CHAPTER 1

Literature review and general introduction

Animal welfare is a very important component of farm animal production, and viable methods of measuring animal welfare are still under investigation. This thesis proposes a novel approach to the measurement of animal welfare, based on adaptive responses to stressor challenges, which are activated in an attempt to maintain homeostasis. The technical aspects of the literature review which are relevant to a particular experiment are presented prior to a description of that experiment.

1.1 HISTORICAL ASPECTS OF ANIMAL WELFARE

The first formal protection granted to animals in the British legal system was in 1822; a British member of parliament, Richard Martin, presented a successful bill, which offered cattle, horses and sheep protection from cruelty. Martin was also one of the founders of the world's first animal welfare organisation, the Society for the Prevention of Cruelty to Animals, in 1824.

The concepts of farm animal welfare that we are familiar with today, started to develop in the 1960s, when Ruth Harrison's book on what she called "factory farming methods" was published (Harrison, 1964). Harrison criticised the intensive animal production systems developing in the United Kingdom at the time, on issues including feeding programs, indoor production systems, space restriction and the use of antibiotics. Following the publication of Harrison's book, an investigation into the welfare of intensively farmed animals was commissioned by the UK government (Brambell, 1965). The commission was chaired by Professor F.W.R. Brambell, and the Brambell Report, as it became known, made a number of significant insights into the issue of farm animal welfare. As the report was mainly addressing issues brought forward in Harrison's critique, it specifically addressed concerns of overcrowding and degree of confinement, and this was reflected in its recommendations. However, the Brambell Report was the basis for establishment of the Farm Animal Welfare Advisory Committee (which later became the Farm Animal Welfare Council), in 1967. The committee's guidelines have evolved and are now widely recognised as the Five Freedoms of animal welfare, namely: Freedom from thirst, hunger and malnutrition, Freedom from discomfort due to the environment, Freedom from pain, injury and disease, Freedom to express normal behaviour for the species, and Freedom from fear and distress (Farm Animal Welfare Council, 1993). The Five Freedoms provided a base from which to develop models to help us determine how best to define and/or assess animal welfare.

1.2 DEFINING ANIMAL WELFARE

The term 'animal welfare' arose in society to express ethical concern regarding the treatment of animals (Tannenbaum, 1991; Fraser, 1995). However, the meaning of the term has been the subject of much discussion. Both Tannenbaum and Fraser described the 'welfare' of an animal as referring to its quality of life, which involves many different elements such as health, happiness, and longevity to which people attach different degrees of importance. According to Broom (1986), the welfare of an animal is its state as regards its attempts to cope with its environment, by trying to counteract any adverse effects of those conditions. Animals can do this by behavioural modification, and also by activation of the neuroendocrine system, to mobilise energy resources, so that the animal is returned to a state of homeostasis (Fraser and Broom, 1990). An animal's welfare is, therefore, deemed poor if it has difficulty in maintaining this state (McEwen, 1998). Duncan and Petherick (1991) proposed an alternative viewpoint, that animal welfare is dependent solely on the mental, psychological and cognitive needs of animals, because in most cases if these are met, the physical needs will be as well. This promotes a more 'feelings based' approach to animal welfare, which is becoming more prominent as the public become more concerned about the quality of production animals' lives.

1.3 MEASURING ANIMAL WELFARE

Stress is a term commonly used in the study of animal welfare to indicate environmental conditions adverse to the well-being of an animal. Stress may be climatic, due to extreme cold or heat; nutritional, due to deprivation of feed or water; social, resulting from a low

ranking in the social group; or internal, due to physiological disorder such as illness (Stott, 1981). The condition of the stressed animal is regarded as abnormal and undesirable. In 1965, Lee defined stress as denoting the magnitude of forces external to the body, which displace that system from its resting state. This definition highlights the importance of forces which bring about changes in the bodily system in order to maintain a constant internal environment, rather than on unfavourable ambient conditions.

Moberg (2000) has taken a different angle on that of Lee (1965), while still focussing on the internal environment rather than the extremes of external environmental forces, and has defined stress as "the biological response elicited when an individual perceives a threat to its homeostasis", with the threat being the stressor. In Moberg's model of animal stress, there are three stages: recognition of a threat to homeostasis, the stress response, and the consequences of stress. The homeostasis that has been disturbed by the stressor can be re-established by a complex repertoire of responses of the organism. These responses can be measured by researchers in an attempt to determine the severity of a stressor to an animal, and whether or not the animal has maintained homeostasis. If adaptive responses to a challenge are unable to restore homeostasis, the welfare of the animal may be compromised. Moberg's model has been chosen to represent animal stress with regard to this thesis because it is commonly and widely used and accepted. Research in the area of animal welfare has traditionally involved separate study in two main fields; i) studying the behaviour of animals, and ii) measuring physiological responses to challenging conditions. The merits and problems associated with both measures will be discussed in the following sections.

1.3.1 Health

Physical health is the most universally accepted measure of animal welfare. Animals which are sick or injured are generally acknowledged to be suffering, and to have poor welfare (Dawkins, 2003). The issue is not whether good health is necessary for good welfare, but whether good health alone is enough. More and more commonly, good welfare is considered to be about much more than just not being sick; quality of life has also become a consideration. Therefore, additional measures of animal welfare have been sought.

1.3.2 Physiology

Physiological responses to a perceived stressor include activation of the autonomic nervous system (ANS), the neuroendocrine system and the immune system. The ANS controls homeostasis by regulating many biological systems, including the cardiovascular, gastrointestinal, and respiratory systems, and exocrine glands, resulting in changes in heart rate, digestion, respiratory frequency and sweat, saliva and urine production (Moberg, 2000). These changes are mostly involuntary, often short-lived, and can be difficult to measure, therefore, they are not as commonly measured for the purpose of animal welfare research as the neuroendocrine system, and, more recently, the immune system.

The activation of the hypothalamic-pituitary-adrenal (HPA) axis is one of the best known and most consistent neuroendocrine responses to stress (Matteri et al., 2000), whether environmental, physiological or psychological, and for this reason it is also one of the most commonly measured responses for the purpose of determining animal welfare. The main hormones involved in the HPA axis are corticotropin releasing hormone (CRH),

adrenocorticotropic hormone (ACTH) and glucocorticoids. Activation of the HPA axis initiates synthesis and secretion of vasopressin and CRH from the paraventricular nucleus of the hypothalamus, and these in turn stimulate secretion of ACTH from the anterior pituitary. The ACTH stimulates the release of glucocorticoids (e.g. cortisol) from the adrenal cortices, which in turn act back on the hypothalamus and pituitary to suppress CRH and ACTH production in a negative feedback cycle.

Cortisol is one of the main products of HPA axis activation, and is commonly called the 'stress hormone' for that reason. The release of cortisol initiates a series of metabolic effects aimed at alleviating the harmful effects of stress, and is partly regulated through negative feedback to both the hypothalamus and the anterior pituitary, which decreases the concentration of cortisol in the blood once the state of stress subsides. The metabolic effects of cortisol include: promoting glucose metabolism and increasing blood pressure to increase energy availablity, increasing the effectiveness of catecholamines, and suppressing immune responses. Cortisol concentration is often considered to relate directly to animal welfare for these reasons, and is frequently the only measure used to make sweeping judgements on the welfare of animals in different situations. The problem with measuring HPA axis activation is that results vary considerably from one study to the next, are dependent on sampling method and frequency, and sometimes clash seriously with other data; for example, involving production and mortality (Rushen, 1986, 1991). Therefore, although there is no doubt that measuring HPA axis activation continues to be a very important feature of animal welfare research, and its use is strongly defended (for example, by Barnett and Hemsworth, 1990), there is a danger in making general comments on animal welfare based solely on results from studies measuring changes in

cortisol concentration. For example, blood cortisol levels can also increase during courtship, mating and active food acquisition (Fraser and Broom, 1990). The comment by Dawkins in 1976 that "we are still faced with the problem of relating changed physiological state to the animals' subjective feelings of distress" is still relevant 30 years later.

Although HPA axis responses to stress are commonly considered to be short-term responses to acute stressors, there is evidence to demonstrate that in some species at least, there are changes in HPA axis regulation in response to chronic stressors. It is likely that high levels of circulating glucocorticoids over a sustained period in response to a low level stressor are detrimental, and would make the animal unable to mount a physiologically appropriate response to an acute challenge. Physiological down-regulation of the HPA axis would help this. Hauger et al. (1990) demonstrated in rats that exposure to repeated restraint resulted in a significant loss of CRH receptor sites in the anterior pituitary, thereby causing a down-regulation of the HPA axis. There are few studies of domestic species in this field of research, but there is evidence of pituitary regulation in cattle in response to lying deprivation (Munksgaard, 1999; Fisher et al., 2002). There does not appear to be any work of this sort done with sheep.

Long-term responses to stress also include a depression of immune function which is activated via both the HPA axis and the sympathetic nervous system (SNS). Activation of the SNS culminates in the release of catecholamines, including epinephrine and norepinephrine, which help to prepare the body for physical activity in response to a threat. This was first described by Cannon (1927) as the 'fight or flight' response.

Catecholamines also modulate a range of immune functions, including cell proliferation, cytokine and anti-body production, cell destruction and cell trafficking. If the SNS is chronically activated, catecholamines can disrupt regulation of immune function (for a review see Padgett and Glaser, 2003). The HPA axis is involved in immune suppression through the effects of glucocorticoids, of which cortisol is the most important. Sustained release of glucocorticoids can cause: negative regulation or 'containment' of the release of catecholamines (Kvetnansky et al., 1993), impairment of the ability of the gut to clear pathogenic invasion, the inhibition of cytokine production, and more (for a comprehensive review see McEwen et al., 1997). A practical example of the effects of cortisol suppressing the immune system is that the ability of pigs to produce antibodies against tetanus declines as salivary cortisol increases (Broom and Johnson, 1993).

Most of the work in this area has concentrated on rat and mouse models, and is occasionally applied to individual animal welfare studies, for example, chronic heat stress (at 35°C and 55% relative humidity for 6 weeks) suppressed T-cell mitogen-induced lymphocyte blastogenesis in sheep, reflecting immunosuppressive properties (Niwano et al., 1990). However, acute heat stress (35°C for 24 hours) did not significantly alter total or differential numbers of leukocytes in lambs, whereas acute restraint and isolation stress did increase leukocyte numbers, but neither treatment altered the ability of lymphocytes to proliferate in response to mitogens (Minton and Blecha, 1990). This suggests that generally only chronic stressors have an immunosuppressive effect, although the change in leucocyte number during restraint and isolation stress suggests that the effects might occur more quickly in an intense stress state.

1.3.3 Behaviour

A wide variety of behavioural tests were developed in the last century to characterise laboratory animals, mostly rodents, in relation to their responses to stressful or emotional situations. Essentially, the different models involved the exposure of the animals to one or more aversive stimuli, whilst observing their behaviour (for a review see Ramos and Mormede, 1998). Animal welfare research has adopted some of these tests. An example of this is the open-field test developed by Calvin Hall and reported in 1934, to determine effects of environmental manipulation and genetic factors on the emotionality of rodents. The open-field test was first used on large domestic mammals (pigs) by Beilharz and Cox (1967) and has since been regarded as a standard technique for assessing specific types of behaviour of farm animals. Researchers are trying to determine the motivational factors associated with livestock behaviour such as emotional reactivity (fear), exploration and locomotion (Thodberg et al., 1999). The interpretation of such tests can be very difficult, as there are often a range of reasons for an animal to perform one particular behaviour (de Passille et al., 1995).

Other behavioural measures of welfare include comparisons of captive animals with wild or free-ranging animals, such as fowl (Wood-Gush, 1971), measurement of abnormal or destructive behaviours such as stereotypies, which are repetitive, invariant behaviour with no obvious goal or function (Mason, 1991; Lawrence and Rushen, 1993), and comparisons of time budgets to identify behaviour changes. Behavioural responses to stress depend on the situation an animal finds itself in, and also the species of animal. Some animals will freeze in response to a stressor, such as chickens displaying tonic immobility, and some will run or fight. These reactions are controlled by the animals' perception of danger,

which starts the cascade of physiological changes discussed in the previous section. However, behavioural stress responses do not tell us anything about the subjective feelings of animals faced with a serious threat, or how much they suffer when kept in environments very different from where they originated. Objective measurement of the welfare state of animals continues to be a central issue in the study and management of animal welfare (Dawkins, 2003), and it is becoming increasingly necessary to take into account not just the health and stress responses of livestock, but also animals' behavioural requirements. An early study by Hughes and Black (1973), which was stimulated by a recommendation of the Brambell Report on floor types for caged hens, offered a new approach to animal welfare. The authors tested a recommendation for using 'heavy' mesh on cage floors, so that hens could stand comfortably, by giving the hens a choice between four different floor types to determine which one they preferred.

1.3.3.1 Preference tests

The early preference tests of Hughes and Black (1973) were followed by other work with caged hens (for example Hughes, 1975; Dawkins, 1976), and gave animals choices of cage floor types, cage sizes and, a cage or outside run. The results were inconclusive and were clearly influenced by, at the very least, previous experience and presence or absence of conspecifics. Dawkins (1976) admitted that the conceptual difficulties involved in interpreting animal preference were vast but, that it was a suitable subject for investigation. Much earlier Hume (1956), and Russell and Burch (1959), had suggested that measuring animal suffering could be done by determining the extent of motivation of animals to learn conditioned reactions to avoid punishment. Dawkins (1976) expanded this idea by suggesting the possibility of using techniques developed by psychologists to

study animal learning, theorising that this methodology could be used to gain an insight into animals' feelings. This approach assumes a relationship between animal responses and feelings; whereby situations which act as positive reinforcers are pleasant to the animal while those that act as negative reinforcers are distressing to it.

Animals can be trained to 'work' for a reward by learning to perform an operant task, such as hens pecking a key for food (Duncan and Hughes, 1972), heifers pressing a panel to be allowed to lie down (Jensen et al., 2004) and pigs pressing a lever for social contact (Ladewig and Matthews, 1996); the reward can be anything that provides either positive reinforcement or avoidance of negative reinforcement. The benefit of introducing operant conditioning into preference tests, was that it allowed the researcher to control more aspects of an animal's environment, and made it easier to determine what the animal preferred, rather than just where it spent the most time, or how long it took to move from one area into another. Then in 1983, Dawkins introduced and promoted the use of consumer demand methodologies for the study of animal welfare. Dawkins investigated the necessity of litter for hens in battery cages, using a 'trade-off' between litter and food. The hens were deprived of food for 0, 3 or 12 hours, and then given a choice between a wire cage with food, or a cage with litter on the floor but no food. Only the non-deprived hens chose the litter floor, whereas the longer hens had been deprived of food the more likely they were to choose the wire cage with food.

1.3.3.2 Behavioural demand

As long ago as the early 1950s, Skinner (1953) linked economic behaviour and application in operant psychology, although in this chapter the context was using psychology to

predict economic affairs. Later, Castro and Weingarten (1970) pointed out parallels to economic concepts in the schedules of reinforcement that are used in operant psychology, and soon after, several other researchers independently concluded that economic concepts could be applied to behaviour analysis, in particular, Rachlin et al. (1976), Lea (1978) and Staddon (1979).

While preference tests allow for an indication of animal preferences, they give no information about the importance of the chosen resource, or indeed of having a choice. Demand methodologies allow for investigation of animals' motivation to access particular resources, or conversely, avoid particular stressors, demonstrating the importance of the reward. The distinction is important as it has been proposed that if animals will work hard to obtain a resource, it is reasonable to conclude that their welfare will improve if they are able to gain access to that resource (Broom, 1988). In the investigation of animals' motivation, the amount of work required to obtain a reward, which consists of access to a resource (for example, food, social contact or extra space), is increased, and the number of rewards obtained at each workload is captured. Motivation is defined as the rate of change in the number of rewards obtained as a function of the cost or workload. This is also called the 'elasticity of demand' (Lea, 1978; Hursh, 1980). The intensity with which the reward is worked for can also be used to indicate the strength of motivation (Hursh, 1984; Pedersen, 2002). There are various ways of using behavioural demand methodologies to investigate the behavioural priorities that animals have for different resources, and some of these are discussed below.

1.3.3.2.1 Trade-off

Trade-off is a term used to describe the choices animals make when they are constrained by one or more limiting factors such as time or energy resources. Trade-off methodologies are used to quantify behavioural priorities of animals to determine the relative importance of different resources. For example, Matthews and Roberts (2001) varied amounts of food deprivation and lying deprivation for dairy cows, followed by test sessions in which animals could either feed or lie (or neither) but not both at one time. It was found that time spent feeding decreased (by 14%) as lying deprivation increased, and time spent lying decreased (by 34%) as food deprivation increased, so that animals were strongly defending feeding and lying when challenged with a competing high priority activity, although feeding appeared to be accorded a higher value than resting.

Munksgaard et al. (2005) investigated the effect of time constraints on eating, lying and social contact in dairy cows, by varying the amount of time available. The authors reported that the proportion of time the animals spent lying increased, while the proportion of time eating and having social contact remained similar and the proportion of time spent on other activities decreased when available time was limited. However, it was also suggested that time constraints on lying would be more severe than those on eating, because animals have the ability to compensate for limited eating time to some extent by increasing bite size, and rate of feed intake. One of the problems with this methodology is that results are typically difficult to interpret, and the approach is not sensitive enough to determine differences in responses to incremental changes in the same challenge.

1.3.3.2.2 Obstruction or weighted door test

Another method used to measure the strength of hens' motivation for dustbathing, is an obstruction test developed by Widowski and Duncan (2000). Hens were given the opportunity to push through a weighted door to gain access to a dustbathing substrate (peat moss) when they had been either deprived of the opportunity to dustbathe, or not deprived. Additional weights were added on successful opening of the door to see how hard the hens would work to gain access. The measures of motivation used were the maximum weight pushed to open the door and latencies and frequencies of attempts to open the door. The results were inconclusive, and it is possible that the physical strength of the animals under the experimental conditions was being measured, rather than how motivated they were.

1.4 INTEGRATION OF BEHAVIOUR AND PHYSIOLOGY

There are inherent difficulties in using any one of the methods of measuring welfare described above, and it is universally accepted that there is no single measure of welfare (for example Dawkins, 1980; Broom, 1988). The complexity of the problem is generally acknowledged, as is the fact that multiple measurements need to be taken, but which combination of measures should be used, and how discrepancies between them should be resolved, has not been agreed (Dawkins, 2003). Although the scientific study of livestock management issues that have a significant animal welfare component has long included the measurement of both behavioural responses and physiological effects, one of the recurring problems in this area of research is a failure of such studies to adequately link these two forms of measurement.

A few studies have measured behavioural and physiological changes in response to a stressor. Munksgaard and Simonsen (1996) studied the effects of lying deprivation and social isolation on the behaviour and cortisol response of dairy cows. The authors reported that both the lying deprivation and social isolation stressors increased blood cortisol concentrations, but the effect was only significant for lying deprivation. Cortisol concentration also increased in lying deprived cows compared with control cows in a novel arena test. Both lying deprivation and social isolation increased the number of transitions between activities and changed the cows' reactions in tests outside their home environment. In a later study with young bulls, lying deprivation caused consistent behavioural changes indicative of both physical strain and frustration, and an initial desensitisation followed by a normalisation of the pituitary sensitivity to corticotrophin releasing factor (CRF) was observed over the course of the treatment (Munksgaard et al., 1999). These two studies show that behavioural stress responses are correlated to some degree with physiological stress responses. However, apart from the stress response, we do not know what physiological adaptations an animal has to make to cope with a stressor, and we do not know the cost to the animal of activating these responses. We also lack information on the animals' perception of the stressor, and its motivation to remove itself from the stressful situation it is in. The answers to all these questions would help us to gain some insight into how the wellbeing of the animal is affected by the stressor.

A study by Mason et al. (2001) addressed some of these additional questions in an investigation of the possible deprivation suffered by mink in captivity, due to the lack of resources that exist for them in the wild. The authors used a weighted door test to measure the cost animals were prepared to pay for a range of different resources including a water

pool, alternative nest site, toys and an empty cage. They then blocked the access to the water pool, the alternative nest site or the empty cage, and measured cortisol concentrations of the mink to determine how stressed they were when deprived of access to those resources, with food deprivation as the baseline comparison. The minks' urinary cortisol increased by 50% from baseline when deprived of food, 34% when access to the water pool was blocked, and did not increase when denied access to the other two resources. These results suggest that the animals like to have access to the water pool, and they get stressed when access is blocked, but it also suggests that they are prepared to pay for access to the pool, and therefore it can be considered to be an important resource. However, there is still more information to be gained by stressor models, if we widen the net of information capture, and investigate changes in biological costs at different levels of a stressor, and how motivation to avoid the stressor changes with them.

It is proposed in this thesis that measurements of animal welfare should include both behavioural and physiological responses to stressful conditions, and also, that the key to new and objective measures of welfare is in the link between these responses. This is important. It is the examination of the relationship between behaviour and physiology that is novel to this work, rather than the methodology. This approach should help us to understand the underlying causes of the behaviour we observe, and the physiological mechanisms affecting it. The hypothesis for this work was that, while animals may exhibit a preference for the most desirable environment, the price they are prepared to pay to change their environment increases significantly at the point when the environmental challenges represent a significant biological cost for adaptation.

1.5 WELFARE ASSESSMENT MODEL, EXAMPLES AND AIMS

The term 'model' as used in this thesis relates to the measurement of adaptive physiological and behavioural responses to a stressor, to investigate the link between these responses as a measure of welfare. The main determinants for an acceptable model were that the stressor could be increased in increments that would allow for measurement of increasing biological cost of adaptation, and that there was a reasonable expectation that the animals' motivation to alleviate the effects of the stressor would change with changes in the intensity of the stressor. The two examples used to describe the model in detail focus on a heat challenge and feed restriction. Sheep were used as the model animal for this work.

1.5.1 Heat challenge

Heat challenge is a common and important stressor, and can occur during transport or in the seasonal conditions that occur in extensive livestock production in many parts of the world. In controlled conditions, such as the climate controlled rooms which were used for this work, it is possible to accurately change the ambient temperature and humidity in even increments, while keeping all other variables constant. Social stress is also very important in livestock production, however, the problems inherent in controlling all the variables associated with social stress would have made the results very difficult to interpret. Additionally, the physiological changes associated with adaptation to a heat challenge have been well documented, as has the necessity of animals' maintaining a thermoregulatory balance. Although there appears to be no record of sheep working to alleviate heat stress, there is some record of them operating a heater in the cold after

shearing, therefore, it seems likely that they will be prepared to work to avoid the challenge of hot conditions.

1.5.2 Feed restriction

Metabolic homeostasis is also very important for survival, and it is possible to accurately control feed intake of animals while keeping all other variables constant. The metabolic changes associated with restricted feed access have also been well documented, and occur in a sequential order over time. It is probable, therefore, that the motivation of animals to work for food at the different stages of adaptation would change with differing periods of restricted feeding.

Because of the lack of data using sheep for operant studies, the first experiment addressed methodological issues with regard to the design of behavioural motivation experiments. Typically, when operant conditioning is required for an experiment, few animals are used due to the difficulty and time involved in training the animals to perform the required response. Therefore, the extent of individual variation in the motivation of animals is not known, and was the focus for the initial experiment.

CHAPTER 2

Individual variation in motivation to feed

2.1 INTRODUCTION

Behavioural demand methodologies using operant conditioning techniques allow us to quantify the behavioural needs of farm animals (Dawkins, 1983; Matthews and Ladewig, 1994). Such methodologies are based on the assumption that if an animal is highly motivated to gain access to a resource, it is reasonable to conclude that its welfare will be improved if it is allowed to interact with that resource (Broom, 1988).

In operant conditioning studies, animals are trained to perform an operant task, for example pressing a lever a fixed number of times (Fixed Ratio (FR) schedule), to obtain a reward, or access a resource of interest. The measurement taken is the number of rewards obtained at different workloads, and the workload is changed by requiring different numbers of lever presses to be accomplished before a reward is provided. For example, if a resource is very important to an animal, it would be expected that the animal would continue to gain rewards, or access to the resource, even as the number of lever presses required to do so increased. However, if the resource is not very important to an animal,

increasing the workload is likely cause a cessation of lever pressing and therefore resource access. The demand function is the term used to describe the line calculated by plotting the declining number of rewards obtained as a function of the changes in workload (Lea, 1978). If the demand function fits a straight line when plotted in log-log co-ordinates, then the slope of the function is equal to the elasticity (Lea, 1978; Ladewig and Matthews, 1996). Therefore, elasticity is the slope of the demand function, and is also used as a determinant of motivation. Animals are said to be highly motivated to gain access to a particular resource if the number of rewards obtained remains fairly constant as the FR value increases (Matthews and Ladewig, 1986). The intercept of the demand function can also be considered as a measure of work intensity (Hursh, 1984).

Statistical tests attempt to use data from samples to determine if differences or similarities exist in a whole population. Usually, random samples are used, which will tend to mirror the population due to mathematical properties, although different sample sizes lead to different accuracies of measurement. All else being equal, a larger sample size leads to increased precision in estimates of various properties of the population. The power of a statistical test is the probability that the test will reject a false null hypothesis, and as the sample size is increased, power is also increased, and the chances of such an error occurring decreases.

It is common in behavioural demand experiments to use small numbers of animals, and run them through each treatment, as opposed to the traditional scientific procedure of randomly allocating animals to treatments. One reason for this is that the operant training can be time consuming, and sometimes difficult depending on the nature of the task and

the species of animal being trained. In most behavioural research, individual variation constitutes a problem (Ladewig and Matthews, 1996), which is exacerbated by the use of too few animals. We need to ensure we are using enough animals in our behavioural research so that the differences we find are reflective of the general population. Larger numbers of animals are necessary to retain the required statistical power for analysis, when there are large individual differences.

As an example of the use of small numbers of animals for operant research, a study looking at shape discrimination in sheep and calves, used only 3 sheep and 3 calves (Baldwin, 1981). The author noted that there was some variation between the sheep in the number of trials needed to reach the criterion of 3 consecutive sessions with less than 50% incorrect responses. Due to the small numbers of animals, a statistical analysis was not even attempted. In a more recent example, a study using an operant response in an attempt to measure the value sows placed on access to an earth floor, used 6 sows with different parities (1, 4, 4, 4, 6 and 9), essentially resulting in sample sizes of 1, 3, 1 and 1 for the parities of 1, 4, 6 and 9, respectively (Hutson and Haskell, 1990). Not surprisingly, the results were inconclusive.

To my knowledge, there has not previously been an investigation of the individual differences between animals in operant research. Although it is recognised that individual variation would differ according to the reward offered for the operant task, information on individual variation would still assist greatly in the design of operant experiments, by indicating whether to continue with the use of small animal numbers, or use larger numbers and randomly allocate animals to treatments in the traditional scientific

experiment design. Therefore, the aims of this experiment were to investigate the individual differences between a large number of sheep in terms of their motivation (as measured by the slope of the demand function) to work for a food reward, and also to make a graphical comparison between the intercept of the demand function and the mean reward value as a measure of work intensity.

2.2 MATERIALS AND METHODS

This study followed the guidelines established by the University of New England Animal Ethics Committee approval number AEC04-031.

2.2.1 Animals and training

Twenty-four, two-year-old Merino ewes were kept outside on pasture throughout the training, and between experimental weeks. The sheep were brought into an animal handling facility daily for training, and were trained in 10-minute sessions to press a lever for a small (8.5 g) reward of lupin seeds. Lupins are legumes cultivated for forage crops or grain (seeds) and are used for both human and animal consumption. The training process used was the method of successive approximations, whereby each successive approximation to the main goal, in this case pressing the lever hard enough to make it buzz, was rewarded manually by the trainer, who could see the training pen via a mirror set up for the purpose (Figure 2.1). The mean (± standard error of the mean (sem)) time taken for animals to reliably press the lever once for a food reward was 86.25 (± 6.3) minutes over a period of 3 weeks.

2.2.2 Housing and feeding

The experiment was carried out in two rooms which were specially set up with an operant chamber in each (Figure 2.2). The operant chamber was a 2100 × 650 mm pen, which included a lever that could be pressed for a food reward, and a feed container in which the reward was presented (Figure 2.3). Each room housed six animals in individual pens, separate from the operant chamber. Each group of 12 spent two weeks in one of the rooms with a break of 1 week in between.

