates a cascade of signalling events resulting in inhibition of lipolysis in adipose tissue, inhibition of gluconeogenic enzyme expression and the release of glucose from the liver (reviewed by Schenk *et al.* 2008). Therefore, there are several mechanisms by which insulin action is altered as a result of undernutrition. For example, it has been shown that undernutrition enhances the response to insulin in sheep (Sano *et al.* 1999) and insulin sensitivity in other species (Davidson *et al.* 2002). In addition, there are a number of cellular pathways that respond to altered energy availability (for example the PI3 kinase, protein deacetylase sirtuin 1 (SIRT1) and the serine/threonine protein kinase mTOR) and these pathways modulate insulin action (reviewed by Rother *et al.* 2008; Schenk *et al.* 2008). Another pathway by which insulin action can be modulated is by alterations in expression of GLUT4 (glucose transporter in muscle and adipose tissue), which has been associated with the development of insulin resistance (Gardner *et al.* 2005; Ozanne *et al.* 2006). These changes in insulin action cannot be detected by measuring plasma concentrations. Therefore, it is possible that LBC ewes had an enhanced sensitivity and altered insulin signalling as a consequence of long-term food restriction.

In the general introduction, evidence was reported of the involvement of insulin and leptin in reducing food intake, and ghrelin stimulating food intake. However, insulin, leptin and ghrelin concentrations did not appear to be related to feeding motivation in this thesis (Chapter 3 and 4). Plasma insulin concentrations were not different between BCS treatments before and after the feeding motivation period in Chapter 3, even though large BCS effects on feeding motivation were observed. Plasma leptin concentrations were considerably lower in LBC compared to MBC ewes in Chapter 3. However, the difference in feeding motivation was small between LBC and MBC ewes. In Chapter 4, a large BCS loss treatment effect was found on leptin, without observing any differences in feeding motivation. Also plasma ghrelin concentrations had no relation to feeding motivation. However, a major limitation of this thesis was the lack of a statistical method to determine the relationship between endocrine signals and feeding motivation. The main obstacle to statistically determining the relationship between endocrine signals and motivation was that a single accurate measure of motivation is required (e.g., O_{max}). However, the narrow range of costs used in Chapter 2 and 4 makes O_{max} not sufficiently reliable for use as the sole measure of motivation. In addition, obtaining individual animal O_{max} values has been problematic because around 10% of data was lost due to technical failure or ewes obstructing the races in Chapter 3 and 4, making the individual O_{max} of some ewes unreliable. Furthermore (as discussed in Chapters 3 and 4), the sampling points in relation to feeding motivation may not have been ideal for detecting differences in insulin and leptin concentrations, and more frequent samples before or during the daily sessions may have been required. However, collecting blood samples frequently during a feeding motivation test like the one used in this study would be extremely difficult, and any disturbance to the animal could affect feeding behaviour. However, it can be concluded that plasma leptin, insulin and ghrelin concentrations, collected as a single sample on a given morning before food intake, are not good indicators of feeding motivation. Therefore, it appears that plasma concentrations of leptin, IGF-1 and insulin are mainly indicators of BCS and feeding level, but not of the level of hunger experienced. Plasma concentrations of ghrelin were of little use as a marker of both BCS and feeding motivation.

Despite the fact that no changes in feeding motivation were observed that reflected changes in plasma concentrations of insulin, leptin and ghrelin, there is strong evidence that these endocrine signals do affect food intake. However, different endocrine signals interact with each other and combined effects may be complex (Woods *et al.* 1998; Baskin *et al.* 1999; Ueno *et al.* 2004). Furthermore, the effects of insulin and leptin on food intake are indirect through activation of several neuropeptides in the arcuate nucleus with opposing effects (see Chapter 1). It has been shown that intracerebroventricularly (ICV) administered insulin decreases food intake in sheep (Foster *et al.* 1991) and rats (Air *et al.* 2002).

