Behavioural and Electrophysiological Characterisation of Sleep in Sheep and its Application in Animal Welfare Studies

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Thesis Abstract

Major aims in the study of animal welfare are to try and understand the subjective mental experience of animals, to develop methods to assess their responses to changes in mental state and to use this information to enhance animal welfare. One of the most profound changes of mental state observable in all mammals is the change between wakefulness and sleep. Electrophysiological measurements, when combined with behavioural observations, provide a powerful means of characterising the states of sleep and wakefulness of animals. The spectral components of an electroencephalogram (EEG) reflect the differences in the electrical activity of the brain between sleep and wakefulness.

When humans undergo an aversive, stressful, disturbing, or worrying experience during wakefulness, their subsequent sleep can be affected. The present series of investigations examined the hypothesis that sheep exposed to aversive husbandry procedures would experience disturbances to their subsequent sleep. A sleep disturbance might provide an indication of the effect of an aversive husbandry procedure on the mental state of a sheep, that would not otherwise have been detected using conventional methods such as behavioural observation, blood biochemistry and heart rate.

Non-invasive electrophysiological hardware and software developed and used for human sleep studies, was adapted and used to study sleep in sheep. To assess the effectiveness of surface electrophysiological recordings to detect changes in the electrical activity of the brain of a sheep, three validation studies were carried out. They consisted of a) the post-mortem passage of electrical current through the head of a sheep; b) changes in EEG in response to depth of general anaesthesia and c) the EEG responses of a sheep in a sleep posture to an auditory stimulus.

The method was then applied to characterise the sleep of six, housed, adult ewes. Three percent (± 0.2) of a 24-h period was spent in rapid-eye-movement (REM) sleep and 15% (± 2.4) was spent in Non-REM sleep.

Three experiments were undertaken to assess the effects of potentially aversive husbandry procedures on subsequent sleep. These consisted of a) movement to a novel environment; b) an 8-h road transport journey and c) a 29-h space

restriction period (simulating times and space allowances used during road transport). Changes were seen in the distribution, quality and quantity of sleep. Although there were no significant effects on the duration of REM sleep or Non-REM sleep, in two experiments, an increase in the number of REM sleep bouts was seen post-treatment. In all experiments, a post-treatment increase in the percentage of slow waves was seen in Non-REM sleep.

This work provided a greater understanding of the impact of potentially aversive husbandry procedures on rest and sleep in sheep. All three of the potentially aversive husbandry procedures used as experimental treatments were associated with changes in subsequent sleep that may have been indicative of aversive experience during wakefulness. Although the changes in sleep found post-treatment were not large, they were consistent and reliable and therefore the methodology has potential for use in other applied animal welfare studies.

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Declaration

This thesis is of my own composition and the work presented is entirely my own. Any contribution by others to any part of the thesis has been acknowledged.

Fritha Mary Langford
August 2006

In Loving Memory Of
Peter Easdell Dunklin
1925-1989

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First and foremost, I would like to thank my principle supervisor, Dr Michael Cockram. He will be the first to admit that he is not the easiest person to work with but, overall I have enjoyed working with him and shared in his (slightly weird) sense of humour. He has put in so much time to make sure that this project was successful and I can't thank him enough. I also really appreciate the fact that if ever there was a problem with the experiments (be it exploding mains converters, or sick sheep) he was always ready to drop everything and come to help. In addition, I would like to express my gratitude for the hours of work he has done in order to read and comment on the drafts of this tome. I always appreciate a diagram or two, and I can almost read his handwriting now!

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I have had a number of undergraduate and postgraduate students working with me throughout these investigations and all of their help, fun and friendship has been much appreciated. Their roles in analysing some of the data appears in the next section. I'd especially like to thank Anna Hollingsworth and Emma Baxter for their help during the early preliminary trials, our many failures –and successes, and their hours of patient computer and video work when we hadn't really worked out how to use the programmes. Jenny Gibbons deserves a medal for her work on the transport project, she should know by now never to work with electrical equipment! I like to express my gratitude for her assistance in running the experiment and her support when things didn't work out in the way they should. Ishbel Baird, Carline van der Pol

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Description of the Author's Role

Michael Cockram contributed throughout the project to experimental design, choice of analysis tools and the presentation of the data.

Chapter 2-preliminary experiment

Michael Cockram and I were jointly responsible for the experimental designs. Non-purchasable equipment was designed and built by Harry Brash. The experiment was carried out by myself, with help from Anna Hollingsworth and Emma Baxter. I was responsible for all of the electrophysiological analysis, the statistical analysis and the writing of the manuscript. Anna carried out analysis on the effect of the equipment on the behaviour of the sheep (as part of her Zoology BSc). Emma carried out the Lag-sequential analysis in 'Observer' (as part of her MSc).

Chapter 2-validation experiments

Michael Cockram and I were jointly responsible for the experimental designs. The 'Arousal stimulus' experiment was carried out by myself, with help from Emma Baxter. I analysed the electrophysiological data and statistics. The electrical signal experiments were carried out by myself and Hugo Black (with help from Harry Brash in design and Jock McCreacken in practicalities). Hugo and myself jointly analysed the data from these investigations (As part of his Zoology BSc). The anaesthetic experiments were designed by Michael Cockram and were carried out by him (with expert help from Iain the brain surgeon and the staff at the Marshall building). I analysed the electrophysiological data with help from Hugo Black.

Chapter 3 –Novel Enviornment experiment

I was responsible for the experimental design and carried out all of the experiment with contribution from Michael Cockram and help from Emma Baxter, Lesley Smith and the farm staff. I was responsible for all of the electrophysiological analysis, the statistical analysis and the writing of the manuscript.

Chapter 4 –Transport experiment

I was responsible for the experimental design with contribution from Michael Cockram. Myself and Jenny Gibbons carried out the experiment (with help from our drivers). Jenny carried out the analysis of the electromyogram for posture of the sheep during transport (as part of her MSc). I was responsible for all rest of the

electrophysiological analysis including all of the sleep analysis. I was also responsible for all of the statistical analysis and the writing of the manuscript.

Chapter 5-Space Allowance experiment

I was responsible for the experimental design with contribution from Michael Cockram. Myself, Ishbel Baird, Carline van der Pol and Jade Spence carried out the experiment. Ishbel carried out some of the postural analysis during the space restriction period (as part of her Zoology BSc). Carline carried out the analysis of the electromyogram for posture of the sheep during the space restriction period (as part of her MSc). Jade carried out the analysis of the electromyogram for posture of the sheep before and after the space restriction period (as part of her MSc). I was responsible for all rest of the electrophysiological analysis including all of the sleep analysis. I was also responsible for all of the statistical analysis and the writing of the manuscript.

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List of Abbreviations

EEG	Electroencephalogram
EOG	Electro-oculogram
EMG	Electromyogram
ECG	Electrocardiogram
REM	Rapid Eye Movement
Non-REM	Non Rapid Eye Movement
SAS	a statistical programme
GLMM	Generalised Linear Mixed Model
Proc MIXED	Procedure for Mixed Models in SAS
REML	Residual maximum likelihood
CS	Compound Symmetry
se	Standard Error
h	Hour
s	Second
d	Day
min	Minute
L	Light
D	Dark
FFT	Fast Fourier Transform
Hz	Hertz
dB	Decibels
CCTV	Closed Circuit Television

Chapter 1. Sleep in domesticated animals and its application to animal welfare studies: using sheep as a model.

1.1 Abstract

One of the most difficult aspects of the study of animal welfare is understanding the subjective mental experience of animals and how they feel about the situations in which humans use them. Scientific studies of animal welfare attempt to develop methods to assess animal changes in mental state in response to their environment, and to use this information to enhance animal welfare. One of the most profound changes of mental state observable in all mammals is the change between wakefulness and sleep. Sleeping mammals share similar characteristics, which are measurable: specific behaviours, changes in responsiveness to external stimuli, electrophysiological characteristics and neurochemical changes. Although sleep is a ubiquitous behaviour in the life of mammals there has been relatively little research on this topic in domesticated animals. All animals are motivated to sleep and this motivation increases after a prolonged period of wakefulness. In humans, sleep can be affected by what has occurred in the prior period of wakefulness, and this has been demonstrated in some non-human mammals. An important aspect of human sleep medicine is the association between stress and subsequent sleep disturbances. There is potential to examine whether these relationships exist in domesticated animals and to study their significance for animal welfare. Characterising animal sleep and then studying any changes in amount, bout length, distribution or type of sleep after exposure to potentially stressful events can help us understand how animals respond to changes in their environment.

Sheep are animals that are easy to handle. They respond well to habituation and training; and they are a similar size to humans. Taking these factors into consideration, sheep make a good species on which to test the hypothesis that potentially aversive waking experience affects subsequent sleep.

1.2 Introduction

In the study of animal welfare, scientists try to ascertain what animals feel about their experiences (Duncan, 1993). Various methods are used to obtain objective data from animals. These include blood assays of hormones and behavioural observation, but it is difficult to know how these measures relate to the subjective mental experience of animals. One aspect of applied ethology in which there has been little research concerns a behaviour that can tell us something about the mental state of an animal in addition to traditional objective measures of behaviour. This behaviour is sleep (Lima et al. 2005), which is often not considered as a behaviour, but merely inactivity. This attitude is misplaced (Anderson, 1998), as sleep is a very important behaviour in the life of all mammals and is a mental state in itself (if not two mental states –refer to section 1.3.2 below). For some mammals such as cats, sleep is the major behaviour/mental state in which they spend their lives, they may spend around 65% of their lives asleep (Allison and Cicchetti, 1976). Other mammals such as sheep, spend less time asleep. However, sleep still takes up about 15% of a sheep's life (e.g. Ruckebusch, 1972).

The differences between wakefulness and sleep are similar across mammalian species. Differences are measurable through behavioural changes such as adopting sleep specific postures and using particular sleeping sites; there are electrical and neurochemical changes in the brain and differences in the levels of responsiveness to environmental stimuli.

In addition, there is evidence from human sleep medical research that waking experiences and sleep are inter-connected. The motivation to sleep increases as the time since prior sleep increases. Moreover, evidence in humans suggests that waking experience can affect subsequent sleep and that sleep disturbances can affect subsequent waking performance (e.g. Åkerstedt et al. 2000). There is potential to examine whether waking experience and sleep are inter-connected in domesticated animals and to use this information to assist when assessing the welfare of animals.

Non-human mammals do differ widely in the quantity and distribution of sleep compared with humans. Therefore, research into the effects of waking experience on sleep in each new species would have to start with a comprehensive characterisation of sleep in that species, before studying any changes after exposure to potentially

stressful events. The present study uses sheep as an 'example' species: they are easy to handle, respond well to repeated human contact and –usefully for electrophysiological studies– are of a similar size to humans. Taking these factors into account sheep, make a good species on which to test the hypothesis that potentially aversive waking experiences affect subsequent sleep.

1.3 Sleep

1.3.1 *Characteristics of sleep behaviour*

1.3.1.1 Specific behaviour relating to sleep

It is important to remember that sleep **is** a behaviour. However, as most of the measurable activity during sleep occurs in the brain, it is useful to recognise other behaviours that are associated with sleep.

a) Sleeping sites

Many animals choose specific sites in which to sleep. They may use these areas only to sleep, not just for other resting behaviour at other periods during the day. The sleeping sites often act as protection from predators for prey animals; many small mammals sleep in burrows, and monkeys sleep in nests. Sleeping sites usually afford the animal some shelter or comfort (see the description of cleft making by mountain sheep in Geist, 1971). All mammals of the same species will generally choose the same type of sleeping site (Anderson, 1998).

b) Prolonged immobility

A sleeping animal can be recognised by the fact it does not move about during sleep. During sleep there can be minor changes in posture; twitches and –especially in young mammals– suckling, but most mammals (except cetaceans, see below) stay in one place during a sleeping bout (Tobler, 1995).

c) Sleeping postures

Furthermore, the postures adopted by mammals when sleeping are often characteristic and repeated, and they are a reliable indicator that a sleep bout either occurring, is just about to occur, or has just finished. The posture adopted varies according to species –obviously bats hang upside down during sleep, not a posture seen in most other mammals– but generally the posture chosen is a relaxed one, allowing for a reduction in muscle tone with deepening sleep. Some mammals, such

as horses, can undergo light sleep when standing up, but all mammals (except cetaceans) must adopt a relaxed posture (e.g. lying down) during deeper sleep. Whales and dolphins overcome the difficulty of being asleep in the water, whilst needing to breathe air: they are able to sleep with one side of their brain at a time ('unihemispheric sleep' see Lyamin et al. 2002).

Such specific sleeping postures could be used to measure sleep approximately. For example, adult cattle undergo deep sleep lying down with their heads resting on their flanks. Careful quantification of this posture during 24-h could give an approximate distribution and amount of sleep. This could be used to examine differences in sleep behaviour in cattle living in different housing (e.g. differences between those in straw yards and cubicle housing) (Krohn and Munksgaad, 1993). Tromp et al (1990) showed that the three different sleeping postures in the rat were related to specific changes in the spectral properties of the EEG. The authors suggested that with careful characterisation of these postures, some sleep research could be carried out without the use of EEG (Tromp et al. 1990).

In humans, a horizontal posture is essential for undisturbed sleep. Aeschbach et al (1994) showed that humans have a disturbed sleep when made to sleep in an upright seated posture in airline style seating. It is possible that other mammals may experience disturbed sleep bouts if they are unable to perform their preferred relaxed posture.

1.3.1.2 Responsiveness to external stimuli during sleep

In humans, the change from 'consciousness' to 'unconsciousness' in the transition from wakefulness to sleep is the most obvious change in mental state that humans can observe about both themselves and others (Vaderwolf, 1992). There is a change in both awareness and the responsiveness of humans to their environment during the transition from wakefulness to sleep and within the levels of deepening sleep (Williams et al. 1964). Humans and non-human mammals need a higher stimulus (such as increased voltage for a shock stimulus) to provoke a behavioural response, when in deep sleep than in light sleep (Dillon and Webb, 1965; Vaderwolf, 1992). To provoke a response from a sleeping human, the stimulus needs to be either intense, or relevant to the human (e.g. their name –Bastuji et al. 2002). In non-

human animals too, there is a change in the level of awareness and responsiveness from waking to sleeping that can be observed. Auditory stimuli have to be higher in a rat that is sleeping than in one which is awake and there are differences in the arousal threshold level of rats in different stages of sleep (Neckelmann and Ursin, 1993). It is probable that non-human mammals may be similarly easier to awaken using an ecologically relevant stimulus, such as alarm calls, or predator calls, for sleeping prey animals. Van Twyver and Garrett (1972) trained rats to associate an auditory tone with a foot-shock (that they could avoid by a behavioural response), so that the tone became 'meaningful' and 'relevant' to the rats. The arousal threshold was lower during sleep when the meaningful tone was played as compared with a tone that had not been associated with anything (Van Twyver and Garrett, 1972). In addition, there were arousal threshold differences between light and deep sleep when the meaningful stimulus was applied, rats needed a louder stimulus to respond when in deeper sleep.

1.3.2 *Electrophysiological characterisation of sleep*

Sleep is often thought of as merely a period of inactivity. However, the brain remains active throughout sleep. The electrical activity from the brains of mammals can be recorded by means of the electroencephalogram (EEG). (For more details on EEG techniques and equipment, see chapter 2 of this thesis). The changes in electrical activity in the brain result in differences of voltage potentials that can be measured on the surface of the brain, or the surface of the scalp.

The different electrophysiological characteristics of the stages of sleep during the 'normal, healthy, adult', human sleep cycle and a standardised method of scoring them, have been described in detail in the manual edited by Rechtschaffen and Kales (1968) (see sections 1.3.2.1 to 1.3.2.5, below for a summary). Since the publication of the manual in 1968, there has been some debate in human sleep research, as to how 'standard' the criteria given in the Rechtschaffen and Kales' manual are, and how 'abnormal' or 'unhealthy' one has to be before one falls outside of the standard sleep stage scoring (Hobson, 1969; Himanen and Hasan, 2000). However, this debate is outside of the scope of this review and therefore the terminology used in the manual shall be used here to describe 'typical' human sleep stages.

In humans, there are four stages of Non-Rapid-Eye-Movement sleep, sometimes referred to as 'slow wave sleep', 'deep sleep' (or 'light sleep') or 'quiet sleep'; the criteria for each stage defined entirely by changes in the EEG. The 'fifth' stage is that of Rapid-Eye-Movement sleep sometimes referred to as 'activated sleep' or 'paradoxical sleep' defined by both the EEG, eye movements and alterations in muscle tone. For an example of the sleep EEG traces taken during each of the human sleep stages see Figure 1.1 below (page 9).

1.3.2.1 Human sleep stage I

During wakefulness, the EEG of the healthy, adult human shows two basic patterns of electrical activity: alpha (so called as it was the first to be described) and beta activity. During active wakefulness, beta activity (low voltage, irregular activity of 13-30Hz) is the main activity found in the EEG. As the person relaxes, (especially if they close their eyes) alpha activity predominates in the EEG. Alpha activity consists of regular waves of 8-12Hz, the voltage of which is higher than the beta activity, as it is more synchronous. As the person starts to go to sleep, alpha activity becomes interspersed with a few seconds burst of theta activity. Theta activity is consists of waves of 3.5-7.5 Hz. In addition, there are slow eye movements and a general lowering of muscle tone throughout the body. Stage I sleep occupies the first ten minutes of sleep in a healthy human adult. Stage I sleep also occurs for a couple of minutes after arousals during sleep.

1.3.2.2.Human sleep stage II

The second stage of human sleep is very similar to the first, in that it consists mainly of alpha activity interspersed with theta waves. However, it is generally more irregular and the periods of alpha and theta are shorter than in stage I. The main difference between stage I and stage II sleep is the presence of sleep spindles and K complexes in stage II sleep. Sleep spindles are short bursts of waves of 12-14Hz. These bursts last about three seconds and occur two to five times a minutes during stage II sleep. Sleep spindles are present in other stages of sleep, but at a much lower rate than sleep stage II. K complexes are only found in stage II sleep in healthy humans. They consist of a short, single, high voltage waveform and occur once a

minute during stage II sleep. They can occur more frequently if the human is asleep in a noisy or uncomfortable environment (Aeschbach et al. 1994). K complexes occur at the greatest density in the stage II sleep at the start of the night and are found at a lower density at each subsequent bout of sleep stage II (De Gennaro et al. 2000). This follows the pattern of density distribution of the slowest waves in sleep (see below) and therefore K complexes have been postulated as the forerunners of the slow waves that are present in sleep stages III and IV (De Gennaro et al. 2000). Sleep stage II generally lasts for about 15 minutes when a person first goes to sleep.

1.3.2.3 Human sleep stage III

Stage III sleep is the first stage of 'deep sleep' or 'slow wave sleep' in current sleep research terminology. This stage consists of delta activity (30 to 50%, interspersed with some theta activity). Delta waves are high voltage, (i.e. highly synchronous) slow waves of less than 4 Hz. Humans in stage III of sleep are difficult to arouse and will exhibit almost no eye movements and much reduced muscle tone. Humans exhibit sleep stage III for about 20 minutes during the first period of sleep and during this time the percentage of delta waves increases.