The animals were weighed before being housed, and their weights were used to calculate feed on offer. The equation used to calculate weekly feed amounts was:

(sheep weight * 0.09 + 1.4) = MJ of metabolisable energy allowance for maintenance (Ministry of Agriculture, Fisheries and Food, 1977). Adjustments were made for the MJ/kg dry matter (DM) of the feed, and for the daily feed allowance. The metabolisable energy content in the lucerne pellets was calculated at 11 MJ/kg DM (Feedtest, Hamilton, Victoria), and for the lupins 13 MJ/kg DM was used. Total daily rations were 1.2 times maintenance requirements (MR). When a combination of lupins and lucerne pellets was offered, MJ required were used to calculate the relative amounts. Fifty percent of the feed was provided as lupins 20 minutes prior to testing, with the balance fed as lucerne pellets two hours after testing. During testing, animals could work for rewards, each of 8.5 g lupins, and the total reward consumption was subtracted from the remaining feed balance.

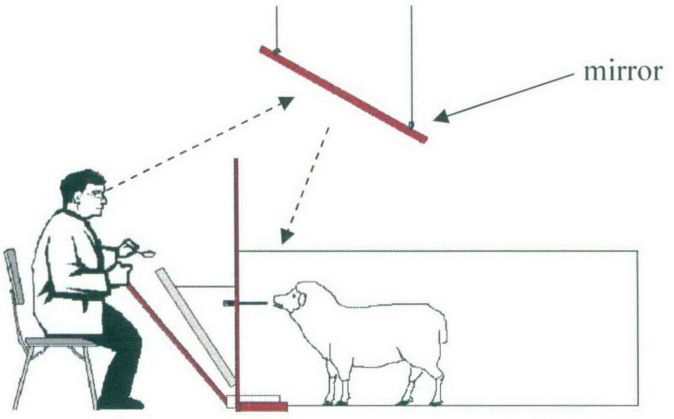


Figure 2.1 Diagram of the training pen. The trainer watches the sheep in the mirror so that rewards can be given precisely when the required behaviour is performed.

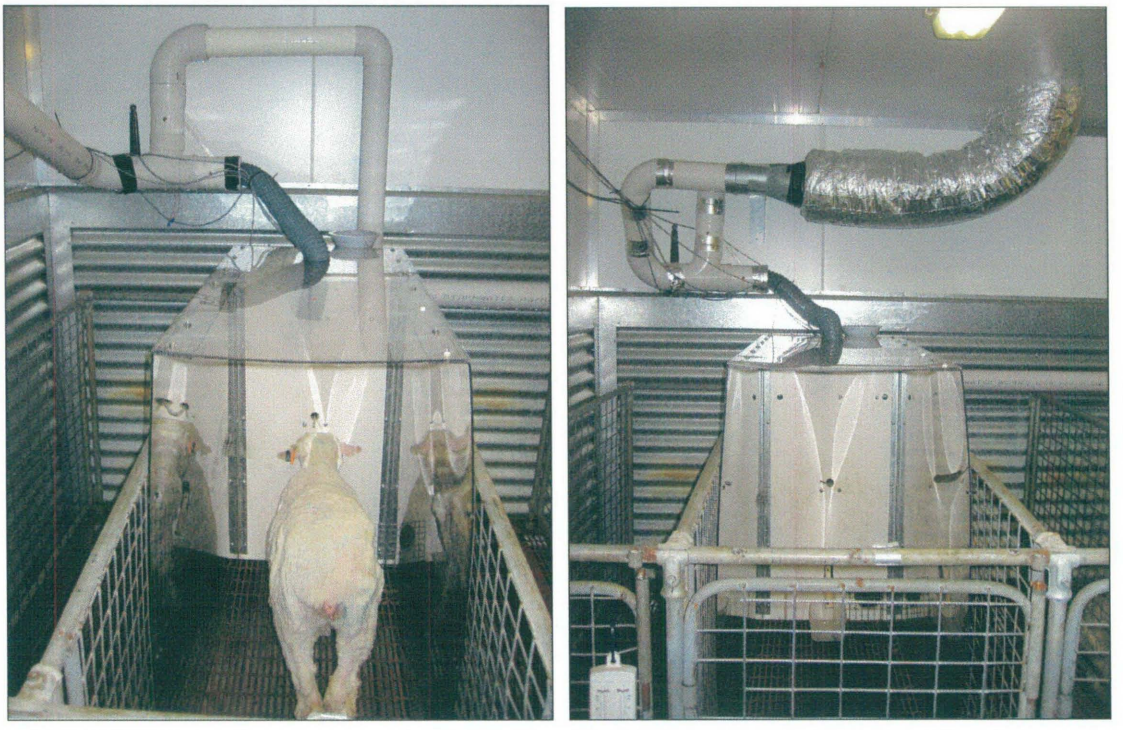


Figure 2.2 The automated operant chamber, with and without sheep, showing the feed container and lever which must be pressed for a food reward.

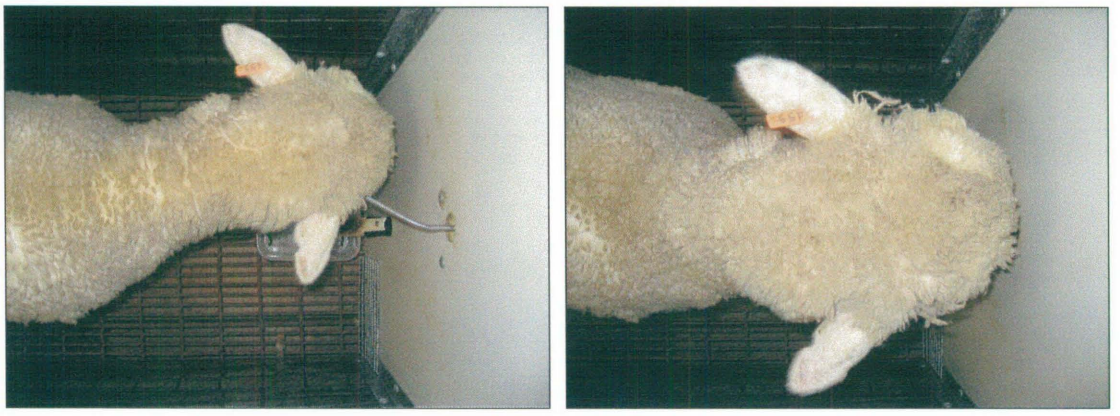


Figure 2.3 A sheep in the operant chamber pushing the lever to gain access to a food reward of 8.5 g of lupins.

2.2.3 Experimental procedure

The animals were split randomly into two groups of 12. One group was taken to the test rooms, and the other group was left on pasture. The following week the two groups were swapped. This was done four times so that each group had been in the test rooms for two weeks with a week on pasture in between. Each group of 12 was brought inside and further split into two groups of six and placed in two separate rooms. This was done on a Sunday morning and a full maintenance ration was fed as lucerne pellets, so that the animals could 'settle in' before testing started on the Monday. The animals were not naïve to the environment.

Testing was carried out over five days, with an ascending sequence of FR values (5, 10, 20, 30, 50) changing daily. The animals were tested in a random order each day. The first to be tested was fed and given 20 minutes to eat. It was then removed from the home pen and placed in the operant chamber. Test sessions lasted for 20 minutes during which time the lever could be pressed for the food reward (see Figure 2.4 for a picture of the feed

delivery system). When a reward was earned, there was a programmed delay whereby the lever ceased to work for 10 seconds to ensure that the animal stopped pressing it and consumed the reward. At the end of the 20 minutes the session ended and the animal was removed from the operant chamber and put back into its home pen after obtaining and consuming the reward it was working towards. The next animal was then placed in the operant chamber, until all animals had been tested.

Purpose-written software and in-house computer systems controlled a buzzing noise associated with a successful lever press, the delivery of the reward after a given number of lever presses, and also collected the data for each session (number of lever presses and number of rewards).

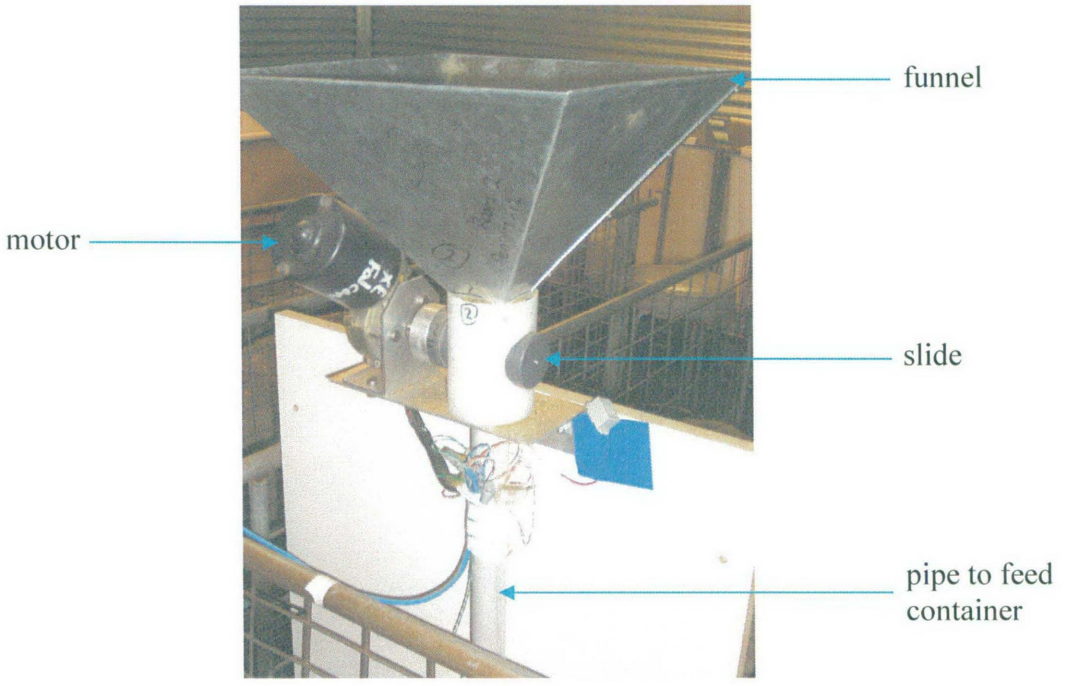


Figure 2.4 The automated feed delivery system; feed was placed into the aluminum funnel filling a hole in the grey plastic slide which was offset with the pipe carrying the food into the container, and when a reward was earned the motor drove the slide over the pipe letting a consistent amount of feed fall into the feed container in the pen.

2.2.4 Statistical analyses

Data for the number of rewards obtained were log transformed, to fulfill the assumption of normality, before performing an Analysis of Variance using Genstat (Lawes Agricultural Trust, 2005). The ANOVA model included terms for Animal within Group + FR + Rep and included all interactions. Further analysis to investigate individual animal differences included fitting the FR value as a covariate to get an average regression value, and assessing the significance of the interaction between the covariate and the individual animal regression values; a likelihood ratio test compared models with and without the Covariate × Animal interaction, and tested the difference between 2 * log likelihood using a Chi square distribution. The least square means from that analysis were used to describe the data further, including giving a measure of intensity. A Quantile-Quantile (Q-Q) plot with 95% confidence bands and a 1:1 reference line was used to assess how well the data approximated a normal distribution, and a range of descriptive statistics are presented.

The data were also calculated as the means of the logarithms of the number of rewards obtained for individual animals for each FR value. Best-fit lines of the form $y = mx + b$ were fitted to the log-log functions for rewards as a function of FR value by the method of least squares. These were then used to compare methods to measure work intensity of individuals, and a range of descriptive statistics are presented for the slope, or elasticity of each demand function.

2.3 RESULTS

There were no significant differences between individuals in their motivation to work for food, as shown by the lack of Animal × FR interaction ($P > 0.05$). When FR was fitted as a covariate rather than a factor, there was still no significant interaction between the covariate and animal ($\chi^2 = 1.32$; $P = 0.25$), confirming that the slopes of reward as a function of FR value did not differ between animals. The mean (sem) of the slopes of the demand functions (elasticity) for all animals was -0.5 (± 0.02), with a range of -0.6 to -0.2 and a standard deviation of 0.10. Elasticity closely approximated a normal distribution according to the Q-Q plot (Figure 2.5). The outlier represents an animal that did not eat the full pre-test feed on the day of the FR 5 test, nor did she eat all of the rewards in the test session that day, but subsequently she behaved similarly to all the other animals and so her data were retained. All other animals throughout the experiment ate all the pre-test feed offered before all test sessions.

The intensity of reward consumption differed significantly between individuals ($P < 0.001$; Figure 2.6). The mean (± sem) intensity of all individuals across all FR values was 24.6 (± 0.90) rewards per test session. They ranged from 15.4 to 32.2 rewards, with a standard deviation of 4.50. The intensity values closely approximated a normal distribution according to the Q-Q plot (Figure 2.7).

Reward consumption declined significantly ($P < 0.001$) at each increase in FR value. For FR values of 5, 10, 20, 30 and 50 mean (back-transformed) number of rewards consumed was 45, 36, 24, 18, and 11 respectively.

A graphical comparison between the intercept of the demand function and the mean reward value (log transformed for comparison), as a measure of individual work intensity, is displayed in Figure 2.8. The data was sorted by the traditional method of using the y-intercept, so the differences between this and the mean reward number can be seen clearly. Although the range is similar, the two methods give quite different results in terms of which individuals worked more or less intensely.

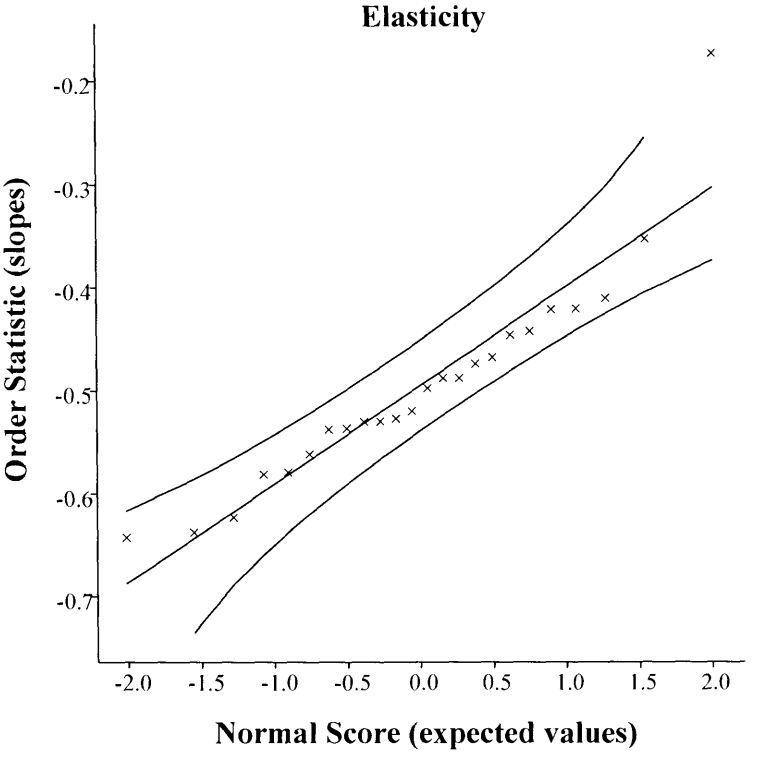


Figure 2.5 Probability distribution plot for the slopes of the demand functions for rewards obtained at all FR values for all animals in log-log coordinates.

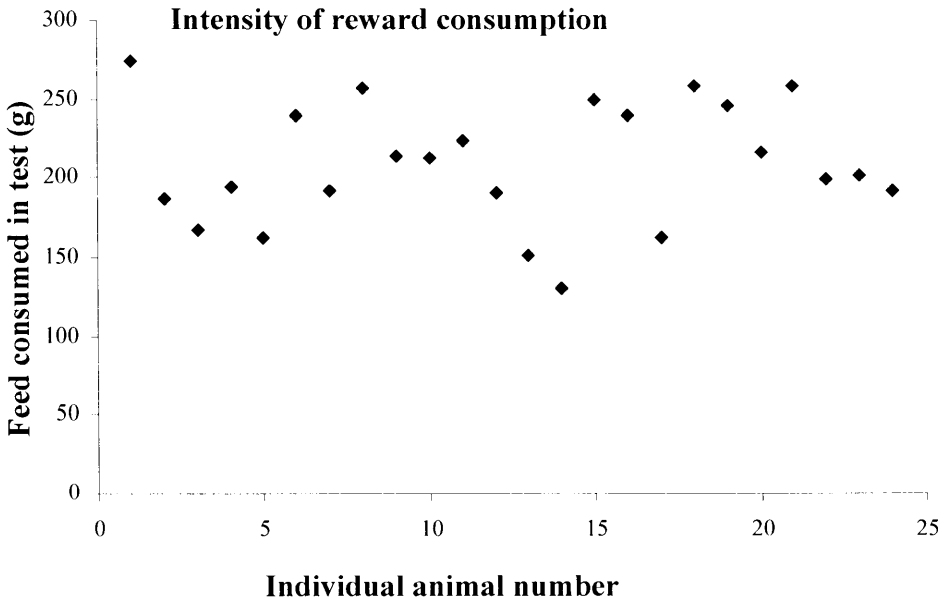


Figure 2.6 Back-transformed means of feed consumed in the test sessions of each animal across all treatments.

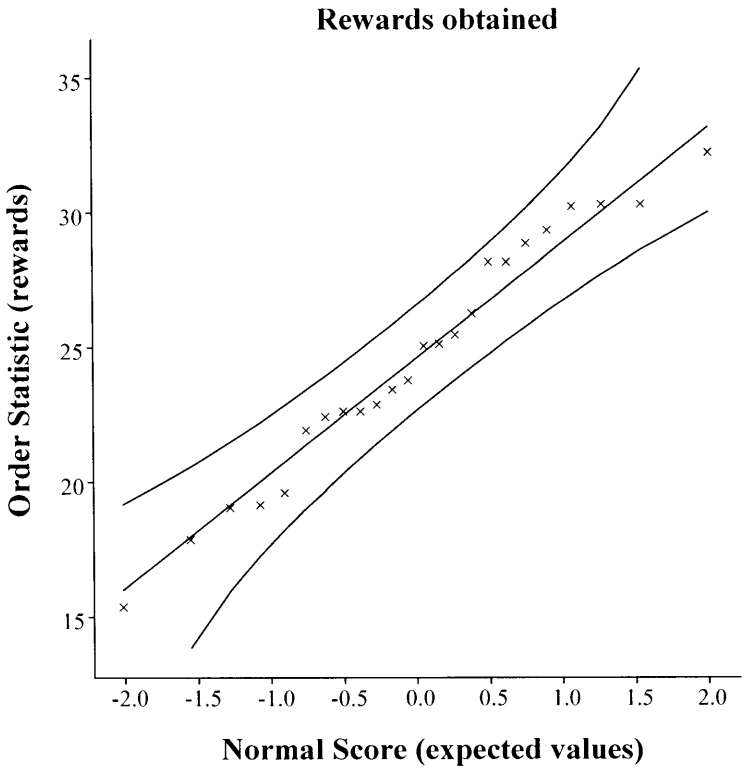


Figure 2.7 Probability distribution plot for the (back-transformed) least square means of the number of rewards obtained in the test sessions of all animals, across all FR values.

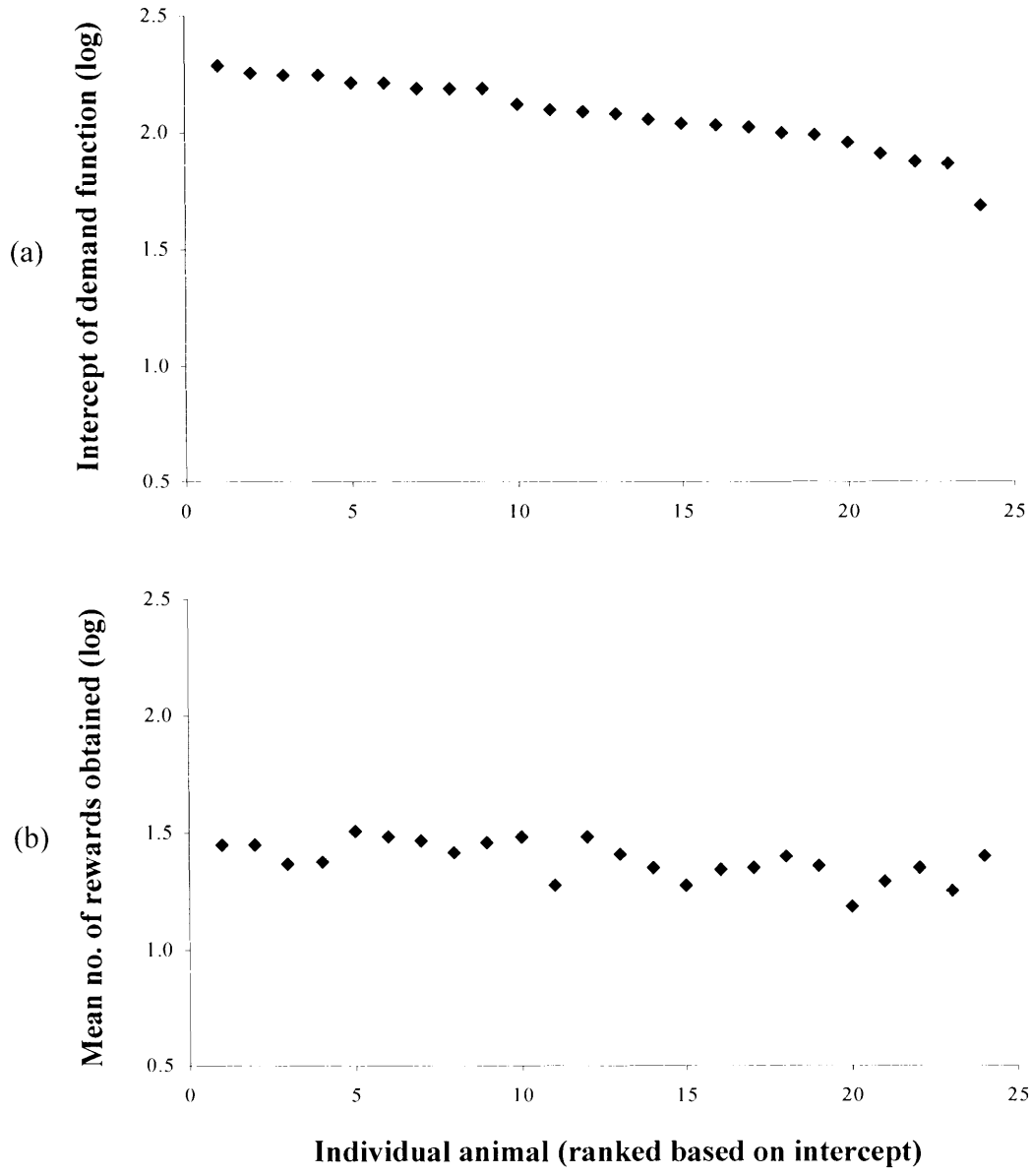


Figure 2.8 Comparison between the intercept (a) and mean reward value (b) as a measure of work intensity. Animals (n = 24) are in the same order in both graphs.

2.4 DISCUSSION

The aim of this study was to investigate the differences between individual Merino sheep in motivation to work for a food reward. The results show that although there were

individual differences in the intensity of work done, there were no individual differences in the motivation of the animals to work for food.

The demand functions in this experiment were obtained in an open economic situation, with food available outside the test sessions. It has been found that when a reinforcer is available immediately before a test, an animal will not work as hard for the resource in the test, and the slope of the demand function will be steeper (Ladewig et al., 2002). It is acknowledged, therefore, that the slopes of the demand functions in the present study may be steeper than if a closed economic situation were used for the tests.

The means for work intensity covered a large range and had a large standard deviation. It is somewhat surprising, therefore, that the elasticity of the demand functions were so similar for all animals. However, Ladewig and Matthews (1996) commented on a similar experience with pigs working for access to straw in a rack, where there were large differences between absolute demand, but only small differences between the slopes of the demand functions. This was not always the case though, as they found very different slopes between animals working for access to straw bedding, probably because animals have the opportunity to perform several different types of behaviour in the straw bedding, such as resting, rooting, eating and chewing. It may be that the motivation of animals' to work for other requirements will not differ either, if they are kept in similar conditions, and if the experiment is designed in such a way that the rewards or stimuli offered can be used in one way only.

It is difficult to compare this study to others, because it appears to be the only work done purely to investigate differences in motivation between animals. Many studies have looked at the demand for food in various animals, such as pigs (Lawrence and Illius, 1989), mink (Hansen et al., 2002), dairy cows (Munksgaard et al., 2005), broiler chickens (Bokkers et al., 2004) and sheep (Jackson et al., 1999). However, this is the first study to use a large number of animals to investigate individual differences in motivation without a food deprivation or other treatment. For example, Jackson et al., (1999) compared two methods for measuring feeding motivation in sheep, and deprived the animals of food for various time periods before testing. Furthermore, the authors report that in one experiment only 3 out of 12 sheep became fully trained to push a panel for a food reward, and in the other experiment only 10 out of 14 sheep became fully trained to push open a door for a food reward. The present study used 24 fully trained animals.

The method of using least square means from the covariate analysis as a measure of work intensity gave more accurate results than using the intercept from the demand function. The intercept is commonly used as a measure of work intensity, but is problematic because it is affected by the elasticity of the demand function. An animal that has consistently worked harder across all FR values may have a lower intercept than another animal which has worked less hard but has a steeper slope. However, the harder working animal will have a higher mean rewards value; therefore it is considered here to be a more useful measure.

In conclusion, the motivation of sheep to work for food at a fixed level of feeding, above maintenance requirements, did not differ between individuals. It follows then that the low

numbers of animals commonly used for behavioural demand experiments should often be sufficient to retain appropriate statistical power. However, it would be recommended that a mean of rewards obtained across all FR values should be used to measure the work intensity of individuals, as this gives a more accurate measure than does the intercept of the demand function, unless slopes are exactly the same for all animals.

CHAPTER 3

Feed restriction

3.1 LITERATURE REVIEW AND INTRODUCTION

In many extensive pastoral areas of world, including Australia, grazing ruminants are subject to recurrent periods of poor nutrition associated with droughts, and seasonal changes in feed quality and quantity. In response to adverse conditions such as restricted feed availability, animals can activate adaptive mechanisms which allow them to maintain homeostasis. Cannon (1929) introduced the term "homeostasis" to describe the product of the "coordinated physiological processes which maintain most of the steady states in the organism". According to Cannon, rapid activation of homeostatic systems preserves the internal environment by producing compensatory and anticipatory adjustments that enhance the likelihood of survival. Adaptive mechanisms activated to maintain energy homeostasis can include changes in both motivational states, such as hunger, and, physiological or metabolic states.

Feed restriction is a stressor which evokes a motivational drive to consume food in order to eliminate the feeling of hunger and restore energy balance. This in turn replenishes the nutrients necessary for survival. Once the food is located and consumed, homeostasis is restored and motivation to feed is reduced, thus maintaining physiological stability

(LaGraize et al., 2004). Therefore, a feed restriction model may be a useful tool to facilitate the investigation of a link between the biological cost of a stressor, and the motivation of animals to alleviate this cost and maintain homeostasis.

3.1.1 Control of energy homeostasis

It is not the purpose of this section to provide a comprehensive review of the complex control of energy homeostasis, but rather to briefly explain the mechanisms as far as they are relevant to this thesis (for recent reviews of energy balance and appetite control see Grill and Kaplan, 2002; Wynne et al., 2005; Kelley et al., 2005).

Energy homeostasis is regulated by neuronal circuits which signal using specific neuropeptides. The arcuate nucleus in the brain receives humoral signals regarding the status of peripheral energy stores, and conveys the information to the lateral hypothalamic area via distinct peptide-coded projections (Elmquist et al., 1999). Levels of circulating factors such as insulin, leptin, ghrelin, and various nutrients have direct and selective access to neurons within the arcuate nucleus, due to low blood-brain barrier integrity at the level of the median eminence and basal hypothalamus (Kelley et al., 2005).

Grill and Kaplan (2002) found that an intact brainstem (using a decerebrate rat model) is sufficient to regulate meal size in response to taste and gastrointestinal feedback, but not to adjust food intake in response to energy balance-related challenges such as food restriction. These feeding-related sequences in brainstem circuitry must act in concert with hypothalamic and forebrain input to reproduce the feeding motivational state in its entirety.