Intravenous administration of insulin also decreases food intake in sheep (Deetz *et al.* 1980; Deetz and Wangsness 1981). However, insulin signalling is complex and involves a cascade of different receptors, proteins and cellular pathways that modulate insulin action (reviewed by Rother *et al.* 2008; Schenk *et al.* 2008). Furthermore, it has been well documented that insulin resistance occurs in mid- to late-pregnancy in peripheral tissues in sheep (Petterson *et al.* 1993; Jaquiery *et al.* 2009). In humans, it has been shown that central (brain) insulin resistance also occurs with peripheral insulin resistance, in particular the brain regions involved in the regulation of appetite and reward (Anthony *et al.* 2006). This complexity of insulin signalling and the development of insulin resistance could explain the lack of relationship between insulin and feeding motivation in this study. In addition, positive relationships between seasonal food intake, body fat, insulin and hypothalamic neuro-peptide Y (NPY) have been found, suggesting that insulin does not reduce NPY during periods of high food intake in sheep (Anukulkitch *et al.* 2009). Therefore, Anukulkitch et al. (2009) suggest that insulin may not directly affect food intake in sheep.

There is also evidence that leptin increases food intake in animals. ICV leptin administration decreases food intake in fully fed lambs (Morrison *et al.* 2001), while intravenous administration does not affect food intake (Morrison *et al.* 2002). In other species it has also been found that ICV administration of leptin decreases food intake, while intravenous administration does not (Ramsey *et al.* 1998). However, peripheral administration of leptin in rats does result in decreased food intake (Campfield *et al.* 1995; Halaas *et al.* 1995; Pelleymounter *et al.* 1995). Therefore, there may be species differences in the peripheral effects of leptin on food intake. Leptin resistance could also occur when peripheral leptin concentrations are high; Ramsey et al. (1998) suggested that peripheral leptin administration can lead to leptin resistance because the leptin receptor facilitating endocytosis across the blood-brain barrier is satiable, which could explain the lack of effect of peripheral leptin administration in high doses.

Finally, the plasma concentrations of leptin and insulin may not be directly related to leptin concentrations or leptin functioning in central brain structures in sheep. For future research it may be useful to measure central concentrations of leptin, insulin and ghrelin as well as the actions of the neuropeptides on which they act. This may provide a clearer relationship between endocrine signals and feeding motivation. Other endocrine signals that were not measured in this thesis could also be involved in the regulation of feeding motivation; for example CCK (Cooper and Dourish 1990), oxytocin (Douglas *et al.* 2007) and adiponectin (Kubota *et al.* 2007) and it would be useful to investigate the relationship between these signals and feeding motivation.

6.7. Metabolic responses to body condition score change

Glucose was a very reliable indicator of BCS in Chapter 3, 4 and 5. Glucose is an important metabolite for both mother and fetus and is relatively stable over time (Bell and Bauman 1997). Glucose was unaltered after the 24 h fast (Chapter 5), the most likely reason for this is that the majority of glucose utilisation is independent of insulin (Weekes *et al.* 2000). Glucose reflected changes in BCS after 12 days (Chapter 5) to 47 days (Chapter 4), suggesting that it is a long-term indicator of feeding level and BCS. These results are in agreement with others who have shown a reduction in glucose after a loss of BCS in pregnant ewes (Luther *et al.* 2007). Glucose concentrations were also unaffected by a 24 h fast in a study by McCann et al. (1986). Free fatty acids (FFA) were also a good indication of body fatness. High concentrations were a reflection of a catabolic state (high concentrations observed in low BCS animals in Chapters 3, 4 and 5) and FFA responded to a change in feeding level between 2 days (Chapter 4) to 44 days (Chapter 5). Caldeira et al. have also shown that FFA is a good indicator of BCS in non-pregnant sheep (2007b, a). In addition, FFA was profoundly increased after the 24 h fast (Chapter 5), which agrees with previous findings (Ingle *et al.* 1973). However, high concentrations of FFA were observed in the control ewes in Chapter 4, suggesting that FFA could also be an indication of moderate-high fat reserves, although this was not observed in the high BCS ewes in Chapter 3. FFA concentrations fluctuated more in all ewes in Chapter 3, which makes interpreting the data more difficult. In particular the high peak observed at day 91 of the high BCS ewes (Chapter 3) is surprising. This data point also corresponds to a drop in glucose and insulin, suggesting that there must have been a (short-term) change in food intake. However, no events that could explain the changes in metabolic markers at day 91 of pregnancy could be found in the data records. Furthermore, both mating groups (samples collected 3 weeks apart) showed the same pattern. Such dramatic alterations in metabolic markers were not observed in Chapter 4 and 5 at this time in pregnancy, suggesting that there was indeed a change in food intake that cannot be explained. Furthermore, plasma concentrations of metabolites collected from the jugular vein were measured in this study, and metabolite concentrations may have been different in the portal vein.