1.3.2.4 Human sleep stage IV

The EEG then becomes almost exclusively delta activity (>50%) and this is known as sleep stage IV. The electrical activity of the brain is highly synchronised (leading to higher voltage EEG traces) with slow cycles, some lasting over a second in duration. This stage lasts for about 45 minutes of the first period of sleep. Humans in this stage lie very still and are the most difficult to arouse. If aroused from sleep stage IV, humans report feeling 'groggy' and confused (Williams et al. 1964).

1.3.2.5 Human Rapid Eye Movement sleep

Until the 1950s, sleep was considered a single state. EEG's used in human sleep research were only run for about an hour as they were expensive and time consuming to analyse. This meant that, unwittingly, only one type of sleep had been recorded. In healthy, adult, humans, the second state of sleep does not appear until after 90 minutes of the first state of sleep. In 1953, rapid-eye-movement sleep (REM

sleep) was discovered by accident, by Aserinsky and Kleitman (1953). They noticed that after 90 minutes of 'deepening' slow-wave sleep an abrupt change was seen in both behaviour and the EEG. The posture becomes completely relaxed, and only eye movements and occasional twitches are seen during this stage. The eyes start to rapidly move back and forth beneath the eye-lids (hence the name). The EEG also changes abruptly, from the slow high voltage waves of sleep stage IV, to what looks very similar to the waking EEG. The REM sleep EEG is desynchronised (i.e. low voltage) and consists of beta activity interspersed with short bursts of theta activity.

Further investigation, using other electrophysiological measurements such as the electromyogram (the EMG –to measure electrical activity generated by muscle contraction) and the electro-oculogram (the EOG –to measure electrical activity from eye muscle movements) showed that the observed behavioural changes had characteristic electrical patterns that could be used to help identify REM sleep. The relaxed posture was shown to be a complete reduction of muscle tone (except for the eye muscles) and the human body becomes paralysed during REM sleep. The EOG recorded the eye movements that occur during REM sleep and they were characterised by high voltage high frequency waves occurring in ten to twenty second bursts. REM sleep lasts for about 20 minutes during the first period of human sleep.

REM sleep is sometimes referred to as 'paradoxical sleep', the EEG contains beta activity which is primarily seen during wakefulness, yet people are very much asleep during this sleep stage. Humans in REM sleep are difficult to awaken, but when woken they report feelings of alertness, quite different from being woken from Non-REM sleep. In addition, when woken from REM sleep humans will usually report that they have been dreaming (only reported in 20% or so of the times when humans are woken from other sleep stages –Bosinelli, 1995).

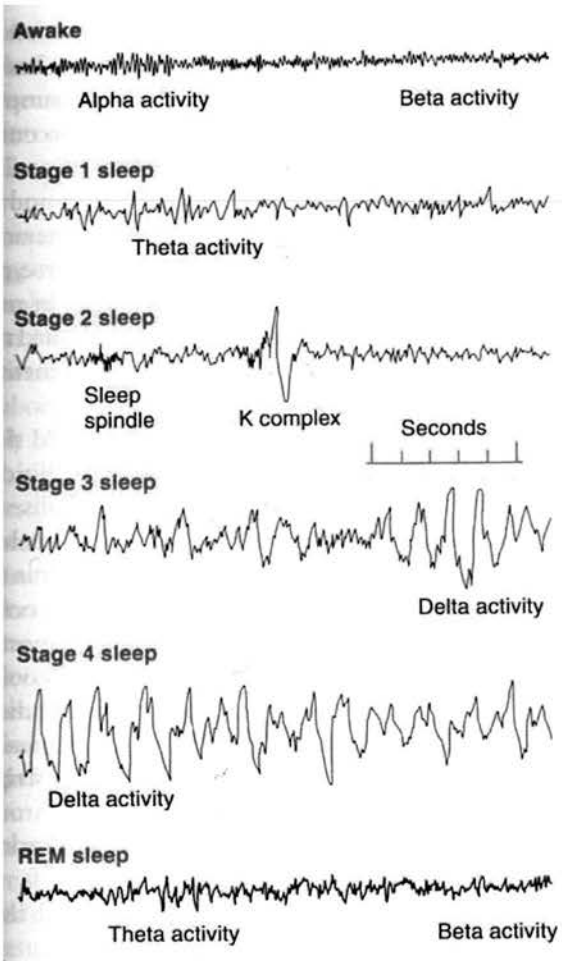


Figure 1.1 EEG recordings of the stages of human sleep. (From Horne, 1988)

1.3.2.6 The human sleep cycle

The sleep stages I-IV are bracketed together as Non-REM sleep. The total sleep cycle of deepening Non-REM sleep and REM sleep takes about 110 minutes during the first period of human sleep. After the REM sleep bout there may be a brief awakening before the person goes back into Non-REM sleep. During the

second and subsequent cycles of sleep the time spent in sleep stage I is very short and the time spent in sleep stage IV becomes shorter over each cycle. The period of time spent in sleep stage II increases over the cycles and REM sleep increases to approximately 30 minutes in each cycle. Each complete cycle lasts around 90 minutes (measured from the middle of one REM sleep bout to the next). Humans tend to go to sleep in one main period during the 24-h, not including very short periods of wakefulness after each bout of REM sleep. Therefore, humans sleep in a monophasic distribution (although the distribution of sleep throughout the day differs according to age –children and the elderly take more naps, and culture –for example, the siesta of the Mediterranean region). The human sleep cycle can also be altered during illness (including mental illness, see section 1.5.3.2 below).

1.3.2.7 Non-human animal Non-REM sleep

Sleep in animals other than humans is also split between two main states: REM and Non-REM sleep. Most of the sleep research in non-human animals has been carried out in the laboratory rat, consequently the rat has the most clearly described sleep behaviour. Other animals commonly used in sleep research are mice, cats and some species of monkeys. Our knowledge of sleep in animals other than these is often as a result of single research papers by diverse research groups (e.g. sleep in the sea lion –Lyamin et al. 2002; sleep in the platypus –Siegel et al. 1999). However, two states of sleep have been found in every mammal and bird (e.g. Ayala-Gurrero et al. 2003) tested, and are therefore likely to be ubiquitous throughout the mammalian and avian classes. I shall concentrate my review of sleep within the mammalian class.

The most detailed sleep stage descriptions are found using invasive EEG techniques, in which electrodes are implanted through the skull and rest on the surface of the dura mater. In most non-human mammal studies using non-invasive EEG techniques, only Non-REM and REM sleep can be differentiated, not stages within Non-REM sleep.

Most researchers do not split Non-REM sleep in the rat into the same four stages as human Non-REM sleep (e.g. Meerlo et al. 1997). Instead, researchers either treat the Non-REM period as one sleep stage, or they split rat Non-REM sleep

into two main stages (often referred to as 'light sleep' and 'slow wave sleep'). Rats tend to exhibit eight to ten minutes of Non-REM sleep, followed by REM sleep. However, some researchers have found sleep spindles during the 'light' stage of Non-REM sleep in rats, analogous to the sleep stage II in humans (this depends on techniques and numbers of electrode channels used, spindles in rats are only seen if electrodes are placed over the frontal and parietal areas of the cortex) (Timo-Iaria et al. 1970; Terrier and Gottesmann, 1978). In fact, Timo-Iaria et al (1970) claimed that rat Non-REM sleep was as complex as human sleep (albeit shorter) and with careful EEG recording techniques at least three distinct stages could be differentiated. Gottesmann (1992) went even further and suggested that altogether there were seven stages of wakefulness and sleep in the rat: alert and non-alert waking; three ever-deepening stages of Non-REM sleep; and REM sleep with and without eye-movements (REM onset and REM sleep).

In cats, Non-REM sleep is also treated as one stage, although like the rat, there seems to be alterations in the spectral properties of the EEG. During any one bout of cat Non-REM sleep (which usually lasts for 25 minutes), approximately half will contain less than 30 % delta waves and half will contain more than 30% delta waves (Jouvet, 1967), possibly indicative of more than one sleep stage. One of the differences between Non-REM sleep in humans and in cats is that at any point in the cat Non-REM sleep, spindles can be observed (Jouvet, 1967).

In primates, Non-REM sleep can be split into the four stages, similar to those of humans (e.g. Kripke et al. 1968). Four Non-REM sleep stages are discernible with invasive EEG techniques, but with non-invasive EEG techniques or behavioural recordings only wakefulness, Non-REM and REM sleep are observed (Balzamo et al. 1998). Squirrel monkeys have been shown to have three distinct stages of Non-REM sleep: stages I and II (including spindle-like occurrences) similar to that of humans and stage III similar to both stage III and IV in humans (Adams and Barratt, 1974). However, the amount of each stage of sleep in non-human primate species differs widely between research groups and this may be due to the method of recording the EEG. Until the mid 1980s, most researchers restrained the primates in 'chairs', so that the electrodes and data acquisition system were not damaged. More recently, techniques have developed to allow primates to be recorded from whilst

unrestrained and in contact with conspecifics (e.g. Breton et al. 1986). Breton et al (1986) discovered that, although all three stages of Non-REM were present in squirrel monkeys that were recorded whilst unrestrained, the distribution of sleep during the 24-h and the relationship between Non-REM and REM sleep was altered significantly compared with restrained animals. This suggests that to get a true representation of animal sleep, the conditions in which the EEG is recorded should be as 'open' as possible, allowing the animal to make choices about when it sleeps and for how long (Videan, 2006).

In most of the mammals commonly used in sleep research (i.e. rats, mice, hamsters, cats and some primates), Non-REM sleep is carried out for approximately 75% of the total time spent sleeping.

1.3.2.8 Non-human animal REM sleep

REM sleep appears very similar in non-human mammals to that of humans. In all the mammals most commonly used for sleep research, REM sleep has a characteristic EEG trace, of low voltage beta activity interspersed with theta and alpha waves. The brain activity is found in conjunction with rapid eye-movements, phasic body twitches and loss of muscle tone. Differences occur in the density of the eye movements during REM sleep and the total and bout durations of REM sleep (Adams and Barratt, 1974). In the mammals commonly used for sleep research, REM sleep is carried out for approximately 25% of the total time spent sleeping. Most researchers report brief arousals in animals after the majority of REM sleep bouts (e.g. in rats –Timo-Iaria et al. 1970; in cats –Jouvet, 1967)

As mentioned above, humans report feeling alert after being woken from REM sleep, even though they need a more intense stimulus to wake them. When rats were woken from REM sleep by a stimulus that they had been trained to associate with a foot-shock, they were more able to quickly and accurately respond and carry out the appropriate trained behaviour to curtail the foot-shock than if they were woken during Non-REM sleep (Van Twyver and Garrett, 1972).

1.3.2.9 Transitional stages

There is a transition process from wake to sleep, in which changes in the EEG are seen. In humans, during relaxed wakefulness prior to sleeping and specifically with the eyes closed, alpha rhythms become predominate on the EEG (Gottesmann, 1996). This may be similar to drowsiness in ruminants, which occurs when the animal is lying down and often during rumination prior to sleeping (Ruckebusch, 1972, see below). The alpha rhythm is not observed in drowsy ruminants, as the jaw movements during rumination make artefact-free EEG recordings difficult.

There are also transitional periods between the sleep stages (especially between Non-REM sleep and REM sleep). These 'transitions' may last for 2-3 seconds in humans but are difficult to define. There is currently more research taking place on the relevance of REM sleep onset periods in relation to mental illness in humans (Bouhuys, et al, 1995). The transitions between sleep stages are also present in other mammals. However, they are even less clear, mainly because they are shorter in duration but also because there are different EEG techniques between humans and other mammals (Glin et al. 1991).

1.3.3 *Neural and Neurochemical characterisation of sleep*

How is it that sleep occurs? Does the nervous system possess a sleep-promoting mechanism? Does one actively go to sleep, or passively cease to be awake? As shown above, sleep is not a passive process for an animal to undergo, and similarly on a neural level, sleep occurs not because neurones are slowing down. Rather it is instead a process where particular neural circuits become active. Moreover, the neurological control of sleep must have some interconnection with the neurological control of wakefulness, and much research has been based on investigating whether there is either a sleep-promoting area or neurochemical in the brain, a wake-promoting area in the brain, or both.

1.3.3.1 Neurological control of wakefulness

In a quest to find what caused wakefulness (and the differing levels of awareness), early studies stimulated different areas of the brains of cats and observed

the subsequent effects (Moruzzi and Magoun, 1949). When stimulated, the reticular formation produced the most alert response in the cats. More recently, brain lesion (e.g. Jouvet, 1972) and brain microdialysis techniques (see Westernik, 1995 for a review) have increased our knowledge of the neural systems involved in wakefulness and sleep. The reticular formation occupies the central area of the brain stem and has two main pathways into the cerebral cortex, one of which projects into the thalamus and the other of which projects into the hypothalamus and basal forebrain (Saper et al. 2001). The reticular formation receives sensory input from the rest of the body, via the spinal chord. There are four main neural systems that relay information from the reticular formation and contribute to wakefulness:

a) The noradrenergic system –neurones in this system fire during wakefulness, and their rate of firing decreases as alertness decreases and further still during Non-REM sleep. Neurones from the noradrenergic system do not fire at all during REM sleep, but start rapidly firing again on arousal to wakefulness (Aston-Jones and Bloom, 1981). This system has connections that branch into many areas of the brain, releasing noradrenaline within the neocortex, the hippocampus, the thalamus, the pons and the medulla. Aston-Jones and Bloom (1981) found that the firing rate of the noradrenergic system concurred with maximum vigilance in rats.

b) The acetylcholinergic system –acetylcholine levels in the cerebral cortex positively correlate with levels of waking activity. Firing rates of neurones in the dorsal pons and basal forebrain are high during vigilance and activity in rats (Day et al. 1991).

c) The serotonergic system –the majority of neurons from this system are located in the raphe nuclei (specific areas of the reticular formation). Peck and Vanderwolf (1991) found that stimulating the raphe nuclei resulted in an EEG trace that was desynchronised and also produced locomotion in rats. The serotonergic neurones are almost completely 'silent' during REM sleep, but have a peak of firing in the first few seconds after a REM sleep bout (Trulson and Jacobs, 1979).

d) The histaminergic system –neurones that release histamine are located in the hypothalamus and have projections to the cerebral cortex and the thalamus. They indirectly are related to waking and activity as they stimulate the acetylcholinergic system (Khateb et al. 1995)

Further regulators of wakefulness are the hypocretins (neuropeptides produced in the dorsal and lateral hypothalamus) (Mieda and Yanagisawa, 2002). Hypocretins (also know as orexin) have been more commonly recognised as being involved with the regulation of mood, appetite, sexual drive and energy homeostasis (Taheri and Mignot, 2002). The link between hypocretins and sleep was first realised during concurrent research into the genetics of the sleep disorder narcolepsy in dogs and a hypocretin-receptor knock-out gene in mice (Taheri and Mignot, 2002). Narcolepsy is a disorder that is characterised by short REM sleep latencies, a tendency for the subject to fall asleep during states of anxiety and a high level of total sleep (Mieda and Yanagisawa, 2002). Canines showing narcolepsy had a mutation in the hypocretin receptor, leading to a loss of protein function. Mice with the knock-out gene also showed the same loss of protein function (Mieda and Yanagisawa, 2002). The loss of protein function, in turn lead to a reduction in the inhibition of sleep.

Intracerebroventricular hypocretin was administered to rats during different phases of the circadian cycle (Espana et al. 2002). The administration took place when the animals were either displaying low-waking levels (light phase) or high waking levels (dark phase). Rats given hypocretin in the dark phase tended to show more waking than vehicle-injected controls. Rats given hypocretin in the light phase showed significantly more waking and waking behaviours than controls (Espana et al. 2002).

1.3.3.2 Neurological control of Non-REM sleep

As previously mentioned, sleep occurs when areas of the brain become active not just when certain areas slow their firing rates and 'switch off'. The main area of the brain that increases activity during Non-REM sleep is the ventrolateral preoptic area (part of the basal forebrain). This area contains neurones that secrete gamma-amino butyric acid (GABA). The axons of the neurones from the ventrolateral

preoptic area project to the locus coeruleus, the raphe nuclei and the hypothalamus (Saper, et al, 2001). The release of GABA in these areas actively inhibits noradrenergic neurones, serotonergic neurones and histaminergic neurones (indirectly reducing firing of the acetylcholinergic system) (Lechin et al. 2004). Electrical stimulation of the ventrolateral preoptic area produced drowsiness and eventual Non-REM sleep in cats (Sterman and Clemente, 1962).

1.3.3.3 Neurological control of REM sleep

REM sleep has similar neuronal activity levels (and desynchronisation levels) as in wakefulness (Paré and Llinás, 1995). During REM sleep, the metabolic rate of the cerebral cortex is as high as in waking (Maquet, 1995). The main differences between REM sleep and wakefulness are the levels of awareness (as shown by arousal thresholds); the paralysis of the body; and the exhibition of rapid-eye-movements and phasic twitching. Within the brain, the main area that is active during REM sleep is the pons. The neural system most active during REM sleep onset and during REM sleep itself is the acetylcholinergic system, once again, similar to wakefulness (Lechin et al. 2004). Acetylcholinergic neurones found within the dorsolateral pons (especially the tegmental nuclei, know as the 'peribrachial area') are particularly active during REM sleep onset. Cornwall, et al (1990) found that neurones within the peribrachial area projected to many brain areas, including the reticular formation, the thalamus, the preoptic area, the hippocampus and the hypothalamus, and to areas of the brain stem involved with eye muscular contraction.

As a sleeping animal moves from Non-REM sleep to REM sleep, a number of observable changes occur:

a) The production of Pons-Geniculate-Occipital waves (PGO waves), which consist of bursts of waves from the pons that spread to the geniculate nuclei and the occipital cortex. There are connections between the peribrachial area and the geniculate nucleus, whereby PGO waves are spread (Sakai and Jouvet, 1980).
b) The EEG becomes desynchronised as the acetylcholinergic system becomes active in the pons, with axons projecting to the thalamus,

exciting the areas of the thalamus that are involved in cortical arousal (similar to wakefulness) (Jones, 1993).

c) The paralysis of muscular activity in the body (apart from breathing and eye movements). Jouvet (1972) showed that the peribrachial area was involved with the paralysis seen during REM sleep when he lesioned the region directly caudal (subcoerulear region) in cats. After lesions had been applied, the cats still went into REM sleep (as shown by EEG and EOG) but did not undergo muscular paralysis. This lead to cats 'acting out their dreams...attacking unknown enemies, playing with absent mice, but not responding to visual or auditory stimuli' (Jouvet, 1972).

d) Rapid eye-movements occur, which is the most obvious sign of REM sleep behaviour to the observer. The rapid-eye movements appear to be produced by the excitation of neurones from the peribrachial area to the tectum, the area which is primarily involved with visual reflexes (Webster and Jones, 1988)

The other differences between REM sleep and wakefulness in the brain are that serotonergic and noradrenergic systems are not involved. Indeed it seems as though REM can only occur in the absence of activity from these two systems (Stresker et al. 1999). Stresker et al (1999) suggested that the timing of REM sleep and the duration of REM sleep bouts may depend on the interaction between the serotonergic and acetylcholinergic systems. Here, serotonin release declines during Non-REM sleep (releasing the REM promoting neurones from serotonin-inhibition) bringing about REM sleep, but also ultimately leading to an increase in acetylcholine in terminal areas, bringing about an eventual end in the REM sleep bout.