Another example of separate feedback mechanisms for feeding motivation is suggested in a recent proposal of a model for the control of energy homeostasis. It suggests that release of the peptide enkephalin from neurons in the striatum of the brain, play a specific role in modulating the rewarding aspects of food intake, which is governed by information about energy balance needs and behavioural state from other parts of the brain (Kelley et al., 2005). When food is encountered, the authors theorised, enkephalin is released within local and efferent motor networks. Although feeding can occur without this event, enkephalin serves to promote the consumption of food beyond energy needs, particularly if the food is energy-dense, via an enhancement of the hedonic impact of food consumption. This ensures that intake will not be limited by immediate energy requirements, but will serve to build up a fat reserve in the event of future low resource availability.

Some support for the above proposal was reported in a study by Will et al. (2004), using rats, either feed restricted or fed *ad libitum*, and either recently fed or not. They investigated the gene expression of proenkephalin and neuropeptide Y (NPY), both of which are peptides involved in feeding motivation. Their results show that enkephalin is specific to short-term feeding motivation, as its expression was low for animals fed recently, and high for those not fed recently, regardless of which animals had been feed restricted. In contrast, the expression of NPY was upregulated by chronic feed restriction, and thus energy deficiency, but was not affected by recent consumption.

Central opioid peptides are also involved in a range of reward-related functions (Wise, 1989). Opiate drugs serve as powerful reinforcers, and have high addictive potential. It is

thought that opioids specifically regulate palatability and the positive hedonic valuation of food (Cooper and Kirkham, 1993; Drewnowski et al., 1995; Glass et al., 1999). Opioids may play a particularly important role during ingestion of feeds high in fat or sugar, which are highly energy dense. Animals treated with opiates will work harder and longer in an operant task for sugar pellets even when not food-restricted (Glass et al., 1999).

In their review of evidence for a neuroanatomical axis for the control of energy balance, Grill and Kaplan (2002) concluded that the results they reviewed are consistent with a distributionist (as opposed to hierarchical) model for the control of energy balance that emphasizes: i) control mechanisms endemic to the hypothalamus and brainstem that use information from around the body to regulate energy balance, and ii) interactions that coordinate adaptive neuroendocrine, autonomic, and behavioural responses to changes in metabolic status.

3.1.2 Adaptive mechanisms of energy homeostasis

3.1.2.1 Behavioural motivation

All organisms require energy intake, among other things, to maintain life. Food, water and other nutrients must be ingested in order to maintain a basic metabolic homeostasis, therefore, the motivation of all animals to eat is very strong. Operant conditioning techniques have been used to measure hunger, by assessing the strength of an animals' motivation to eat. In most cases, the interest in feeding motivation has been to determine whether livestock animals, which were bred for an increased appetite, suffer when feed is restricted, (for example pigs, Lawrence et al., 1988; Day et al., 1996; Ramonet et al.,

2000; and broiler hens, Savory and Lariviere, 2000). However, feed experiments using operant conditioning techniques with sheep are rare.

In a study comparing methods to investigate feeding motivation in sheep, it was found that food deprived sheep were more motivated to work for food than sheep which were not food deprived (Jackson et al., 1999). However, there was no difference in motivation to work for food when animals had been deprived of food for different lengths of time (6, 12, 18 and 24 hours). It may be that 24 hours was not long enough for any difference in motivation to be evident. Additionally, the authors commented that the push-door method that was used may not have been sensitive enough to differentiate between the different durations of feed restriction. Therefore, in the present study, much longer time periods of feed restriction were used (1, 3, 5 and 7 days) to optimise the possibility of determining the effects of different lengths of feed restriction on feeding motivation.

3.1.2.2 Metabolic adaptation

When insufficient nutrients are absorbed due to feed restriction, lipolysis is induced or increased. Lipolysis increases concentrations of non-esterified fatty acids (NEFA; Annison, 1960), and their carrier albumin (Klinhom et al., 2006). The NEFA are broken down and carried to the liver, where they are further broken down to form acetate. Due to an eventual lack of propionate, which is the major precursor for gluconeogenesis and is also required to break acetate down to yield energy, acetate molecules build-up and combine to make acetone, acetoacetate and beta-hydroxybutyrate (BOHB). Therefore, NEFA, albumin and BOHB should be useful indicators of metabolic adaptation to restricted feeding.

Crossbred sheep restricted to 82% of metabolisable energy (ME) required for maintenance, for 1 month, were compared with *ad libitum* fed sheep (Sano et al., 1999). The feed restricted animals had higher plasma concentrations of NEFA than the non-restricted animals, and the concentrations decreased on initiation of feeding. Plasma NEFA levels were also found to be at the minimum after feeding, and the maximum before feeding, in Merino wethers (Annison, 1960; Trenkle and Kuhlemeier, 1966). The increase in NEFA concentration coincided with a decrease in rumen acid concentration (Trenkle and Kuhlemeier, 1966). Plasma concentrations of NEFA were also found to have a significant diurnal variation in dairy cows (Nielsen et al., 2003). As NEFA concentrations increased during fasting in sheep, the blood concentration of glucose decreased slowly (Trenkle and Kuhlemeier, 1966), and the glucose turnover rate was reduced (Sano et al., 1999).

Blood glucose concentration in ruminants has been reported to increase, decrease or remain unchanged in response to feed restriction. An early study on glucose utilisation in sheep, showed that plasma glucose concentration declined over a 48-hour period after feeding in sheep, as measured at 4-5 hours, 24 hours and 48 hours (Annison and White, 1961). Conversely, blood glucose was found to increase over a 2 to 10-hour post-feeding period, and return almost to pre-feeding values by 24 hours in adult sheep (Manns and Boda, 1967). In steers fasted for 8 days, glucose concentration decreased from day 0 to day 2 before stabilising (Rule et al., 1985), and the greater the volume of high or low-roughage feed consumed, the higher the plasma glucose concentration in yearling rams (Evans and Buchanan-Smith, 1975). A more recent study, conducted by Sano et al. (2007) on the effects of dietary intake and cold exposure on glucose metabolism in sheep,

compared the effect on blood glucose concentration of diets either medium or high in metabolisable energy content. The authors reported that there was no difference in concentrations and pool size of plasma glucose between the two dietary treatments. Glucose is the most important carbohydrate in biology, and is used as an energy source in most organisms, including ruminants. It is possible, therefore, that changes in glucose concentration are strongly defended, perhaps by the utilisation of a precursor other than propionate, when feed is restricted and the availability of propionate decreases.

Beta-hydroxybutyrate represents approximately 70% of total blood ketone bodies (Leng, 1965). It arises from butyrate produced in the rumen, and from free fatty acids from adipose tissue (Leng and West, 1969). Steers which were fasted for 8 days showed an increase in BOHB from day 0 to day 2, with little change thereafter (Rule et al., 1985). This could be because blood BOHB does not necessarily reflect nutritional status in the long term. Animals kept at 3 different levels of nourishment (unlimited intake, liveweights reduced by 7 kg and liveweights reduced by 10 kg) over a 9 month period did not have different concentrations of BOHB in their blood (Farrell et al., 1972).

Cortisol is known to be released by HPA axis activation in response to a stressor, as discussed in Chapter 1. However, the information available regarding whether or not a cortisol response occurs with feed restriction is not conclusive. For example, cortisol concentration of ewes did not change in response to a 5-day feed deprivation (Kiyma et al., 2004), whereas a study investigating metabolic and endocrine responses to a feed restriction of 1.35 × MR over a 5-week period, found that feed restricted wether lambs had increased concentrations of plasma cortisol compared to control lambs (Ekpe and

Christopherson, 2000). It seems that the difference in these two results could be attributed to the differences between acute and chronic stress, although the acute stressor would be more likely to increase cortisol concentration than the chronic stressor. For example, on fasting 40-week-old sheep for 78 hours, Nagatani et al. (2000) found that cortisol concentration increased after only 64 hours of fasting.

Cortisol may also influence feeding motivation by impairing the action of leptin, a hormone with a key role in the regulation of energy intake and energy expenditure (Howe et al., 2002). Solano and Jacobson (1999) presented evidence to suggest that, in mice at least, glucocorticoids may be a more important regulator of food intake than leptin. Leptin inhibits food intake by signalling satiety, whereas glucocorticoids, such as cortisol, reduce sensitivity to leptin and can affect both appetite and fat mobilisation. This means that if cortisol concentration is increased by exposure to a stressor, leptin sensitivity may be reduced, causing an increase in the motivation of animals to feed.

3.1.3 Conclusions and aims

Short-term adaptation to feed restriction (hours or days) is clearly linked to behavioural motivation, because animals are motivated to eat as part of a normal daily cycle. Long-term adaptation (weeks or months) is less likely to be linked to behavioural motivation, because if feed is restricted over a long period there is no adaptive benefit in being highly motivated to eat, and other adaptive processes will be necessary. Therefore, what was required for this study was measurement of medium-term metabolic adaptive processes, whereby homeostasis is maintained in between the daily feeding cycle and long-term adaptation. However, the complexity of the control and regulation of metabolic

homeostasis is such that an orderly procession of changes, for short to long-term adaptation, is unlikely to occur. For this reason, the decision was made to measure metabolic processes that are linked with fat mobilisation, such as NEFA, albumin and BOHB rather than those linked with appetite, such as leptin and enkephalin. Glucose was measured because it is so important for energy production, and cortisol was measured to see if the HPA axis was activated during the feed restriction of sheep in this study.

The aim of the first experiment in this chapter was to investigate the motivation of sheep to work for food after different periods of food restriction. The hypothesis was that animals would be more highly motivated to work for food the longer they had been exposed to a food restriction regime. The aim of the second experiment was to define the metabolic and stress related effects of the same feed restriction periods. The overall aim of the chapter was to develop a model whereby animal motivation to alleviate the known costs of hunger after those periods of feed restriction could be linked to physiological changes.

3.2 BEHAVIOURAL MOTIVATION TO ALLEVIATE NUTRITIONAL STRESS

3.2.1 MATERIALS AND METHODS

This study followed the guidelines established by the University of New England Animal Ethics Committee approval number AEC05-065.

3.2.1.1 Animals and housing

Twenty-four two-year-old Merino ewes that had previously been trained to press a lever for a food reward were used for this experiment (see Chapter 2 for details of training). The animals were kept in their treatment groups in outside pens in between treatments. During treatments they were housed in individual pens of 750 × 1500 mm. The same room contained two operant chambers for the test procedure (Figure 3.1). Each operant chamber was a 900 × 2500 mm pen, with the addition of a lever enabling animals to work for food. Water was freely available in all situations.

3.2.1.2 Feeding

The animals were weighed before each treatment condition, and their body weights were used to calculate feed amounts (see Chapter 2 for more information on feed calculations). They were all fed 1.2 × MR before a treatment condition, and treatment groups 1-3 were fed the same during recovery. Treatment group 4 was fed at an increased level of 2.4 × MR during recovery, to assist with regaining weight lost during the treatment condition.

Figure 3.1 The two operant chambers with a lever to press for a food reward, the hopper which was filled with lucerne pellets at the beginning of each session, and feed and water containers.

3.2.1.3 Experimental procedure

Animals were assigned to one of four treatment groups (N = 6 per group), balanced by weight. The groups corresponded with treatment conditions of 1, 3, 5 or 7 days of food restriction at the level of 0.3 × MR. As only two animals could be tested at one time, the treatment conditions were staggered so that the appropriate deprivation and recovery periods were utilised. The 7-day food restricted animals were given a minimum of 7 days to recover before the next treatment condition. Testing was carried out on the completion of each treatment condition (see Figure 3.2). Each test consisted of an animal being put into the operant chamber for a 24-hour period, for testing at one of the fixed ratio (FR) values. These were 5, 10, 20, 30 and 50, and changed each time an animal was tested, so

that every animal was tested at each FR value in an ascending order. Each food reward consisted of a small amount (approximately 11 g) of lucerne pellets.

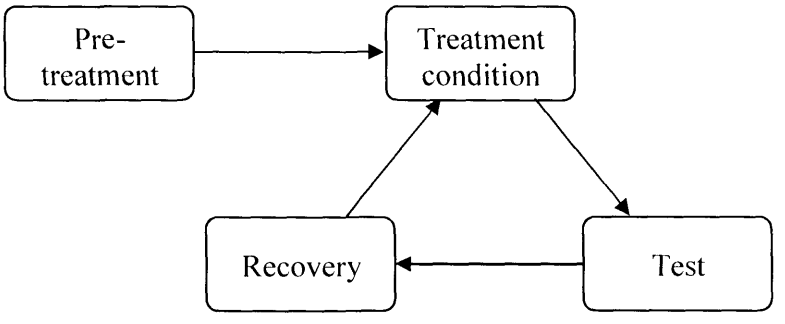


Figure 3.2 Each animal went through the treatment condition and test phases of the experiment 5 times, once for each FR value.

An additional FR value of 80 was added a month later. The same animals had undergone the same treatment conditions for a subsequent experiment, with the only difference being that they were catheterised and blood samples were taken throughout the treatment condition period.

Purpose-written software and in-house computer systems controlled the buzzing noise associated with a successful lever press, the delivery of the reward after a given number of lever presses, and also collected the data for each session (number of lever presses and number of rewards).

3.2.1.4 Statistical analyses

A linear mixed model analysis was performed on the number of rewards obtained using Genstat. The model included Treatment + FR + Treatment × FR as fixed terms, with

Animal included as a random term. Because differences in motivation between treatments was of particular interest, a further model was run comparing the demand function, or slope (log Rewards × log FR) for individual animals, between treatments. The model included Treatment as a fixed term and Animal as a random term. The Wald statistic for each of the fixed terms and their interactions for both models was tested for significance against a chi-square distribution.

The data were also calculated as the means of the logarithms of the number of rewards obtained for individual animals for each FR value. Best-fit lines of the form $y = mx + b$ were fitted to the log-log functions for rewards as a function of FR value by the method of least squares. Some descriptive statistics are presented for the slopes of the demand functions. In addition, the 24-hour test sessions were split into blocks of 3-hour time periods, so that the pattern of response could be seen for the different treatments.

3.2.2 Results

There were no significant differences between treatments in the animals' motivation to work for food as measured by the slopes of the lines in Figure 3.3 ($P > 0.05$), and also the direct slope by treatment comparison ($P > 0.05$). Figure 3.3 also presents some Treatment × FR interactions. The mean (± sem) of the slopes of the demand functions for all animals in each treatment was 0.08 (± 0.064), -0.08 (± 0.052), -0.14 (± 0.093) and 0.04 (± 0.108) for the 1, 3, 5 and 7-day treatments respectively. Figure 3.4 presents the mean number of lever presses performed at all FR values and all feed restriction levels. In a 24-hour test session, the highest number of rewards gained was 268, by a 5-day treatment animal

working on FR5, and the highest number of lever presses performed was 10,640 by a 1-day treatment animal working on FR80. The slopes show that the demand for food was inelastic at all the levels of restricted feeding in this study. Figure 3.5 presents the number of rewards obtained, and feed consumed, in time blocks for each feed restriction period. It shows that the pattern of responding was very similar between the treatments, with the majority of the responses occurring in the first 6 hours, regardless of treatment. The mean amount of food consumed in each test period after the 1, 3, 5 and 7-day feed restriction periods were 1272, 1133, 948 and 995 g, respectively.

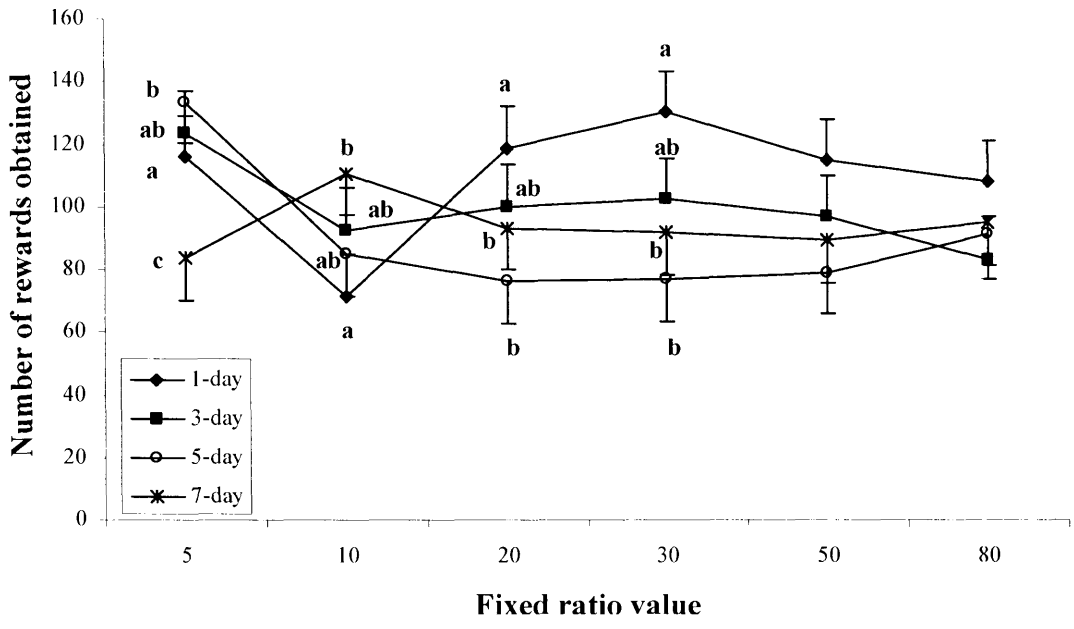


Figure 3.3 Mean (+ or – sem) number of rewards obtained by all animals after 1, 3, 5 or 7 days of feed restriction. Within fixed ratio value, means without a common superscript are significantly different ($P < 0.05$).

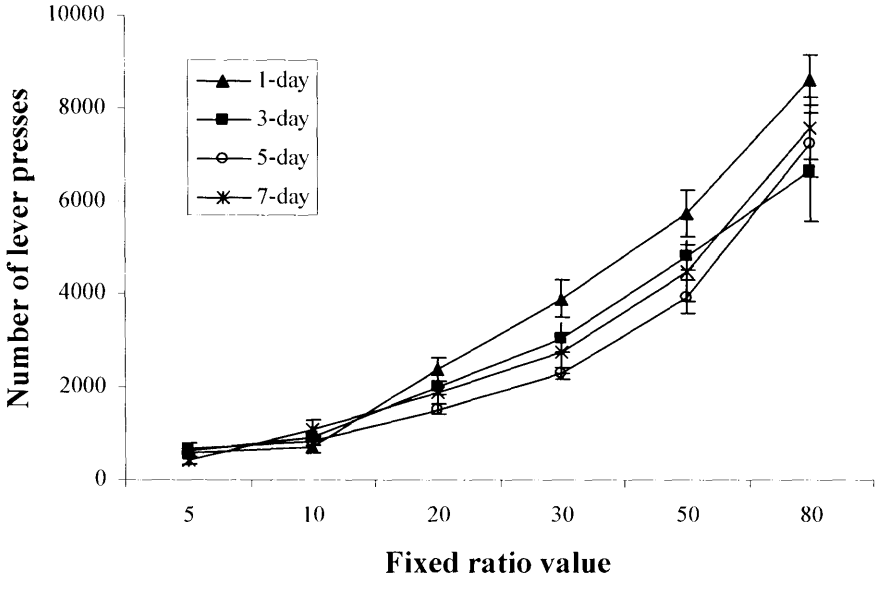


Figure 3.4 Mean (± sem) number of lever presses performed by all animals after 1, 3, 5 or 7 days of feed restriction.

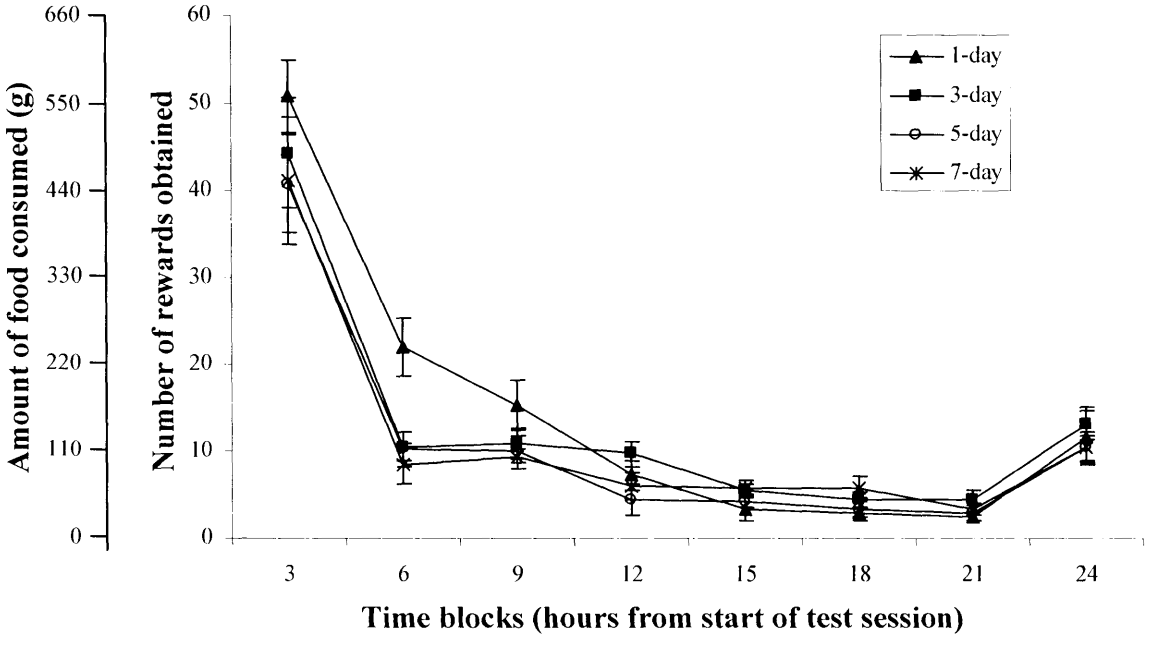


Figure 3.5 Mean (± sem) number of rewards obtained (and grams of food eaten) by all animals after 1, 3, 5 or 7 days of feed restriction, split into 3-hour time blocks to examine the pattern of responses (and food consumption) over time.

3.3 METABOLIC ADAPTATION TO FEED RESTRICTION

3.3.1 MATERIALS AND METHODS

This study followed the guidelines established by the University of New England Animal Ethics Committee approval number AEC05-065. The experiment followed the previous experiment in the chapter, and used the same animals in the same treatments.

3.3.1.1 Animals and housing

Twenty-four two-year-old Merino ewes were used for the experiment. They were housed in individual pens of 750 × 1500 mm throughout the treatment period of the experiment, and at other times were kept in groups outdoors. They were fed lucerne pellets, and water was freely available at all times.

3.3.1.2 Feeding

The animals were weighed before each treatment was imposed, and their live-weights were used to calculate feed requirements. They were all fed 1.2 × MR before the treatment commenced.

3.3.1.3 Experimental procedure

Animals were assigned to one of four treatment groups (N = 6 per group) in the previous experiment, and these were maintained for this experiment. The groups corresponded with treatment conditions of 1, 3, 5 or 7 days of feed restriction at the level of 0.3 × MR.

Each animal had a vinyl catheter (internal diameter 1.0 mm; Biocorp Australia Pty Ltd, Huntingdale, Victoria) inserted into the jugular vein under local anaesthetic (Lignocaine 20; 20 mg/ml lignocaine hydrochloride, Troy Laboratories Pty, Smithfield, New South Wales) using a 13 gauge hypodermic needle. The catheters were covered by an elastic bandage to protect them, and were checked and flushed twice daily with heparinised sterile physiological saline. Blood was collected into two different S-Monovette tubes (Sarstedt Australia Pty, Technology Park, South Australia) in the morning and evening for analysis of glucose (2.6 ml tube with fluoride salt as a glycolysis inhibitor), albumin, NEFA, BOHB, total protein, urea nitrogen and cortisol (9 ml EDTA coated tube). In addition, a morning sample was collected into a 6 ml EDTA coated tube for analysis using a Cell Dyn 3500 haematology unit (Abbott Diagnostics, USA) to give counts of white and red blood cells, neutrophils and lymphocytes, and levels of haemoglobin and haematocrit.

Blood samples were taken every 12 hours (am and pm) over the treatment period, and also 1 day prior to treatment as a baseline. Morning blood samples were obtained before the animals received their daily feed. Animal body weights were recorded before and after the feed restriction treatments.

3.3.1.4 Laboratory assays

Plasma cortisol concentrations were determined using a commercial radioimmunoassay (Spectria Cortisol RIA, Orion Diagnostica, Espoo, Finland), adapted and validated for ovine plasma. Human serum standards were used as provided or diluted in phosphate-buffered saline (PBS), and 20 µl of standard, control or unknown sample pipetted into anti-cortisol antibody-coated tubes. Five hundred microlitres of the provided ^{125}I-labelled

cortisol tracer (diluted 1 in 2 in PBS) was then added before incubation for 2 hours at 37°C. Tubes were decanted, washed once with 1 ml of distilled water and counted for 1 minute in a gamma counter. Parallelism between the standard and unknown samples was demonstrated by serial dilutions of two ovine plasma samples: the calculated slopes of the binding vs. log cortisol concentration or dilution curves for the cortisol standards and the two samples were -0.172, -0.165 and -0.131, respectively. The mean recovery of added cortisol to ovine plasma was 102% and the sensitivity of the assay was 2.5 nmol/l. The stated cross-reactivities of the anti-cortisol antibody with corticosterone, cortisone, dexamethasone, prednisolone and prednisone were 0.2, <0.1, <0.1, 45.3 and 0.3%, respectively.

The intra-assay coefficient of variation (CV) for samples containing 136.7, 63.3 and 27.7 nmol/l cortisol were 7.7, 8.3 and 8.3%, respectively. The inter-assay CV for the same samples were 9.3, 3.1 and 4.4%, respectively.

Albumin, total protein, BOHB, Glucose, NEFA and urea nitrogen were analysed using an AU400 clinical autoanalyser (Olympus, Japan). Quality control (QC) standard means and their intra-assay CV are presented in Table 3.1.

Table 3.1 Mean QC standards and intra-assay CV for all biochemical analyses run in the Olympus AU400 autoanalyser.

	Mean QC standards		Intra-assay CV (%)	
Metabolite	high	low	high	low
Albumin (g/l)	44.5	25.1	2.5	3.4
BOHB (mmol/l)	2.5	0.9	3.8	6.8
Glucose (mmol/l)	15.1	3.4	2.7	2.4
Total protein (g/l)	72.2	47.7	1.9	2.4
Urea (mmol/l)	22.0	3.3	1.6	5.2
NEFA (mmol/l)	1.0	0.5	4.4	6.3

3.3.1.5 Statistical analyses

Statistical analyses were performed using REML linear mixed models in Genstat (Lawes Agricultural Trust, 2005). The cortisol data was log transformed to follow a normal distribution before analysis. The models for albumin, NEFA, BOHB, glucose, total protein, urea nitrogen and cortisol estimated the effects of the fixed terms Time (am or pm) within Day within Treatment. The haemotology data was collected once daily and thus did not have a Time term, otherwise the analysis was the same. Animal was included as a random term in all analyses. Baseline was included in the fixed model as a covariate (or the average of baseline values if more than one was available). Baseline was not significant for cortisol, BOHB and NEFA concentrations, or the white blood cell and neutrophil counts, and was therefore omitted from the final model of those analyses. Data for albumin, NEFA, BOHB, glucose, total protein, urea nitrogen, cortisol and all the haemotology data from the last day of each treatment was also analysed estimating

treatment effects only. The difference between before and after treatment body weights was analysed, also estimating treatment effects. In all models run, the Wald statistic for each of the fixed terms and their interactions was tested for significance as a chi-square distribution. The predicted means for cortisol were back transformed for presentation.

3.3.2 RESULTS

3.3.2.1 Metabolites

Plasma albumin concentrations increased on the second day of the 3 and 5-day feed restriction treatments (Figure 3.6; $P < 0.05$), but not the 7-day treatment. Plasma concentrations of NEFA increased on the 2nd (3-day and 7-day treatments) and 3rd (5-day treatment) days of feed restriction (Figure 3.7; $P < 0.05$). There was also a diurnal effect whereby the am samples had a higher plasma NEFA concentration than the pm samples on every day for all treatments (Figure 3.8; $P < 0.05$). Plasma BOHB concentration increased on the 2nd (3-day treatment), 3rd (7-day treatment) or 4th (5-day treatment) day of feed restriction (Figure 3.9; $P < 0.05$). The only significant difference between am and pm samples for plasma BOHB was a higher concentration in the morning than the afternoon on day 3 of the 5-day feed restriction treatment (Figure 3.10; $P < 0.05$).