β-hydroxybutyrate (β-HBA) was an indicator of BCS in Chapter 3, where high concentrations in β-HBA were observed in the HBC ewes after day 105 of pregnancy. β-HBA was also increased in mid-pregnancy in the FL ewes of Chapter 4. No differences in β-HBA were observed in Chapter 5 between BCS treatments, the reason for this is not clear. Others have found that plasma concentrations of β-HBA increase with food restriction in pregnant ewes (Charismiadou *et al.* 2000; Banchero *et al.* 2006). β-HBA concentrations increased in response to the 24 h fast in Chapter 5, which is in agreement with a study by Heitmann et al. (1986). Urea was not a reliable indicator of BCS, except for showing some difference between BCS treatments in Chapter 3. However, protein may not have been limited in the studies presented in this thesis, even in low BCS ewes. The concentrate diet (Chapter 3 and 5) and lucerne chaff diet (Chapter 4) were relatively high in protein, which may explain the lack of BCS effect on urea concentrations. Therefore, the most reliable metabolic indicator of BCS was glucose, followed by FFA. β-HBA was a less reliable indicator while urea was the least useful.

6.8. Body condition score and environmental challenges

In Chapter 5, ewes were subjected to an acute cold challenge in mid-pregnancy in order to assess the ability to maintain energy homeostasis and to induce adaptive metabolic and stress responses in ewes with different BCS. The major result of this study was the considerably delayed and reduced cortisol response in the first 60 minutes in the LBC compared to the MBC and HBC ewes. Cortisol is a component of the HPA-axis and involved in the stress response to challenges. Stress occurs when the homeostasis of the animal is threatened or perceived to be so (Chrousos 2009). The classical stress response has many consequences, including increased energy mobilisation and decreased energy storage, gluconeogenesis, enhanced cardiovascular tone, stimulation of immune functions, inhibition of reproduction, decreased appetite and a heightened state of alertness and cognition (Sapolsky *et al.* 2000; Chrousos 2009). All these responses allow the animal to react behaviourally and physiologically to the challenge and increase its survival chances. However, long or repeated activation of the HPA-axis and high levels of cortisol due to exposure to the same stressor may have negative consequences for the animal. For example, chronic stress may lead to anxiety and depression, hypertension, immune or metabolic disorders (Chrousos 2009). Therefore, constant activation of the HPA-axis and high levels of cortisol are not beneficial to the individual. Therefore, a mechanism may be in place to protect the individual from these negative consequences of HPA-axis activation.