1.3.4 *Circadian rhythms of sleep and wakefulness*

Humans and non-human animals undergo sleep cycles, which may be monophasic or polyphasic depending on the species. However, all animals follow a 24-h pattern in their sleep and wake cycles. There are two main controls of an animal's daily activity rhythms: light and an internal 'clock' (Lavie, 2001). The endogenous rhythm (stabilised by the internal 'clock') has been shown to run slightly slower than 24-h in many studies. Therefore, in the absence of light the

biological rhythms of an animal may 'free-run' on an approximately 24-and-a-bit cycle, and regular activities (such as sleep) will occur a little later every day. (It was thought until recently that all animals free-ran on a 25-h internal 'clock' but these results have been refuted as it seems even the dimmest light can act on the internal 'clock'-Czeisler et al. 1999.) Light acts on the internal 'clock' to maintain the exact 24-h rhythm and synchronises the endogenous rhythm with the exogenous world. Light is known as a Zeitgeber ('time giver' from the German).

The suprachiasmatic nucleus of the hypothalamus was found to be the location of the internal 'clock' in rats. Lesions of this area result in rats that sleep in many polyphasic bouts throughout the 24-h (rather than in the light period only). However, the amount of sleep is not affected, just the distribution (Ibuka and Kawamura, 1975). Individual neurones of the suprachiasmatic nucleus have a genetic material transcription-translation feedback loop that maintains an approximately 24-h clock (Reppert and Weaver, 2002). This endogenous cycle is synchronised to the external light/dark cycle by light signals that are received by the retina, and then information is transmitted via the retino-hypothalamic tract to the suprachiasmatic nuclei of the hypothalamus (Saper et al. 2005). Neural activity (as measured by Fos protein synthesis) occurs in the suprachiasmatic nucleus after exposure of the retina to light (Saper et al. 2005). The suprachiasmatic nucleus 'clock' can be reset by exposure to light during darkness periods and by behavioural arousal (Mistlberger et al. 2003).

The internal 'clock' or 'circadian pacemaker' located in the suprachiasmatic nuclei controls the rhythmic production of melatonin in the pineal gland (Sumová et al. 2002). A synaptic pathway from the suprachiasmatic nuclei leads to the superior cervical ganglion, which releases noradrenaline onto the pinealocytes of the pineal gland, stimulating the production of melatonin. Melatonin synthesis occurs in the dark and is inhibited by light. There is some evidence that daytime administration of melatonin has a time-related sleep promoting affect in humans. If melatonin was given early in the day, there was no effect on sleepiness; but when given in the early evening, people reported feeling extremely sleepy (Lewy et al. 1992). Bubenik et al (2000) suggests that endogenous melatonin induces sleep in pigs, although the

production of the melatonin measured in this study was associated with the gastrointestinal tract rather than the pineal gland.

The suprachiasmatic nucleus contains a high density of melatonin receptors, allowing melatonin to be involved in the feedback loop of the circadian clock. Endogenous melatonin, released into the bloodstream and the cerebrospinal fluid from the pineal gland is received into the suprachiasmatic nuclei (Turek and Gillette, 2004).

There are many mammalian genes that change in the suprachiasmatic nuclei with differential exposure to daylight and other zeitgebers. These are altered by photoperiod in sheep (Lincoln et al. 2003a) and hamsters (de la Iglesia et al. 2004). In sheep, the same genes are also altered by photoperiod when they occur in cells in the pars tuberalis (in the anterior pituitary gland). Therefore, are associated with longer circannual timing (Lincoln et al. 2003b). The pars tuberalis in sheep has the highest density of melatonin receptors of any area of the brain and the circadian rhythm of melatonin secretion results in differential prolactin secretion during the seasonal changes (Barrett et al. 2003). Sumová et al (2002) found that the expression of the *Per-1* gene product in rats was more variable under natural day light conditions than under artificial short- or long-days. This led to a more variable production in melatonin receptors in the suprachiasmatic nucleus.

However, light is not the only zeitgeber for the internal 'clock'. An animal has to be able to respond to different environmental conditions, other than merely the day length. Rats have been shown to change their activity patterns in relation to the accessibility of food, even though their underlying suprachiasmatic nucleus 'clock' mechanism remained unaltered (Stephan, 2002). Therefore, the day-to-day behaviour of animals remains flexible: it depends on the environmental conditions and the experience of the animal (Saper et al. 2005)

1.3.5 *Possible functions of sleep*

All vertebrates undergo a period or several periods of reduced vigilance during the 24-h period (Meddis, 1975). This period of reduced vigilance also includes a reduction of movement and muscle activity. During this period active feeding does not occur (Meddis, 1975). In every mammal and bird species tested,

some of these periods of reduced vigilance contain the changes in the normal EEG to a pattern resembling the characteristic changes from wakefulness to sleep in humans (see above) (Tobler, 1995). Animals exhibiting the changes in EEG also display the changes in behaviour described in section 1.3.1 above, which can include lying down, resting of the head on an available surface and closing of the eyes and a reduction in responsiveness to external stimuli. These animals can be said to be sleeping.

All mammals share certain characteristics of sleep; they all show both REM and Non-REM sleep. REM sleep and Non-REM sleep are positively correlated: animals which show a large amount of Non-REM sleep will show a large amount of REM sleep (Siegel 1995). The REM sleep period is always approximately 25% of the total sleep exhibited by the adult species (Horne, 2000; Hendricks and Morrison, 1981). Non-REM sleep is often accompanied by relaxation of the body. However, REM sleep is accompanied by a complete loss of muscle tone in the limbs, the neck and the face (with the exception of some marine mammals, such as the beluga whale, that exhibit unihemispheric sleep -Lyamin et al. 2002). REM sleep forms a greater proportion of sleep in young animals than in adults (Horne 2000). For example, neonatal cats spend 75% of their total sleep time in REM sleep as compared with 25 % in adult cats (Hendricks and Morrison, 1981).

A comparison of sleep across non-human taxa is beyond the scope of this thesis but, for a very good review of the total sleep time and sleep bout durations for over 150 species is given in Campbell and Tobler (1984). It is still necessary to look at the way different animals sleep to understand the evolution of sleep behaviour and try to identify the function(s) of sleep. Allison and Cicchetti (1976) carried out a study that correlated the amount of sleep an animal exhibited with ecological variables (such as animal size, likelihood of being preyed upon and the size of the social group) and found that large prey animals showed a negative correlation between body size and total sleep time. Predators exhibited a longer period of sleep than prey animals and REM sleep time was associated with predatory danger: from the data collected, the less likely a species was to be preyed upon (e.g. a lion), the longer the total time of REM sleep in a 24-h period. However, predators also consume more energy per meal than prey animals and therefore they can afford to

spend more time idle than most herbivores. Mammals that need to remain vigilant such as sheep will spend less time sleeping than animals, such as rabbits that can hide and can also consume more energy in shorter periods of time. Therefore, sleep may be an efficient way for small herbivores to remain inactive and safe from predators in between foraging bouts (Allison and Cicchetti, 1976). It must be noted that large prey animals generally need to spend a large proportion of the day foraging for food and therefore may not 'have time' for sleep (Elgar et al. 1988; Lima et al. 2005).

1.3.5.1 Functions of Non-REM sleep

There are several views on the function of Non-REM sleep. It has been suggested that Non-REM sleep (especially deep slow wave Non-REM) allows physiological restoration; in producing slow rhythmical waves the brain is leading to some sort of recuperation process that is needed from the rest of the day's activity (Inoue et al. 1995). In rats, total sleep deprivation for a prolonged period (>12 days) has debilitating effects (such as hair loss, tissue breakdown and weight loss) on the body; it has been argued that these effects are directly a product of the lack of a recovery period afforded by sleep (Tobler, et al. 1983; Cai, 1995). However, some authors have noted that by design, all sleep deprivation experiments are stressful and lead to fatigue of the muscles, which could increase the likelihood of debilitating effects (Freemon, 1971). Moreover, most of the debilitating effects are on the body. Cognitive function remains unimpaired after sleep deprivation, which argues against Non-REM sleep performing a brain repair function (Cai, 1995).

Another study has focussed on the ability of specific areas of the brain to rest. It is argued that although most of the body and brain can be in a state of relaxation during wakefulness, this is not true in the case of the cerebral cortex (Drummond et al. 2000). Drummond et al (2000) showed that 35-h sleep deprivation in humans had an effect on the subject's ability to carry out a verbal learning task. The prefrontal cortex is the area which is most active during verbal learning tasks. After sleep deprivation performance on the task was reduced by 45 % and activation (as shown by MRI scans) of the prefrontal cortex was higher than in normal sleep

controls (Drummond et al. 2000). The prefrontal cortex is not inactive during Non-REM sleep, but is the area where most slow waves are sourced (Muzur et al. 2002). The slow waves found in the EEG during deep sleep may, in some way, provide rest for the prefrontal cortex (Muzur et al. 2002). In addition, during slow wave sleep the blood flow to the cortex is reduced (Maquet, 1995).

Non-REM sleep may also be involved with memory processing (Cai, 1995). A study determined that in humans, only the hippocampal cells active during wakefulness were involved in the slow-wave production during Non-REM sleep (Pavlides et al. 1988). In behavioural research, it has been found that rats housed in enriched environments increase their learning and memory ability, and also increase the amount of Non-REM sleep compared with those from barren environments (Cai, 1995).

Meddis (1975) and Berger and Phillips (1995) suggested that sleep allows the animal a period of inactivity, which can then enable the body to rest, reducing energetic output and thus increasing efficiency. Metabolic rate and body temperature fall at sleep onset in the rat and the change between sleep and waking accounts for the biggest variation in body temperatures (range of 2.29°C) (Berger and Phillips, 1995). In humans, the metabolic rate during Non-REM sleep is on average 25 % below that of the waking rate (Berger and Phillips, 1995).

Non-REM sleep probably evolved earlier than REM sleep, as similar electrophysiological patterns to Non-REM sleep are seen in fish and amphibians, whereas REM sleep is absent from these groups (Siegel, 1995). The selective pressures that allowed Non-REM sleep to develop and be maintained throughout the vertebrates might never be fully understood (Kavanau, 2002). It is probable that sleep developed out of restful wake states (Kavanau, 2004), especially as complex vision and the need to consolidate memories had already developed.

1.3.5.2 Functions of REM sleep

It has been suggested that REM sleep probably evolved later than Non-REM sleep (Kavanau, 2002). Modern day reptiles show some signs of desynchronised EEG during sleep and it is found in all mammals (including monotremes –Siegel et al. 1998 and 1999) and birds (Siegel, 1995).

One proposed function of REM sleep is known as the 'sentinel hypothesis', based on studies showing that animals (including humans) are able to be extremely alert after arousal from sleep (see above section on responsiveness during sleep). Moreover, although arousal thresholds are generally high during REM sleep, animals are able to respond quickly to relevant stimuli. In fact, recent research has shown that humans are able to process complex cognitive information while in REM sleep (such as syntax tasks –Cote et al. 2001). However, this hypothesis has flaws: the arousal stimulus needed to awaken an animal from REM sleep is very high (except for certain relevant stimuli). In addition, one would expect prey animals to undergo more REM than Non-REM sleep for this hypothesis to be true, whereas for adult animals, Non-REM 'outweighs' REM sleep by three to one (Lima et al. 2005).

REM sleep, like Non-REM sleep may also have a recuperative function. Selective sleep deprivation experiments conducted on rats, and involving only REM deprivation (know as partial sleep deprivation), show that without REM sleep a rat suffers physical effects similar to those caused by total sleep deprivation (Brunner et al. 1990). However, humans on tricyclic antidepressant drugs (eg Phenelzine), which reduce REM sleep to almost zero, have been shown to live for months and even years at a time without exhibiting REM sleep (Horne, 2000). No ill effects have ever been reported as being due to REM sleep deprivation in such patients (Horne, 2000).

Perhaps the function of REM sleep is involved in the development of the brain. Young animals all have a significantly higher amount of REM sleep than adults of their species; foetal animals a higher amount still (Horne, 2000). Perhaps REM sleep offers stimulation to the developing brain that would otherwise be lacking in the uterus or egg (Mirmiran, 1995). Perhaps REM sleep allows for the development of neural connections in the foetal brain (Horne, 2000).

Several researchers have suggested that complex memory consolidation and learning either occurs, or at least has a two-way relationship with REM sleep (e.g. Sejnowski, 1995; Smith, 1996; Maquet, 2001). When people have been asked to learn a task, their subsequent sleep contains more REM sleep bouts (Laureys et al. 2001). Rats will show more REM sleep bouts after learning a maze for food rewards than handled controls, indicating the increase in REM is connected to learning rather than the stress of the procedure (Smith and Rose, 1997). Moreover, if selectively

deprived of REM sleep after learning a task, humans are less able to recall the task accurately than controls (Smith, 1996). This may be one of the reasons why young mammals have more REM sleep than adults as they constantly have to take in new information and learn new skills when interacting with the environment.

An important aspect of REM sleep, at least in humans, is that it is within this mental state that the majority of dreams occur. In addition, the majority of REM sleep episodes in humans include dreaming. However, dreams do occur in Non-REM sleep in humans (Bosinelli, 1995). Many people assert that dreams in themselves hold some sort of function and may help to interpret the previous day's events and improve memory. Some people believe that we sleep in order to dream. However, caution must be exercised as there is as yet no evidence for any such theories (Horne, 2000).

Whether or not non-human animals dream, or if their dreaming experience is different from ours, is at present speculation. There is however evidence that other mammals do experience dreamlike occurrences during REM sleep. As mentioned above, Jouvet (1972) investigated the muscle paralysis in REM sleep in cats by creating lesions in the areas of the brain that block muscle activity. The cats performed recognisable behaviours (such as predatory pouncing) during REM sleep, but were as unresponsive to external stimuli as non-lesioned controls during REM sleep. Jouvet (1972) described the cats as 'acting out their dreams'. The same phenomenon occurs in humans with the brain degenerative disorder known as REM sleep behaviour disorder. Humans with this condition will act out behaviours during REM sleep (quite different from sleep walking). The behaviours seem to relate well to the dreams reported by the subjects (Ferini-Strambi et al. 2005)

1.4 Sleep in sheep

1.4.1 *Rest and activity behaviour in sheep*

Sheep, like all mammals, have a complex pattern of activity, restful-wakefulness and sleep (Campbell and Tobler, 1984). In the wild, as they are vulnerable to predation, sheep have a trade-off between remaining vigilant for predators and all of the other behaviours they carry out. Sheep live, like many other prey animals, in a large social group, in which each individual can assume that,

when it is feeding, or engaging in any other non-vigilant behaviour, a further individual remains vigilant for predators (Frid, 1997). However, as sleeping requires a reduction in awareness, the amount of time a prey animal such as the sheep can sleep is shorter than many other animals (Elgar, 1988). The amount of time that sheep can sleep is also restricted by morphology, habitat and food sources. Sheep are unable to climb trees (although the wild sheep is very adept at climbing in rocky areas –Geist, 1971), and unable to dig burrows, and therefore must sleep out in the open. The natural habitats of the wild sheep are mountainous scrubland and alpine areas, and even when there are woodlands, sheep are unlikely to enter further than the fringes (Geist, 1971). Sheep forage for grasses and other low lying herbaceous plants, which are foodstuffs that require long time periods to eat and to forage for, so a wild sheep is unlikely to be able to eat its fill in one meal. All of these factors influence the amount of time sheep can rest and sleep and the distribution of rest and sleep over 24-h.

Studies of wild sheep (e.g. Geist, 1971; Langbein et al. 1996), hill sheep (e.g. Arnold, 1984) and sheep on lowland pasture (e.g. Bueno and Ruckebusch, 1979; Tobler et al. 1991) all note the same major behaviour pattern. Sheep start to graze at dawn and graze for several hours, they then rest and ruminate during the late morning interspersed with grazing. Sheep have a second major grazing period in the afternoon and evening (interspersed with ruminating) and become more inactive after sunset (although with some grazing, Langbein et al. 1996). If the day is particularly hot, sheep may spend more of the daylight hours inactive (standing idle) and more of the night active (grazing) than in cooler climates (Squires, 1971). Tobler et al (1991) also found a drop in activity of sheep occurred between 1200-1500h on warm days.

Tobler et al (1991) measured activity levels in sheep and could indirectly record REM sleep episodes by the complete cessation of measurable movement (corroborated by video evidence). They found that sheep at pasture only went into REM sleep for periods of 2-6 minutes at night. In the daylight, wild sheep spend approximately 26 % of the time resting, some (unquantified amount) of this time is spent lying in a relaxed posture which may have been sleep (Bowns, 1970). Bueno and Ruckebusch (1979) showed that sheep at pasture rested for approximately 40 %

of 24 hours mainly at night or in the middle of the day, between the two major grazing periods.

Sheep do not just lie down and go to sleep where they have been feeding; specific areas are sought out: high ground, free from trees and shrubs (Bowns, 1970). Some shelter making behaviour has been observed: both wild and hill sheep actively rub certain areas of hilly ground to make clefts to lie within affording some shelter from the wind (Geist, 1971 and Munro, 1962 for wild and hill sheep respectively). Mountain sheep tend to rest as a group, in which some animals ruminate, whilst others lie alert or with lie their head down on the ground (Geist, 1971). Sheep leave these bedding grounds daily, just before sunrise to start grazing. Sheep generally return to the same bedding areas each night and throughout the year –except in winter– when they seek shelter at lower levels (Geist, 1971).

1.4.2 *Sleep and rest in farm animals*

Balch (1955) investigated behaviour, rumination and digestion, in order to evaluate sleep in cattle. Cattle never seemed to be asleep in the presence of the observer; they lay down in a characteristic posture, with their head resting on their flank, but would only lie with their eyes closed for a period of a few minutes. Balch concluded that because ruminants need to remain in an upright posture, sleep was either not possible at all, or if so, only very short periods of light sleep would take place.

Bell (1960) used electrodes implanted onto the surface of the brain to record the brain electrical activity of goats during the times when the goat was lying down in a relaxed manner. Slow waves were seen during this posture and a greater intensity of auditory stimulus had to be used to achieve arousal than when the slow waves were not seen on the EEG. However, as the goats had their eyes partially open whilst there were slow waves seen on the EEG, Bell (1960) suggested that there was no loss of consciousness in this state in the goat. Bell also reported a similar pattern of slow wave EEG during rumination in the goat, but as no electromyogram of the jaw muscle was recorded, it is impossible to say whether this was due to brain electrical activity or artefact from the jaw muscle as later suggested by Klemm (1966). No REM sleep was seen in this study, but as no EOG was used to record eye

movements, and as the EEG during REM sleep has similar characteristics to wakefulness, this is perhaps unsurprising. Bell concluded that although goats sleep, they do so unlike other non-ruminant animals, and he suggested that rumination replaced the need for sleep. Klemm (1966) refuted the work of Bell by showing REM sleep in goats using the simultaneous recording of the EEG, the EMG from the neck and the EOG. Klemm (1966) found that during rumination, the implanted EEG was obscured by artefact, but EEG analysis of the gaps between chewing indicated that the animal was awake, not showing slow waves as had been suggested by Bell (1960).

Merrick and Scharp (1971) studied resting behaviour in cattle using the EEG (but did not record the EOG or EMG). They described three levels of rest in cattle: standing with eyes open; lying down with head erect and eyes open; and lying down with head supported on flank and eyes partially closed. Each of these resting states showed an increasing percentage of slow waves on the EEG, but the authors asserted that as the eyes of cattle were not seen to be closed that the animals could not be showing 'true sleep'. They argued that the results found by Klemm (1966) should not be extended from goats to cover all ruminants.