Mean plasma glucose concentration was lower during the 3-day treatment than the 1, 5 or 7-day treatments (Figure 3.11; $P < 0.05$). There was also a diurnal effect, with the pm plasma glucose concentration higher than the am concentration every day for all treatments (Figure 3.12). The difference was significant ($P < 0.05$) on all days of the 1

and 3-day treatments, 4 days of the 5-day treatment (day 3 not significant) and 2 days of the 7-day treatment (days 3 and 5 not significant).

There were no differences in plasma concentrations of total protein in the 3-day feed restriction treatment (Figure 3.13; $P > 0.05$), although there was an increase on day 2 of the 5-day treatment, followed by a decrease on day 3 ($P < 0.05$). Plasma total protein concentration increased on day 3 of the 7-day treatment ($P < 0.05$), then remained stable. Significant diurnal effects occurred on days 3 and 4 of the 5-day treatment, and on days 1 and 2 of the 7-day treatment (Figure 3.14; $P < 0.05$). Plasma concentrations of urea nitrogen decreased on day 3 in the 3, 5 and 7-day feed restriction treatments (Figure 3.15; $P < 0.05$). Urea nitrogen concentration was significantly higher in the am than the pm samples in the 1-day treatment, on days 1 and 3 of the 3-day treatment, and on day 1 of the 5-day and 7-day treatments (Figure 3.16; $P < 0.05$).

3.3.2.2 Cortisol

Plasma cortisol concentration decreased to a low at day 3 in the 3 and 5-day feed restriction treatments and day 4 in the 7-day treatment (Figure 3.17; $P < 0.05$). There was also a significant diurnal effect in cortisol concentration during the 1-day treatment, on days 3 and 4 of the 5-day treatment, and on day 2 of the 7-day treatment (Figure 3.18; $P < 0.05$).

3.3.2.3 Haemotology

There were no significant day within treatment effects for the counts of white and red blood cells, neutrophils and lymphocytes, or levels of haemoglobin or percent of haematocrit ($P > 0.05$).

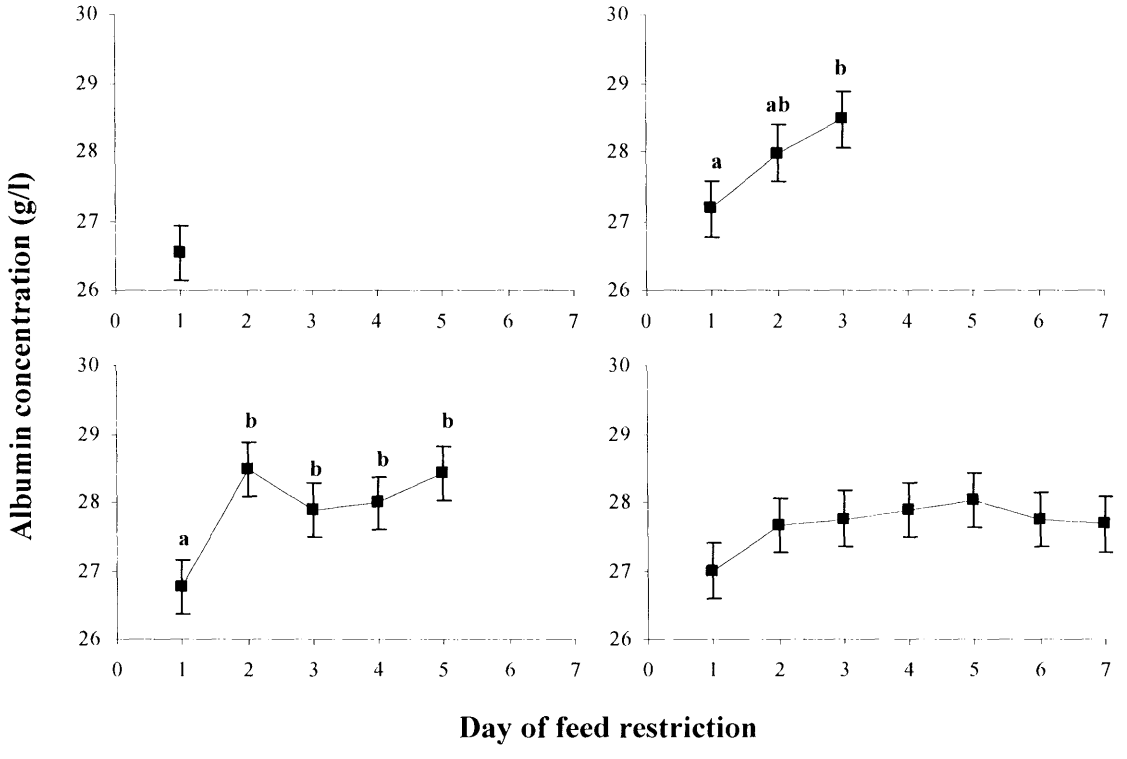


Figure 3.6 Predicted means (± sem) for plasma albumin concentration, during the four feed restriction treatments (1, 3, 5 and 7 days). Within treatment, means without a common superscript are significantly different ($P < 0.05$).

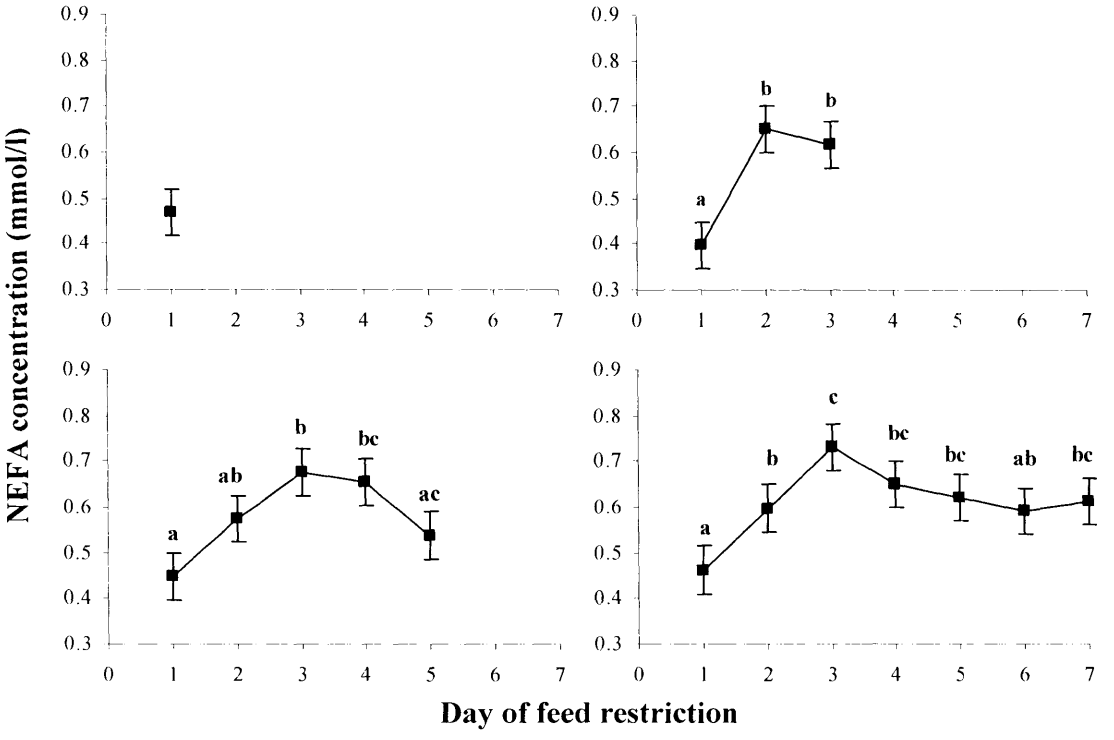


Figure 3.7 Predicted means (± sem) for plasma NEFA concentration during the four feed restriction treatments (1, 3, 5 and 7 days). Within treatment, means without a common superscript are significantly different ($P < 0.05$).

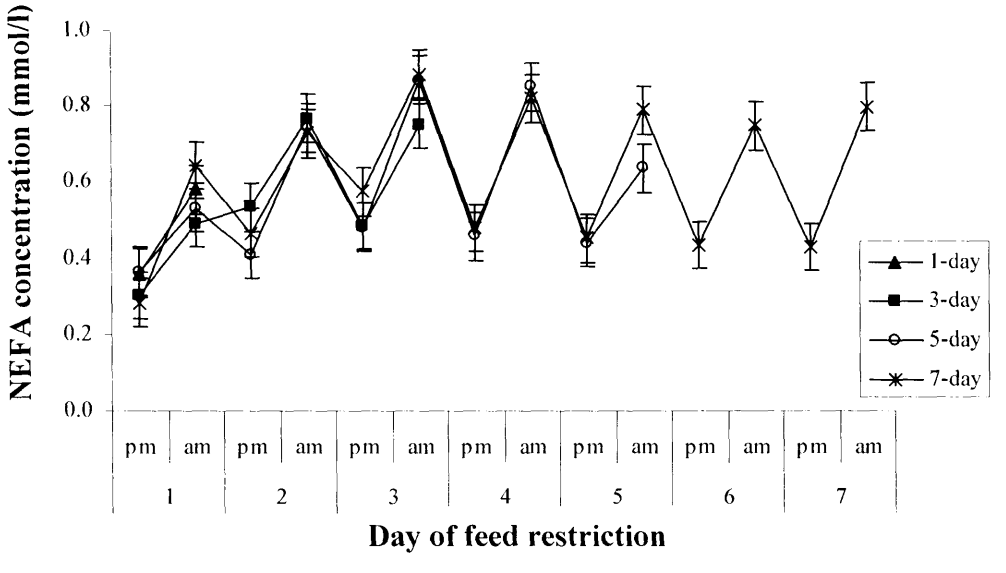


Figure 3.8 Predicted means (± sem) for plasma NEFA concentrations at 12-hour intervals during the four feed restriction treatments (1, 3, 5 and 7 days).

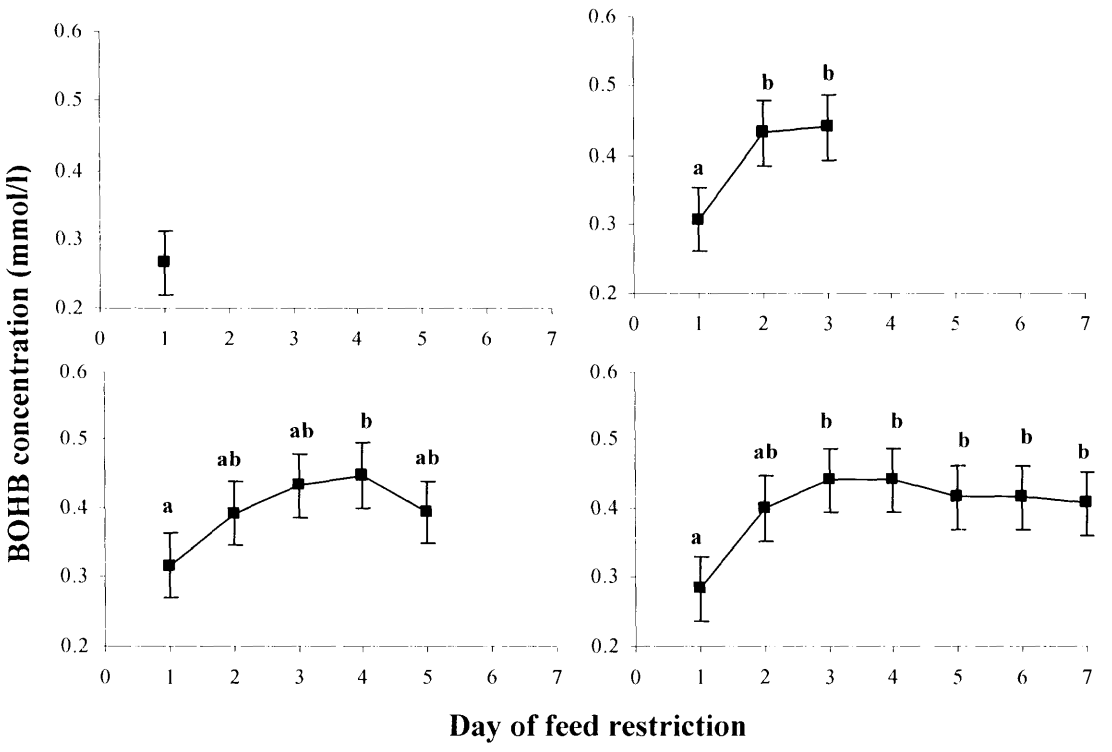


Figure 3.9 Predicted means (± sem) for plasma BOHB concentration during the four feed restriction treatments (1, 3, 5 and 7 days). Within treatment, means without a common superscript are significantly different ($P < 0.05$).

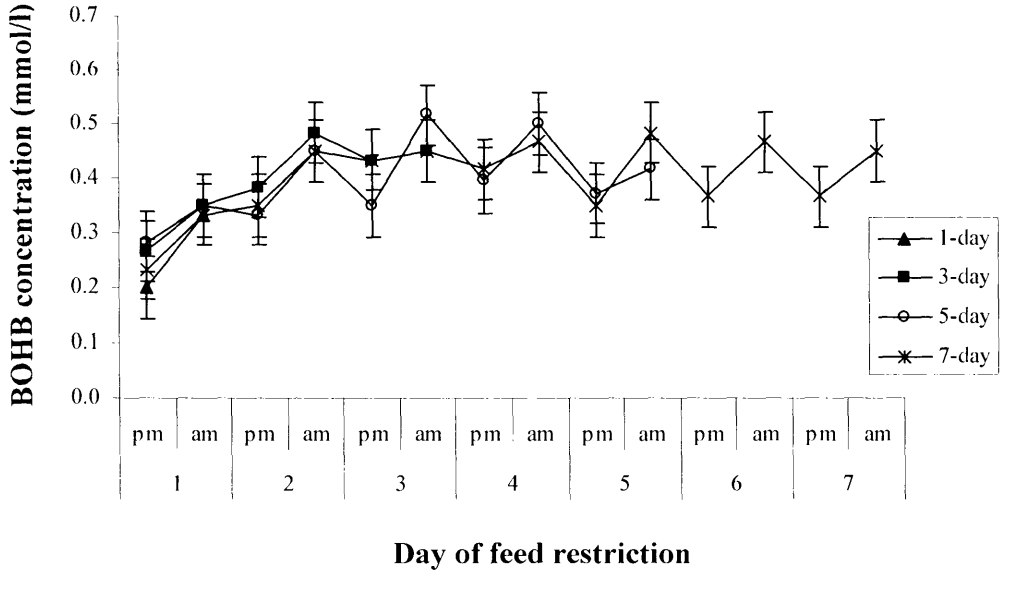


Figure 3.10 Predicted means (± sem) for plasma BOHB concentrations at 12-hour intervals during the four feed restriction treatments (1, 3, 5 and 7 days).

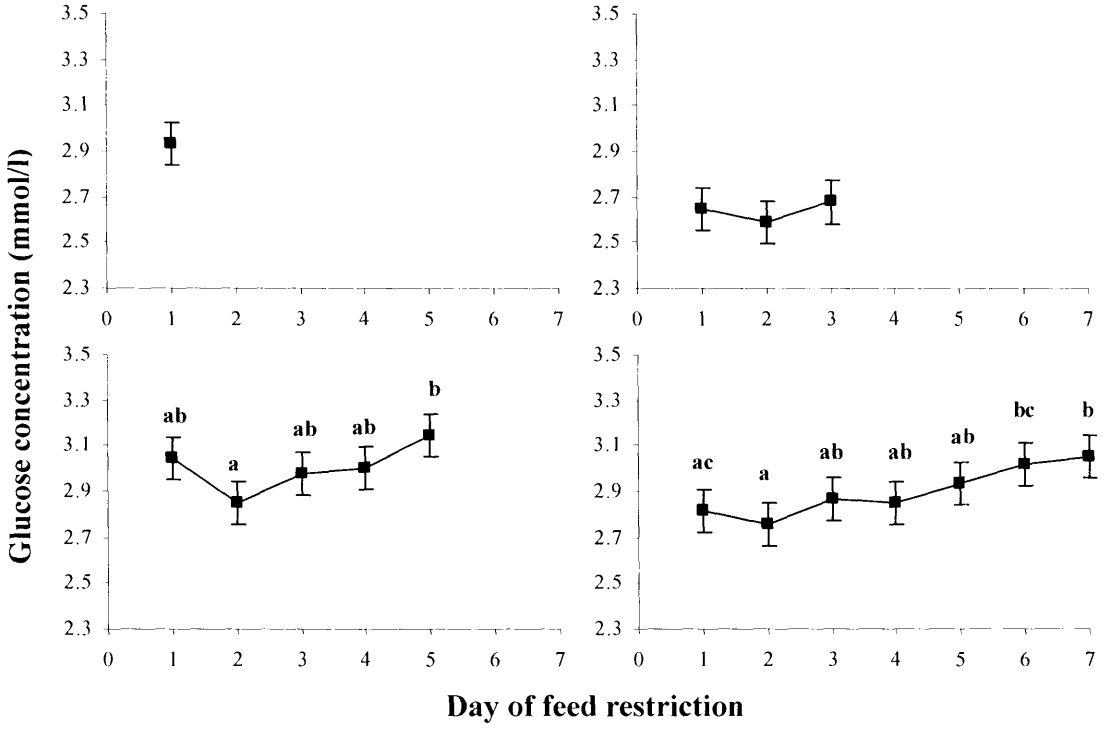


Figure 3.11 Predicted means (± sem) for plasma glucose concentration during the four feed restriction treatments (1, 3, 5 and 7 days). Within treatment, means without a common superscript are significantly different ($P < 0.05$).

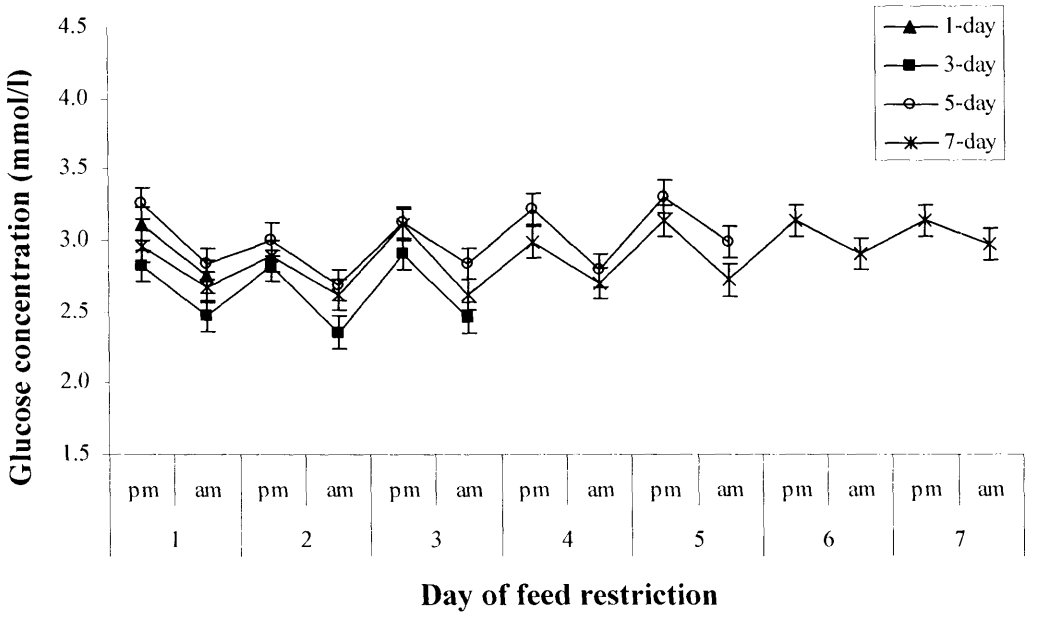


Figure 3.12 Predicted means (± sem) for plasma glucose concentrations at 12-hour intervals during the four feed restriction treatments (1, 3, 5 and 7 days).

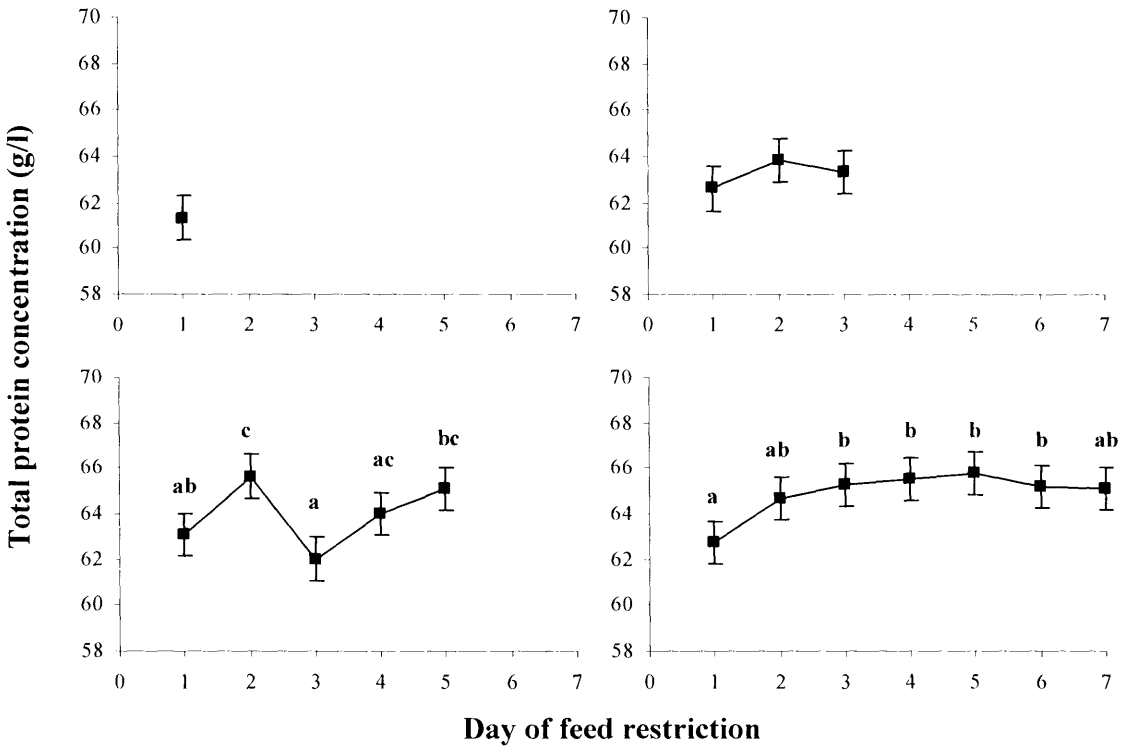


Figure 3.13 Predicted means (± sem) for plasma total protein concentration during the four feed restriction treatments (1, 3, 5 and 7 days). Within treatment, means without a common superscript are significantly different ($P < 0.05$).

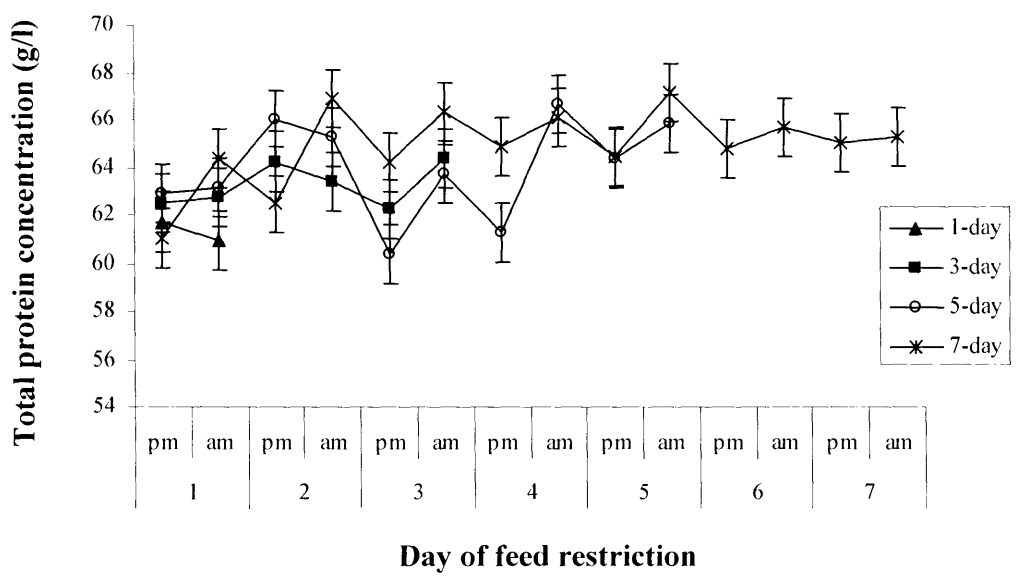


Figure 3.14 Predicted means (± sem) for total protein concentrations at 12-hour intervals during the four feed restriction treatments (1, 3, 5 and 7 days).

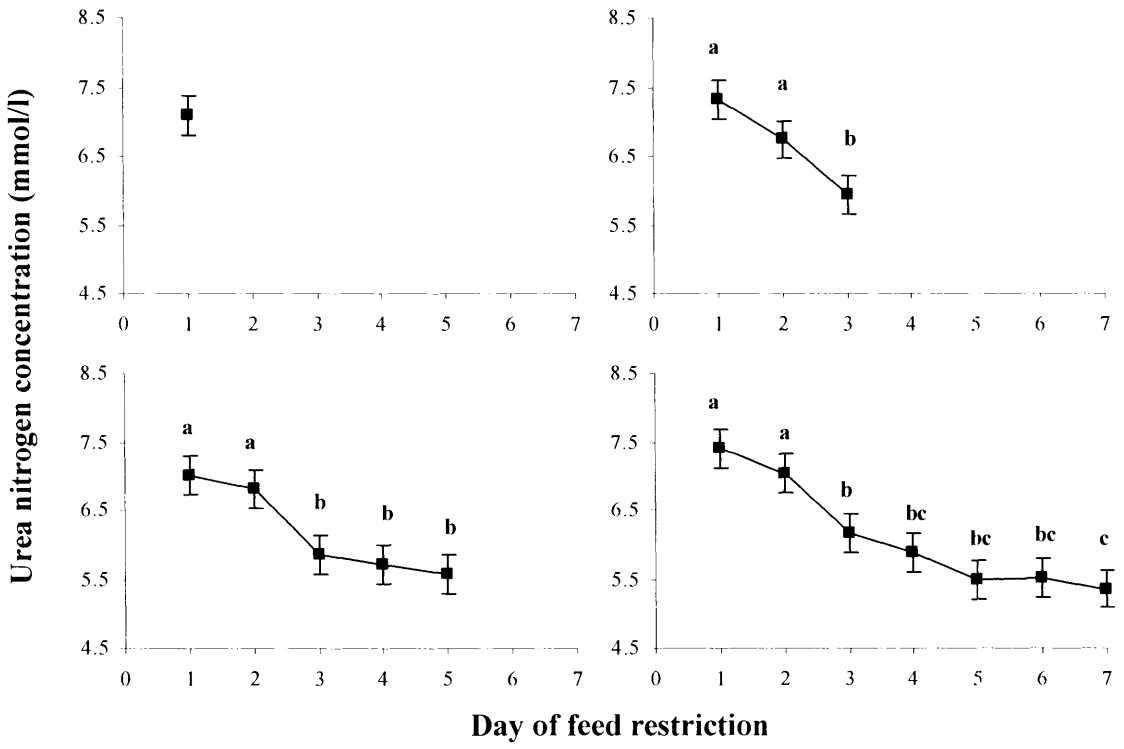


Figure 3.15 Predicted means (± sem) for plasma urea nitrogen concentration during the four feed restriction treatments (1, 3, 5 and 7 days). Within treatment, means without a common superscript are significantly different ($P < 0.05$).