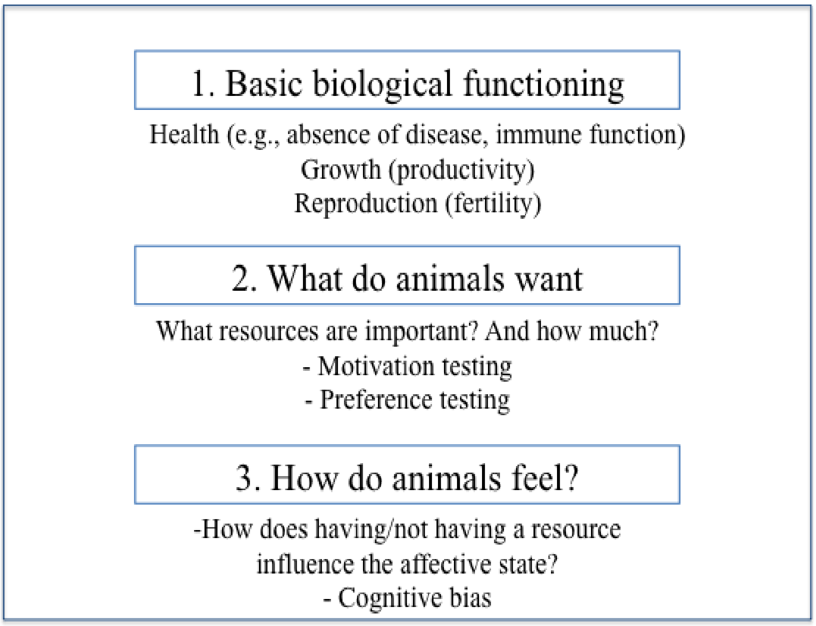
It has been shown that repeated exposure to the same stressor leads to habituation of the HPA-axis in a number of different species, including rats (Armario *et al.* 1988; Girotti *et al.* 2006), dogs (Beerda *et al.* 1999) and cows (Ladewig and Smidt 1989). This habituation manifests itself by a reduced HPA-axis response to the stressor, and cortisol levels in chronically stressed animals are usually not much different from control animals. In the case of a severe stressor (immobilisation or restraint in rats), only one exposure is sufficient to induce habituation of the HPA-axis while other types of stressors (insulin-induced hypoglycemia) do not lead to habituation after a single exposure (Armario *et al.* 2004). It is not clear what the mechanisms are behind this adaptation to repeated stress. Some studies have shown a reduction in neural activity (measured by c-fos staining) in the paraventricular nucleus of the hypothalamus and perhaps other brain regions, which may possibly be mediated by glucocorticoids (Girotti *et al.* 2006; Arnhold *et al.* 2007). However, brain regions involved in emotional processing (including amygdala and limbic cortex) may also be involved in the habituation (Armario *et al.* 2004). Girotti et al. (2006) propose that integrative brain regions in the context of repeated exposure to the same stressor can rapidly decrease neural responses to the primary sensory stimulus. This change in sensory gating would then produce an attenuated response to a reoccurrence of that stressor (habituation). This habituation of the HPA-axis after repeated exposure to the same stressor would protect the individual against the negative consequences of prolonged HPA-axis activation and elevated glucocorticoids. Interestingly, in the studies presented in this thesis, basal cortisol concentrations were only different between BCS treatments in Chapter 3, but not in any of the other studies. The reduced cortisol response to the cold challenge suggests that undernutrition leads to a habituation of the HPA-axis. A study by Jaquiery et al. (2006) showed that undernourished pregnant ewes had a lower cortisol response to an arginine vasopressin/corticotrophin releasing hormone (AVP/CRH) challenge, providing strong evidence that undernutrition indeed leads to habituation of the HPA-axis. Poore et al. (2010) showed that undernutrition has long lasting consequences for the HPA-axis in sheep; lambs undernourished between 12 and 25 weeks of age had a significantly reduced adrenocorticotrophin (ACTH) response to a corticotrophin releasing factor (CRF)/AVP challenge at 1.5 years of age, while the cortisol response remained unchanged. However, no altered cortisol or ACTH responses after undernutrition were found when lambs were exposed to an isolation transport challenge (Poore *et al.* 2010). Furthermore, studies in non-pregnant cows (Fisher *et al.* 2002) and pregnant gilts (Spoolder *et al.* 1996) did not find an effect of undernutrition on the cortisol response to a CRH and ACTH challenge, respectively. It is likely that the effects of undernutrition on HPA-axis responses depend on the severity and duration of the undernutrition period, and that the HPA-axis response to the acute challenges depend on the type and severity of the challenge.