In 1973, Bell and Itabisashi recorded the EEG and EOG, to identify sleep and wakefulness in sheep and goats. They found slow waves and also REM sleep during periods of recumbency. They also found slow waves during rumination (but, again, no EMG was taken from the jaw and inter-chewing EEG was not recorded separately) and showed that slow-wave sleep is frequently found directly after a period of rumination, suggesting that rumination can be associated with drowsiness that preceeds sleep in sheep and goats (Bell & Itabisashi 1973).

In a major study of sleep in ruminants (and other farm animals), Ruckebusch (1972) suggested that drowsiness be classified as a period of non-alert wakefulness rather than alert wakefulness or sleep. He showed that both cattle and sheep go through cycles of alert wakefulness, drowsiness, slow-wave sleep and REM sleep. Ruckebusch's study showed that the muscle tone loss during sleep was different between cattle and sheep. Sheep exhibited a gradual loss of tone and cattle a more distinct drop in muscle tone with the onset of sleep. Both ruminant species always had their eyes closed during REM sleep, and the periods of REM sleep could last for

approximately five minutes in both species. It had been suggested that ruminants ruminate instead of sleep (Balch, 1955; Merrick and Scharp, 1971). However, Ruckebusch (1975a) found that during REM sleep there is a slowing of the rumen and reticular contractions in sheep. Ruckebusch (1975a) concluded that the relationship between REM sleep and the slowing of 'gastric motility' (i.e. reticular and rumen contractions and the process of rumination) is an "independent consequence of a common central nervous system activity" and not a causal relationship. It was suggested that both species can ruminate during alert wakefulness and drowsiness and may even progress into light slow wave sleep during rumination (Ruckebusch 1972).

The authoritative research on sleep in sheep was carried out by Ruckebusch (1972). This is the study from which most subsequent investigations into sleep in mammals quote when describing sleep in sheep and cattle (e.g. Meddis, 1975; Campbell and Tobler, 1984; Elgar, 1988; Tobler, 1995). He found that in a period of 24-h sheep spend 70 % standing (including locomotion), and 30 % lying down. He showed, by using implanted electrodes to record the EEG from three sheep, that in a period of 24-h sheep were awake for 84 % of the time, were in Non-REM sleep for 13.6% of the time and were in REM sleep for 2.4 % of the time (Ruckebusch, 1972). His results show the small amount of total sleep in a 24-h period that is found in sheep in comparison to humans; sheep sleeping for about half the total time that humans do (approximately 33 %) (Fisch, 1999).

Ruckebusch (1972) found that sheep spent up to 70 % of their time standing and would ruminate whilst in this posture as well as when recumbent. The figure recorded for standing in sheep by Ruckebusch was somewhat higher when compared with figures of up to 50 % in other, behavioural studies of sheep (Fordham et al. 1991, Done-Currie et al. 1984). This difference in behaviour may have been due to the confinement of sheep in metabolic crates during the study by Ruckebusch. In addition, Ruckebusch (1972) found that sheep remained standing for 60 % of the night period (12-h). Tobler et al. (1991) found that sheep at pasture, during the summer, are in a lying down posture (including lying down ruminating) for up to 65 % of the night and that standing and activity increases after dawn, reaching a peak at midday. Sheep in stalls showed resting behaviour at night and in periods during the

day where humans were not present in the stall area, but activity levels at night were higher in stalls than out at pasture. Activity levels of sheep in the stalls were at a maximum when there was human activity in the stall area (Tobler et al. 1991). Tobler et al. (1991) suggested that sheep at pasture might spend more time active during the night at times of year when the dark period is longer than in the summer. Das (2001) found that in stall-fed sheep, 25 % of the day sheep rested in a standing posture ('Loafing') and 25 % of the day was spent lying down (including 9 % sleep). The other 50 % of the day was spent 'active' that included lying down and ruminating (Das 2001). Sleeping was observed behaviourally, when the animals were lying down with their eyes closed. A problem with the studies outlined above is an inconsistency in terminology. It is difficult to determine the comparison of time budgets in sheep between studies as different research has different definitions for 'active' and 'inactive' behaviours.

There have been many studies into sleep in foetal and neonatal lambs, using sheep as a model for human foetuses and babies. These have tended to be applied studies, concentrating on hypoxia in foetuses (e.g. Abrams et al. 1991; Koos et al. 2001) and neonates (Cohen et al. 1997; Johnston et al. 1998) and reactions of the foetuses to various pharmacological agents (Morrison et al. 2001; Nicol et al. 2001). However, these studies are useful for research into sleep and animal welfare, as they contain information on techniques of sleep recognition in sheep (including the automatic scoring of sleep in sheep -Grant, et al, 1995). In addition, they give information on the sleep of neonatal lambs that would be useful in future animal welfare studies of the neonatal lamb (amounts of REM and Non-REM sleep, development of sleep patterns and associated physiological changes e.g. Anderson et al. 1998; Schmidt et al. 2000).

1.5 Relevance of the studies of sleep to animal welfare

1.5.1 *The scientific study of animal welfare*

The concept of animal welfare started out, not in the realm of science, but out of moral concern for animals, out of ethics. It is difficult to define animal welfare. The concept of animal welfare is not a strictly scientific one; animal welfare cannot be defined within strict parameters of scientific terms in the same way as it is

possible to define physical properties such as 'force'. There has been an evolution of the term welfare, from the primary usage: 'well-being, happiness; health and prosperity' (Oxford English Dictionary, 1996) to a regard for animal welfare, defined by Hughes (1976) as 'A state of complete mental and physical health, where an animal is in harmony with its environment'. It is a common belief that when we take an animal into captivity, gaining absolute power over whether it lives or dies, we then have the moral responsibility as far as is practical to look after it while it is alive and kill it in the most humane way (Sandøe and Crisp, 1997). Accepting that an animal is sentient and that it has an interest in its own survival (let alone an interest in increasing the pleasurable experiences in life and decreasing the unpleasant) would mean that we give animals moral standing. For a review on the relationship between animal welfare science and animal ethics see Fraser (1999).

Animal welfare has often come to be thought of as a scale that can be applied to an animal or group of animals in any particular situation, in which the welfare can be thought of as very bad or as very good or as something in between. The majority of definitions that have been produced in the last 20 years are definitions that include welfare as a scale from good to bad. Broom (1986) proposed that 'the welfare of an individual is its state as regards its attempts to cope with its environment. This state includes how much it has to do to cope, the extent to which it is failing to cope, and its associated feelings'. This definition, similar to Hughes' above, is centred on the biological balance that exists between an animal and its environment. An animal that can cope with its environment may have satisfactory welfare and an animal that is failing to cope would be considered to be in a state of poor welfare.

Of course, the assessment of an animal's welfare is carried out by humans, using human judgement. Therefore, animal welfare can most technically be defined as the state 'which is considered by human observers to be consistent with the consensus of current human knowledge of the best interests of the animal' (Hill and Sainsbury, 1990).

Many scientists working in the field of animal welfare have concluded that welfare is not just an issue of homeostasis of the animal, but about the animal's feelings about its situation. These can be overall negative 'to be concerned about animal welfare is to be concerned with the subjective feelings of animals,

particularly the unpleasant subjective feelings of suffering and pain' (Dawkins, 1988). However, this definition does not really allow for the recognition of good welfare. Should we only be concerned about improving bad welfare and not about the subjective feelings of pleasure and happiness? Similarly, Duncan (1993) based his definition of animal welfare on the subjective: 'It is generally agreed that welfare is a term which cannot be applied sensibly to the 'lower' animals or to plants, but only to sentient animals. Since 'sentient' means capable of feelings, the argument is developed that welfare is solely dependent on what an animal feels'. There is debate here, about how one can recognise sentience. Duncan (1993) also states 'neither health nor lack of stress nor fitness is necessary and/or sufficient to conclude that an animal has good welfare. Welfare is dependent on what animals feel'.

The problem with trying to define animal welfare in scientific terms is that the whole idea of welfare is based on values: 'the welfare of animals refers to their quality of life, and this involves many different elements, such as health, happiness and longevity, to which different people attach different degrees of importance' (Duncan and Fraser, 1997)

Animal welfare scientists attempt to investigate the experience of animals, primarily when the animals are affected in some way by humans (e.g. they are captive in zoos, are farmed for food, they are kept as pets, are used as experimental animals, or they are wild animals that come into human contact). Moreover, animal welfare scientists attempt to investigate the experience of animals in an unbiased and reliable way, "submitting concepts referring to subjective experience to objective measurement and evaluation" (Wemelsfelder, 1997).

Many scientists think that the study of consciousness, awareness and emotions, especially of non-human animals, are outside the remit of science and should be left to philosophy (for discussion see Fraser, 1999). In fact, Tinbergen (1951) –one of the founders of ethological science –stated that:

"subjective phenomena cannot be observed objectively in animals, it is idle either to claim or to deny their existence" (Tinbergan, 1951)

However, as discussed above, the most important aspect of animal welfare science (and the one that makes it more far reaching and interdisciplinary compared with

pure veterinary or animal science) is that it attempts to deal with the subjective feelings of animals (Wemelsfelder, 1997).

What are emotions? Emotions are the internal processes within the brain during which an animal will assess the environment and their internal state on the basis of past experience and present stimuli (Wiepkema and Koolhaas, 1992). Emotions allow an animal to make judgements about objects and situations, that enables the animal to increase its biological fitness (Cabanac, 2002). There are arguments that describe the experience of emotion as the phenomenon to be explained, the physiological and behavioural changes as mere expressions of the emotional state. Salzen (1998) argues that the behaviour is the primary phenomenon and the experience is a secondary consequence of self-perception of the behaviour. Most authors agree that emotions are internal processes originating in the brain that allow an animal to make decisions about the world but, that emotions are different (and lesser in complexity) to thinking (abstract internal processes, not necessarily connected with the external world) which is possibly the preserve of humans and other great apes alone (Piggins and Phillips, 1998).

Awareness and consciousness are even more difficult to define, let alone to study scientifically. There are different levels of awareness, and the majority of animal welfare scientists would give mammals the levels of 'cognitive awareness' (neural processing of sensory inputs, or memory resulting in a flexible response – Sommerville and Broom, 1998) and 'assessment awareness' (the ability to assess a situation in relation to itself –Sommerville and Broom, 1998) (Salzen, 1998). Most non-human animals would not be given the level of 'executive awareness' (an understanding of goals and intentions) (Piggins and Phillips, 1998). It is also important to remember species differences mean that some forms of sensory input (e.g. auditory or olfactory inputs) will predominate over others, depending on species (Heffner, 1998; Sommerville and Broom, 1998).

Discussions on consciousness suffer from the different usage of the word 'consciousness' between disciplines (Searle, 1992). For example, in medical terminology there are levels of consciousness ranging from fully alert to unconscious in a coma (the Glasgow coma scale). In psychology, 'the conscious' and 'the unconscious' have meanings connected to the available and unavailable parts of the

self (e.g. the writings of Freud –Gay, 1995). Within this review, I have taken consciousness to mean that the animal has awareness of its environment and itself within that environment (similar to Kendrick, 1998), so a reduction in consciousness occurs when the animal goes to sleep. An animal that was in 'deep' Non-REM or REM sleep would have a low level of conscious awareness, but they are still able to adaptively respond to a stimulus, especially if the stimulus is relevant and therefore they are not unconscious (Batsuji et al. 2002). A completely unconscious animal would be one which would be under general anaesthetic and could not respond to external stimuli. I am not assuming that to be conscious an animal has to have self-awareness and/or theory of mind.

Obviously, I do not have the space within this review to thoroughly dissect all of the arguments for and against animal awareness and consciousness. However, there are aspects of conscious awareness in humans that lend themselves to scientific study. One of the easiest changes in consciousness that humans can notice about themselves and others is the difference between wakefulness and sleep. This is one of the changes in consciousness that can be recorded in non-human animals by objective means. Paré and Llinás (1995) suggest that wakefulness and REM sleep are basically the same state in terms of most physiological measurements, the main differences (apart from the paralysis of muscles) lie in the lack of responsiveness to sensory stimulation.

1.5.2 *The assessment of animal welfare*

The most important issue in the scientific study of animal welfare is: how to objectively and scientifically assess the welfare and therefore the subjective state of an animal. We can objectively assess whether or not an animal is physically healthy, and this goes part of the way in assessing animal welfare (e.g. Fitzpatrick et al. 2006). However, as discussed above, we also need to be able to assess whether an animal is psychologically healthy and has what it needs and wants to maintain that physical and psychological health (Dawkins, 2003). There is no simple 'measure' of welfare, no one factor that in its presence or absence makes the welfare of an animal 'good' or 'bad'. There are several methods of gathering evidence about animal welfare, behaviourally and physiologically. There are a few important factors to be

vigilant for when attempting to assess welfare including: stress and distress (both acute and chronic); pain; physical health problems; and –especially if one is trying to assess good welfare as well as bad– positive experience. Some of these factors are particularly relevant to the investigation of sleep in relation to animal welfare and are reviewed below.

1.5.2.1 The recognition of stress

Stress, somewhat like 'welfare', is difficult to define, it is the biological response obtained when an animal perceives a threat to its homeostasis (Moberg, 2000). Reviews of stress and animal welfare include Moberg (2000) and Wiepkema and Koolhaas (1993).

A stress response can be defined as the physiological and pyschological changes within an animal, in a response to a stressor -the threat (or perceived threat) and Moberg (2000) suggests that when the stress response is 'bad enough' to alter the animal's welfare, the animal is suffering from distress. The huge majority of stress perception and responses do not alter an animal's welfare, as they do not alter an animal's biological function (Wiepkema and Koolhaas, 1993) (Figure 1.2). The stressor is perceived by the central nervous system on the basis of inputs from the sensory nervous system. The stressor does not actually have to be a 'real' threat to the animal, just perceived as one, for the stress response to happen. The stressor is evaluated in the central nervous system and a biological response is developed. The response can be either behavioural; an autonomic nervous system response; a neuroendocrine response; a response within the immune function; or a combination of the four types of response.

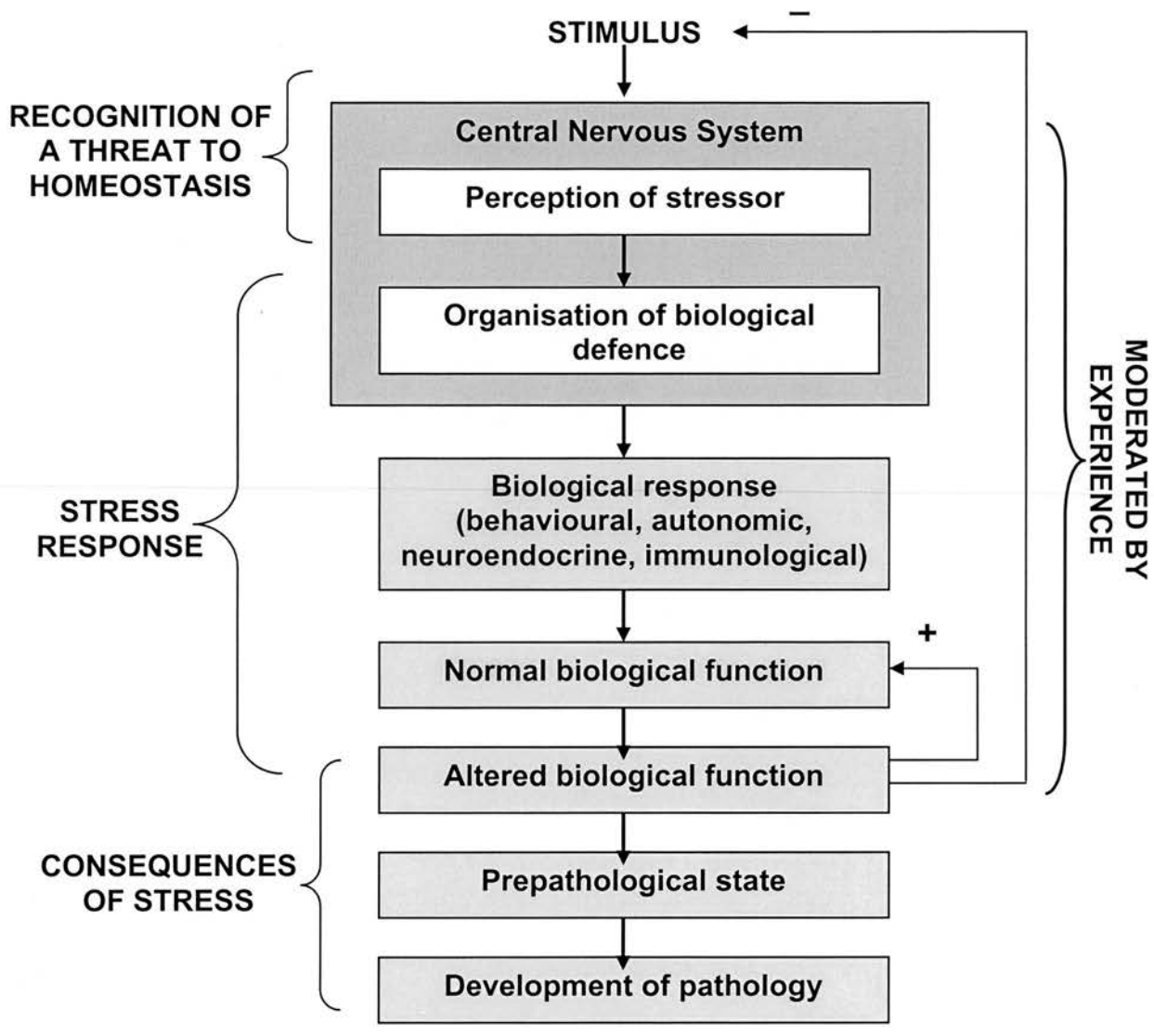


Figure 1.2. A model of the biological response of animals to stress (from Moberg, 1999).

Most stressors can be avoided by responding behaviourally, e.g. moving away from the stressor. Where animals are captive, the behavioural response to a (perceived or actual) threat may be impossible or curtailed (Wiepkema and Koolhaas, 1993). Many stress responses also include an autonomic nervous system component, resulting in changes in heart rate and blood pressure. These responses enable the animal to take quick or drastic action to avoid the stressor (e.g. the increase of blood flow to the muscles enabling a prey animal to run away from a predator). Both behavioural and autonomic nervous system responses to stressors do

not necessarily alter an animal's welfare (especially if the responses are short-term – Moberg, 2000).

A neuroendocrine response may have long-lasting effects for the animal. The pituitary gland secretes hormones in response to stressors and most studies that include hormonal assays to 'measure' stress are investigating the hypothalamic-pituitary-adrenal (HPA) axis. The hormones that are secreted in response to stressors, for example adrenal glucocorticoids, have wide implications within the body and can affect reproduction, immunological function and sleep (Möstl and Palme, 2002).

The difficulty with trying to measure corticosteroid secretion as a way of recognising stress in animals is that occasional blood sampling does not give a clear picture of the underlying physiology of the animal (Rushen, 1991), let alone the fact that blood sampling in itself is stressful for the animal (Möstl and Palme, 2002). Corticosteriod sectretion is an irregular and pulsing, so that even a sampling frequency of two minutes may not show the distribution of secretion (as shown in sheep by Engler et al. 1989). Cortisol secretion has a species specific circadian rhythm that varies among individuals, and without having a full baseline profile of cortisol secretion over 24-h (rather than a single baseline sample), the relationship between the post-treatment sampling and the baseline cannot be ascertained (Schmidt-Reinwald et al. 1999).