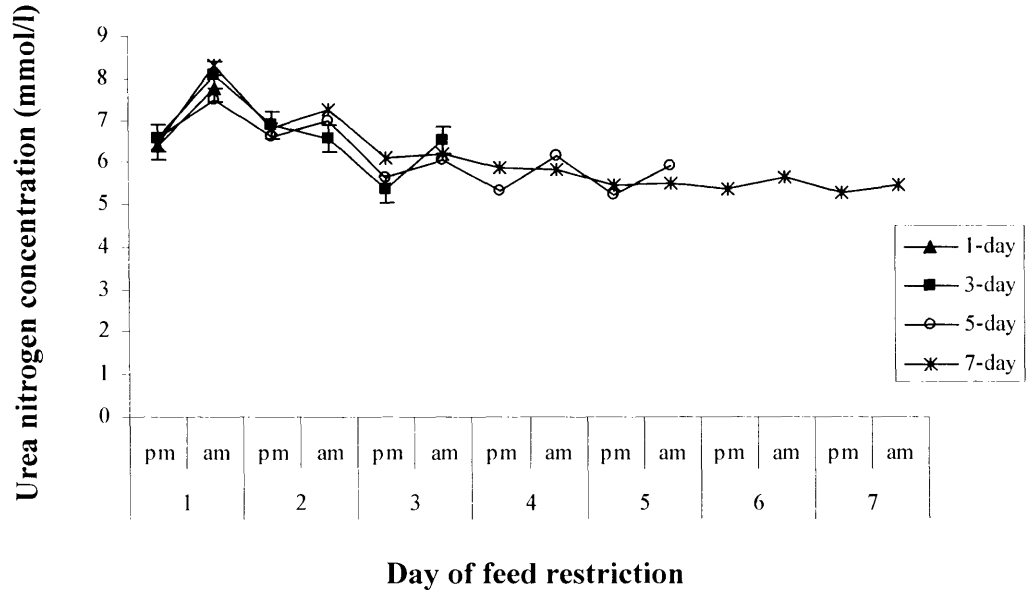


Figure 3.16 Predicted means (± sem) for plasma urea nitrogen concentrations at 12-hour intervals during the four feed restriction treatments (1, 3, 5 and 7 days).

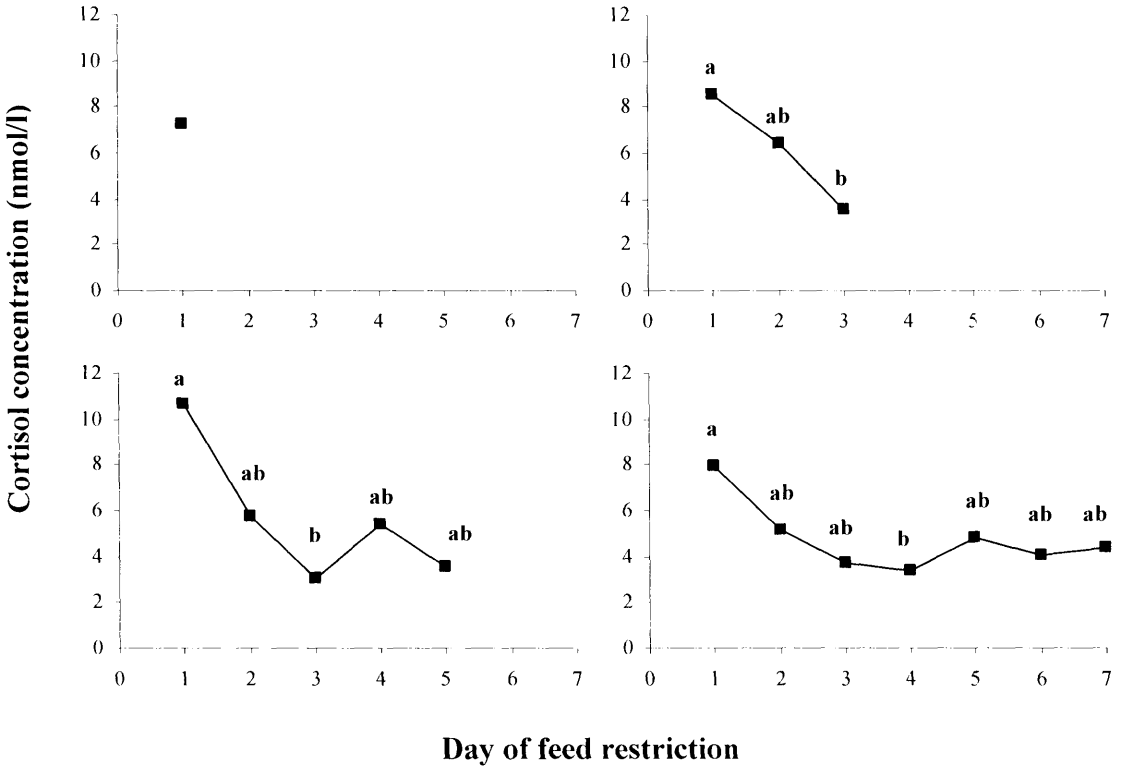


Figure 3.17 Predicted back-transformed means for plasma cortisol during the four feed restriction treatments (1, 3, 5 and 7 days). Within treatment, means without a common superscript are significantly different ($P < 0.05$).

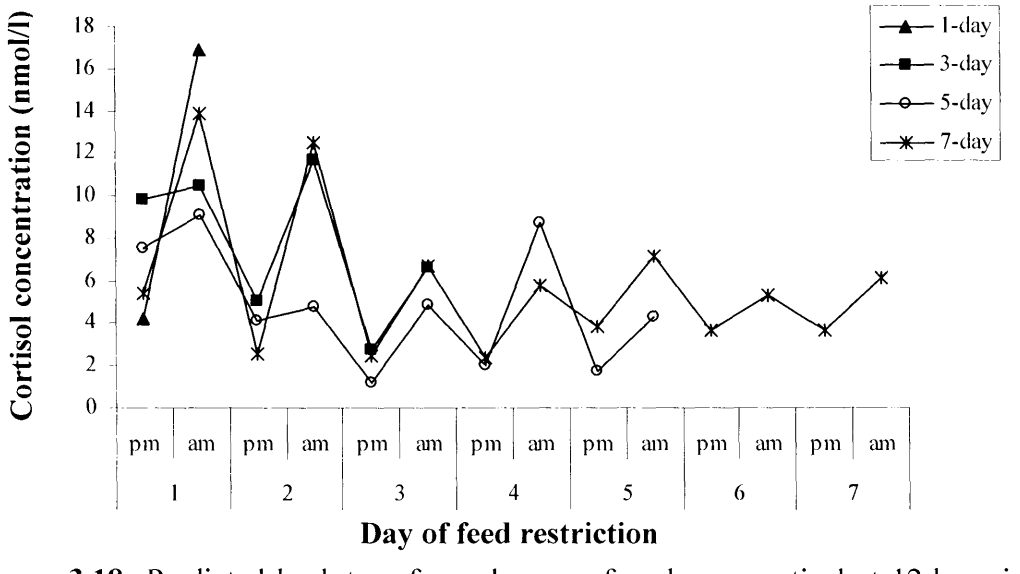


Figure 3.18 Predicted back-transformed means for plasma cortisol at 12-hour intervals during the four feed restriction treatments (1, 3, 5 and 7 days).

3.3.2.4 Treatment comparison

Means for plasma concentrations of albumin, globulin, total protein, BOHB, cortisol, glucose, NEFA and urea nitrogen, as well as counts of white and red blood cells, neutrophils, lymphocytes, and levels of haemoglobin and haematocrit, are presented in Table 3.2. Mean plasma albumin concentration was significantly higher at the end of the 3-day than the 1-day treatment, then lower at the end of the 7-day treatment. There was also a difference in the plasma concentrations of BOHB, but again without any clear pattern. Plasma concentrations of urea nitrogen were significantly higher at the end of the 3, 5 and 7-day treatments, than they were at the end of the 1-day treatment.

3.3.2.5 Live-weight

All animals lost weight during the experiment. The average weight (± sem) of all animals before any treatment was imposed was 39.0 (± 0.8) kg. Animals in the 5 and 7-day treatments lost significantly more weight than those in the 1 and 3-day treatments (Figure 3.19; $P < 0.05$).

Figure 3.19 Predicted means (+ sem) for total weight loss during each of the four feed restriction treatments (1, 3, 5 and 7 days). Means without a common superscript are significantly different ($P < 0.05$).

Table 3.2 Mean (sem) concentrations of blood variables on the last day of each treatment of 1, 3, 5 or 7 days of feed restriction.

	Treatment			
Response variable	1-day	3-day	5-day	7-day
Total protein (g/l)	61.40 (1.180)	63.02 (1.210)	65.09 (1.160)	65.06 (1.150)
Albumin (g/l)	26.2^{a} (0.35)	28.2^{c} (0.36)	28.1^{bc} (0.35)	27.2^{ab} (0.37)
Non-esterified fatty acids (mmol/l)	0.47 (0.062)	0.62 (0.060)	0.54 (0.060)	0.61 (0.062)
Beta-hydroxy butyrate (mmol/l)	0.27^{a} (0.047)	0.44^{b} (0.048)	0.40^{ab} (0.045)	0.41^{b} (0.046)
Glucose (mmol/l)	2.96 (0.134)	2.66 (0.135)	3.16 (0.132)	3.06 (0.132)
Urea (mmol/l)	7.17^{a} (0.303)	6.05^{b} (0.300)	5.67^{b} (0.303)	5.51^{b} (0.312)
Cortisol (nmol/l) *	7.49	3.73	3.28	4.46
White blood cells (10^6/ml)	4.78 (0.646)	6.25 (0.648)	5.46 (0.648)	4.92 (0.683)
Red blood cells (10^9/ml)	7.47 (0.288)	7.52 (0.297)	7.43 (0.288)	7.16 (0.301)
Neutrophils (10^6/ml)	1.79 (0.538)	2.93 (0.582)	2.76 (0.545)	2.27 (0.610)
Lymphocytes (10^6/ml)	2.33 (0.157)	2.22 (0.154)	1.95 (0.154)	2.05 (0.157)
Haemoglobin (g/dl)	8.86 (0.330)	8.84 (0.337)	8.82 (0.331)	8.44 (0.340)
Haematocrit (%)	25.22 (0.940)	25.23 (0.960)	25.05 (0.940)	24.36 (0.960)

^{abc} Within row, means without a common superscript are significantly different ($P < 0.05$).

* Means presented for cortisol are back-transformed. No standard errors are available.

3.4 DISCUSSION

3.4.1 Behavioural motivation to alleviate nutritional stress

The aim of this study was to investigate differences in Merino sheep in motivation to work for a food reward following four different lengths of restricted feeding (1, 3, 5 or 7 days). The results show that there were no differences between the treatments in the motivation of the animals to work for food, and also that the demand for food is inelastic regardless of the length of the restricted feeding period prior to testing.

The animals in this study strongly defended the level of food intake across all workloads. This was expected, as demand for food is known to be inelastic in many species, (for example pigs; Matthews and Ladewig, 1994). What was less expected, however, was that there was no difference between the treatments, not only in elasticity of demand, but also in the pattern of response. It seems that animals in all treatments ate with similar intensity, as the greatest amount of food was consumed in the first 3-6 hours after entry to the test pen. In hindsight, the results from this work make intuitive sense, as all the animals were hungry, with even the least restricted treatment group still only having eaten a fraction of the amount required just for maintenance, in the last 24 hours. It has been suggested that the motivation of sheep to feed increases after only 6 hours without food (Jackson et al., 1999), although at that stage it was almost certainly a short-term hunger motivation rather than a longer-term motivation for energy homeostasis.

It is important to note that the total amount of feed consumed in the test period was more than the animals' daily requirements. In total they consumed 166, 148, 124 and 130% of their daily ration (1.2 × MR) in the 1, 3, 5 and 7-day feed restriction treatments, respectively. Also, the longer the feed restriction was, the less was consumed in the first 6 hours. This may be to do with the shrinkage of the rumen after such a long period of time with little food intake, and the 7-day treatment animals made up for some of the difference between 13 and 18 hours after the test period began. However, it is also possible to look at the pattern of responses in another way. Perhaps the initial intense period of responding is showing the motivation to eat to satiety as part of the daily hunger cycle, which would be reflected by the 1-day treatment animals consuming more than their daily requirements in the first 6 hours. In comparison, the 7-day treatment animals only consumed about 70% of their daily ration in the first 6 hours. As hunger declines with food restriction, it is possible that the longer period of restriction is starting to reflect the motivation to return to energy homeostasis, rather than a short-term effect of hunger. If this is the case, some longer feed restriction periods might help to tease out the differences between short, medium and long-term regulation of motivation to feed.

3.4.2 Metabolic adaptation to feed restriction

The aim of this experiment was to define the metabolic and stress related effects of incrementally increasing feed restriction periods. The results show that although there were treatment differences in concentrations of albumin, BOHB and urea nitrogen on the last day of treatment, these were all within normal ranges (Kaneko et al., 1997). This means that sheep were in a similar metabolic state at the end of all the treatments, possibly

because most of the metabolic changes occurring over time took place in the first 24 – 48 hours of the feed restriction period.

The plasma concentrations of NEFA and BOHB increased early in the feed restriction periods, indicating the initiation of lipolysis due to insufficient nutrient intake. This was the adaptive response required for a successful feed restriction model. Glucose concentration is very tightly regulated, and as such, was not expected to change greatly in response to the feed restricted periods in this study. However, there was an initial drop, although not significant, followed by a slow increase in plasma glucose concentration in the two longest treatment periods. The initial drop was consistent with some early work by Trenkle and Kuhlemeier (1966), which showed that glucose concentrations slowly decreased over a 48-hour period after feeding, although no subsequent concentrations were reported. The slight increase in glucose concentration over the next 3 to 5 days in the present study, is not inconsistent with a study carried out by Sano et al. (1999), which did not indicate any difference in plasma glucose concentration with feed restriction, but the glucose turnover rate decreased. A decrease in turnover rate could have contributed to the slight increase in glucose concentration in this study.

The results for total protein concentration were varied, with a sharp decline on the 3rd day of the 5-day treatment, but not in the 3 or 7-day treatments. However, all 3 treatments increased plasma total protein concentration initially. This result agrees with a study on hepatic protein synthesis in sheep, which found a non-significant increase in the concentration of total protein during a fasting period of 3 days, compared with a fed condition (Connell et al., 1997). However, Connell et al. also reported an increase in

plasma albumin concentration which was not seen in the present study. This difference can possibly be explained by the difference in severity between the two studies. A complete fast, although only for 3 days, is not providing the liver with any dietary protein with which to make albumin, whereas in the present study, although the steadily decreasing concentration of blood urea nitrogen indicated a diminishing supply of protein to the liver as the period of feed restriction increased, animals were fed a small amount daily and thus were not subject to a complete lack of protein intake.

Plasma cortisol concentration decreased in the first 3 days of feed restriction before becoming stable at a low level, suggesting an initial increase. This is a common pattern, usually associated with handling stress as animals are moved and conditions changed. Cortisol concentrations often differ across studies investigating the same effects (see Rushen, 1991 for a brief review). For example, Ekpe and Christopherson (2000) found that plasma cortisol of wether lambs was increased by a feed restriction of 1.35 × MR compared to *ad libitum* feed access, although the increase in cortisol seems unlikely to be due to the feed restriction as it was very moderate. The results of a study with a more severe feed restriction regime support this conclusion; mature ewes were either fasted for 5 days, or in a control group with *ad libitum* access to feed, and there was no treatment difference in serum concentrations of cortisol (Kiyma et al., 2004).

There was some diurnal variation in plasma concentrations of cortisol, BOHB, glucose, total protein and urea nitrogen. However only plasma concentrations of NEFA were significantly different in the morning to the afternoon every day, with the highest levels in the morning before feeding. This diurnal effect on NEFA has also been described in dairy

cows on a feed restriction of 65% of *ad libitum* dry-matter intake (Nielsen et al., 2003). In the present study, the variation was probably caused by the small amount of feed given in the morning after sampling. This is in agreement with results for Merino wethers in which NEFA concentration was at its highest level before feeding, and depressed after feeding (Annison, 1960).

The haematological data presented in this experiment appears to add little to the ability to interpret the results of the experiment, and therefore is discussed no further.

3.4.3 Conclusions

The requirement for the animals to activate adaptive mechanisms in order to adapt to the restricted feed intake, necessary for feed restriction to be to be useful as a model, was fulfilled. Even though the metabolic changes were moderate, they still showed the adaptive mechanisms at work. The same did not occur with the animals' motivation to return to a balanced state of nutrition. Animals at all levels of feed restriction were similarly motivated to maintain a level of feed intake above maintenance requirements. It may be necessary to attempt to separate adaptive mechanisms that drive feeding motivation, in response to feed restriction, into three categories in order to improve this model; a short-term daily hunger cycle, a medium-term adaptive process which activates behaviour, but is directed towards energy balance rather than gut-fill, and a long-term adaptation during which increased motivation to eat is no longer useful. The mechanisms measured would be chosen to reflect those categories. A range of feed restriction periods covering the three categories should identify the metabolic mechanisms activated at each stage, and the associated changes in motivation to feed.

CHAPTER 4

Heat challenge

4.1 LITERATURE REVIEW AND INTRODUCTION

An animal's environment is regularly changing, which means that there is a need to constantly adapt to the changes, in order to maintain a metabolic equilibrium, or homeostasis. This adaptation often has a price, which Moberg (2000) called the biological cost of stress. When the cost is too high because the adaptive systems available are either overworked, fail to shut off after the initiating event is over, or even fail to respond adequately to the challenge, the welfare of the animal can be considered to be compromised (McEwen, 1998).

One of the aspects of the environment that is constantly changing is temperature conditions, and mammals deal with these changes by having mechanisms to maintain body temperature within tightly controlled limits. Normal body temperature of mammals ranges from approximately 36 to 40°C, and declines with advancing age (Bianca, 1968). In sheep the range is approximately 38 to 40°C, and death is possible if temperature rises to 42°C. It is imperative, therefore, that body temperature is maintained within the normal range, and the mechanisms for doing this include both behavioural and physiological adaptations.

4.1.1 Mechanisms of thermoregulation

In mammals, heat is transferred to the body from the environment, by radiation, convection and conduction, all of which an animal has little control over, except in the way of behavioural responses such as seeking shade and wallowing in mud for protection from the sun. The animal does have some control over the evaporation of water, which occurs from the skin and the respiratory passages (Bianca, 1968). Respiratory heat loss is the most effective means of thermoregulation for sheep at high environmental temperatures (Hales and Brown, 1974; Gomes da Silva et al., 2002), even though evaporation decreases with increasing air humidity, becoming zero at a relative humidity of 100% (Bianca, 1968). Sheep also have a low level of cutaneous heat loss which remains steady over a range of conditions, possibly due to the thick cover of the fleece making an increase in cutaneous heat loss inefficient (Brockway et al., 1965).

The mechanisms of thermoregulation are activated in a sequential manner in conditions of a rising environmental temperature. Initially, animals seek behavioural means to control temperature, for example they will lie on a cool surface if one is available and lose heat by conduction. The next mechanism to be activated is vasodilation, whereby blood flow to the skin is increased, particularly to areas which have a high surface to volume ratio such as ears, legs and tongue. This allows heat to dissipate more easily from the body by radiation and convection. Following vasodilation, evaporation is increased. For respiratory evaporation to increase, the volume of air moved per unit of time over the moist surfaces of the respiratory passages must increase, which is accomplished by rapid shallow breathing, or panting. Under severe heat stress, sheep can change from panting to a slower deeper breathing called 'second phase breathing' which is less efficient. Hales and

Webster (1967) reported a peak respiratory frequency in sheep of 300 breaths per minute at ambient temperatures of 55 to 60°C, which dropped to 200 breaths per minute after 80 to 95 minutes of heat exposure. Similarly, Maskrey et al. (1981) reported an average of 275 breaths per minute at 42°C, dropping to 158 breaths per minute before the end of the heat challenge. If all the available mechanisms of heat loss fail to restore thermal equilibrium, body temperature will start to rise and there is usually an associated decline in appetite and thyroid activity which eventually contribute to a lowering of heat production.

The physiological system that regulates temperature in sheep consists of 3 basic components: sensors, a thermostatic control unit and thermoregulatory effectors. The thermoregulatory control centre lies in the hypothalamus in the mid-brain. Central thermosensors are also located there, although possibly not exclusively so. Thermal information received centrally leads to the arrest or correction of an actual displacement of core temperature. By increasing ambient temperature while separately measuring blood and skin temperature, Bligh (1959) was able to show a simultaneous rise in skin temperature and respiratory frequency of sheep, before a change in blood temperature was detectable. This suggests that the control of temperature in sheep is partly anticipatory, which can be measured by respiration rate and skin temperature, and partly reactionary, which can be measured by feed and water intake and body temperature.

4.1.2 Measuring adaptive mechanisms of heat stress

The process of adaptation to a heat challenge in mammals includes reduced feed intake (Silanikove, 1992), raised respiration rates (Yousef, 1985), reduced heart rates (Blazquez

et al., 1994), and activation of the hypothalamic-pituitary-adrenal (HPA) axis (Christison and Johnson, 1972; Ghani, 1988; Minton and Blecha, 1990; Parrott et al., 1996). These mechanisms of adaptation, along with the behavioural motivation they activate, are discussed in the following sections.

4.1.2.1 Behaviour

Behaviour is an integral part of the stress response for many animals, and is often the first response initiated. It is also the most biologically economical response (Moberg, 2000), and can involve the animal simply removing itself from a threat, such as seeking shade in hot weather, or may require a more energetic response such as fleeing from a predator. Behaviour is a fundamental mechanism by which animals regulate body temperature (Weiss and Laties, 1961).

Most of the operant studies that have been conducted on behavioural thermoregulation have used laboratory animals, although some work has been done with pigs and sheep. Pigs have been reported to turn heaters on at ambient temperatures below, but not above, 25°C (Baldwin and Lipton, 1973). Interestingly, 25°C is the approximate temperature at which pigs enter their thermoneutral zone, in which the ambient temperature makes minimal metabolic demand on the animal (Mount, 1968). Thus, it could be concluded that pigs are capable of choosing the ambient temperature which benefits them the most.

Shorn sheep have been reported to place their muzzle through a slit in their cage to break a photoelectric beam, whereby a 1.8 kW array of infra-red lamps suspended above the cage came on and remained on for as long as the beam was broken (4 sheep; Baldwin, 1975).

The sheep had the lamps on for approximately 500, 200, 50 and 10 minutes at 0, 10, 20 and 25°C, respectively. Additionally, sheep were exposed to either 900 or 1800 W intensity lamps for 24-hour periods, and broke the infra-red beam for 402 and 209 minutes, respectively, thus compensating accurately for changes in the intensity of the radiant heat. Therefore, it appears that sheep also show a preference for a particular ambient temperature. However, the importance of this to the animal, combined with the biological costs of adaptation if the preferred conditions are not available, remain unknown. Although a large volume of work has been carried out in the area of animal preferences, little work has been done on thermoregulatory preference in any livestock species, and almost no preference or behavioural demand work has been done at all with sheep.

4.1.2.2 Physiology

4.1.2.2.1 Respiration rate

Increasing the rate of respiration is the main mechanism of thermoregulation for sheep in hot conditions, and evaporative heat loss through panting accounts for 60 to 80% of total heat loss when sheep are exposed to elevated temperatures and humidity (Hales and Brown, 1974). The increase of respiration rate in sheep in response to increasing temperature and humidity has been well documented (Lee and Robinson, 1941; Hales and Webster, 1967; Hales and Brown, 1974; Maskrey et al., 1981). Hales and Brown (1974) found that at air temperatures above 25°C, respiratory frequency increased with increases in both temperature and humidity, but changes in rectal temperature were relatively small over the range of air temperatures measured (5, 15, 25, 35 and 40°C).

The range of respiration rates reported for sheep are many and varied, and of course they are closely associated with the specific conditions of each experiment. One of the highest reported was for Welsh Mountain sheep at 42°C, when the relative humidity was increased from 20% to 75%; the respiratory frequency increased from 160 to 420 breaths per minute in 150 minutes (Bligh, 1963). The same breed at the same temperature of 42°C had a maximum respiration rate of only 190 breaths per minute when the relative humidity was at 12%, showing the importance of humidity on the frequency of respiration in sheep (Bligh, 1959). A comparison of Merino and Omani sheep found that at a temperature and humidity index value of 72, Merino and Omani sheep had a mean respiration rate of 50 and 34 breaths per minute, respectively. When the temperature and humidity index value increased to 93, Merino and Omani sheep increased respiratory frequency to 128 and 65 breaths per minute, respectively, showing definite breed differences with the more adapted Omani being less responsive (Srikandakumar et al., 2003). Merino sheep in hot and humid conditions have been recorded to have average maximum respiration rates of 240 to 300 breaths per minute (Lee and Robinson, 1941; Hales and Webster, 1967; Maskrey et al., 1981).

4.1.2.2.2 Feed and water intake

Heat stress reduces feed intake of ruminants (Baile and Forbes, 1974), especially when the food contains a high fibre content (Bhattacharya and Hussain, 1974). Reducing feed intake is an adaptive mechanism used by ruminants to prevent further hyperthermia (Abdalla et al., 1993), because ingestion of food increases heat production, with rumen micro-organisms contributing up to 10% of an animal's basal heat production (Bianca, 1968). Increasing water intake is also adaptive, allowing for increased evaporative heat loss

through panting as discussed in the previous paragraph, and, in recently shorn sheep, also through cutaneous evaporation (Klemm, 1962; Thwaites, 1985). An investigation into the effects of varying conditions of air temperature (24 to 45°C) and humidity on ingestive behaviour of 32 shorn or unshorn Polwarth ewes, found that shorn sheep had a significantly higher water intake than unshorn sheep, and that feed intake was affected by the proportion of roughage to concentrate in the diet of both shorn and unshorn animals (da Costa et al., 1992).

In a 6-month duration study designed to examine breed differences in acclimatisation of sheep to intense and dry summer heat, Monty et al. (1991) found that feed consumption declined and water intake increased in St Croix, Karakul and Rambouillet sheep as the hot season progressed. There was no change in live-weight associated with the decline in feed intake, and blood glucose levels were maintained at or above cool season levels in the St Croix and Rambouillet sheep. These sheep were able to adapt to the hot conditions in the long-term, but short-term measurements were not taken.

4.1.2.2.3 Cortisol

It was initially found in the 1960s by Robinson and Morris (1960) that plasma cortisol increased during the first days of exposure to a hot environment. Later, Tilton et al. (1975) used mature ewes to measure the diurnal variations in cortisol concentrations, and patterns of cortisol release during either control conditions (18°C) or heat exposure (maximum of 31°C). Blood samples were taken every 4 hours for a 76-hour period, and then for a further 3 weeks. Cortisol concentration differed in control (2.53 ng/ml) and heat exposed (6.29 ng/ml) animals in the first 3 days of heat exposure, but thereafter this difference

declined. Due to information lacking in this account, such as the total number of animals used, the frequency of blood sampling after the initial 76 hours, and the cortisol concentration of control and heat exposed animals after the first 3 days, it is impossible to determine the usefulness of the results in comparison with other work. However, the study did show that cortisol concentration was affected by heat exposure to some extent, and the measurement of cortisol has been common in studies of heat stress since then.

Cortisol concentration was studied to indicate the stressfulness of hot environments for livestock using temperature alone, before it was recognised that humidity could be as important as heat due to its impact on evaporative loss, and it became necessary to include humidity in evaluations. For this reason the temperature-humidity index (THI) which combines the effect of ambient temperature and humidity on animals was formulated (Ingraham et al., 1979). Guerrini and Bertchinger (1982) looked at the effect of high temperature (29 to 32°C) and high humidity (89 to 97%) on plasma cortisol in sheep. Unfortunately, these authors took the first blood samples 7 days after the heat treatment commenced, so it was impossible to know if there had been an increase of plasma cortisol early in the treatment period as expected. What they did find was that by day 7, plasma cortisol had decreased to levels below that of the animals in control conditions (19 to 23°C and 54 to 61% RH). This agreed with work of Tilton et al. (1975) who suggested that the depression in cortisol values was possibly an adaptation of the animals to stressful conditions.