In undernourished pregnant sheep, repeated exposure to novel environmental or chemical challenges lead to a reduction in the HPA-axis response (Jaquiery *et al.* 2006; Poore *et al.* 2010). Another study in cattle has also shown a decrease in the stress response to a novel stressor (ACTH challenge) after chronic stress exposure (tethering) (Ladewig and Smidt 1989). However, a number of studies in rats have shown that chronic or repeated exposure to the same stressor enhances, rather than reduces, the response to a novel stressor (Armario *et al.* 1988; Hellriegel and D'Mello 1997; Bhatnagar and Dallman 1998; Ma *et al.* 2008). These authors suggest that the ability to maintain sensitivity to a novel acute stressor is an important survival mechanism for chronically stressed animals. The discrepancy between results (i.e., reduced vs enhanced response to acute stress in chronically stressed animals) may be due to the different types of stressors and challenges used. It has been suggested that the degree of habituation to a stressor and the responses to a novel stressor may depend on the type, duration and intensity of the stressor (Armario *et al.* 1988; Retana-Márquez *et al.* 2003; Armario *et al.* 2004).

In order to improve animal welfare, it would be valuable to further investigate the relationship between chronic and additional novel (acute) stressors. The acute cold challenge in this thesis was relatively short, and ewes were able to release sufficient glucose and FFA in order to maintain energy homeostasis and T_{core}. However, longer exposure to cold, or perhaps different types and combinations of novel stressors may lead to more severe consequences in ewes with low BCS. Other stressors could potentially also lead to chronic stress, for example, unstable social environments due to constantly changing individual animals between flocks. Different types of chronic stressor could potentially affect the response to different acute stressors (e.g., cold exposure, restraint or transport). However, little is known about the interaction between chronic and acute stress in sheep and their impacts on welfare.

6.9. Wider implications for animal welfare

In this thesis, the effects of hunger and metabolic functioning on the welfare of food restricted ewes have been discussed extensively. However, there are many other potential welfare issues in New Zealand and Australian farming systems. In order to assess welfare objectively and accurately in a wider context (i.e., farm or farming system), I would like to propose three steps of welfare assessment following on the ideas by Fraser (2009b, a) and Mendl (2001). These are outlined in Fig. 6.2 with step 1 assessing the basic biological functioning of the animals. This includes assessing whether the animals are healthy, growing and reproductive. The absence of disease is one of the main determinants of animal welfare and is an important component of animal welfare legislation in many countries (Webster 2001; FAWC 2009). The measurement of biological functioning should be a first step in assessing overall welfare, because of the large impacts on animal welfare if biological functioning is not sufficient. Step 2 involves asking animals what they want (Dawkins 1988, 2006). This could be done by preference testing to rank what resources are most important to the animal, and by assessing the strength of the motivation for a particular resource (Kirkden and Pajor 2006; Jensen and Pedersen 2008). Finally, the third step involves assessing the affective state of the animal. This relates to how having or not having a resource for which a high motivation/preference exist determines the affective state of the animal (either positively or negatively). Assessing the cognitive bias (Paul *et al.* 2005; Doyle *et al.* 2010), appraisal (Desire *et al.* 2002) and anticipatory behaviour (Spruijt *et al.* 2001) are currently the only methods that have the potential to provide direct insight into the affective states of animals. Therefore, this top-down integrated approach may be useful in accurate assessment of animal welfare. However, the outcome of the three steps is likely to depend on the type of husbandry system (for example, extensive or intensive) and will differ between species because different species may have different needs and preferences.



1. Basic biological functioning

Health (e.g., absence of disease, immune function)
Growth (productivity)
Reproduction (fertility)

2. What do animals want

What resources are important? And how much?
- Motivation testing
- Preference testing

3. How do animals feel?

-How does having/not having a resource
influence the affective state?
- Cognitive bias

Figure 6.2. Three important steps for assessing animal welfare (see explanation in text).