Furthermore, trying to 'measure' stress is difficult, not least because the way an animal responds to a stressor (i.e. the type of response used) and the magnitude of the response can be affected by many variables related to the individual animal. Animal temperament, early experience, learning and its emotional or physiological state (e.g. pregnancy) all may affect how an animal responds to a standard stressor. In addition, whether the animal has any control or can predict the stressor will also affect the stress response (Weiss, 1972).

Moberg (2000) argues that in terms of assessing animal welfare, it is not the stress response that is important, but the alteration in biological function. Prolonged or severe stress may have a high biological cost (i.e. the response to the stressor may divert energy from the normal function to the stress response). When the biological cost is high enough to alter biological function the animal's welfare is adversely

affected and the animal is suffering from distress. If the stressor is particularly severe, the animal is weak (e.g. neonatal lambs), or the stressor is of a chronic nature, then the animal can be in a prepathological state, which can further develop into a pathological state. Here the animal may have reduced immune competence and be unable to fight off pathogens, or the animal may develop abnormal behaviours; both would be seen as a pathology in the model proposed by Moberg, (2000). For discussion of the relationships between stress responses and sleep see section 1.5.3.1 below.

Chronic stress describes the ongoing response to a prolonged or repeated stressor. When acute stressors are repeated and the recovery period between each one does not allow the animal to fully recover from the previous stress response, then the animal may suffer chronic stress, and may have altered biological function as a result. Each individual stress response may be minor and not result in the alteration of biological function, but with the build up of stressors over time, the animal's biological reserves cannot replenish and distress can occur (Dwyer and Bornett, 2004). Recognition of chronic stress may be more difficult than recognition of acute stress, especially if the stressors are not identified due to their minor nature. In situations of chronic stress the animal may no longer react to stressors in a behavioural fashion, and may become withdrawn, -a condition known as learned helplessness (Wemelsfelder, 2005). Chronic stress may affect animals in a similar way to the symptoms of depression in humans. Chronic mild stress experiments are often carried out with laboratory animals as models for human depression. For more information on the relationship between chronic stress, depression and sleep see section 1.5.3.2 below.

1.5.2.2 The recognition of pain

The recognition of pain in non-human animals is an extremely important topic in animal welfare, as pain is one of the negative experiences that can cause animal suffering (Dawkins, 2003). Details of pain mechanisms and the discussion of pain in non-human animals is beyond the scope of this review and can be found elsewhere (e.g. Bateson, 1991). Clearly, being able to assess when an animal is in pain, whether it is experiencing minor or severe pain and being able to assess

whether the pain is short-term or chronic, would greatly assist in the assessment of animal welfare (Molony and Kent, 1997).

1.5.2.3 Positive mental experiences

Much of the current literature in the field of animal welfare science is based on the recognition and alleviation of suffering and assessment of poor welfare. Whilst the ultimate alleviation of suffering might be a goal for the animal welfare scientist, it may overlook what might be important to the individual animal (Dawkins, 2006). Is it good enough to reduce suffering without thinking about the animal's positive mental experiences? Some aspects of animal welfare assessment try to look at what the animal wants, such as in preference testing. However, the choices available to the animal may not always enhance its well-being, but merely give it the best of a poor lot (Cabanac, 2005). Preference testing can be enhanced by allowing animals to 'rank' choices, e.g. healthy rats will prefer water with sweeter molecules in it, i.e. polycose>maltose>sucrose>glucose etc. Cabanac (2005) argues that behaviours that are beneficial to the animal in terms of biological fitness should be pleasurable (and certainly are pleasurable for humans):

"The hedonic dimension of sensation and consciousness that allows the optimisation of behaviour...maximisation of pleasure produces useful behaviours"

The useful behaviours in question are those that increase fitness, including feeding, sexual behaviour and sleeping (Cabanac, 2005).

1.5.3 *The potential use of sleep to help assess animal welfare*

Studies within human sleep medicine have shown evidence that waking experiences and subjective feelings can affect subsequent sleep quality and quantity, and that disturbances to sleep can affect subsequent waking performance. Sleep medicine is now a serious discipline and hospitals and medical centres often have dedicated sleep centres. However, even though when animals are used as models for humans, and their sleep has been affected in a similar fashion to humans, sleep and its relationship to waking experience is often not taken into consideration when trying to assess animal welfare. During normal husbandry procedures, sheep undergo experiences that they may find stressful (in the short-term e.g. shearing –Hargreaves

and Hutson, 1990c, or over a longer period, e.g. transportation –Cockram et al. 2000) or painful (e.g. castration in lambs –Molony and Kent, 1997) and these procedures may result in an alteration in subsequent sleep. Sheep may also be put into situations in which they are deprived of sleep and rest for long periods (e.g. during long transport journeys –Cockram et al. 1996) and this could affect how they respond to subsequent stressors. Understanding the relationships between sleep and waking experiences in sheep could assist in our assessment of their welfare.

1.5.3.1 The relationships between sleep and stress

In order to investigate the hypothesis that waking experience can affect the sleep of sheep, it is important to understand the relationship between sleep and stress. Human sleep can be disturbed by waking experiences (reviewed by Van Reeth et al. 2000) -surveys of human populations have found that one in four adults report that they experience sleep disruption and daytime related tiredness at some point during a year (Kalimo et al. 2000) the most common cause of non-pathological sleep disruption was job-related stress. Åkerstedt et al (2002a) found that both 'psychological stress' (e.g. worrying about work) and 'physical stress' (e.g. long working hours) were strongly associated with disturbed sleep and impaired awakening. Kirkegaard Thomsen et al (2003) showed that having negative feelings about the following day affected sleep by increasing the number of awakenings over the night, giving evidence to suggest an emotional modulation of sleep. Komada et al (2001) showed that psychological factors were associated with difficulty in falling asleep. Major stressful events, leading to post-traumatic stress disorder in humans have also been shown to affect sleep years after the original stressor (Germain and Nielsen, 2003; Otte et al. 2005). The above research indicates an association between psychological stress in humans and sleep disturbance. However, surveys do not determine how waking experience affects sleep in humans and non-human animals.

The hormones secreted by the HPA axis have a relationship with sleep that is not just related to the stress response: there is a circadian pattern to the secretion of HPA hormones, and they may have a sleep regulatory function apart from the stress response related functions (Friess et al. 1995). Cortisol secretion, for example, is at

its lowest during the first hours of sleep in humans, coinciding with the period of maximum stage IV Non-REM sleep. During the second half of the sleep period, cortisol secretion is increased, coinciding with the period of increased REM sleep in humans. Whether there is a connection between the sleep stages and cortisol secretion is open to some debate. Cortisol secretion usually recommences at the start of the second bout of REM sleep in humans (Fehm and Born, 1991). However Follenius et al (1992) show that changes in sleep stage presentation (achieved by short auditory arousal stimuli) do not significantly affect the timing of cortisol secretion. Späth-Schwalbe et al (1991) suggested that wakefulness induced secretion of cortisol; that during a normal night's sleep in humans, microarousals occur in the second half of the night, increasing cortisol secretion; which increased again when people were awake in the morning, before lowering during quiet wakefulness. Therefore, the work by Follenius et al (1992) must be questioned as they used arousal stimuli to affect the states of sleep. During the first half of the night in humans, growth-hormone-releasing-hormone secretion is at its highest, and the high concentration of growth hormone may affect/inhibit the secretion of corticotropin-releasing-hormone (Holsboer et al. 1988). This results in a reduction of plasma cortisol levels in the first half of the night as discussed above (as reviewed by Steiger et al. 1998 and Steiger, 2002).

A similar pattern of growth hormone, corticotropin-releasing hormone and corticosterone secretion exists in the sleep period of the rat (Vázquez-Palacios et al. 2001) and the adult pig (cortisol secretion Ruis et al. 1997). Subcutaneous injections of doses of corticosterone were found (in the higher doses) to have an alerting response (more wakefulness and less Non-REM sleep) in rats during the first 3-h of the sleep period; REM sleep was not affected (Vázquez-Palacios et al. 2001). Other rodents studies have found that CRH is related to waking (e.g. Ehlers et al. 1986 -as reviewed by Opp, 1995) and in the regulation of waking *per se* (Opp, 1998; Chang and Opp, 2001) and this provides evidence for the hormonal mechanism for stress-induced changes to sleep (Chang and Opp, 2002). The action of the hormones that can be secreted, and the activation of the autonomic nervous system, in a stressful situation is to prepare the animal for a flight response (Moberg, 2000). This in itself could be expected to decrease sleep (in the short term) and increase vigilance

(Steiger, 2002). However, in laboratory studies of animals subjected to stressors, the relationship between sleep and stress are extremely complex, stressors seemingly both increasing and decreasing sleep, with both or either REM or Non-REM sleep being affected (reviewed by Cespuglio et al. 1995).

The majority of laboratory ('non-clinical') research into the effects of stress during waking on subsequent sleep has been carried out on rats and mice. Webb and Friedmann (1971) attempted to modify the sleep patterns of rats by subjecting them to a variety of waking experience and found very little change in the overall sleep amounts or pattern. However, since the late 1970s, researchers have been investigating the subtle effects of waking experience on rats.

An experiment to look at stress and its effect on sleep in rats used a controllable and uncontrollable foot shock as the stressor (Kant et al. 1995) similar to that used by Weiss (1972). One group of rats could pull a chain to escape the shock while the other group was yoked to the first and received a shock that could not be controlled. The foot shock was applied many times during the 14 day stress period, but the majority of the shocks were avoided by rats pulling the chain. Prior studies had shown that plasma corticosteroid levels took 10 days to return to baseline in the controllable stress group and 14 days to return to baseline in the uncontrollable stress group. The authors expected that any change in sleep would follow a similar pattern.

After the first day of the shock test the total amount of sleep decreased significantly and the REM sleep decreased by four times that of the baseline amount. The total sleep levels were not significantly different from the baseline by day two of testing. The REM sleep was not significantly different from the baseline by day four in the controllable stress group and by day two in the uncontrollable stress group. The rats that could control the shock experienced more of a change in the sleep pattern than those which had no control. Rats would then exhibit a higher amount of sleep during a recovery period ('rebound' sleep) and this was higher in the controllable shock group as opposed to the uncontrollable shock group. Interestingly, these results are the opposite to the classic study of stress-related gastric ulcers: rats that could control a shock had fewer ulcers then rats that experienced the same, uncontrollable shock (Weiss, 1972).

However, the most striking change in sleep observed by Kant et al (1995) was the change in the rat's circadian sleep pattern. In both stress groups, sleep decreased in the light phase of the day (the animals were kept on a 12hL:12hD regime) and increased during the dark phase of the day, and this change in circadian rhythm lasted throughout the experiment (both the testing and the recovery period). The change in circadian rhythm lasted at least 21 days, longer than the researchers previous 'measures' of stress. One problem with this experiment is attempting to separate the effects of the stressful procedure from those of the pain of the foot shocks on sleep alterations, although, the authors show that the majority of the foot shocks were avoided.

Moreover, the rats in the experiment by Kant et al (1995) remained in the shock chamber during the test period. Pawlyk et al (2005) showed that the decrease in REM sleep after foot shock training was only seen in rats that remained in the familiar test chamber post-training. Rats that were moved to a familiar neutral chamber showed *increases* in REM sleep post-training. These results showed that changes in sleep were not only due to the physiological responses to the pain/stress/learning of the foot-shock training, but also modified by psychological factors.

Sanford et al (2003) investigated the change in sleep in mice during foot-shock association (with an auditory signal) training. During four days of training, REM sleep was always shown to decrease compared with baselines. The mice also showed reduction in REM sleep after presentation of the auditory signal alone, and this response could be seen for two days after the presentation of the signal (although there were mouse strain differences in the response). This indicated that sleep in mice was affected by both the stressors/painful stimulus itself and the psychological association (anticipation and fear) of the stimulus. The same group of authors showed decreases in REM sleep after exposure of mice to an open-field test (again with strain differences seen in both the response and the recovery - Tang et al. 2004).

Koehl et al (2002) suggest that there may be circadian variation in sleep disturbance depending on when the stress is applied in a 24-h period. They had two treatment groups of rats housed on a 12hL:12hD regime. The rats were restrained for 1-h. The restraint was either the first hour of the light phase, or the first hour of the

dark phase. The EEG was recorded for the 23-h post restraint. Both groups showed an increase in REM sleep as compared with the controls and this increase was due to an increase in the number of REM sleep bouts, rather than an increase in bout length. Rats that were restrained at the start of the dark phase showed the increase in REM sleep from 3-h after the termination of the restraint. Those that were restrained at the start of the light phase, showed an increase in REM sleep from 9-h after the termination of the restraint; most of the change in sleep occurring in the dark phase. Therefore, the increase in REM sleep after a short restraint stress seemed to be consistent in occurring in the dark phase. The authors did not suggest why the circadian variation in sleep changes after stress should occur.

Dewasmes et al (2004) found an increase in the number of bouts of REM sleep during the dark phase after a 1-h immobilisation stressor. They also found differences in the inter-REM-sleep-bouts post-treatment as compared with baselines but no difference was seen in the amount or bouts lengths of Non-REM sleep. Bonnet et al (1997) also found increases in the number of REM sleep bouts after an immobilisation stressor and correlated this increase with the initial secretion and resulting active re-uptake of serotonin and the increase (without rapid re-uptake) of a corticotropin-like intermediate lobe peptide in the dorsal raphe nucleus (see section 1.3.3 above).

One hypothesis of the function of sleep is a recovery process of the central nervous system from prior wakefulness (e.g. Marinesco et al. 1999). Meerlo et al (1997 and 2001) carried out a series of experiments on rats to determine whether changes in sleep seen after a stressful event depend on the duration of that event or a combination of duration and the 'emotional experience' of the animal during that event. Prolonged wakefulness results in a high proportion of slow-waves in Non-REM sleep (Borbély and Tobler, 1996). One group of rats were gently handled for a period of 1.5-h to keep them awake. The second group of rats were subjected to a 1-h stressful social interaction (with 0.5-h handling, to match the controls). The social interaction consisted of placing the rats into the home cage of a larger, aggressive rat for 1-h (and this stressor had previously been shown to affect behaviour and circadian rhythms of temperature control – Tornatzky and Miczek, 1993). A significant increase in the proportion of slow waves seen in Non-REM sleep was

seen in both groups as compared with baseline levels (Meerlo et al. 1997). The increase in slow waves was significantly higher and lasted for longer in the social interaction group than in the handled controls. All the Non-REM sleep seen in the 24-h post treatment was characterised by an increase in low frequency waves in the defeated rats than in the controls and the baseline. There was no effect on total sleep time in either group; the change was only in what the authors define as sleep 'intensity' (Meerlo et al. 1997).

In a follow-on study, Meerlo et al (2001) found that the increase in Delta waves in Non-REM sleep after a stressful waking experience persisted over time. Socially defeated animals were gently handled for 5-h following the 1-h of social defeat (controls gently handled for 6-h). The increase of slow waves between the treatment and the control groups was still seen (albeit at a smaller magnitude than the previous experiment) (Meerlo et al. 2001). Further studies have shown that the relationship between hormones released during the social stressor and the relationship with sleep are complex. Vaanholt et al (2003) used β-endorphin deficient mice and showed that β-endorphins were involved in the direct behavioural and thermoregulatory responses to social stress, but were not involved in the changes of sleep.

Meerlo et al (2001) suggested that some stressors may be associated with an increase in arousal and alertness and that this could 'inhibit the occurrence of sleep'. Other, perhaps more 'emotionally extreme' stressors could build up a sleep debt, increasing both the motivation for sleep and the 'intensity' of the sleep experienced (Meerlo et al. 2001). Papale et al (2005) carried out a series of different stressors (22-h of restraint, intermittent foot shocks, swimming and cold) on rats to investigate the effects on sleep. They found that each type of stressor altered sleep in a different way, and only cold stress had no effect on subsequent sleep.

There has been very little research on the effects of stressors on sleep in farm animals. Ruckebusch showed that changes in environment (1975b) and parturition (1975c) had effects on sleep in cattle. Cattle showed increased fragmentation of sleep after changes in housing. Ruckebusch showed that cattle reduced their total sleep time after parturition and again when the calf was removed, although it should

be noted that only two cows were used in the experiment, so conclusions are difficult to draw.

It is important to remember that learning may be involved in all of the stress and sleep experiments, and that learning in itself can produce changes in sleep (Laureys et al. 2001). Schiffelholz and Aldenhoff (2002) provided rats with novel objects in familiar environments and found that there were post-treatment increases in REM sleep. The authors suggested that the increases in REM sleep were due to learning rather than stress, as observation of behaviour showed very little stress-related behaviour and much exploratory behaviour.

1.5.3.2 The relationships between sleep and subjective mood

One of the challenging questions in animal welfare research is that of whether or not animals can become depressed in a similar way to humans. Depression is a clinical term, which comes about in humans as a result of unrelenting stress, thus reducing the ability to cope (Wirz-Justice and Van den Hoofdakker, 1999). Depression in animals is a relatively new area of animal welfare research and may be linked with boredom resulting from barren laboratory and farm environments (Wemelsfelder, 2005). The effects of chronic mild stress regimes (repeated mild acute stressors) in non-human animals are often used as models for human depression and the behavioural alterations in rats after a regime of chronic mild stress are similar to those seen in humans (Grønli et al. 2004). Whether these responses would be seen in animals such as the sheep, remains to be seen.

In humans, it is known that moods, particularly clinically depressive moods, are associated with particular differences in sleep pattern as compared with mentally healthy people (Kirkegaard Thomsen et al. 2003). Moreover, people with sleep disorders (such as sleep apnoea) are more likely than healthy people to suffer from depression (Vandeputte and de Weerd, 2003). The changes in the sleep/wake pattern in humans diagnosed with depression are mainly a shortening in the latency to exhibit REM sleep and an increase in the frequency of REM sleep (Rotenberg et al. 2002) (different sleep alterations are seen in different psychiatric disorders, such as obsessive-compulsive disorder –Hohagen et al. 1994). The quality of REM sleep is also affected by depression; patients with clinical depression have a greater density

of eye-movements within eye-movement bursts in REM sleep bouts (Douglass et al. 1992; Buysse et al. 2001). In addition, Röschke and Mann (2002) showed changes in the spectral qualities of the EEG during Non-REM sleep in depressed and healthy humans. The Non-REM sleep had a higher amount of delta/beta oscillations and theta/beta oscillations than the healthy controls (leading to a lower proportion of the slowest waves). The authors suggest that the change in the oscillations that occur during Non-REM sleep in depressed people might impair the function of Non-REM sleep (Röschke and Mann, 2002).

Further evidence of the inter-relatedness of sleep and depression is shown by the fact that humans can have their depressive mood improved by a night of sleep deprivation (Adrien, 2002). Specifically, reducing the amount of REM sleep (partial sleep deprivation) has the short-term consequence of improving mood in clinically depressed patients (Cartwright et al. 2003). The anti-depressant response to REM sleep deprivation seems to be mediated by tiredness: those people who were the least tired (by self-report) were the ones who had the greatest benefit from REM sleep deprivation, regardless of the level of prior depressive state (Bouhuys et al. 1995). One of the side-effects of tricyclic anti-depressant drugs is the reduction in REM sleep –leading some authors to suggest that this is a drug action rather than merely a side-effect (Wirz-Justice and Van den Hoofdakker, 1999).