Rapid and short lived increases in cortisol concentration due to heat stress is a common finding: for example, Ghani (1988) was interested in the effect of protein intake on plasma

cortisol concentration, but also compared this in acute and chronic treatments of heat exposure of lambs. Once again, cortisol levels were increased on the third day of heat exposure (32°C) to nearly 2.5 times higher than at the control temperature (18°C), and once again cortisol concentrations were lower after a month of heat exposure than they were at the control temperature. Similarly, Minton and Blecha (1990) found that although cortisol concentrations increased rapidly in lambs exposed to 24 hours of heat stress (35°C), they decreased back to pre-treatment levels within 12 hours of removal from the heat. In agreement with this, Parrott et al. (1996), using hot (35°C) and cold (7°C) temperatures as treatments found that cortisol concentrations increased initially, but quickly (6 hours later) declined and remained stable for the rest of the 48-hour period.

It would be very difficult to determine stress levels in sheep in a chronic heat challenge model using cortisol concentration alone. It is possible that at least some of the increase in cortisol concentration which commonly occurs at the very beginning of a treatment and is short lived, actually reflects a response to changing conditions, for example environment, pen mates and even the sampling procedure, rather than just the heat challenge itself (Parrott et al., 1996). For this reason it is necessary to also measure other physiological responses which may help to define the effects of heat stress on sheep in the conditions of this study. One of these responses which may be informative is that of plasma prolactin concentration.

4.1.2.2.4 Prolactin

Prolactin is a pituitary hormone well known for its role in reproduction, although there is evidence to indicate that it may be involved in other physiological and metabolic

processes such as thermoregulation. For example, in a study by Parrott et al. (1996), prolactin concentration increased as sheep entered a hot treatment (35°C) and remained high for the following 48 hours, but did not increase at all in a cold treatment (7°C). A similar result had been reported earlier by Hooley et al. (1979).

Salah et al. (1995) carried out a study in which 6 of 12 lambs were treated with bromocryptine (CB154), a prolactin secretion inhibitor, after which all were exposed to extreme temperatures (43 to 44°C) for about 18 days. The authors found that all lambs increased their respiration rate in response to the temperature treatment, and control lambs maintained their body temperature for the first period, whereas treatment lambs had elevated rectal temperatures (Salah et al., 1995). The authors suggest that it was the prolactin and not the bromocryptine that had a direct role in regulating body temperature under their elevated environmental temperatures.

Prolactin may influence both emotional responses and HPA axis activity. This neuropeptide has been shown to participate in the regulation of maternal behaviour, grooming, and food intake in mammalian and non-mammalian species. A possible involvement of prolactin in stress response mechanisms is suggested by the findings that prolactin acts as an endogenous anxiolytic and reduces HPA axis responses to stress in the male and female rat (Torner et al., 2001). For example, during 10 minutes of forced swimming, prolactin-treated rats showed a more active stress-coping strategy; with the latency to float prolonged, and the time spent struggling increased. Brain prolactin was also shown to regulate ACTH secretion in the same study; infusions of ovine prolactin directly to the brain caused a shift of HPA axis activity toward elevated basal ACTH

plasma concentrations and an attenuated ACTH secretory response to exposure to an emotional stressor.

Infusion of prolactin directly into the paraventricular nucleus of the hypothalamus or the posterior pituitary, was reported to decrease cortisol responses in sheep in the presence of a dog (Cook, 1997), but it had no dampening effect on increased heart rate. The author suggested that the reason prolactin suppressed cortisol might be because high pulsatile levels of cortisol can disrupt the lactation process, although the same effect of prolactin was seen in non-lactating sheep.

The main role of increased prolactin secretion in response to stress is thought to be to counteract suppressive effects on the immune system of stress-induced increases in glucocorticoids (Davis, 1998), thereby maintaining the competence of the immune system (Fujikawa et al., 1995). Fujikawa et al. (1995) investigated whether stress-induced serum prolactin can act on the central nervous system, and reported that restraint stress in the water caused a rapid and sharp elevation of the serum prolactin level in the rat and markedly induced the expression of a prolactin receptor mRNA in the choroid plexus of the brain.

4.1.3 Conclusions and aims

The physiological adaptive mechanisms that sheep activate in response to heat challenge have been well documented, and although only a small volume of work has been reported on behavioural thermoregulation of the species, it suggests that sheep will actively seek to maintain a homeostatic body temperature. What is not known is the link between these

two responses, physiological and behavioural, and the importance of them with regard to each other; how does the animal regulate the use of each response in order to keep the biological costs of adaptation to a minimum?

Therefore, the aim of the first experiment in this chapter was to investigate the motivation of sheep, held at a range of ambient temperatures, to open a door to a cool pen. The hypothesis was that the motivation of animals to enter a cool area from a hot one, would increase at the point where the biological cost of adaptation to the heat challenge increased significantly. The aim of the second experiment in the chapter was to define the biological cost of adaptation that the animals were subject to in the previous experiment, with the same heat challenge levels used. The intention for the chapter was to use the example of heat challenge to investigate the links between physiological and behavioural adaptation, with particular focus on how the use of specific adaptive mechanisms changed as the biological costs of adaptation increased.

4.2 BEHAVIOURAL MOTIVATION TO AVOID A HEAT CHALLENGE

4.2.1 MATERIALS AND METHODS

The study was approved by the University of New England Animal Ethics Committee, approval number AEC06-025.

4.2.1.1 Animals

Initially, 19 six-month-old female Merino lambs were trained for use in the experiment. Twelve of the lambs were kept for further training and moved inside into pens, and the rest were left on pasture. Six animals were fully trained as experimental animals and the remaining six were used as companions during both training and testing. They were each fed 900 g lucerne pellets daily.

4.2.1.2 Training

The lambs were handled daily before training began to habituate them to people in close proximity. They were then put into individual indoor pens and habituated to more handling, pens and the concentrate feed.

Training sessions were held daily, 5 days per week, in one of two climate rooms. The animals were moved from their home pens into individual pens in the climate rooms for training sessions. A period of habituation to the rooms, pens and noise and movement of

the equipment was allowed. The climate rooms were to be used for the experiment, but at this stage were maintained at approximately 20°C and 25% relative humidity (RH).

When the lambs appeared at ease in the new environment, the temperature was increased to approximately 35°C and 70% RH, and the training to push a door panel for access to a cool pen (20°C) began. The training process used was the method of successive approximations. In this method, each successive approximation to the main goal, in this case pressing the door panel hard enough to make it buzz (and therefore open the door), was rewarded by the trainer remotely opening the door to the cool pen and letting the subject remain inside for a period of 20 minutes to cool down.

When the lambs repeatedly stood by the cool pen door exploring, rather than elsewhere in the pen, and entered the cool pen immediately upon the door opening, it was decided that they required a more immediate and obvious reward in order to learn to push the panel. Additional daily training began for panel pushing. A perspex panel, that looked the same as the door to the cool pen, was made to slot into the front of the home pens. It was connected to the computer program, and had a door panel with buzzer, a feeder and a feed container attached. This training used successive approximations also, and the lambs quickly learned to push the panel for a food reward.

In order for the animals to transfer their learned behaviour of pushing the training panel, to pushing the cool pen door panel in the climate rooms, some crushed and moistened lucerne pellets were smeared onto the panel of the training apparatus, and then onto the panel in the climate rooms. The animals soon associated the panel in the climate rooms

with the training panel, and pushed the panel and entered the cool pen. Although to begin with the animals were almost certainly pushing the cool pen door panel in expectation of a food reward, they never received food in the climate rooms during training, and yet they continued to open the door and enter the cool pen.

The last step was practice, with the animals successfully pushing the panel to get access to the cool pen without any smell of food on the panel, and an increase in the number of panel pushes (FR value) required to open the cool pen door.

4.2.1.3 Equipment

One pen in each of the climate rooms was fully enclosed in perspex (1200 x 1200 x 910 mm high), with a perspex sliding door at the end (700 wide × 850 mm high; Figure 4.1). On the front of the door was a panel (90 × 95 mm) with a micro switch under it which activated the opening of the door when the programmed number of panel pushes was accomplished. The pen had cool air continuously cycled through it and was maintained at a temperature of approximately 20°C. It also had rubber mats on the floor over the wire mesh so that the cool air was retained.

The layout of the climate rooms is depicted in Figure 4.2 showing the location of the companion pens, the treatment animal pen and the cool pen. The temperature and humidity in the climate rooms was electronically controlled and monitored.

Figure 4.1 A sheep is shown in the cool pen on the left, and a companion animal on the right is beside the cool pen. There was a companion animal on each side of the cool pen at all times during the training and experiment.

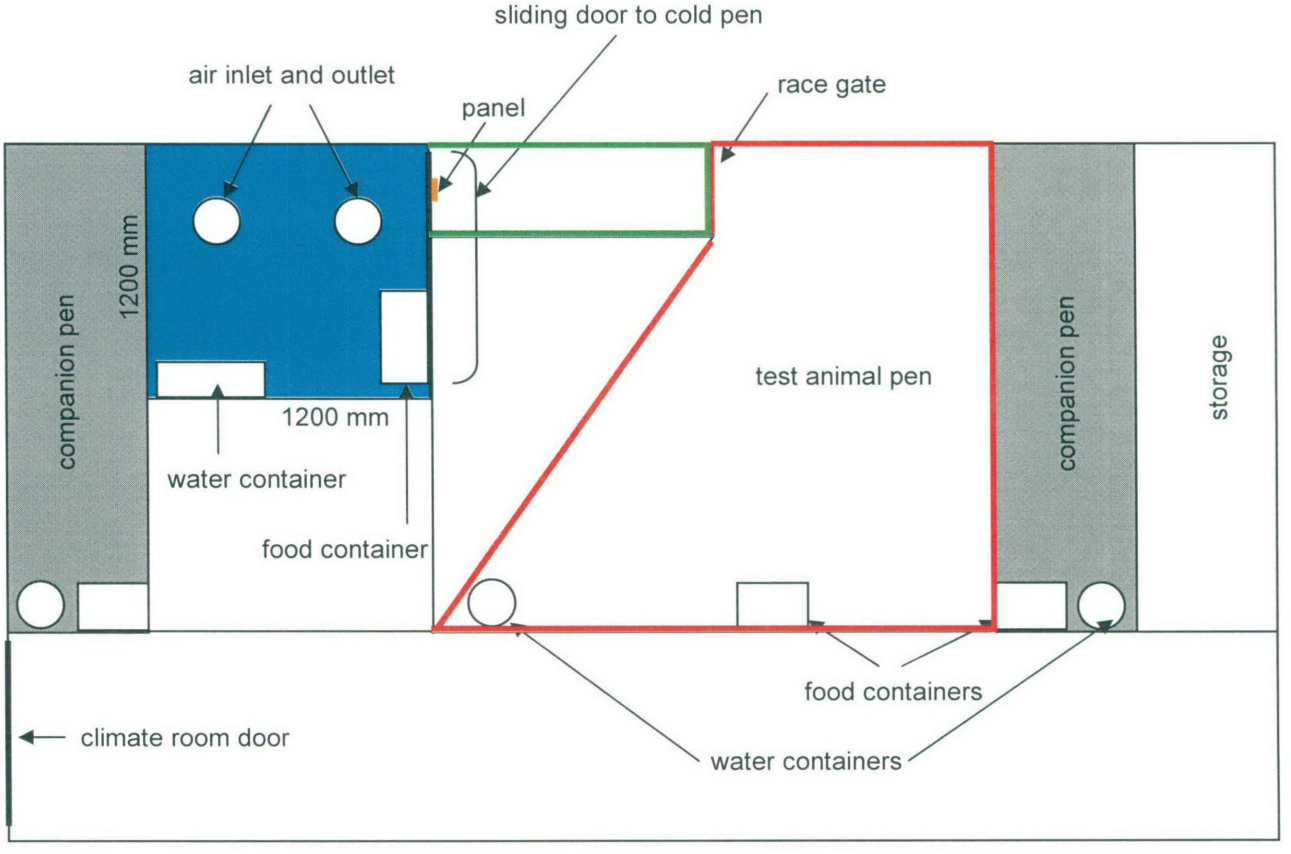


Figure 4.2 Diagram of the climate rooms used for the experiment.

4.2.1.4 Experiment

Each animal was exposed to all four heat challenge treatments of 20, 25, 30 and 35°C with 70% RH. Two animals were tested each day, one in each room, in a 9-hour session. The treatment conditions were the same in both rooms, with the order of the treatment conditions assigned randomly. Room and test order for each FR within each treatment were randomly assigned to animals. Within each treatment, four FR values were used: 1, 4, 10 and 25.

The 2 test animals of the day, along with 2 companion animals in each room, were moved into the climate rooms at 0800 hours and fed. At 0900 hours the door to the race was opened (enabling access to the cool pen door and panel), and remained open for the next 8 hours. Fifty percent of each daily feed for the treatment animals was available in the main pen and the other 50% in the cool pen. Water was available in both. Animals were removed from the cool pen 20 minutes after entering it. The time each reward was earned, and the number of rewards earned, was recorded automatically in each session. Respiration rates were manually counted and recorded at 5 time periods; 1, 3, 5, 7 and 9 hours after entry to the climate rooms, for all treatment and companion animals.

Purpose-written software and in-house computer systems controlled the buzzing noise associated with a successful panel push, the number of panel pushes required to open the cool pen door, and the opening and closing of the cool pen door. The same computer system collected the data for each session (number of panel pushes and number of rewards).

Due to concern regarding the use of the mat in the cool pen for lying, there being no mat outside the cool pen, one of the conditions was repeated at the end of the experiment. All animals were run through one more test session at 20°C, on a FR10 schedule of reinforcement, with no mat in the cool pen.

4.2.1.5 Statistical analyses

4.2.1.5.1 Rewards

A REML linear mixed model analysis was performed on the number of rewards obtained using Genstat (Lawes Agricultural Trust, 2005). The model included Treatment + FR + Treatment × FR as fixed terms, with Animal as a random term. The Wald statistic for each of the fixed terms and their interactions was tested for significance as a chi-square distribution. There was no Treatment × FR interaction, so the term was omitted from the final model.

The data were also calculated as the means of the logarithms of the number of rewards obtained by all animals in each treatment, at each FR value. Best-fit lines of the form $y = mx + b$ were fitted to the log-log functions for rewards as a function of FR value by treatment, using the method of least squares.

The data for the number of rewards obtained during the 20°C treatment at FR4 with and without a mat in the cool pen, was analysed in Genstat using a paired-sample t-test. It was calculated using a one-sample t-test with the null hypothesis that the mean of 'mat' minus 'no mat' was equal to zero.

4.2.1.5.2 Respiration rates

Two models were used for analysis of the respiration rate data. One included Group (Treatment or Companion animal) for comparison, and the other omitted Group and all the companion animals as they were not exposed to the FR schedules, nor did they have access to the cool pens. Time 1 was not included in the analysis for either model, as the treatment had not yet started and treatment animals did not have access to the cool pen door.

For the treatment and companion animal comparison, a REML linear mixed model analysis was performed on the respiration rates. The model included Treatment + Group + Treatment × Group as fixed terms, with Animal as a random term. The Wald statistic for each of the fixed terms and their interactions was tested for significance as a chi-square distribution.

For the treatment animal analysis, data were log transformed before again performing a REML linear mixed model analysis. This time the model included Treatment + FR + Time + Treatment × FR + Treatment × Time + FR × Time + Treatment × FR × Time as fixed terms, with Animal as a random term. The Wald statistic for each of the fixed terms and their interactions was tested for significance as a chi-square distribution. The interaction terms for Treatment × Time, FR × Time and Treatment × FR × Time were not significant, therefore they were omitted from the final model. Means were back transformed for presentation.

4.2.1.5.3 Latency

Latency was used as an indication of how motivated the animals were to enter the cool pen. It was the number of minutes it took for an animal to re-enter the cool pen, after it was removed following a 20-minute cooling period. Data were log transformed before performing a REML linear mixed model analysis. The model included Treatment + FR + Treatment × FR as fixed terms, with Animal as a random term. The Wald statistic for each of the fixed terms and their interactions was tested for significance as a chi-square distribution. However, there was no Treatment × FR interaction, so this term was omitted from the final model. Means were back transformed for presentation.

4.2.2 RESULTS

4.2.2.1 Rewards

Animals entered the cool pen more often with each increase in temperature ($P < 0.05$; Figure 4.3). The number of cool pen entries remained the same over the first three fixed ratio (FR) values, or workloads, but decreased on the fourth when 25 panel pushes were required (FR25; $P < 0.05$; Figure 4.4). There was no interaction between Treatment × FR, indicating that the demand functions did not differ significantly between treatments (Figure 4.5). Looking at the heat challenge treatments separately, the number of rewards obtained remained approximately constant for each treatment, with regression slopes close to zero, as the FR value increased. The absolute values of the slopes measure the elasticity of demand, and the average elasticity for all treatments was 0.07.

There was no significant difference in the number of rewards obtained with and without the rubber mats in the cool pen (t = -0.68; P = 0.53) at a temperature of 20°C and a workload of FR10.

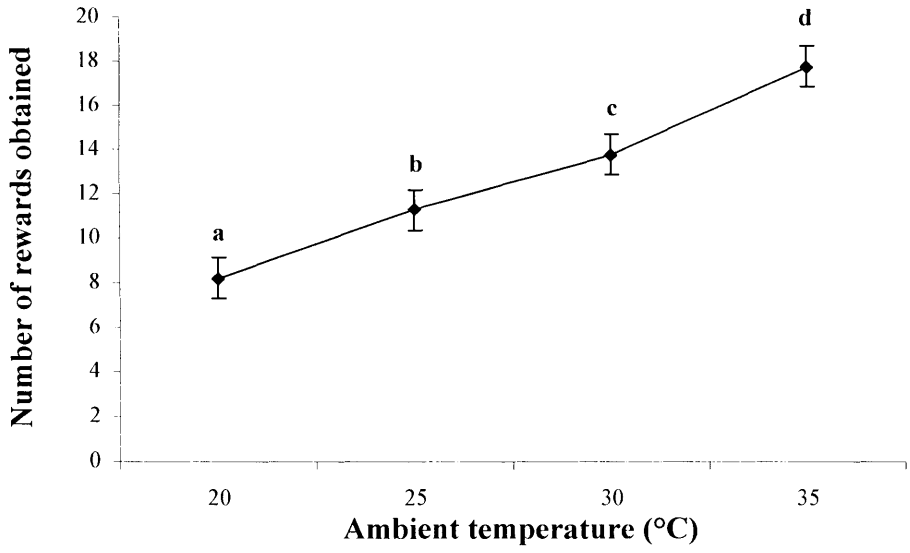


Figure 4.3 Mean (± sem) number of rewards obtained in the test sessions at a heat challenge of 20, 25, 30 or 35°C. Means without a common superscript are significantly different ($P < 0.05$).

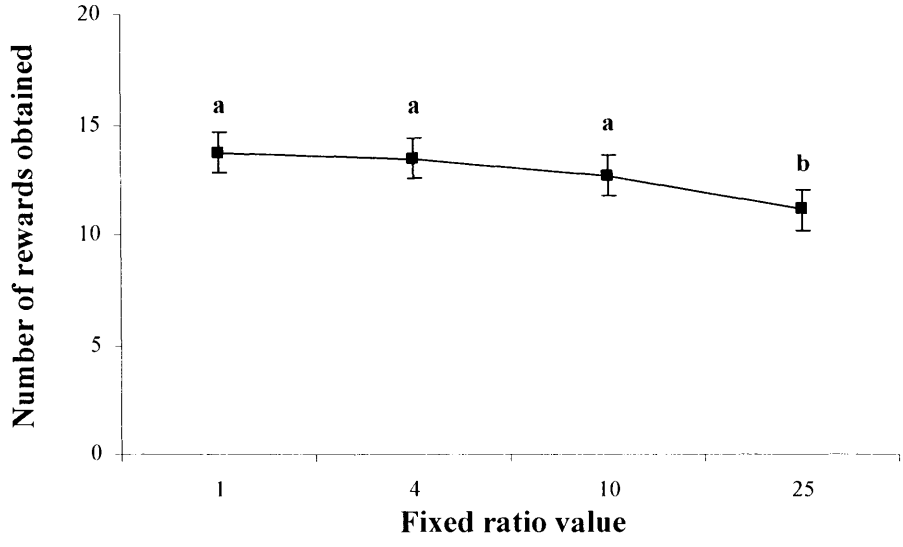


Figure 4.4 Mean (± sem) number of rewards obtained in the test sessions at each fixed ratio value. Means without a common superscript are significantly different ($P < 0.05$).

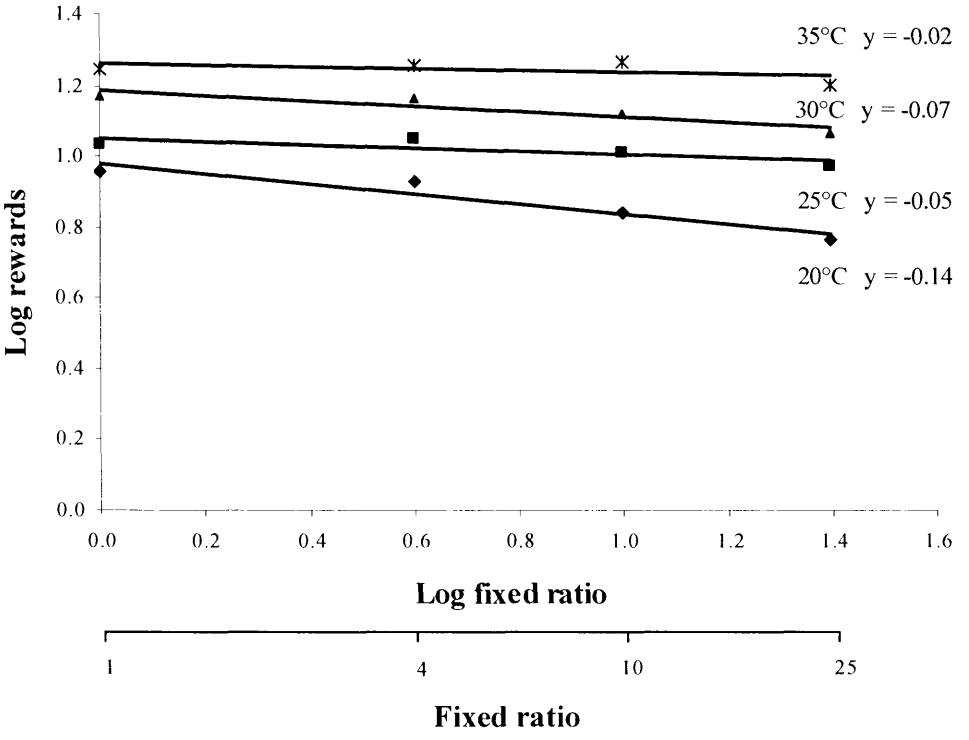


Figure 4.5 Demand functions for cool pen access at each heat challenge of 20, 25, 30 or 35°C (y = slope, or elasticity coefficient).

4.2.2.2 Respiration rates

The respiration rates of the companion animals (without access to the cold area) were higher with each increase in temperature ($P < 0.05$), while those for the treatment animals stayed relatively low, showing the effect on respiration rate of having access to the cool pen (Figure 4.6). A Treatment × FR interaction (Table 4.1; $P < 0.05$) indicated that respiration rates of treatment animals increased at FR25 at the 25, 30 and 35°C temperatures. At 30°C there was an additional increase at FR4.

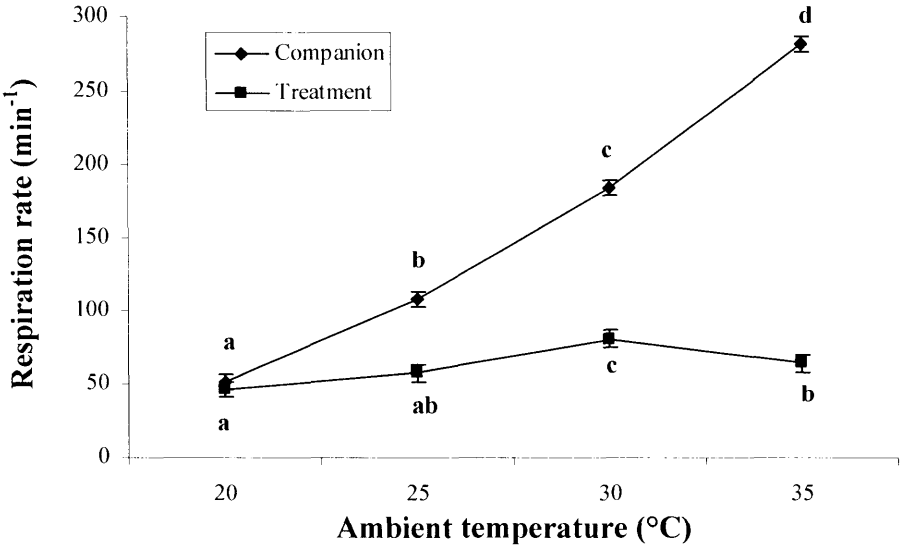


Figure 4.6 Mean (± sem) respiration rates for the treatment animals, and the companions which did not have access to the cool pen. Within group (Companion or Treatment), means without a common superscript are significantly different ($P < 0.05$).

Table 4.1 Predicted (back transformed) means for respiration rate (min^{-1}) of treatment animals (with access to the cool pen), at each of the 4 fixed ratio values during a heat challenge of 20, 25, 30 or 35°C.

	Fixed ratio value			
Treatment	FR1	FR4	FR10	FR25
20°C	43.6	38.9	41.8	45.3
25°C	41.2^{a}	45.7^{a}	48.2^{a}	74.1^{b}
30°C	42.3^{a}	74.5^{b}	79.6^{bc}	97.9^{c}
35°C	40.5^{a}	51.4^{a}	49.5^{a}	76.4^{b}

^{abc} Within row, means without a common superscript are significantly different ($P < 0.05$).

4.2.2.3 Latency

The time taken to enter the cool pen after the last exit decreased as the temperature increased (Figure 4.7; $P < 0.05$). However, the latency did increase at the highest workload of FR25 (Figure 4.8; $P < 0.05$). There was no Treatment × FR interaction.

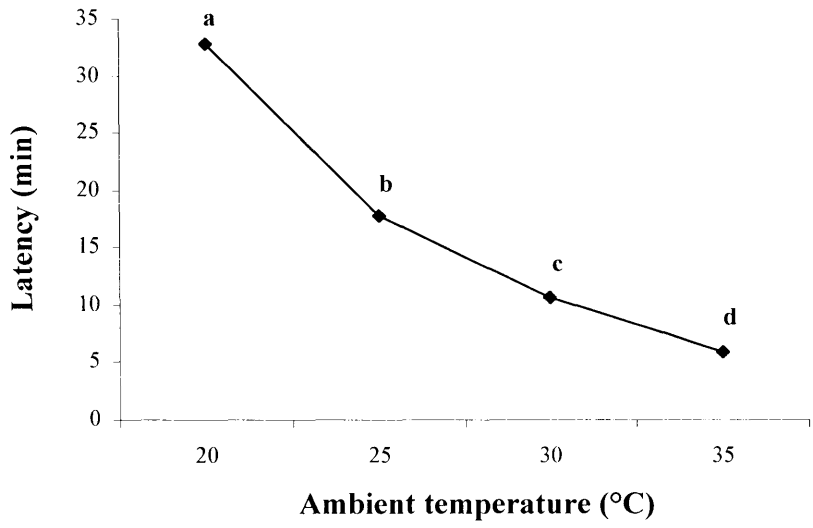


Figure 4.7 Predicted (back transformed) means for the latency (min) of the treatment animals to enter the cool pen after the previous exit during a heat challenge of 20, 25, 30 or 35°C. Means without a common superscript are significantly different ($P < 0.05$).

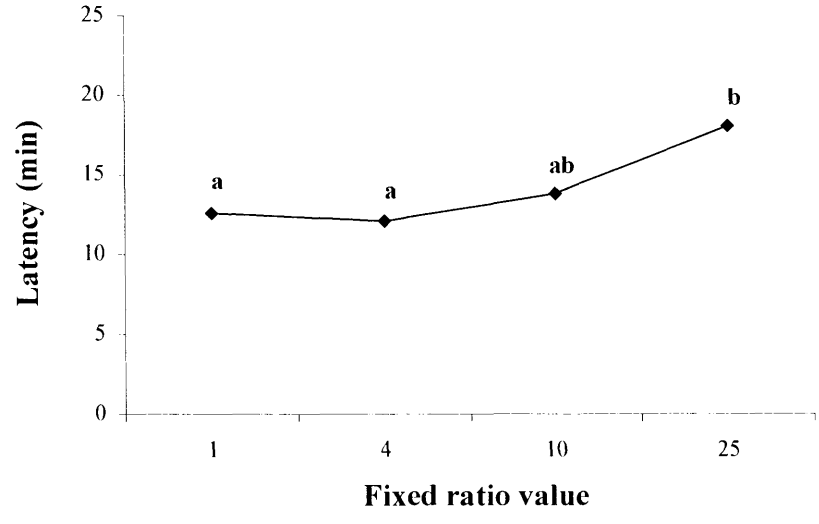


Figure 4.8 Predicted (back transformed) means for latency at each of the 4 fixed ratio values. Means without a common superscript are significantly different ($P < 0.05$).