The welfare of the ewes in this thesis could be reasonably judged according to step 1 (basic biological functioning). The ewes were generally healthy in all experiments and no clinical signs of disease were observed. However, in future experiments it would be valuable to assess the immune function of ewes with different BCS. Even when there are no clinical signs of disease, there may be differences in immune function of animals in different metabolic states (for examples and reviews see: Douglas *et al.* 1998; Houdijk *et al.* 2001; Pond 2005) that could potentially make animals more susceptible to disease when exposed to a pathogen. The reduced cortisol response to the acute cold challenge could have potential effects on immune function and other aspects of biological functioning, although the actual effects and implications need to be investigated. All ewes gave birth to twins (Chapter 3 and 5) or singleton lambs (Chapter 4). There were some stillbirths, but numbers were not high enough to do a statistical analysis and no obvious relationship between stillbirths and BCS treatment could be observed.

The birth weights of the lambs tended to be different between BCS loss treatments in Chapter 4; however, lambs born to the FL ewes had the highest birth weights. A tendency for decreased weaning weights was found in lambs born to LBC ewes (Chapter 3), suggesting that growth of the lambs born to low BCS ewes may have been decreased. However, a larger number of lambs is necessary to assess the effects of birth weight and weaning weights more accurately. Other studies have also shown that undernutrition in pregnancy may lead to reduced birth weights (Redmer *et al.* 2004; Kenyon *et al.* 2009a), which are associated with reduced lamb survival (Clarke *et al.* 1997; De Blasio *et al.* 2007). The long-term consequences of low BCS during pregnancy on reproductive performance in subsequent years have not been studied in this thesis. There is some evidence that low BCS during pregnancy has long-term effects on reproductive performance; ewes with a BCS 2 at mating in year 1 produced about 20 lambs per 100 ewes less in year 2 compared to ewes with a BCS of 3 at mating in year 1 (Oldham and Thompson 2004). In dairy cows, there is also some evidence that a high BCS at calving leads to a smaller loss in LW/BCS post-calving and an earlier attainment of successful pregnancy (Roche *et al.* 2009). This suggests that low BCS during pregnancy may have long-term consequences on reproductive performance.

The feeding motivation methodology used in this study mainly addressed step 2; what do animals want, and how much do they want? However, only the motivation for food was assessed, and not for other potentially important resources. In terms of hunger, a clear answer to step 2 has been provided and it can be concluded that low BCS and fasting both lead to a profound increases in feeding motivation, but a low feeding level that does not lead to a loss in BCS does not increase feeding motivation. It can also be concluded that animals prefer to obtain food near *ad libitum* levels. However, it could also be “asked” what type of food animals prefer, and if motivation and preferences change with different physiological states. Furthermore, different durations of long-term food restriction over different physiological states and their effects on feeding motivation have not been explored in this thesis. In this thesis, long-term food restriction was a period of 7 (Chapter 3) to 13 weeks (Chapter 4) during which ewes lost 1 BCS, and all ewes were fed *ad libitum* at lambing. However, lactation is also a very metabolically demanding period and it would be important to assess feeding motivation of ewes with different BCS at that time. It would be valuable to determine the impact of a pregnancy completed on a low BCS on feeding motivation during lactation and in the following year. There is some evidence that BCS indeed has long-term consequences for food intake, because ewes with a previously low BCS had an increased food intake compared to ewes with a previously high BCS, even though the previous differences in BCS had disappeared (Sibbald and Rhind 1997).

The third step is the most difficult to assess. Currently, there is very limited information on affective states in animals, and how animals perceive their own situations and environment. In terms of hunger, the best methodology currently available is to ask animals how much food they want, and perhaps what type of food they prefer. However, this does not provide direct insight into the affective state of animals when access to food is denied (see also section 6.3). Future research could focus on assessing affective states in sheep with a high motivation for food, for example by testing for cognitive bias (Paul *et al.* 2005).