Depression in humans is associated with a reduction of serotonin in the brain. Serotonin has been shown to be involved in sleep regulation (see section 1.3.3 above). Serotoninergic systems are active during wakefulness and inactive during sleep, especially inactive in the transition to, and during REM sleep (Adrien, 2002). Adrien (2002) suggests that if there is a reduction of serotonin associated with depression, then the transition and continuation of REM sleep is facilitated. The increase in rapid-eye-movements is probably caused by an increase in Pons-Geniculate-Occipital (PGO) waves, though as PGO waves can only be measured by implanted electrodes they have not been seen in humans (Douglass et al. 1992). It can be speculated that the activity in the PGO-wave generating cells of the peribrachial pons are involved with the reduction in serotonin (Buysse et al. 2001). However, the neurobiology of depression and its relationship with sleep is not well understood (Checkley, 1996; Nestler et al. 2002). Further investigations into the

association between chronic stress, depression and the HPA axis and its effects on the circadian rhythm are necessary.

Cheeta et al (1997) carried out experiments to induce depression-like-symptoms in rats, in order to investigate alterations in sleep. There are two main methods of recognising depression in humans. First: the subject self-reporting feeling of depression, obviously impossible to directly record in non-human animals. Second: a change in behaviour including a reduction in pleasure inducing activities, known as anhedonia. Anhedonic behaviour can be induced in animals by a regime of chronic mild stress. In the study by Cheeta et al (1997), rats were subjected to a series of mild stressors (including, 24-h lighting, 6-h water deprivation, 12-h tilting the cage, etc) for 35 days. There was no effect of stress on latency to enter Non-REM sleep as compared with the baseline. There was a significant reduction in the latency to enter REM as compared with baseline. There was a tendency for the stressed animals to have a higher amount of REM sleep than the baseline. Cheeta et al (1997) suggested that animals showing anhedonia, similar to humans with depression, may have an associated reduction of dopamine in the brain, and that it is the depletion in amines that affects the latency of REM sleep. Supporting this is the fact that drugs which deplete amines have also been shown to shorten REM latency in humans (Berkowitz et al. 1990). Cheeta et al (1997) conclude that both stress-induced anhedonia and stress-induced sleep changes may share common neurophysiological mechanisms. Grønli et al (2004) carried out a very similar experiment, and found both anhedonic reduction in the intake of sucrose and changes in sleep including: increased REM sleep, increased number of arousals and a decrease in slow waves during Non-REM. Comparable sleep patterns were seen in a genetic model of depression in mice. The 'helpless' mouse had more REM sleep and more awakenings than 'normal' mice. The different sleep pattern could be 'treated' with anti-depressant drugs and became identical to the pattern exhibited by the 'normal' mice (El Yacoubi et al. 2003).

In addition, exercising an animal that is 'depressed', or giving an animal tricyclic antidepressant drugs has been shown to have the same reversal effect on the 'depression-related' sleep pattern as it does in humans (Sarbadhikari, 1995; Sarbadhikari et al. 1996; Cheeta et al. 1997).

Sleep is not just related to negative moods and depression in humans but is also related to positive moods. People who were rated as happy, also self-reported that they slept well. In addition the majority of people view sleep as a positive experience saying 'they look forward' or 'enjoy' going to sleep (Dement, 2000). It is possible that non-human animals may have similar positive feelings about sleep.

1.5.3.3 The relationships between sleep and pain

Details of pain mechanisms and the discussion of pain in non-human animals is beyond the scope of this introduction. However, there is evidence that painful conditions can disturb sleep and pain is an important area in animal welfare studies so that a brief discussion is merited.

It is well documented in human medicine that sleep can be affected by aversive experiences such as a painful condition and medical illness (reviewed by Moldofsky, 2001). Human patients with a painful condition provide a clear example of how sleep can be disrupted by pain. Drewes et al. (1998) studied the EEG during sleep and wakefulness of patients with rheumatoid arthritis and found that patients with arthritis were more likely to experience shorter, more fragmented, sleep bouts than healthy controls. The EEG showed that there was a higher percentage of alpha waves in the stage III and IV sleep in the arthritic patients (Drewes et al. 1998). The authors suggested that, as alpha waves are characteristic of wakefulness, this may be caused by an 'internal arousal activity' which is disrupting normal sleep (Drewes et al. 1998). Hospitalised burn patients also have sleep problems (Raymond, et al. 2001). It was found that patients reporting a poor nights sleep had a higher intensity of pain the next day, but there was no evidence that the most painful days were followed by poor sleep at night. However, 75 % of the patients reported poor sleep on a regular basis (Raymond et al. 2001).

There are also relationships between sleep and pain in non-human animals. Rats with (chemically induced) adjuvant arthritis showed a significant reduction in REM sleep, a reduction in the highest amplitude slow-wave sleep and a lowering of the amplitude of slow waves throughout the sleep periods (Landis et al. 1989). The rats with arthritis could not sustain long periods of sleep. Cats that had received

formalin injections showed 'pain related behaviours' and decreased sleep, particularly Non-REM sleep as compared with handled controls (Moldofsky, 2001). When injections were no longer given, pain related behaviours were reduced after 3-h, but Non-REM sleep was reduced for the following 24-h. This experiment suggests that changes in sleep patterns are more long-term than other behaviours that may be useful in assessing animal welfare.

Onen et al (2001) showed that the relationship between pain and sleep is a two-way relationship. They deprived rats of REM sleep and then tested for pain sensitivity. They found that rats that had been deprived of REM sleep had lower thresholds in response to minor pain and increased their behavioural responses to electrical stimulation compared with handled controls (Onen et al. 2001). After sleep recovery, Onen et al (2000) found that the thresholds for pain had returned to pre-sleep deprivation levels.

1.5.3.4.The relationships between prior sleep deprivation, waking experience and subsequent sleep

It has been shown above that the waking experience of an animal can affect its subsequent sleep. In what respect can disturbed sleep affect the subsequent waking behaviour, responses to subsequent stressors and the following sleeping periods?

There are two types of sleep deprivation: total sleep deprivation, in which the subject is kept awake for a period and partial sleep deprivation, in which a subject is allowed to sleep but is woken every time he/she enters REM sleep (reducing overall REM sleep by 98 % -Brunner et al. 1990). Cajochen et al (1999) found that after 40-h total sleep deprivation in humans, the recovery period consisted of a lower proportion of REM sleep than the baseline, and increased in stages III and IV Non-REM sleep. Non-REM sleep also increased after 24-h total sleep deprivation, but differed in the amount of increase depending on when the deprivation and recovery periods started, day or night. The increase was highest at the start of the night as compared with the day (Tagaya, 2002). Scott et al (2006) found that a 30-h total sleep deprivation resulted in lowering of reaction times indicative of impaired cognitive function.

Sleep restriction (partial deprivation) in humans has been shown to reduce the latency of sleep in the following sleep period (Brunner et al. 1990). After two nights of 4-h sleep, humans showed an increase in total sleep time for three consecutive days as compared with baselines. The first night of the recovery period consisted of a lower proportion sleep stages I and II and a higher proportion of sleep stages III, IV and REM sleep (Brunner et al. 1990).

Ozturk et al (1999) studied the effects of sleep deprivation on the immune profile in humans. There was a decrease in the CD16+ 'Natural Killer' lymphocyte cells after 24-h of sleep deprivation in comparison to the baseline values, and this lower level was maintained after 48-h of sleep deprivation. The count of CD16+ cells returned to baseline levels after 24-h of recovery period (Ozturk et al. 1999). With respect to animal welfare studies, it is important to know whether sleep deprivation can lead to increased susceptibility to infection.

The original studies into sleep deprivation claimed that rats would die if deprived of sleep for a little over a week. These original claims have now been refuted, as the method of sleep deprivation was extremely stressful and there was no way of separating the effects of sleep deprivation from the effects of stress (Rechtschaffen et al. 1983). Allan Rechtschaffen and colleagues developed a less stressful method of total sleep deprivation known as the 'disk over water' method. Here, there were control and treatment rats both experiencing the same conditions, except that the disk would turn when the treatment rat started to go to sleep, forcing both animals to walk. Even with the relatively lower-stress sleep deprivation method, treatment rats did show outward signs of pathology during a 33-day sleep deprivation period; all increased their food intake but also lost body mass and three died (Rechtschaffen et al. 1983, see also Rechtschaffen and Bergmann, 1995). Everson (1995) found that immune system integrity was affected by prolonged total sleep deprivation, allowing lethal organisms to enter the blood stream of rats, eventually leading to their deaths.

Just 24-h total sleep deprivation can have long lasting effects on the rat. Borbély et al (1984) found that the rebound EEG after 24-h sleep deprivation was characterised by increased slow waves seen in Non-REM, but a reduction in the total time spent in Non-REM sleep and an increase in time spent in REM sleep. Similar

results were seen by Schwierin et al (1999), except that Non-REM sleep increased in the sleep rebound period and the increase in both REM and Non-REM sleep was seen to last for four days post-deprivation. Rats deprived of sleep were found to have an increase in extracellular serotonin levels (as measured by microdialysis) during the deprivation day, and these elevated serotonin levels remained higher than controls during the recovery period (Lopez-Rodriguez et al. 2003). Sleep deprivation also results in an increase in HPA axis activity (plasma ACTH and corticosterone concentrations –Sgoifo et al. 2006).

REM sleep deprivation (partial deprivation) alone is a stressor in rats as demonstrated by a corticosterone response (Suchecki et al. 1998 and 2002). The studies used different methods of REM sleep deprivation to control for differences in the stress of the procedure. The authors concluded that the HPA axis responded to the sleep deprivation over and above the reaction to the stressful procedure (Suchecki et al. 2002).

Kennedy (2002) studied the effects of REM sleep deprivation on behaviour in rats. The rats were trained to press levers to gain food pellets. REM sleep was deprived by the use of a pedestal over water. The REM sleep deprivation procedure lasted for 24, 48 or 96-h. The numbers of successful lever presses were significantly lower in rats that had been REM sleep deprived for 96-h. There was no difference in lever pressing behaviour after 24 and 48-h of REM sleep deprivation compared with the baseline (Kennedy, 2002). The author suggests that the positive reinforcement involved with the lever pressing task would mean that differences after a short period of REM sleep deprivation would not be shown, as the rats would be very motivated to perform the task. When the task was associated with negative reinforcement, the rats spent more time sleeping during the task period and lever pressing tended to show a reduction after all REM sleep deprivation amounts.

Meerlo et al (2002) showed that total and partial sleep deprivation not only acted as a stressor and caused activation of the HPA axis, but also altered the reaction to subsequent stressors. This is an important study for animal welfare as sleep deprived animals may often be further challenged with stressors and may have altered coping abilities with these after a period of sleep deprivation. For example, sheep may be deprived of sleep and rest during long distance road transport, and

when reaching their destination they will have to cope with additional stressors: a novel environment and mixing of social groups. Rats were sleep deprived by means of a slowly rotating wheel; controls confined to a non-rotating wheel (Meerlo et al. 2002). Both sleep deprived and control rats were subjected to a restraint stress for 30 minutes, 4-h after the sleep deprivation. Blood samples were taken before the sleep deprivation, after 1, 6, 24 and 48-h of sleep deprivation and before, directly after and 45 minutes after the restraint stress. The concentration of plasma corticosterone was significantly higher after 6-h of sleep deprivation than in the controls and remained elevated until the end of the 48-h sleep deprivation (Meerlo et al. 2002). After the 4-h recovery period, there was no difference in corticosterone concentrations between the control and sleep deprived rats. However, sleep deprived rats showed a significantly smaller ACTH response to the restraint test than the control rats (corticosterone concentrations were not significantly different). It is possible that the sleep deprivation resulted in increased adrenal sensitivity as the corticosterone concentration of the sleep deprived rats was the same as the control rats, whereas the ACTH concentration was much reduced. Sleep deprivation not only caused a rise in HPA axis activity during and shortly after the stressor, but also affected the response to a subsequent novel stressor (Meerlo et al. 2002).

In a similar study, rats that had been subjected to 48-h total sleep deprivation were subsequently (4-h post-deprivation) subjected to a 15 minute restraint stress (Sgoifo et al. 2006). Blood samples were taken during the sleep deprivation period, and before and after the restraint test. ACTH and corticosterone were elevated during the sleep deprivation as compared with the controls and corticosterone remained elevated throughout the sleep deprivation period. Both sleep-deprived and control rats experienced elevation of corticosterone after the restraint stress. The ACTH response to the restraint test was attenuated in the sleep deprived rats (Sgoifo et al. 2006). The authors suggested that the attenuated ACTH response may have reflected a decreased input from higher brain centres, including a decrease in the secretion of corticotropin-releasing hormone from the hypothalamus, paralleled with an increase in adrenal sensitivity to ACTH.

Sleep deprivation experiments can be unintentionally flawed by the stressful procedure to deprive the animal of sleep (Rechtschaffen and Bergmann, 1995;

Suchecki et al. 1998). Interestingly, human studies do not find an increase in HPA axis responses after sleep deprivation, unless they are asked to perform a physical task to remain awake (Ozturk et al. 2002). Perhaps being consciously aware of the reason for sleep deprivation means that it is not a stressful event for humans.

There has been very little published research into sleep deprivation in farm animals. Ruckebusch (1974) carried out an experiment to determine the effects of REM sleep deprivation on the subsequent sleep bouts in cattle. Recumbency, and therefore REM sleep, (as Ruckebusch found cattle can exhibit light Non-REM sleep while standing if forced to do so, the cattle were able to lean on the strap that prevented lying) was prevented for 14-h each day for four weeks, 20-h/day for two weeks and 22-h/day for two weeks. In the first four weeks the sleeping pattern of the cattle had adapted within five days so that a similar total amount of REM sleep to the baseline period (no lying restriction) was seen, but it occurred during the day when lying was permitted (Ruckebusch, 1974). In the four weeks, REM sleep was much reduced (and absent in the 22-h/day deprivation weeks). There was an increase seen in Non-REM sleep during this time. The bouts of Non-REM decreased in length as compared with the baseline period. On the fourth day post-deprivation rebounds were seen in both Non-REM and REM sleep. Fragmentation of Non-REM sleep was reduced (i.e. bouts increased in length) and REM sleep showed double the number of episodes compared with baseline values. This included sleep during the day, although by the fifth day post deprivation sleep only occurred at night (Ruckebusch, 1974). The author suggests that cattle can adapt quickly to a reduction of lying behaviour. Interestingly, Ruckebusch notes that the cattle became more aggressive towards humans by the end of the experiment, which may indicate psychological stress. It would be interesting to carry out such an experiment in conjunction with objective measures in changes of behaviour to assess the psychological changes that occur during the experiment.

1.5.3.5 The relationships between sleep and fatigue

Fatigue is not a simple phenomenon: there are many emotional, behavioural and cognitive factors which build up to the subjective feeling of fatigue (Dirnberger et al. 2004). There is a difficulty in the use of terminology and there can be

confusion between feeling sleepy and feeling fatigued (Loge et al. 1998). Sleepiness is defined as the increased feeling and propensity to go to sleep. Sleepiness in humans is affected by posture, and the amount of physical and mental activity being undertaken, as well as the duration of wakefulness (Johns, 2000). People will report that they do not feel sleepy during a physical or mental task, especially if they have to maintain an upright posture during the task. Sleepiness is therefore different from fatigue.

Muscle fatigue is the physiological event, occurring after a physically demanding task, and it is caused by the build-up of lactic acid in the muscles during anaerobic respiration. Physical fatigue is the emotional feeling that muscle fatigue can bring on, and it can be described as a feeling of weariness, weakened and having a depletion of energy (Pigeon et al. 2003). People report that they feel physically fatigued during and after exercise tasks.

Mental fatigue is more difficult to define, and easily confused with sleepiness. People report feeling mentally fatigued during mental tasks and this requires rest, rather than sleep, to recover (Johns, 2000). When humans are mentally fatigued they tend to report that they have trouble 'thinking clearly' and may have difficulty completing tasks that require motivation or attention (Lichstein et al. 1997). However, if people are in a recumbent posture while undertaking a mental task, then they are more likely to report increased sleepiness than when in an upright posture. (Postural changes raise the body core temperature and this possibly has an alerting effect –Matsumoto et al. 2002; Caldwell et al. 2000). Mental and physical fatigue can also both be described as 'tiredness', in which rest is needed, but not necessarily sleep. A common term used when people report fatigue is that they feel 'exhausted' (Hartz et al. 2003) (but these descriptions are also synonymous with sleepiness –Pigeon et al. 2003). However, fatigue does have a relationship with sleep (Dawson and McCulloch, 2005). Lichstein et al (1997) showed that humans with sleep disorders (such as sleep apnoea syndrome) can have a build up of a feeling of fatigue over time if the sleep disorder is not treated. In addition, humans that reported mental fatigue (cognitive impairment) were shown to be more likely to suffer from disturbed sleep (Åkerstedt et al. 2004).

Fatigue (other that the physiological measurements of muscle fatigue) in non-human animals is an under-researched area. The problems with studying fatigue (definitions, confusions between sleepiness and other forms of tiredness, etc) are increased by the inability of non-humans to self-report their feelings. Studies using demand functions for rest and lying behaviour (such as that carried out on dairy heifers by Jensen et al. 2005) may be a suitable method to use to assess fatigue and its implications for animal welfare.

1.5.3.6 An overview of using sleep to help assess animal welfare

It has been shown that sleep can be altered after waking events (e.g. Kant et al. 1995; Koehl et al. 2002; Sanford et al. 2003). The alteration in the sleep pattern may depend on the waking event, and whether the experience is chronic or acute (e.g. Meerlo et al. 2001). The alteration in sleep pattern is also species specific and may depend on the pattern of sleep that the animal exhibits under 'normal' conditions (e.g. Ruckebusch, 1974). It has also been shown that an alteration in sleep pattern, or a change in the amount of sleep available, may have an effect on an animal's response to subsequent waking experience, and may change an animal's responses to stressful events (e.g. Meerlo et al. 2002). Furthermore, research suggests that giving an animal a recovery period after sleep deprivation or disturbed sleep can allow them to return quickly to baseline levels (e.g. Ruckebusch, 1974). Therefore, if it is possible to recognise sleep and characterise the pattern of sleep in animals in their natural environment, before changes in a captive environment, or before potential stressful experiences, then sleep can be used as a tool to assess the effects of changes in environment and the application of stressors in animals. If animals are forced to change their sleep pattern, or are unable to perform enough sleep, this may compromise welfare (Marinesco et al. 1999). Conversely, if an environment allows an animal to perform sleep without disturbance to 'normal' patterns than it may be of benefit to the animal's welfare. Similarly, if an animal's sleep is disturbed due to a stressful event, it may be important for that animal to undergo a period of sleep recovery, allowing its sleep to return to baseline levels, reducing the likelihood of stress becoming distress (Moberg, 2000).

One problem in determining the effects of waking experience on sleep is that most research in this area does not comprehensively record all possible changes that could be taking place. For example, some studies concentrate on the amounts of REM sleep observed, others on the effects of stress on the circadian rhythm and the change in frequency of the Non-REM sleep after stress. Many sleep experiments use the EEG to determine wakefulness, REM and Non-REM sleep, but these are usually visually scored without spectral analysis. Therefore, they lose potential information (such as the increase in delta waves in Non-REM sleep found by Meerlo et al. 2001) on the effects of aversive experience on sleep. With advances in digital polysomnography and computer assisted scoring, spectral analysis of the EEG should become more widely utilised. Without looking at all of the possible changes in sleep, a complete picture of how stress affects sleep will not be possible.