4.3 PHYSIOLOGICAL ADAPTATION TO A HEAT CHALLENGE

4.3.1 MATERIALS AND METHODS

The study followed the guidelines established by the University of New England Animal Ethics Committee, approval number AEC03-150.

4.3.1.1 Animals and design

Sixty 12 to 18-month-old Merino ewes were housed indoors in individual pens placed alongside each other, and with open mesh dividers, throughout the experimental period and were offered *ad libitum* lucerne pellets. All animals were weighed prior to housing, and the group was split into 4 groups consisting of 15 animals each, balanced for weight. The 12 animals in each group that settled best into the new environment were chosen to continue. After two weeks of habituation to indoor housing and daily handling, animals were randomly assigned to one of two groups of 6, each of which was put into a separate climate controlled room (Table 4.2). Both rooms were set at a thermoneutral temperature (20°C) for the first week (baseline) and then one of four levels of heat challenge (a different level in each room) for the next 5 days. Relative humidity was kept at 70% throughout. Both rooms were set on a 14:10 hour light:dark cycle with lights on at 0700 hours. After the 2-week period in the climate rooms, all 12 animals were moved back to the animal house for a further week.

Table 4.2 Schedule of animal movement, grouping and treatments during the experiment showing baseline and heat challenge temperatures (in bold).

Week	Animal house	Climate room 1		Climate room 2		Animal house
	pre-treatment	No. sheep	Temp	No. sheep	Temp	post-treatment
1	(grp 1&2)					
2	(grp 1&2)					
3	(grp 3&4)	6 (grp 1)	20°C	6 (grp 2)	20°C	
4	(grp 3&4)	6 (grp 1)	**20°C**	6 (grp 2)	**30°C**	
5	(grp 5&6)	6 (grp 3)	20°C	6 (grp 4)	20°C	(grp 1&2)
6	(grp 5&6)	6 (grp 3)	**25°C**	6 (grp 4)	**35°C**	
7	(grp 7&8)	6 (grp 5)	20°C	6 (grp 6)	20°C	(grp 3&4)
8	(grp 7&8)	6 (grp 5)	**30°C**	6 (grp 6)	**20°C**	
9		6 (grp 7)	20°C	6 (grp 8)	20°C	(grp 5&6)
10		6 (grp 7)	**35°C**	6 (grp 8)	**25°C**	
11						(grp 7&8)

4.3.1.2 Procedures

All animals were weighed before being taken to the climate rooms, and again on their removal. Once in the climate rooms, each animal had a vinyl catheter (internal diameter 1.0 mm; Biocorp Australia Pty Ltd, Huntingdale, Victoria) inserted into the jugular vein under local anaesthetic (Lignocaine 20; 20 mg/ml lignocaine hydrochloride, Troy Laboratories Pty, Smithfield, New South Wales) using a 13 gauge hypodermic needle. The catheters were covered by an elastic bandage to protect them, and were checked and flushed twice daily with heparinised sterile physiological saline (105 IU/ml heparin/0.9% saline). Blood was collected into 10 ml S-Monovette tubes (Sarstedt Australia Pty Ltd, Mawson Lakes, South Australia) coated with either heparin or EDTA depending on the

intended analysis. Temperature loggers ('Minilog', Vemco Ltd, Nova Scotia, Canada) were mounted on blank (non-progesterone) controlled intravaginal drug releasing (CIDR) inserts (CIDR-G™, InterAg, Hamilton, NZ; Figure 4.9) and inserted into the vagina of each animal.

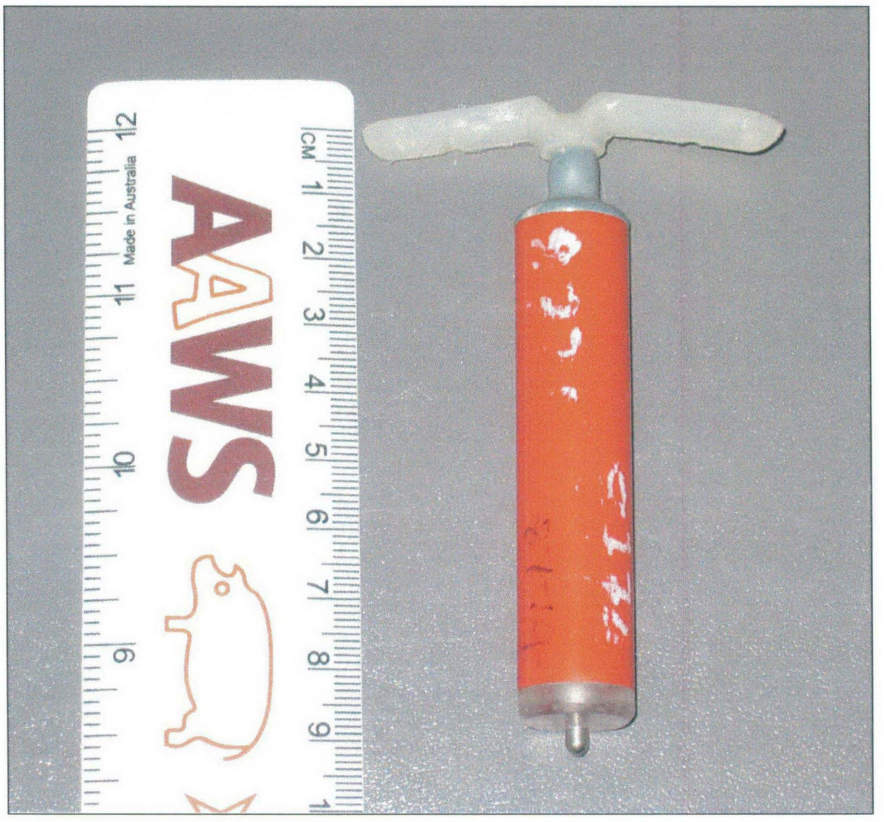


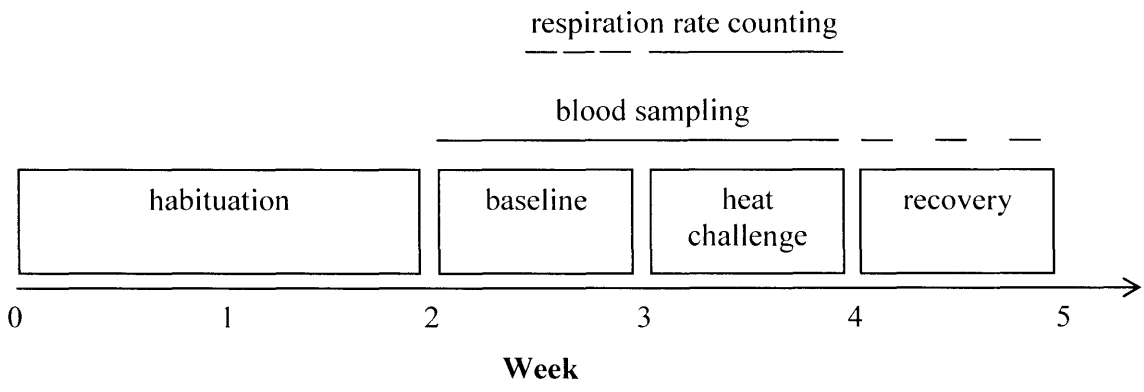
Figure 4.9 Temperature logger mounted on a sheep CIDR to hold it in place in the vagina.

4.3.1.3 Measurements

Individual feed and water intakes were measured daily in the climate rooms; measured volumes were provided and any remainder was measured the following day. Respiration rates were measured twice daily at 0800 and 2000 hours during the heat challenge week,

with baseline measurements taken at 0800 hours on each of the last 3 days of the baseline week (see Figure 4.10).

Figure 4.10 Timeline of the habituation, baseline, heat challenge and recovery periods showing when blood sampling and respiration rate counting occurred.



Baseline blood samples were collected at 0800 hours on the last 3 days of the baseline week, and at 0800 and 2000 hours during the heat challenge week. Samples were collected into two tubes in the morning, one for haematology and one for the rest of the analyses, and one tube in the evening. The samples collected for haemotology were gently mixed on collection, and processed within 2 hours using a Cell Dyn 3500 haematology unit (Abbott Diagnostics, USA) to give cell counts and concentrations for standard white blood cells, neutrophils, lymphocytes, red blood cells and haemoglobin. The samples collected for analysis of cortisol, prolactin and haptoglobin included the same baseline samples as for the haemotology, with the addition of evening samples (2000 hours) during the heat challenge week. Additional blood samples for more comprehensive cortisol and prolactin analyses were taken (0, 0.5, 1, 1.5, 2, 3, 5, 7, 11, 15 and 27 hours from heat on) during the heat treatment week. Controls for the additional samples were taken at the same time

points as the additional samples from 0800 hours on the second to last day of the baseline week. All non-haemotology blood samples were gently mixed, then stored on ice until processing. They were centrifuged and the plasma removed and frozen at -18°C until it was assayed. The vaginal temperature loggers automatically recorded body temperature at 5-minute intervals.

4.3.1.4 Laboratory assays

4.3.1.4.1 Cortisol

Plasma cortisol concentrations were determined using a commercial radioimmunoassay (Spectria Cortisol RIA, Orion Diagnostica, Espoo, Finland), adapted and validated for ovine plasma (Chapter 3). The intra-assay CV for samples containing 148.4, 70.8 and 32.5 nmol/l cortisol were 7.7, 9.2 and 9.6%, respectively. The inter-assay CV for the same samples were 12.6, 7.4 and 10.6%, respectively.

4.3.1.4.2 Prolactin

Plasma concentrations of prolactin were assayed in duplicate by double-antibody homologous RIA as previously described by McNeilly and Andrews (1974). The samples were assayed in duplicate 10 µl aliquots and the limit of detection was 0.6 ng/ml. The assay included 6 replicates of 3 control samples containing 13.3, 5.4 and 4.0 ng/ml which were used to estimate the intra-assay CV; which were 2.8, 4.1 and 6.3%, respectively.

4.3.1.4.3 Haptoglobin

Haptoglobin was assessed using a modified technique of Jones and Mould (1984), based on the principle that the haptoglobin/haemoglobin complex initiates a peroxidase reaction

which releases oxygen from introduced hydrogen peroxide, oxidising colourless guaiacol to a brown coloured tetraguaiacol.

Plasma samples were diluted in PBS, if necessary, to bring their optical density to within the linear range of the known standards. Plasma samples and standards (50 µl) were added in triplicate to wells of a microtitre plate (Sarstedt Australia Pty Ltd, Mawson Lakes, South Australia). An equal volume of methaemoglobin solution (0.12 mg/ml in 0.15 M NaCl solution) was added to all wells and lightly tapped to mix. Following incubation for 10 min at room temperature, 100 µl of guaiacol solution (Sigma Aldrich) was added (13.64 g guaiacol, 366 ml IM acetic acid and 384 ml MilliQ water; pH 4.0) and mixed (3 parts guaiacol with 2 parts 0.02 M H_2O_2). The absorbance was read at 450 nm on a plate reader after 3 minutes, and the haptoglobin concentration was automatically calculated. The inter-plate CV for quality control samples containing 0.015, 0.041 and 0.081 mg/ml were 9.4, 8.2 and 10.0%, respectively.

4.3.1.5 Statistical analyses

Feed intake, water intake, respiration rate and body temperature data were analysed using R statistical software (R Development Core Team, 2004). Individual period means were calculated for baseline and heat challenge periods, and analysed using a linear model, with baseline values as a covariate. The baseline values were taken on the last 3 days of the baseline week, the final one being 2 days prior to the start of the heat challenge. The raw means are presented as they have more biological significance than the adjusted means from the analysis.

Animal live-weights were analysed in Genstat (Lawes Agricultural Trust, 2005) using a paired 2-sample t-test for differences in weight at the beginning and end of the heat challenge period.

Cortisol, prolactin, haptoglobin and the haemotology data were analysed in Genstat using REML mixed models for repeated measures, with Treatment, Time, Treatment × Time and Rep as fixed effects. An average of baseline values for each variable was added to the fixed model as a covariate where appropriate (intensive prolactin, red blood cells, neutrophils, lymphocytes, haemoglobin and neutrophil:lymphocyte ratio). Data were log or lambda transformed as necessary to normalise distributions before analysis. The Wald statistic for each of the fixed terms and their interactions was tested for significance as a chi-square distribution before means were back transformed for presentation.

4.3.2 RESULTS

4.3.2.1 Feed and water intake, respiration rate and body temperature

Means for feed intake, water intake, respiration rate and body temperature during the four levels of heat challenge are presented in Table 4.3. When compared with the 20 and 25°C treatments, feed intake decreased ($P < 0.05$) and water intake increased ($P < 0.05$) as the temperature increased to 35°C. There were no significant changes in mean (± sem) animal live-weights ($t = 0.25$; $P > 0.05$), which were 31.7 (0.55) kg before treatment, and 31.7 (0.64) kg after treatment. Body temperature increased significantly from the 25°C treatment to the 30°C treatment ($P < 0.01$), and increased significantly again at 35°C ($P < 0.001$). The increase in body temperature was immediate (within 24 hours) in the two

hottest treatments, and in the 35°C treatment body temperature was still increasing at the end of the 5-day heat challenge (Figure 4.11). Of the variables presented in Table 4.3, respiration rate was the most sensitive to the heat challenge, differing significantly at each temperature increase ($P < 0.05$). Mean body temperature on the other hand rose significantly only between 25 and 30°C and between 30 and 35°C.

Table 4.3 Mean (sem) daily feed intake, water intake, respiration rate and body temperature responses during heat challenge of 20, 25, 30 or 35°C.

	Treatment			
Response variable	20°C	25°C	30°C	35°C
Feed intake (g)	1135^{a} (27.8)	1012^{a} (30.8)	1017^{ab} (90.4)	869^{b} (21.0)
Water intake (ml)	3201^{a} (123.1)	3110^{a} (84.0)	3620^{ab} (310.4)	4051^{b} (247.2)
Respiration rate (min^{-1})	100^{a} (4.4)	132^{b} (3.4)	184^{c} (2.6)	216^{d} (5.4)
Body temperature (°C)	39.3^{a} (0.03)	39.4^{a} (0.01)	39.8^{b} (0.01)	39.9^{c} (0.06)

^{abcd} Within row, means without a common superscript are significantly different ($P < 0.05$).

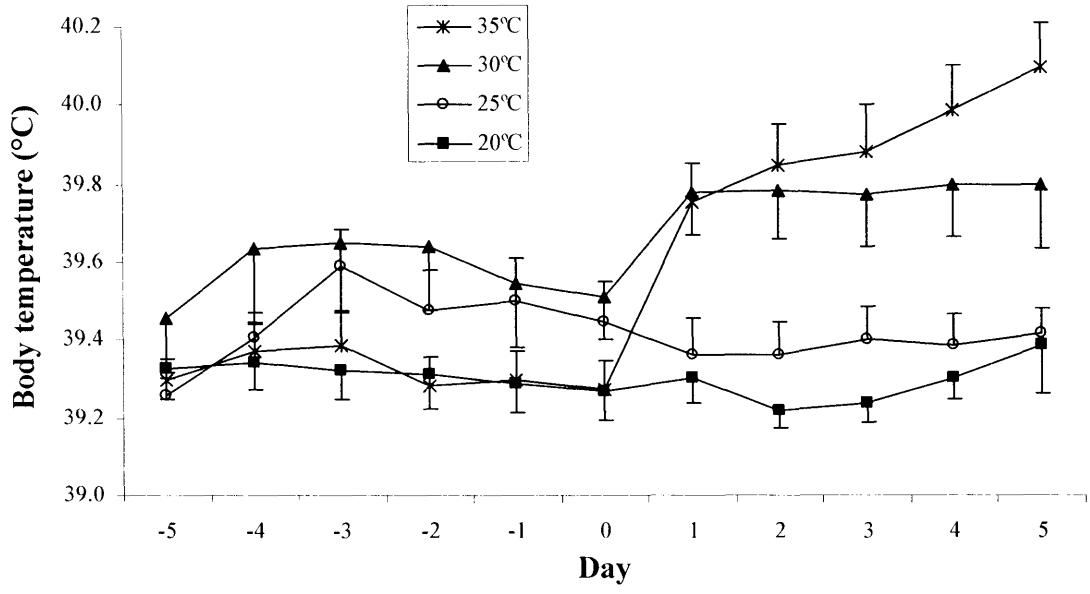


Figure 4.11 Mean (+ or – sem) internal body temperatures during periods of baseline (days -5 to 0; 20°C) and heat challenge (days 1 to 5) at 20, 25, 30 or 35°C.

4.3.2.2 Cortisol

There were no significant effects of treatment on mean plasma cortisol concentration ($P > 0.05$). There was a significant Time × Treatment interaction (Table 4.4; $P < 0.05$), but no consistent pattern was apparent. The additional 24 hour intensive sampling did not show any significant differences between the treatment periods, although there was a decrease in concentration over time during the baseline period ($P < 0.001$).

Table 4.4 Predicted (back transformed) means for plasma cortisol concentration (nmol/l) at 12 hour time points over 5 days during a heat challenge of 20, 25, 30 or 35°C.

		Treatment			
Time		20°C	25°C	30°C	35°C
Day 1	am	10.4^{a}	15.7^{a}	2.6^{b}	11.0^{a}
	pm	6.0	7.7	4.8	5.6
Day 2	am	3.6^{a}	14.2^{b}	5.5^{ab}	10.2^{b}
	pm	4.1^{ab}	4.3^{ab}	2.7^{a}	8.6^{b}
Day 3	am	10.8^{a}	8.4^{a}	2.8^{b}	10.8^{a}
	pm	8.5^{a}	2.2^{b}	13.5^{a}	7.4^{a}
Day 4	am	6.4	15.0	7.1	11.9
	pm	7.5^{a}	4.1^{ab}	9.1^{a}	2.4^{b}
Day 5	am	10.4	10.3	11.6	7.5
	pm	4.8^{a}	6.6^{ab}	15.8^{b}	5.8^{ab}

^{ab} Within row, means without a common superscript are significantly different ($P < 0.05$).

4.3.2.3 Prolactin

There was a significant Time × Treatment interaction for mean plasma prolactin concentration ($P < 0.05$), which remained low over the five days of heat challenge at the 20 and 25°C temperatures, but increased significantly at 30 and 35°C ($P < 0.05$; Figure

4.12). In addition to these data, Figure 4.13 shows the results of the intensive sampling period of 27 hours from the time of the heat being turned on. Plasma prolactin concentration was significantly higher in the 30 and 35°C treatments than the 20 and 25°C treatments within 2 hours ($P < 0.001$). The peak concentration was recorded in the 35°C treatment 7 hours after the heat was turned on, and was significantly higher than the other three treatments ($P < 0.001$). It remained that way for 10 hours before starting to decrease.

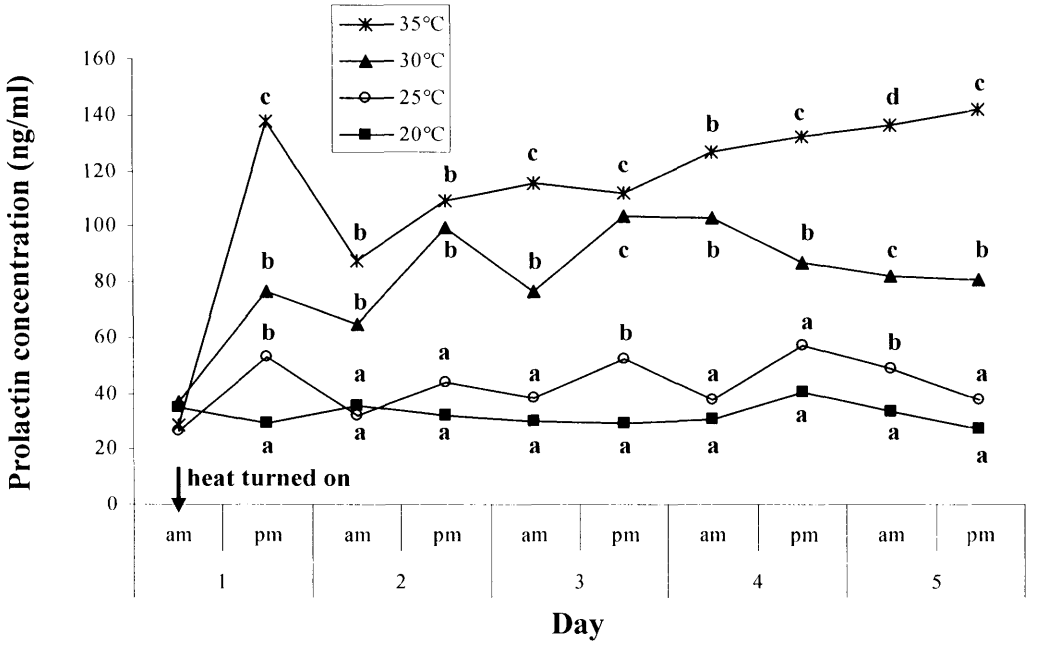


Figure 4.12 Predicted (back transformed) means for plasma prolactin concentration (ng/ml) at 12 hour time points during 5 days of a heat challenge at 20, 25, 30 or 35°C. Means within each time point, without a common superscript, are significantly different ($P < 0.05$).

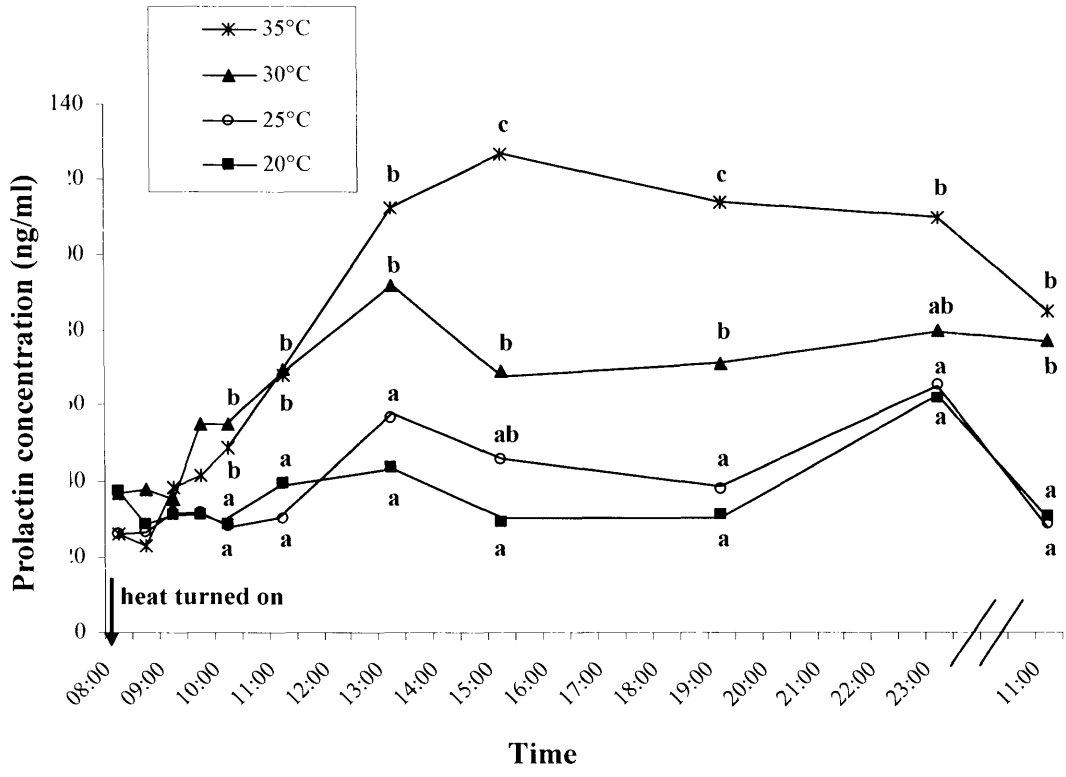


Figure 4.13 Predicted (back transformed) means for plasma prolactin concentration at 11 time points during the first 24 hours of heat challenge at 20, 25, 30 or 35°C. Means within each time point, without a common superscript, are significantly different ($P < 0.05$).

4.3.2.4 Haptoglobin

Mean plasma haptoglobin concentration decreased over time ($P < 0.001$), and there was a significant Time × Treatment interaction ($P < 0.05$; Figure 4.14) although treatments differed at only two time points on day 1 and day 5.

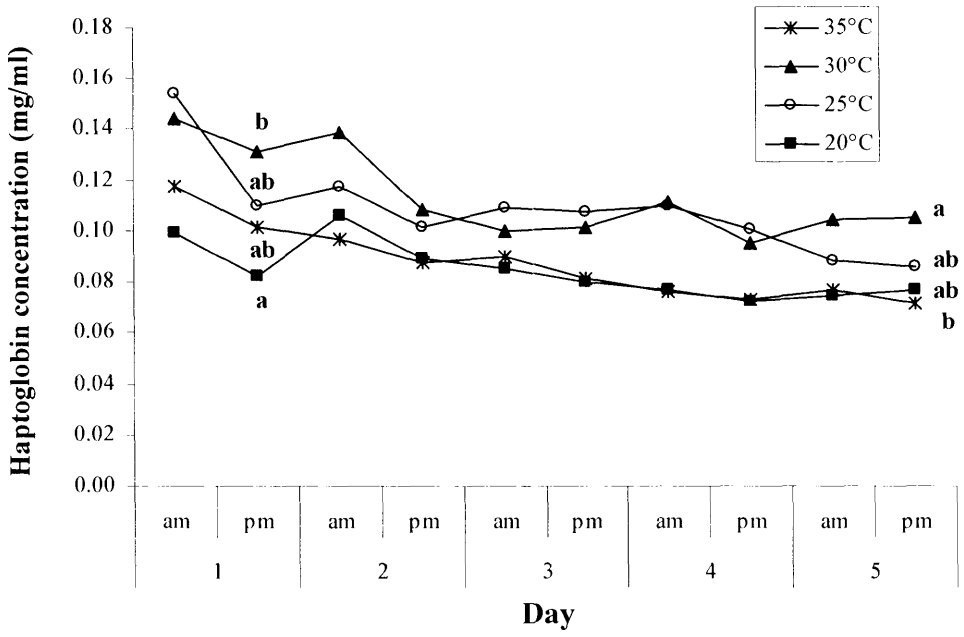


Figure 4.14 Predicted (back transformed) means for haptoglobin concentration (mg/ml) at 12 hour time points during 5 days of a heat challenge of 20, 25, 30 or 35°C. Means within each time point, without a common superscript, are significantly different ($P < 0.05$).

4.3.2.5 Haemotology

Data related to counts of white and red blood cells, neutrophils and lymphocytes, as well as haemoglobin levels and the neutrophil:lymphocyte ratio are presented in Table 4.5. The neutrophil:lymphocyte ratio increased from the 25°C treatment to the 30°C treatment ($P < 0.05$), but then dropped again at 35°C. There were no significant effects of treatment on any of the other haematology variables ($P > 0.05$). Time effects are presented in Table 4.6. There was a decrease in red blood cell count over the first three days ($P < 0.001$) before stabilising. Lymphocyte counts were higher on days 3 and 4 than on day 1 ($P < 0.05$). There was a significant Time × Treatment interaction ($P < 0.05$) for haemoglobin which decreased over time (Figure 4.15). However, there were only two treatment differences

and they were between 25 and 30°C on day 1 ($P < 0.05$) and between 30 and 35°C on day 5 ($P < 0.05$).

Table 4.5 Mean (sem) white blood cell, red blood cell, neutrophil, lymphocyte and haemoglobin responses during heat challenge of 20, 25, 30 or 35°C.