6.10. Conclusions

1. The behavioural demand methodology was successful in detecting differences in motivation between fasted and fed ewes, as well as between ewes with different BCS. When using behavioural demand methodologies, it is important to use a wide range of costs in order to obtain a full demand curve, so that measures of motivation can be accurately assessed. The number of rewards consumed was most successful in detecting differences in feeding motivation between ewes with different food restriction levels, O_{max} was the second most useful measure and P_{max} was the least able to detect differences in motivation.
2. Short-term (24 h) food restriction increased feeding motivation compared to *ad libitum* fed control ewes. Long-term food restriction (leading to a loss of

BCS) increased feeding motivation compared to *ad libitum* fed control ewes, and tended to increase motivation compared to ewes fed to maintain BCS. However, compared to control ewes that were fed to maintain BCS, feeding motivation was not increased when the feeding level was reduced to 50% of previous feeding levels and no BCS loss had occurred. Therefore, the effects of long-term food restriction leading to a loss in BCS most likely lead to the largest increase in the subjective experience of hunger.

3. Metabolic and endocrine signals were good indicators of short-term food restriction (fasting) and long-term food restriction (changes in BCS). Glucose and FFA were the most accurate metabolic indicators of BCS, while leptin and IGF-1 were the most reliable endocrine indicators of BCS. The rate of BCS loss also had major impacts on metabolic and endocrine responses, with a fast loss in BCS resulting in larger responses while responses to a slow loss were not different from ewes maintaining BCS. Therefore, pregnant ewes are metabolically better able to adapt to a slow rather than a fast BCS loss.
4. Plasma concentrations of endocrine signals did not reflect the level of feeding motivation. Reasons for this were not clear, but may stem from lack of a single accurate measure of feeding motivation, lack of a statistical method to test the relationship between endocrine signals and feeding motivation, and alterations in leptin and insulin signalling and action (i.e., changes in tissue responsiveness and sensitivity).
5. Ewes with a low BCS were able to maintain energy balance and internal body temperature in response to an acute cold challenge, but showed lower and delayed metabolic responses compared to medium and high BCS ewes. Cortisol responses were reduced and delayed, which implies that low BCS leads to down-regulation of the HPA-axis. Low BCS ewes may therefore have a reduced ability to adapt to environmental challenges.
6. Food restriction lead to an increase in hunger and a reduction in the metabolic state, which is likely to reduce the welfare of pregnant ewes.

Appendix A.

Mean number of rewards consumed and distance walked (m) during the feeding motivation tests by the ewes from Chapter 2, 3 and 4. For details on procedures and standard errors see the corresponding Chapters.

			Cost (m)					
		Treatment	2.0	6.6	12.1	22.0	40.0	P_{max} and O_{max}
Chapter 2	Mean number of rewards	Control	299.3	220.0	285.9	181.1	157.1	77.7
		Fasted	383.2	376.1	348.7	275.8	248.6	88.8
	Mean distance walked	Control	598.6	1452.0	3459.3	3985.1	6285.9	7338.2
		Fasted	766.4	2482.6	4219.7	6066.8	9943.7	12327.0
			2.0	7.2	13.8	26.4	50.0	P_{max} and O_{max}
Chapter 3	Mean number of rewards	LBC	549.9	524.7	529.1	442.6	261.7	49.1
		MBC	532.6	506.2	469.9	333.1	170.1	35.5
		HBC	258.8	225.0	46.3	86.7	3.7	12.4
	Mean distance walked	LBC	1099.9	3777.6	7301.3	11685.0	13086.9	13347.2
		MBC	1065.1	3644.7	6484.9	8793.1	8502.7	9185.7
		HBC	517.6	1620.2	638.6	2289.4	182.8	1521.8
Chapter 4	Mean number of rewards	Control	571.9	423.1	441.7	341.7	228.0	69.3
		SL	499.4	496.2	438.0	326.9	208.6	54.0
		FL	518.4	503.3	449.5	334.8	232.6	46.7
	Mean distance walked	Control	1143.8	3046.2	6095.8	9022.1	11397.7	12132.3
		SL	998.8	3572.3	6043.7	8630.8	10427.6	10320.1
		FL	1036.9	3624.1	6203.3	8837.5	11627.6	11458.8

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