1.5.4 *What welfare questions can be tackled by studying sleep in sheep?*

This review of the literature of the relationships between waking experience and sleep has shown that non-human animals react to waking experience in a number of ways; that the relationship is a multifaceted one. However, it can be shown that both the physiological and psychological effects of stressors can alter subsequent sleep. There is some evidence that this occurs in farm animals as well as in laboratory rodents (e.g. Ruckebusch, 1975b). It is probable that similar physiological and psychological stress responses in sheep would alter subsequent sleep. Some of the welfare issues for sheep and implications for sleep in sheep are discussed below.

Sheep are generally farmed in an 'extensive' manner (in a broad sense) – usually outdoors. From a public perspective, outdoor farming can appear to be very good for sheep welfare, in comparison to pigs, or chickens, which are often housed indoors in 'factory-like' conditions. However, the welfare of sheep can be compromised and many examples of poor welfare in sheep are connected with extensive means of production.

Sheep have been domesticated for at least 8000 years (Clutton-Brock, 1999). Like other species that have been successfully domesticated, they have a number of behavioural traits that make them able to be domesticated, such as: flocking

behaviour; leadership and following behaviours; and precociousness of the young (Clutton-Brock, 1999). However, when sheep are farmed in extensive conditions, they may be unused to human contact. Many husbandry procedures, such as shearing, require human contact, and sheep unused to such contact may find the procedures more stressful then sheep that have been handled on a regular basis. Studies have shown (Hemsworth and Barnett, 2000) that some physiological parameters, for example heart rate, that increase in sheep during potentially stressful procedures, are lower in those sheep that have experienced gentle handing prior to the procedure, than in sheep with little previous handling experience. Therefore, sheep that are unused to regular human contact may experience poorer welfare in association with husbandry procedures then sheep that have regular human contact.

Even seemingly innocuous, non-invasive procedures such as shearing can have an effect on sheep welfare. The procedure is carried out while the sheep is in a sitting position, in which it struggles less than in a standing position. This posture may cause anxiety for a sheep as it is very vulnerable, even though behaviourally it is less reactive (Lynch et al. 1992). The procedure is noisy and involves very close human contact. The sheep is often out of visual contact with its conspecifics and social isolation for a sheep has been shown to be stressful (Hargreaves and Hutson, 1990d). Restraint stressors in rats can affect the subsequent sleep for over 24-h post restraint (e.g. Papale et al. 2005) and it is likely that sheep would find husbandry related restraint stressful. It could be hypothesised that subsequent sleep may be affected in the sheep.

Sheep have to make the journey to market or to slaughter. The journey can involve many novel and potentially fearful stimuli. Sheep are rounded up and grouped; -the groups may well include unfamiliar animals- (Williams, 1999), and then they are loaded. As the transportation usually takes place at a low space allowance per sheep, there is restricted room to lay down to rest (Cockram et al. 1996). The journey can be long (14-h travel without food and water, a 1-h break with food and water provided and a further 14-h travel is the maximum allowed under European law - Council Directive 98/290/EEC, The Protection of Animals during Transport) and may involve a complete change in climate. Bruising and other injuries can occur (Rushen, 1996; Knowles, 1998). Sheep suffer stress and may be

partially, or totally, deprived of sleep during transport. This may lead to sleep rebound and alterations to the circadian rhythms.

The wild ancestors (and the wild species of the genus *ovis*) of the domesticated sheep were prey animals and so have a wide repertoire of predator avoidance behaviour. The domesticated sheep generally exhibits a 'dampening' of these behaviours. For instance, they have a reduced flight distance in comparison to wild relations (Clutton-Brock, 1999). However, the domesticated sheep still behaves as a prey animal, and this can have a negative affect on their welfare. Prey animals may show pain-masking behaviours so as not to exhibit weakness to possible predators. For example, sheep will graze 'normally' with a broken leg (Bateson, 1991). It could be argued that if the animal does not show pain behaviours (such as, vocalisations, or withdrawal behaviour) and continues to graze, it does not feel pain. But, if the animal is in pain, and if we are unable to see the subtle changes in behaviour, the animal's welfare would be compromised (Bateson, 1991). Sheep are stoical animals and this may in itself lead to compromises in welfare.

Up 50% of sheep in the UK have some degree of lameness caused by infections such as foot-rot and interdigital dermatitis (Williams, 1999). This may be in part due to the difficulty the stockperson has in checking over their stock on a frequent basis under extensive farming conditions. Lameness is an expressed behaviour by way of a change in gait and weight bearing (i.e. the limp) occurring in response to an injury or infection in the foot (Welsh et al. 1993). It is probable that the animal 'limps' because it is in pain and has an aversion to placing the foot on the floor and bearing weight in a normal manner. The low economic value of ewes can lead to reluctance among stockpeople to call out a veterinarian for treatment. This can be a problem during lambing as many serious complications, such as uterine prolapse, can be 'fixed' by the stockperson, potentially leading to pain and infection in the ewe and possible loss of the lamb (Scott et al. 1995). Sleep is affected by painful conditions in both humans and rats (e.g. Landis et al. 1989) and it is possible that sheep with lameness may also have altered sleep patterns.

In the UK, lambs are routinely castrated and tail-docked up to 12 weeks of age without the use of anaesthetic or analgesic (Williams, 1999). Lambs that have been castrated or tail-docked in this way show a variety of abnormal behaviours that

indicate that they are suffering from acute pain for up to 6-h after the procedure (Kent et al. 1995). This can lead to a reduction in suckling in some lambs and, if castration is carried out when the lamb is still receiving colostrum from its dam, the reduction in intake may increase the lamb's susceptibility to infection. The intermittent presence of similar behaviours for up to six weeks after castration may be indicative of chronic pain (Molony and Kent, 1997). If techniques to record the EEG in sleep are developed small enough to be fitted to unrestrained, fully conscious neonatal lambs, the EEG could be used to investigate the effects of chronic pain on sleep in lambs (e.g. Ong et al. 1997).

Sheep are also commonly used as an experimental animal and this can raise other welfare issues. Sheep are similar in size to humans and are therefore used as a model for humans in experimental surgery. The lamb shares some characteristics with human babies: birth weight, the number of young born from each pregnancy, and these allow the sheep to be used as a model in experimental foetal research (e.g. Hecker, 1983; Abrams et al. 1991). The welfare issues specific to experimental sheep are similar to other animals kept for experimentation. These include the stress of the experimental procedure itself, restricted diet, restraint and possible social isolation. Sheep used in experiments may be housed in apparatus to enable the collection of faeces and urine. The metabolic crates restrict the movement of the sheep and allow for standing and lying, but do not allow turning round or much movement (Hecker, 1983). There is evidence for the development of abnormal behaviours and stereotypies in sheep after penning for a prolonged period of time (Done-Currie et al. 1984).

1.5.5 *Possible difficulties with using sleep in animal welfare studies*

There are three main difficulties in using sleep as a method of animal welfare assessment: technical difficulties in recording sleep from animals; understanding the complex inter-relation between experiences during wakefulness and subsequent sleep alterations; and ascertaining how important sleep is to the animal.

The technical difficulties with recording and measuring sleep in non-human animals are being reduced as digital technology increases. The size of data acquisition devices has reduced in recent years, and with micro-technology it will

probably continue to reduce. It is possible to record the EEG from mice using a two-channel data acquisition and telemetry system, which can be placed intraperitoneally. Ethical concerns during animal welfare studies may lead to the preference of non-invasive electrophysiological techniques, and as Giovagnoli et al. (1996) have shown, these are already available for larger animals. Non-human animals bring other difficulties (e.g. the effect of the attachment of electrophysiological equipment on the behaviour of animals –Storch et al. 2004) to the recording of the non-invasive EEG which are reviewed in chapter 2 of this thesis. However, it seems that recording sleep in non-human animals will become easier as advanced technology leads to smaller equipment sizes and improved reliability.

Understanding how sleep, wakefulness, and mental states interact with one another is a more difficult problem to overcome. It seems that sleep and wakefulness interact in a subtle manner. Webb and Friedmann (1971) reported no significant changes in sleep (totals in 24h and Non-REM: REM sleep ratios) in rats after potentially stressful and fearful experiences during wakefulness. However, their experiments have since been repeated and changes in spectral properties and latencies to sleep have been reported (see section 1.5.3 above). The difficulties understanding sleep and wakefulness are exacerbated within non-human animals, as many changes in sleep in response to waking experiences are species specific. Careful planning and controlling of experiments to ensure any change in sleep is due to the variable being tested is extremely important in animal sleep research, especially as we do not understand all of the complex interactions between experiences during wakefulness and sleep.

When humans have disturbed sleep patterns, they report changes in subjective mood and changes in ability to remember things and learn new tasks. When asked to evaluate the importance of a 'good night's sleep', most people rate it very highly in comparison to other activities (Åkerstedt et al. 2004). Although sleep is important to humans, we do not yet know how important sleep is to other animals. As discussed above, all mammals and birds sleep, and some animals spend two thirds of their lives sleeping. It may seem as if it is obvious that sleep is important to them. However, this may only be the case when food is plentiful or predators are few (or possibly only shown in the barren environment of the laboratory). What about the animals

that sleep so much less than humans, such as sheep? Demand function experiments may be the answer to finding out how important sleep is to non-human animals (Dawkins, 1983). The difficulty in undertaking such experiments is that usually, the animal is made to work for the reward of performing the behaviour. Therefore, sleepiness or fatigue may confound the demand function by making the animal less able to work for the reward of sleep. Building on the framework used to assess the requirements for lying and rest in dairy cows as set out by Jensen et al (2004 and 2005) may provide a way of experimentally ascertaining how important sleep is to non-human animals.

1.6 Conclusions

Human studies and some non-human animal work have shown that prior waking experiences affect sleep (e.g. Ruckebusch, 1975; Meerlo et al. 2001). The minimum that can be said about this relationship is that the physiological control of sleep in humans and some non-human animals has been affected by the physiological consequences of the waking experience. Sleep, therefore, could be a valuable tool in studies to assess animal welfare. The qualities and duration of any post-experience sleep disturbance could be recorded to assess responses of the animal to that experience. In human studies, sleep disturbance after waking experiences have strong emotional content and are affected by subjective feelings, not merely physiological differences. It could be speculated that non-human animal sleep disturbances after waking experiences could also be due, at least in part to emotional changes in the animal.

Signs (such as sleep disturbance) of emotional change in animals after experiences are not necessarily indications of welfare problems. Sleep may be altered by an emotional reaction, even after that reaction has passed and the animal has returned to 'normal'. On the one hand, sleep may not be as important to other animals as it is to humans, especially animals that spend less time sleeping than humans. On the other hand, sleep in animals that are only able to sleep for short periods, might be even more important and inelastic (with regard to demand functions) than it is to humans.

Research into how aversive experiences affect sleep could expand our understanding of how animals react to such experiences. Reliable techniques to record the EEG and other electrophysiological measures on animals outside of the laboratory need to be developed to enable sleep to be used as a tool for assessing animal welfare. A technique that is able to record many parameters continuously for a long time period has the advantage of being able to assess these parameters without human interaction disturbing the animal. Most husbandry techniques, such as housing types, have a profound, and potentially long lasting affect on animals. One of the biggest problems in animal welfare studies is how to assess the long-term effects of animal husbandry on animal feelings. It is possible that chronic mild stress, which could occur under most husbandry regimes, could lead to symptoms similar to that of depression humans (as in the laboratory, chronic mild stress regimes in rats are used as a model for depression in humans). Such symptoms are not easy to record using current welfare assessment techniques. Perhaps a measure of sleep disturbance, which seems so closely connected with depression in humans, may provide animal welfare studies with a method of assessing long term chronic stress in animals.

Studies have been undertaken to determine the effects of potentially aversive experiences on lying and resting behaviour, but there are few studies that record sleep specifically. However, recording lying behaviour itself (especially in detail) will provide information about the ability of the animal to rest. If animals are unable to lie down, then sleep deprivation is a likely outcome, which potentially leads to a change in the animals' ability to cope with future stresses (Meerlo et al. 2002).

What is clear, after reviewing the literature on sleep and waking experience, is that any research into sleep disturbance after waking experiences in a non-human animal has to proceed from a thorough understanding of sleep in that species. As the literature is quite sparse for some species, researchers may need to spend the time to learn about the fundamentals of sleep in their chosen species, before applied studies can begin. In the following chapters, I introduce a novel methodology for recording the sleep of sheep and use it to measure the 24-h profile of sleep in sheep. I then go on to use the methodology to record the sleep of sheep during and after potentially aversive husbandry procedures.

Chapter 2. General Methodology

2.1 Abstract

Six Dorset ewes were used in a preliminary study to recognise and characterise sleep in sheep using a non-invasive, electrophysiological technique. Harnesses were developed to carry and affix an Embla data-logger to the back of the sheep for 24-h, ambulatory electrophysiological recordings. Individual fibre-glass helmets were fashioned for each sheep to protect the electrodes that were attached to the face and head. Three, 24-h simultaneous behavioural and electrophysiological (electroencephalogram EEG, electro-oculogram EOG and electromyogram EMG) recordings were made from each sheep. Posture was recognised using the leg and neck EMGs. Eating and rumination were recognised from the jaw EMG. Spectral analysis (frequency and amplitude) of EEG and EOG recordings was undertaken to recognise sleep and wakefulness and to quantify the time spent in REM and Non-REM sleep. The mean (s.e.) results expressed as a percentage of a 24h period were: REM sleep 2.8 (0.21) and Non-REM sleep 14.5 (2.38). These were consistent with studies using invasive methods.

Three validation experiments were conducted. An auditory arousal stimulus was used to find differences in the frequency and amplitude of EEG traces before and after waking. Changes in the sleep power bands, the frequency (fast Fourier transform) and the power spectrum of the EEG, before and after stimuli indicated waking from sleep. Post-mortem material was used to assess the ability of the non-invasive electrodes to record an EEG. A sine wave signal of known frequency was generated into the brain of an electrically-isolated sheep head and electrodes were used to record from the scalp. Three sheep were also put under terminal anaesthesia at different anaesthetic depths and the EEG traces were recorded from skin surface electrodes on the scalp and ball electrodes placed on the surface of the dura. The amplitude of the EEG recordings from the brain electrodes was considerably higher than that recorded from the scalp. During anaesthesia, the frequency spectrum of the EEG recorded by both scalp and brain electrodes was within the same order of magnitude.

EEGs recorded from non-invasive electrodes in conjunction with behavioural observation from videos, gives more information about the time budgets and circadian rhythms of sheep than behaviour alone.

2.2 Introduction

2.2.1 Aims

- To develop a non-invasive method of recognising sleep in sheep with simultaneous behavioural observation and electrophysiological recordings.
- To validate the reliability of the non-invasive electrophysiological techniques to record an EEG that could be used to differentiate between sleep and wakefulness in sheep.

2.2.2 Electrophysiology and sleep

The EEG is a record of the spontaneous electrical activity of the brain. The potentials vary in polarity in a rhythmical way, with fluctuations confined to a narrow bandwidth between 0.5Hz and 50Hz. However, amplitudes can range from a few microvolts to several hundred millivolts (Speckmann and Elger, 1987). When the EEG is recorded from electrodes placed on the surface of the scalp, the electrical activity that is recorded comes mainly from the cerebral cortex from a depth (in humans) of approximately 5mm with an average amplitude of 100μV (Peters et al. 1988). Although the EEG recorded using electrodes placed on the surface of the brain also mainly records activity from the cerebral cortex, such electrode placements can record electrical activity from other areas of the brain. This is because there is less attenuation in signal, and electrodes can be placed closer to, or even subdurally, on other brain areas (Lopes da Silva. 1991).

The skin-surface recorded EEG arises from two sources: synaptic activity of cortical neurons and changes in membrane potential of glial cells (the supporting tissue of the brain, composed of highly branched fibrous cells). The release of neurotransmitters at a synapse allows selective movements of ions through the post-synaptic membrane. These transmembrane currents result in local changes in ionic concentrations, both intracellularly and extracellularly, which result in the formation of dipoles. A dipole consists of a separation of positive and negative charges leading

to a temporary redistribution of positive and negative charges in the cellular medium. Extracellular and intracellular ionic currents flow between the dipoles because both media are excellent electrical conductors. The extracellular current gives rise to the recorded EEG from electrodes placed on the surface of the scalp (Speckmann and Elger, 1987).

The pyramidal cells of the cerebral cortex are an important neuronal source of the EEG. Their dendrites are long and arranged in parallel. Therefore, post-synaptic potentials can occur in one part of a cell while other, remote parts are quiet. The dipoles formed cause currents to flow, which will have a greater effect on an electrode (on the surface of the brain and on the scalp) than those of smaller neuronal cells. The glial cells contribution to the EEG results from potential changes in cortical neurons causing temporary changes in extracellular potassium ions. These in turn produce passive depolarisation in membrane potentials of glial cells. Because of glial cell morphology, a depolarisation of one glial cell can spread the potentials to other glial cells, amplifying the change in potential initiated by the neurons (Speckmann and Elger, 1987).

Individual post-synaptic potentials are too small to be detected by the EEG electrodes. However, there may be approximately 40,000 synapses on a single pyramidal cell, therefore there are millions of synapses within recording range of a surface electrode. There is a degree of synchrony among synapses which produces the rhythmically changing potentials seen on the EEG (Fisch, 1999).

When mammals are awake, the electrical activity produced in the brain gives a high frequency, low amplitude, and low synchronous EEG output (known as beta activity, 13-30Hz). In addition, in unrestrained animals, there is a certain degree of muscular movement (depending on the animal and the activity levels, see below), which makes recording an artefact-free EEG from electrodes placed on the surface of the scalp very difficult during wakefulness. However, during sleep, the electrical activity produced within the brain is different from that during wakefulness and there are fewer artefacts associated with muscular movement (see Chapter 1 for a review of sleep types and stages). In a mammal undergoing Non-REM (or slow-wave) sleep, the electrical activity in the cerebral cortex becomes more synchronous, resulting in a lower frequency (below 7Hz) and a higher amplitude. A relatively large area of the

cortex (particularly the parietal lobes) is involved, making the recording of the scalp surface EEG easier (Lopes da Silva. 1991). When a mammal undergoes REM sleep, the EEG is similar to that of wakefulness. However, there is a complete reduction in muscle tone (coupled with rapid eye movements), which means that the EEG (although similar to that of the waking EEG) is relatively easy to recognise if other electrophysiological correlates (such as the EOG and an EMG) have been recorded simultaneously.

2.2.3 Electrophysiological methodologies

2.2.3.1 Electrodes

There are four main electrode shapes used in electrophysiology: screw shaped, ball shaped, needle shaped and cup or disc shaped. Screw electrodes are screwed into holes drilled into the skull, allowing the electrode to have contact with the dura surface. Ball shaped electrodes are usually used for recording the EEG when implanted within the brain or when placed on the brain surface. Needle shaped electrodes can be used within the brain or under the skin. All of these types of electrodes have to be within conductive tissue to ensure maximum conductance (Schiff, 1974). Cup, or disc shaped electrodes are attached to the skin surface using an adhesive substance. Either this adhesive is conductive in itself, or the cup part of the electrode has to be filled with conductive gel (ionic gel) (Taheri et al. 1994).