	Treatment			
Response variables	20°C	25°C	30°C	35°C
White blood cells (10^6/ml)	6.4 (0.08)	7.2 (0.02)	7.3 (0.05)	6.4 (0.14)
Red blood cells (10^9/ml)	7.7 (0.15)	8.4 (0.14)	7.4 (0.09)	7.5 (0.14)
Neutrophils (10^6/ml)	2.0 (0.10)	2.2 (0.03)	3.2 (0.05)	2.2 (0.08)
Lymphocytes (10^6/ml)	2.9 (0.07)	3.1 (0.03)	2.5 (0.05)	2.7 (0.05)
Haemoglobin (g/dl)	9.2 (0.14)	9.6 (0.14)	8.6 (0.09)	8.9 (0.16)
Neutrophil:Lymphocyte	0.8^{ab} (0.05)	0.8^{a} (0.02)	1.3^{b} (0.05)	0.9^{ab} (0.03)

[ab] Within row, means without a common superscript are significantly different ($P < 0.05$).

Table 4.6 Mean (sem) red blood cell and lymphocyte counts for all treatments (20, 25, 30 or 35°C) over the 5-day period of heat challenge.

	Day				
Response variables	1	2	3	4	5
Red blood cells (10^9/ml)	8.2^{a} (0.16)	7.8^{b} (0.16)	7.6^{c} (0.15)	7.6^{c} (0.17)	7.5^{c} (0.15)
Lymphocytes (10^6/ml)	2.7^{a} (0.13)	2.8^{ab} (0.12)	2.9^{b} (0.12)	2.9^{b} (0.13)	2.7^{ab} (0.12)

[abc] Within row, means without a common superscript are significantly different ($P < 0.05$).

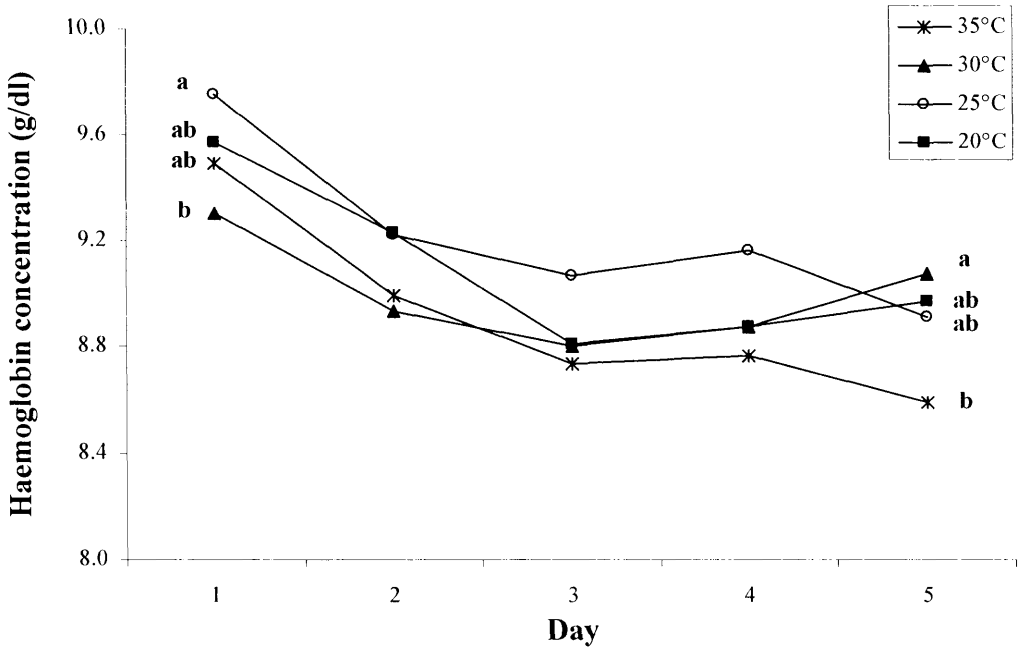


Figure 4.15 Predicted (back transformed) means for blood haemoglobin concentration (g/dl) during 5 days of heat challenge at 20, 25, 30 or 35°C. Means within each day, without a common superscript, are significantly different ($P < 0.05$).

4.4 DISCUSSION

4.4.1 Behavioural motivation to avoid a heat challenge

The number of times the sheep entered the cool pen increased with each increase in temperature, suggesting that the animals were using the cool pen to avoid the ambient temperature of the room, and therefore avoid the necessity for adaptive responses. This was confirmed by both the respiration rate results, which showed an increase at each temperature for the companion animals (without access to the cool pen), but no increase for the treatment animals (with access to the cool pen), and also the results for latency to re-enter the cool pen which decreased markedly with each temperature increase.

It is likely that the sheep worked to get into the cool pen because it was the least costly adaptive mechanism available for maintaining body temperature within its thermoneutral range. The respiration rates of the animals tend to confirm this, as respiration rates increase early on in a challenge, facilitating the maintenance of a healthy body temperature. It is clear that in this study respiration rates were kept low by the time spent in the cool pen, and that in turn would have stopped body temperatures from increasing above normal.

The lack of difference in motivation to enter the cool pen at the different temperatures was unexpected, although perhaps not so surprising, as maintaining a thermoneutral body temperature, at a particular ambient temperature, would require the same amount of time in the cool pen regardless of the work required to get there. This may mean that maintaining body temperature is more important than the cost of doing so, as long as the energetic cost associated with performing the required task does not itself cause an increase in body temperature. This could explain the drop in work intensity at FR25, because performing the greater number of panel pushes may indeed have caused an increase in the animals' body temperature, especially at the higher ambient temperatures. Because the net energetic efficiency and thermoregulatory efficiency of panting is very high in sheep subjected to moderate heat stress (Hales and Brown, 1974), it may be that a combination of panting and panel pressing for cool pen access is more efficient at the higher workload of FR25 than just panel pressing for cool pen access alone.

It is important to note that although there was no difference in motivation between the treatments, the elasticity coefficients for cool pen access were less than 1 (inelastic),

which means that the demand for cool pen access was high, regardless of increases in workload (Lea, 1978). The actual values of the elasticity coefficients in the present study were 0.14, 0.05, 0.07 and 0.02 for the 20, 25, 30 and 35°C treatments, respectively. In comparison, a study looking at demand curves for pigs working for access to food, social contact and a door opening stimulus reported mean elasticity coefficients of 0.02, 0.49 and 0.63, respectively (Matthews and Ladewig, 1994). Results from the present study also agree with results from Chapter 2, in which the average elasticity coefficient for food was 0.02 for sheep. Food is obviously a very important commodity for animal survival and thus is commonly used as the standard against which to measure the importance of other resources to animals (Dawkins, 1983; Matthews and Ladewig, 1994). Therefore, this study highlights the importance to sheep of being able to avoid the challenge of hot, humid conditions, particularly those above 25°C.

The latency measurements are necessarily related to the total number of cool pen entrances. It is no surprise then, that latency to re-enter the cool pen was longer at FR25. One explanation could be that the energetic requirements of pushing the panel were higher at FR25 than at the other FR values, as has been previously discussed in relation to the decrease in work intensity at FR25. Another explanation could be that the longer latency at FR25 reflects the time taken to perform that number of panel pushes. However, observation of the animals working would tend to suggest otherwise. The sheep knew their task well, and they could perform it either quickly or slowly. If performed quickly, 2 to 3 panel pushes per second would not be unusual. So it is true that it would take longer to work at FR25 than the other FR values, but not so much longer that it would affect the results over the 8-hour test period. This interpretation is supported by the fact that the

difference between the time taken to push the panel 1 and 10 times would be similar to the difference between the time taken to push the panel 10 and 25 times, yet there were no differences in the number of rewards earned at FR 1, 4 or 10.

The use of the cool pen at the lowest temperature treatment, during which the room was at the same temperature as the cool pen, was more than expected and requires explanation. The animals spent an hour in the treatment rooms before they were allowed access to the cool pen door. During that time they had consumed half of their daily feed. The other half was in the cool pen, thus, when they had access to the door, they worked to get in at a similar intensity at all treatments and all FR values. Although not ideal, the effect of the initial motivation to feed would have been the same across all treatments and FR values and so should not affect the overall results of the experiment. It should also be noted that the 900 g fed during the test days did not take long to eat.

It is also possible that cool pen use at the control temperature was associated with boredom. A similar event was reported by Matthews and Ladewig (1994) whereby pigs worked almost as hard to open a door to an empty room as they did for social contact. These authors suggested that the restrictive environment the pigs had been living in meant that the smallest stimulus change could have been very important. If that were true for the pigs, then it could also be true for the sheep in this study, as they too were kept in a very restricted environment over the period of the experiment. This theory is also corroborated by observations of animals working to open the door a number of times before entering the cool pen, most commonly during the 20°C treatment.

A concern arose about the fact that there was rubber matting on the floor of the cool pen (for temperature retention) and not on the floor outside of the cool pen. It appeared from observation that the animals might be going into the cool pen to lie down because it was more comfortable to lie on the mat. However, the results of an extra condition added to the experiment, whereby the mat was removed from the cool pen, showed that this was not the case. The animals entered the cool pen about the same number of times with and without the mat.

4.4.2 Physiological adaptation to a heat challenge

There were three main findings from this experiment. Firstly, the respiration rate results showed that there is some biological adaptation required from the sheep even at low levels of heat challenge; respiration rates were higher at 25 than 20°C. Secondly, the biological cost of adapting to the heat challenge presented, increased significantly at 30°C. This was illustrated by changes in respiration rate, body temperature and plasma prolactin concentration, which at 30°C were all significantly different from lower temperature treatments. Finally, body temperature and plasma prolactin concentration demonstrated that the animals did not adapt to the 35°C heat challenge, as values were still increasing at the end of the treatment period.

The results for feed and water intake in this study were consistent with the findings of other experiments investigating the effects of heat stress on sheep (e.g. Monty et al., 1991; da Costa et al., 1992; Abdalla et al., 1993). The reduction of feed intake is particularly significant in this study as sheep are more likely to decrease roughage than concentrate intake in hot conditions because digesting roughage increases heat production (da Costa et

al., 1992), and only concentrate was provided here. The decrease in feed intake under an increased heat load is due to reduced appetite, but feed utilisation is also increased (Silanikove, 1992), which may be why no weight loss occurred in this study.

Respiration rate in the present study was the most sensitive indicator of heat challenge, as was also found in the study by Lowe et al. (2002). The increase of respiration rate in sheep in response to increasing temperature has been well documented (Lee and Robinson 1941; Hales and Webster, 1967; Hales and Brown, 1974; Maskrey et al., 1981). A large range in both temperature and humidity have been studied with respect to effects on respiration rate in sheep and other animals. For example, Hooley et al. (1979) exposed Merino ewes to hot temperatures of 45 to 52°C (30 to 81% RH) for 8 to 10 hours per day, for 11 days, and found average respiration rates to be 164.7 breaths per minute. In comparison, respiration rates in the present study averaged 216 breaths per minute in the 35°C (70% RH) treatment; they were probably higher than in Hooley's study because in this study treatment was continuous for 5 days. Hales and Webster (1967) exposed Merino wethers to 40 to 60°C for 120 minutes with low humidity. They found that respiration rates peaked at 300 breaths per minute before dropping to 200 breaths per minute, reporting that animals breathed more slowly and deeply at the lower rate. This was termed second phase breathing by Bianca (1962) and was not observed in the present study. It may occur only when the environmental challenge is severe (Hales and Webster, 1967; Hales and Brown, 1974), and may also indicate that the biological cost of adaptation in that situation is extreme. In the present study, respiration rate results suggest that there is some biological cost to adapting to even a mild environmental challenge, as they increased from the 20 to the 25°C treatment.

Body temperature is the defining parameter with regard to biological cost, as most mammals die when deep body temperature reaches 42 to 45°C (Bianca, 1968). Body temperatures in the present study increased significantly in the 30°C treatment, within 24 hours of the heat being turned on, but stabilised thereafter and remained stable over the heat challenge period. At 35°C body temperatures increased daily, and were still increasing on the last day of the heat challenge. This is similar to findings of Lee and Robinson (1941) whereby rectal temperatures in sheep kept rising in a 41°C and 45% RH heat challenge treatment. Also, Lowe et al. (2002) reported an increase in the rectal temperatures of lambs up to and over 42°C in a heat challenge of 33°C and 85-100% RH. In that study also, body temperatures were increasing at the end of the treatment period of 12 hours. Although the animals were adapting to the heat challenge at the lower levels in this study by increasing water intake, decreasing feed intake and increasing respiration rate, they were unable to adapt to the hottest challenge by these methods as was indicated by the body temperatures not stabilising at 35°C. It is uncertain whether body temperatures would have stabilised if the heat challenge had lasted for longer.

The impact of the heat challenge in the present study on plasma cortisol concentration was almost non-existent, but previous studies in this area have reported different results. It was first found in 1960 (Robinson and Morris) that plasma cortisol increased during the first days of exposure to a hot environment, and that has since been backed up by other studies. Ghani (1988), for example, investigated short and longer term effects of a heat challenge on the plasma cortisol concentrations of Merino lambs. After measuring baseline cortisol concentrations at an average of 3.12 ng/ml at 18°C, lambs were exposed to 32°C for 3 days, after which time average cortisol concentrations were 7.14 ng/ml. However, after 30

days at 32°C, average cortisol concentrations of the lambs were only 2.02 ng/ml. Similarly, Minton and Blecha (1990) found that although cortisol concentrations increased rapidly in lambs exposed to 24 hours of heat challenge at 35°C, they decreased back to pre-treatment levels in 12 hours. One explanation for the difference found between the present study and previous work, is that the short-lived increases in cortisol in previous studies could reflect a response to changing conditions, environment and the sampling procedure rather than the heat challenge (Parrott et al., 1996). In support of this theory, the actual concentrations of plasma cortisol found in the present study were not much above baseline concentrations in the previously mentioned work, perhaps because in this study the animals had catheters inserted for the blood sampling, and they were very used to human handling. The consistently low levels suggest that there was no activation of the HPA axis in this study. It is likely that adaptations such as changes in feed and water intake and respiration rate were instead used to reduce the impact of the heat challenge.

Studies examining changes in prolactin concentration in relation to heat stress in sheep have found that it increased rapidly and stayed high over the period of heat challenge (Hooley et al., 1979; Parrott et al., 1996). The results of this study reinforce that finding. What has not been so clear before, however, is that the level of increase in plasma prolactin concentration is dependent on the severity of the heat challenge. The stress-induced increase in prolactin is not only seen for heat stress, although like many other stress responses it is specific to certain types of stressors. It does not change in response to a cold challenge for example (Parrott et al., 1996), but it does rise rapidly and sharply in rats as a response to restraint stress water (Fujikawa et al., 1995), and with electrical stimulation stress in sheep (Wolinska-Witort et al., 1986). It has been suggested by Salah

et al. (1995) that prolactin has a direct role in the regulation of body temperature in response to heat. They treated lambs with bromocryptine, a prolactin secretion inhibitor, and then exposed them to extreme temperatures (43 to 44°C) for 18 days. All lambs increased their respiration rates in the hot conditions, but, control lambs maintained their body temperatures, whereas treated lambs had elevated body temperatures. The authors proposed that normal prolactin secretion allowed the untreated lambs to regulate their body temperature, and that the bromocryptine interfered only by inhibiting prolactin release.

The profile of prolactin increase in the present study supports the conclusion that there is a biological cost to animals of adaptation to a heat challenge at the 30°C level, as that was where plasma prolactin increased, and, like respiration rate and body temperature, prolactin increased further at 35°C. Similar to body temperature, plasma prolactin concentration stabilised or decreased towards the end of the heat challenge period for all treatments except 35°C, where it was still increasing on the last day.

Haptoglobin can be used as a measure of the acute phase response, which is an innate body defence seen during acute illness and involves the increased production of certain blood proteins, termed acute phase proteins, of which haptoglobin is one. Haptoglobin concentrations increase in relation to acute infectious conditions, but were thought not to increase in non-infectious conditions in cattle (Skinner et al., 1991), and sheep (Skinner and Roberts, 1994). However, Murata and Miyamoto (1993) have reported that serum haptoglobin concentration increased in calves due to the stress associated with 2 days of road transport, and found a correlation between serum haptoglobin concentrations and

suppression of lymphocytes. These authors suggested that evaluation of serum haptoglobin concentration may offer a diagnostic aid to monitor the vulnerability of cellular immune function in calves which may appear after transportation. In contrast, Arthington et al. (2003) tried to determine whether haptoglobin concentration could be used as a reliable indicator of the stress-response of calves to weaning, transportation and commingling, but were unable to come to a conclusion, saying that further research was necessary. Skinner and Roberts (1994) used a level of 0.2 mg/ml of haptoglobin as being within the normal range, and as all plasma haptoglobin concentrations in this study were well below this range, it was concluded that haptoglobin was not a useful measure to indicate the biological cost of heat stress in sheep.

There was little change in the blood haematology variables measured in this study, which is similar to the results of other studies of heat challenge in sheep. Decreases in red blood cell count, white blood cell count and haemoglobin have been reported in a heat challenge study using a hot treatment of 25 to 46.5°C (on average 36.4°C), but none of the changes were significant (da Silva et al., 1992). In the present study there was a change in neutrophil:lymphocyte ratio that was statistically significant, however it is unlikely to be biologically significant because it increased at 30°C but then decreased at 35°C.

The adaptive changes made by the sheep at the different levels of heat challenge show that the levels used are ideally suited to incremental increase in stressors; they can be physiologically described, and therefore correlated to behavioural change. Although the behavioural motivation of the animals did not change with increasing heat challenge, use of the cool pen did increase with increasing ambient temperature, even at high workloads,

confirming the importance to the animals of maintaining a thermoneutral temperature. This result confirms the validity of the use of the model to test the relationship between behavioural motivation and physiological adaptation.

At only a 5°C increase in ambient temperature from thermoneutral (20°C), the respiration rate of the sheep increased, but if they had access to the cool pen they worked to spend enough time in it to completely negate that rise in respiration rate. However, after a week at 25°C, no other adaptive mechanisms had been activated, so the animals were not in danger of losing thermoregulatory equilibrium. Another small increase in ambient temperature, up to 30°C, and respiration rates rose again. In addition, feed intake decreased, water intake increased and body temperature rose; the biological cost to the animals of maintaining homeostasis has increased. Although body temperature increased early on in the heat challenge, it stabilised over the rest of the week, so the animals managed to adapt to a heat challenge of 30°C. Given the opportunity to work for access to the cool pen at 30°C, the animals spent 58% of the time available in that pen, and the increase in respiration rate was negligible, suggesting that no other adaptive mechanisms would have been required. When the ambient temperature increased to 35°C, feed intake decreased again, and further increases occurred in water intake, respiration rate and body temperature; at this level of challenge body temperature continued to increase until the animals were removed from the hot conditions. Sheep with access to the cool pen at 35°C worked at all four workloads to spend 86% of the available time in the cool. Once again, the associated respiration rate changes were negligible.

These heat challenge experiments indicate that the adaptive mechanisms required by sheep increase with increasing temperature, as does the biological cost to the animal of using those mechanisms. In addition, they have contributed valuable information about how hard the sheep are prepared to work to avoid the costs associated with having to activate the adaptive mechanisms. Each of these approaches is useful on its own, but there is increased value in studying them together. For example, sheep were prepared to work to get into the cool pen and spend up to 160 minutes inside, at 25°C. Should we take that to mean that they need access to a cool area at an ambient temperature of 25°C? It can be argued that we should not, because the only adaptive mechanism they had activated at that temperature was increased respiratory frequency, which is an efficient and low-cost way for the animal to lose heat. Looking at both of these approaches at the same time is invaluable for assessing the welfare of animals in particular situations; addressing their physiological requirements along with their choices and reinforcement mechanisms.

4.4.3 Conclusions

The aim of this series of experiments was to investigate the motivation of sheep to work for access to a cool pen, from a room heated at each of four different levels of heat challenge, for which physiological adaptive changes were also determined. The hypothesis was that the motivation of animals to enter the cool pen would increase at the point where the biological cost of adapting to the heat challenge increased significantly. This appeared not to be the case, as there was no difference in motivation to enter the cool pen at the different temperatures. However, there was an increase in work intensity with each temperature increase, suggesting that the animals were motivated to avoid the cost of having to adapt to the heat challenge, which increased with increasing temperature. This is

confirmed by the elasticity coefficients from the demand functions, which show an inelastic demand for access to the cool pen, signifying that the thermoneutral environment was very important to the animals, and comparable to the commonly used standard of demand for food.

Heat challenge was a suitable stressor to use to examine the links between the behaviour and physiology of Merino sheep in a stressful situation. The changes in the 'biological cost' of adaptation at each level of heat challenge were illustrated clearly, with the respiration rate alone changing at 25°C, body temperature and plasma prolactin concentration as well as respiration rate changing at 30°C, and feed and water intake, respiration rate, prolactin concentration and body temperature changing at 35°C. Also very significant was the fact that prolactin concentration and body temperature did not stabilise in the 35°C treatment which indicates that animals did not adapt to this heat challenge level. Although it was intended that the motivation of the animals would be used to determine the importance of avoiding the heat challenge, it appears that thermoregulation is too important to the animals and, therefore, a lack of differences in motivation at even the low levels of challenge were observed. This model is still very useful, however, as the number of times the animals went into the cool pen increased with increasing heat challenge, and this itself must indicate their motivation to avoid the heat, and thus fits in with the requirements of the model.

CHAPTER 5

General discussion

The commonly measured physiological responses of animals to stress, for example activation of the HPA axis and blood concentration of cortisol, are only a small part of the adaptive mechanisms activated in response to a stressor. The reason they are so commonly measured is because they are activated in response to a range of stressors, although not all. In order to objectively measure the welfare of production animals, the diversity of responses to any situation needs to be recognised, and therefore, a wide variety of measurements of different aspects of animals' physiological and behavioural responses will probably be necessary. This thesis suggests a model on which to base the choice of measures, namely using adaptive mechanisms which are vital to maintaining homeostasis.

In addition to just physiological or metabolic homeostasis, animals' perception of their situation is important. For example, an animal will activate responses to a perceived stressor, whether or not there is actually any danger present. An approach was required that could measure adaptive responses, both physiological and behavioural, and determine the relative importance of each. Therefore, the main objective of this thesis was to investigate the link or links between physiological and behavioural adaptive responses to a stressor. This has been achieved by investigating two stressors, feed restriction and heat challenge, which have validated the approach of measuring physiological adaptive

mechanisms in response to a stressor and examining the importance to the animal of avoiding the cost of using those responses.

The term 'biological cost' has been used here to indicate that during a stressor challenge, the mechanisms of adaptation that are activated beyond the usual daily requirements to maintain homeostasis, come at a price. The fact is, we do not know exactly what the 'cost' is to an animal, to mount a response to a stressful or challenging situation, even though we know a great deal about the processes themselves. Usually 'biological cost' is associated with evolutionary fitness, such as the ability to survive and produce offspring. The examples used in this study did not involve long-term stressors, but both feed restriction and heat challenge could affect survival and reproduction in the long-term, therefore the term 'biological cost' is considered appropriate.

The behavioural demand methodology used to measure the motivation of animals in both examples, may not have been entirely appropriate for measuring motivation to feed after restricted food intake, as it was applied in Chapter 3, because the initial drive to eat is not regulated by a need to return to a balanced energy state, but rather by a daily cycle of eating to satiety. However, it may be possible to investigate motivation to return to homeostasis by feeding the animals their daily ration after the feed restriction period, and then measuring their motivation to compensate for lost energy over the following 24 hours. If the feed restriction periods were long enough, and sufficiently different to detect differences in short, medium and long-term changes in motivation, the model could be validated.

The outcomes of the examples used in this thesis may be affected by the adaptive mechanisms that are activated in response to a particular stressor. It is possible that the mechanisms measured may not be directly, or indirectly, linked to motivation for stressor avoidance, in which case it would not be the physiology – behaviour link that was being examined. In that case, it might be concluded that motivation to alleviate the stressor is high because of the biological cost of a particular adaptive mechanism, whereas it is really the cost of an unmeasured mechanism that is determining the behaviour. For example, it was difficult to differentiate between the adaptive mechanisms that were directed towards energy replenishment in Chapter 3, and those that represented the daily hunger cycle. A study focussed on differentiating between these would make the feed restriction model more useful, and help in the interpretation of the results.

The example of heat as a stressor challenge in Chapter 4 described the physiology – behaviour link better than the feed restriction example, because the adaptive mechanisms in response to the heat challenges were activated in a sequential manner, and the behavioural response of using the cool pen increased with increasing intensity of the stressor. However, in this model too, there was a problem with the behavioural demand method used to measure motivation. In order for the method used in this model to show an increase in motivation with increasing stressor intensity, as was expected, the animals would have had to stop working for access to the cool pen at the lower temperatures when the workload increased. The fact that this did not occur only means that the animals were strongly motivated to access the cool area for a certain proportion of the time available at a certain temperature, regardless of the cost. Use of the cool pen did decrease slightly, but significantly, at FR25, suggesting that perhaps it was not the method of measurement at

fault, but rather the choice of workloads. The respiration rates of the treatment animals also increased at FR25 in the 25, 30 and 35°C treatments, indicating that at this workload the animals were having to make a choice about which of the available mechanisms to use to maintain thermal balance. The assumption is that they will choose the least biologically costly option, which it certainly appears that they did. Therefore, if some higher workloads were included, the animals may be forced to make more choices about which responses to make, and even more information would be gained on the link between physiology and behaviour, and the relative cost to the animals of each available response.

The use of work intensity as a measure motivation was relevant in the individual variation study in Chapter 2, although this was not recognised at the time the experiment was conducted. An animal which works harder than another, and obtains more rewards, must be more motivated. This should be taken into account, and it is for this reason that I would advocate using larger numbers of animals in behavioural demand studies. This may be being recognised by researchers, although not explicitly stated, as in recent studies numbers of animals tested has generally increased. It may have been beneficial to address the issue of work intensity in the design of the feeding motivation study, perhaps by feeding a measured amount and comparing how quickly it was consumed between treatments, although, having that information would not have helped to determine motivation to restore energy balance, which was what was required for the feed restriction model. However, a measure of work intensity should be considered as an indication of the strength of motivation in the heat challenge study, because the number of rewards obtained increased significantly with each increase in temperature, even though they barely changed with workload.

Therefore it can be concluded:

- That the technique of measurement of behavioural motivation should include a measure of work intensity, particularly when the use of, or access to, a resource is defended strongly, and that mean number of rewards obtained is a better measure of work intensity than the y-intercept of the demand function.

- That it is necessary to measure the adaptive mechanisms which drive behavioural motivation, so that the relative importance of each can be defined by changing stressor intensities and required workloads.

- That the behaviour – physiology link with respect to adaptive mechanisms activated in response to a stressor, provides a novel approach to the measurement of animal welfare.

The conclusions imply that this model for measuring animal welfare, a combination of measurements of adaptive responses to a stressor, could be applied to many different conditions of concern in the livestock industries, for example: animal transport, cage or pen size, handling, isolation and many more. With regard to transport, as an example, there are many things going on at one time that an animal has to adapt to; such as the stress of handling, feed and water deprivation, close contact with conspecifics and often extremes of heat or cold. Measurements of acute stress responses from the HPA axis, combined with metabolic adaptive responses, respiration rates if possible, body

temperatures and other adaptive mechanisms specific to the stressor being imposed, if taken at sequential time points along the journey, should indicate how well the animals are able to adapt, and when the biological costs of adaptation increase significantly, such that the animal struggles to maintain homeostasis. Testing the motivation of the animals to avoid or alleviate the stressful situation could be achieved by training an operant response relevant to the stressor, for example, one that allows the animals to escape the conditions by leaving the pen or room.

Further investigation will be necessary into the adaptive mechanisms that influence behavioural motivation, so that the behaviour – physiology link being investigated is relevant to the stressor imposed. For example, the adaptive mechanisms used by sheep to maintain thermoregulatory balance are relatively well known, when compared to those activated to maintain energy balance in the medium to long-term. If the adaptive mechanisms which are relevant to maintaining homeostasis in response to a particular stressor are not already clear, they may have to be defined before this model can be used to determine an animal's welfare with respect to that stressor.

Future work involving the development of this model might include incorporating some emotional component, to investigate how an animal feels about being in a particular situation, in addition to the biological costs. It may be possible to use a component of the behavioural demand methodology used here, such as the intensity with which an animal responds in order to avoid or escape from a stressor, combined with a physiological measurement such as prolactin concentration, which is known to increase in response to some emotional stressors.

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