Silver is often used as an electrode material, as it has a high conductance, stainless steel, gold and tin can also be used but have lower conductance. However, pure silver electrodes can produce electrical artefact when introduced to the conductive medium (such as gel). A coating of silver-chloride reduces the ion exchange and therefore reduces the electrode based artefact (Webster, 1984).

The EEG can be recorded from anywhere where the brain's electrical activity can be picked up by sensors (i.e. the electrodes). Electrodes can be implanted into the brain, to record from structures within the brain's interior. Electrodes can be placed on the surface of the dura, recording from the cortical structures beneath the electrodes. In both of these cases, the electrode leads can either be brought out through the skull and scalp to allow for data acquisition (usually held in place with dental cement), or the leads can be brought out through the skull and passed

intradermally to attach to an internally positioned data acquisition device (usually placed intraperitoneal). When electrodes are implanted in the brain or on the surface of the brain, the EEG subject has to undergo surgery under general anaesthetic for electrode placement. Electrodes placed in or on the brain can be needle, screw or ball electrodes. Electrodes can record the EEG that is transmitted through the skull, by being placed either under the skin (needle electrodes), or attached to the skin surface (cup or disc electrodes). Electrodes that are implanted under the skin in most cases require general anaesthetic during placement, but local anaesthetic may also be used to place needle electrodes (e.g. Strain et al. 1986). Needle electrodes are commonly used to record the EMG and the ECG during long term electrophysiological studies or recording during exercise (e.g. Adams and Barratt, 1974). When electrodes that are attached to the scalp surface are used, the EEG subject does not need to undergo surgery for electrode placement as each individual electrode takes seconds to attach to the skin and shouldn't cause any pain. Skin surface cup/or disc electrodes can be used to record other electrophysiological variables such as the EMG and ECG during sleep.

There are a number of advantages with using non-invasive EEG techniques:

(a) The electrodes can be attached swiftly and do not require the subject to be anaesthetised;

(b) This means that the EEG can be recorded directly post-attachment as there need not be a recovery period (however, EEG subjects may need an acclimatisation period to get used to wearing the electrodes and associated equipment and animal subjects may need to get used to the handling procedures needed to place the electrodes);

(c) Compared with invasive techniques, there is a reduction in the risk of infection, as there is no need to break the skin;

(d) Non-invasive EEG techniques may not need a Home Office licence in the UK;

(e) The procedures do not require specialised staff to perform the attachment operation;

(f) Therefore, non-invasive EEG techniques are cheaper than invasive techniques;

(g) The animals need not be euthanased after the procedure and can be re-used;

(h) These techniques are ethically more acceptable: animals need not be euthanased and the techniques do not cause pain, distress or lasting harm.

Modern, high impedance acquisition techniques improve the skin-surface EEG, as the skin no-longer requires the harsh abrasion needed with data acquisition units with lower impedance (Boone, 1996); without the harsh abrasion (just thorough skin cleaning) the risk of infection is decreased further. There are a number of disadvantages with using non-invasive EEG techniques as compared with electrodes implanted into the skin or on to the brain surface, and these are discussed in section 2.2.3 below.

There are two main types of electrode placements: monopolar and bipolar. Both types involve using the information received at two electrodes, as the acquisition system for the EEG relies on a differential. A monopolar placement exists when there is (for each electrode channel) one electrode placed in an electrically active position on the head, and a second is placed in an indifferent (neutral) position elsewhere. The indifferent electrode is placed somewhere that is as neutral as possible with regards to brain potentials, such as on the ear or the base of the neck. In the monopolar placement, the EEG that is recorded is mainly that of the brain electrical activity beneath that electrode (Fehmi and Sundor, 1989).

For bipolar placements, both electrodes of the pair that form the differential are placed in active positions on the head. The EEG that is recorded is that of the activity from the area of the brain between the two sites. The bipolar electrodes also require a reference electrode in a neutral position; however there can be one reference electrode for several channels of EEG with this type of placement. The signal on the reference electrode is subtracted from each of the input electrodes: this removes signals which are present on all three electrodes, thus removing most of the muscular artefact (Fehmi and Sundor, 1989).

Bipolar electrodes need careful positioning: this requires balancing the maximum area of the brain between the two electrodes and the minimum diffusing action by recording too large a section of brain. If the electrodes are too closely positioned (less than 20mm for electrodes placed on the surface of the scalp), then 'crosstalk', conduction across the skin between the two electrodes can happen (Fisch,

1999). In addition, if electrodes are close, there is the possibility of there being an insufficient difference in the potentials produced from the brain under the electrodes, and therefore the resulting EEG would have a very low amplitude trace. The most commonly used positioning of EEG electrodes (or 'montage') on the scalp in humans is the International Federation 10-20 system. Here, the electrodes are placed evenly over the top of the scalp, starting with three electrodes on the midline.

The reference electrodes also require careful positioning: an optimal site would be where the electrical potentials change the least in comparison to the potentials to be recorded from the EEG pairs. In a study undertaken to assess whether sternovertebral, ear, knee, ankle or nose positions for reference electrodes were best for use when recording auditory evoked potentials in humans, Wolpaw and Wood (1982) found that the sternovertebral position, although variable between subjects, was the most indifferent (potential) site and was therefore the best for evoked potential measures.

2.2.3.2 Data Acquisition

Differential amplifiers are used to amplify the difference in the voltage between the two electrodes in a pair. It is the magnitude of the difference between the electrodes, and the degree of amplification specified by the user that determines the amplitude seen on the EEG trace.

The signal can be processed by analogue or, more recently by digital means. In the analogue system, the signal may be filtered, added or subtracted by a combination of resistors and amplifiers. These transform the original signal that can be discharged into a galvanometer that deflects a mechanical device which in turn affects a pen, that draws a graph of amplitude (y-axis) by time (x-axis) on continuous paper (Blum, 1998). In the digital system, the original signal is amplified, filtered and then transformed into a series of discreet digital values. These values can be used to build up a picture on a computer of the EEG in both the time domain and the frequency domain (Harner, 1988). The frequency components of the recorded EEG are determined by the bandwidth of the filter and the rate of digitisation; the filter bandwidth has to be chosen to allow the frequency of the EEG to be passed but to reject frequencies that represent artefacts (Zapulla, 1990). This means that non-biological artefacts are rejected. However, if other electrophysiological variables are

recorded alongside the EEG, then filtering at this stage cannot remove biological artefacts (because the filter bandwidth will have to be wide enough to allow EMG frequencies to pass through). The rate of data sampling is governed by the Nyquist theorem (Nilsson et al. 1993) which states that the sampling rate needs to be twice the highest frequency of the variable being recorded. For example, when recording the EEG with a maximum frequency of approximately 50Hz, the sampling rate needs to be at least 100Hz.

2.2.2.3 Recording the EEG with other electrophysiological variables

Using other electrophysiological recordings alongside the EEG enables artefact identification and aids the identification of sleep stages. The EMG can be recorded from many muscle areas on the body and enables the viewer to recognise and label biological artefacts appearing on the EEG trace, such as limb movement and muscle activity. Similarly, the EOG can be used to recognise blink and eye movement related artefacts that appear on the EEG trace. Furthermore, the recording the ECG helps in the identification of cardiac-related artefact. When all the biological artefacts have been recognised and labelled, the EEG can be analysed using spectral analysis, taking care to use artefact-free portions of the trace for the analysis. In addition, post-hoc filters can be used to remove common high frequency (or in the case of the ECG, low frequency) artefacts from the EEG trace before analysis commences (e.g. Anderer et al. 1999; Tong et al. 2001).

Ever since Aserinsky and Kleitman (1953) reported spontaneous eye-movements when subjects were sleeping, the EOG has been used in the recognition of REM sleep, especially as the high frequency, low amplitude waves found in the REM sleep EEG can resemble the awake EEG. In addition, the EMG from the neck is often used in sleep studies to recognise the level of muscle tone during sleep. In Non-REM sleep, muscle tone is relaxed compared with wakefulness. However, in REM sleep a complete reduction in muscle tone is observed on the EMG trace. Moreover, the EMG trace can also be used to record the phasic twitching seen in REM sleep (Steriade and Hobson, 1976). Other physiological changes occur during sleep that can be measured using electrophysiology alongside the EEG. The heart rate changes during different sleep stages (in humans, lowering from resting rate during Non-REM

sleep and raising from resting rate during REM sleep) and this can be measured using the ECG (Welch and Richardson, 1973; Aldredge and Welch, 1973). In addition, the respiration changes during sleep (lowering from resting rate during Non-REM sleep and raising from resting rate during REM sleep) and can be measured using the EMG recording the muscle activity of the intercostals between the ribs, or by recording the temperature difference of inhaled and exhaled air by the nostrils (Martin et al. 1990)

2.2.4 Comparing invasive and non-invasive electrophysiological methods

When recording the EEG using electrodes placed on the skin surface, the electrical signal from the brain has to travel through the brain, cerebro-spinal fluid, bone and skin to reach the electrodes. The activity recorded by these electrodes is different from that recorded by electrodes placed directly on the brain (or dura) surface (Cooper et al. 1965). In humans, the decrease in amplitude between potentials recorded from electrodes on the surface of the brain and those recorded from electrodes on the scalp can be 60 times, although the average difference in amplitude is only three times (Cooper et al. 1965). The higher level of attenuation of amplitude occurs when neuronal activity is mostly asynchronous, whereas synchronous activity is recorded by the scalp electrodes with little attenuation in amplitude (Abraham and Ajmone Marsan, 1958). This difference in the amount of attenuation has its origin in the way in which synchronicity of neuronal activity is achieved. A simplistic illustration would show that when the neuronal activity is synchronous, there is a large amount of activity from one or more areas of the cortex, which is 'going in one direction'. When activity is asynchronous, the activity is not found in one or more particular areas of the cortex, but is carried out over a wider area and the signals are 'going all over the brain'. To use a simile borrowed from Carlson (7th Edition, 2000): synchronous activity is like the noise made by people saying the same thing at the same time, asynchronous activity, on the other hand is like people saying different things at slightly different times: even if the original noise made is at the same level, the synchronous noise 'sounds louder' than the asynchronous noise. The signals received by electrodes placed on the skin surface are diffuse and cannot be pinpointed to a particular area of the cortex when compared with electrodes implanted within the brain or on the surface of the dura. In humans, the method of countering the diffuse

aspect of skin surface EEG is to use many electrodes over the skin surface to pick up differences in electrical activity from many areas of the cerebral cortex (Fisch, 1999).

The other main difference between invasive and non-invasive techniques is that implanted electrodes suffer less from muscular artefact and movement artefact than skin surface electrodes. Muscular artefacts are related to head, limb, jaw and more general body movements such as breathing. They are caused by the electrical differential created by muscular activity, which can be picked up as the signal can travel through conductive material such as the skin and body fluids. Muscular artefacts can cause intermittent, or continuous high frequency activity, which can partially, or completely obscure the EEG from electrodes placed on the scalp surface (Anderer et al. 1999).

Movement artefacts are produced by the stretching and relaxing of the skin, upon which the electrodes rest. There is a natural skin potential of about 30mV inside and outside the barrier layer of dead cells that occurs above the living tissue (Webster, 1984). When the skin in stretched, due to movement (and even if there are no directly underlying muscle action potentials), the potential across the barrier layer of the skin can reduce by up to 5mV. One method of reducing the skin potential when using non-invasive electrophysiological techniques, is to clean and gently abrade the skin, removing much of the barrier layer, before electrode attachment (Webster, 1984).

2.2.5 The use of remote recording devices for unrestrained animals

2.2.5.1 Types of remote recording device

In order to understand the behaviour of free-ranging (and wild) animals many types of remote recording devices have been developed. The simplest of these devices are radiocollars, which are transmitters attached to a collar round the neck (or to a harness round the body) of an animal. A pulse signal is transmitted at radio frequency, which can then be picked up by a receiver and pinpointed. This allows individual animals to be tracked and their position ascertained (e.g. Cypher, 1997).

The recording device does not always have to be attached to the animal to be measured. Langbein et al (1996) developed an 'activity data-logger' to determine the presence and temporal patterns of wild sheep at a salt-lick. This device consisted of a

Passive Infrared Sensor that recorded temperature differences between animals and the surrounding environment and the movement of animals. The sensor was able to relay information to the data-logger, which could make recordings for up to 80 days. Here, the device did not disturb the animals, but lacked the precision needed for most research, as any large animal moving within range of the sensor would be recorded.

Many studies have attempted to measure activity using remote devices. Ruckebusch and Bueno (Ruckebusch and Bueno, 1978; Bueno and Ruckebusch, 1979) recorded activity and ingestive behaviour in sheep and cattle in the field using vibracorders. The vibracorders consisted of a pendulum connected to a stylus that marked paper when the pendulum moved. The vibracorders were attached to the animals on leather harness and could record movement over 24-h periods. This method seems robust to weather conditions and was used continuously over a period of six months (the paper had to be changed daily).

Champion, et al (1997) developed an activity lying/standing sensor based on a mercury tilt switch, hanging below the body of a sheep, where the mercury completed an electrical signal when the animal was standing, but was tilted to such an angle when the animal lay down that the signal connection was broken. A similar device was added to the animal's leg, so that movement could be detected. As the sensors were hanging below the body of the animal, these devices may not be suitable for very active, or highly inquisitive animals, such as pigs. However, the study only lost five out of 68 recordings to damage.

More recently, Müller and Schrader (2003) have developed an accelerometer activity recorder to record movement and therefore activity in dairy cows. Here the device was affixed around the leg of the cows and could stay in place continuously, recording at 1 min intervals for 10 days. The device had 64 KB of digital memory to hold this information. A very similar device was used in a study of free-ranging reindeer for up to 12 months (Van Oort et al. 2004). In this study, the accelerometers were embedded in silicon and enclosed in a metal container and attached to a collar, also equipped with radiotracking equipment.

Telemetry is the process in which data are captured by instrumentation on an animal and a signal of that data is transmitted to a remote receiver, where it can be traced, recorded and analysed. Radiotelemetry has been used to record the heart-rate

of cattle in a housing system, where ECG electrodes and a pulsating transmitter were attached to cattle using a harness and a data acquisition apparatus was placed within the animal house (Lefcourt et al. 1999). Stermer et al (1980) used a small radio telemetric device that could be swallowed by cattle, in order to record deep body temperature during road transport - here the receiver system was attached to the trailer. These devices offer advantages: they do not affect the cattle behaviour, and they do not require much time to position. Internal devices also have disadvantages: they may not arrive in the correct place internally (the rumen varies in temperature); there is no way of altering the equipment once it is inside the animal; and devices can be lost out of the gut if care is not taken.

Radiotelemetry can be used in recording the EEG from unrestrained animals in the laboratory. This means that animals do not have to be handled as frequently and restraint can be kept to a minimum (Adams and Barratt, 1974). If the EEG is recorded using traditional methods, active animals such as monkeys need to be restrained (e.g. Balzamo et al. 1998). In more recent primate and rodent-sleep studies, small data acquisition and transmitting devices have been developed that can be implanted intraperitoneally (e.g. Crofts et al. 2001; Louis et al. 2004). Each device has only one to three channels for electrophysiological data acquisition. However, this is enough for two EEG channels and an EOG or ECG channel. A data receiver and recorder can be placed in a convenient place in the home pen of the animal (usually covering the floor of a rodent cage, or placed under the common sleep site in a primate cage) (Crofts et al. 2001).

2.2.5.2 The use of the skin surface EEG to record from free-ranging animals

There are few studies where the non-invasive, surface EEG has been recorded in unrestrained animals outside of the laboratory, whereas surface EEG techniques are relatively common for studies of humans in their home environment. The low numbers of studies may be because of technical difficulties of recording the EEG on animals (as humans, even young children can be asked to be careful with electrodes and electrode leads). However, Giovagnoli et al (1996) has successfully recorded the surface EEG from unrestrained horses in their stable. The electrodes on the head of the horses were connected via a cable to a miniature recorder which contained a

magnetic tape cassette to store the signals and could be attached to a surcingle around the horse's body. The tapes could store approximately 24-h of information and the results could be viewed on a PC (Giovagnoli et al. 1996).

In contrast to the situation with non-human animals, the majority of research and clinical studies carried out with human subjects have used electrodes placed on the skin surface to record the EEG (e.g.Chapotot et al. 1998). Furthermore, many EEG studies on humans can be carried out using ambulatory techniques in the person's home, or even while carrying out extreme exercise (e.g. Finnegan et al. 1985). The human has a number of morphological features which are advantageous when attempting non-invasive techniques of EEG recording. In comparison to other animals, humans have large brains, with large surface areas (for example, a human brain mass is 1.5kg and a sheep brain is between 140 and 200g). Humans have a large, domed skull to house the brain leading to a substantial area of cranium for electrode placement. Therefore, many electrodes can be used to record the EEG from the skin surface in humans; even neonates can have multiple channel EEG recordings (Dyson et al. 1984). Moreover, the head morphologies of humans and other primates are associated with less muscular artefact then in non-primates. The ears of primates are small and relatively immobile when compared with many other animals, including sheep. Therefore, muscular artefacts from ear movements are rare on the primate skin surface EEG. Also, the human jaw muscles are small when compared with carnivores (e.g. dogs have large *temporal* muscles compared with humans) and ruminants (e.g. sheep have large *masseter* muscles) (Frandson and Spurgeon, 1992) and therefore muscular artefacts from jaw movement are less important in the human skin surface EEG.

In addition, human behaviour is advantageous for skin surface EEG recordings as humans tend to exhibit a monophasic pattern of sleep; that is to say that sleep occurs in one long bout during 24 hours with no substantial period of wakefulness during the sleep period. When humans are sleeping there are very few muscular or movement artefacts. Therefore, skin surface electrodes can be temporarily adhered to a human in order to record the sleep EEG, in the knowledge that dislodgement due to movement and muscular artefact will be kept to a minimum. Most non-human animals used in sleep research sleep in a polyphasic pattern (i.e.

they exhibit multiple small bouts of sleep during 24 hours and may show substantial wakefulness during the period of sleep). For example, rats show most sleep during the light phase of the 24-h period, but during this time they can undergo approximately 10 bouts of sleep interspersed with periods of activity (Borbély et al. 1975). Therefore, it would be difficult to use non-invasive electrophysiological techniques in rats to record the EEG without muscular artefact and a likelihood of electrode dislodgement due to movement.

2.2.5.3 Technical problems with remote recording devices

Remote recording devices are often subject to technical problems, some of which cannot be corrected as the animals are wild and need to be recaptured. Other problems are based on the recorder's inability to cope with the rigours of long-term attachment to a free-ranging animal. Van Oort et al (2004) found that eight out of 46 accelerometers, which were used to record activity from free-ranging reindeer, had incomplete or unusable data after seven to 12 months of use.

The method of attachment to the animal can also be a source of technical problems. Baldock et al (1987) attempted to record 24-h ECG from unrestrained sheep at pasture using non-invasive skin surface electrodes and a tape-recording system. They found that there was a low success rate (55%) in achieving seven-hour recordings using such electrodes, as one or both of a pair of electrodes was likely to become detached from the skin during the recording. The authors then tried subcutaneous electrodes to record the ECG and found these stayed in place reliably and seemed (although no inferential statistics are offered) less affected by electrode movement and muscular artefact compared with the skin surface electrodes (Baldock et al. 1987).

One of the main difficulties with recording the surface EEG in free-ranging animals (such as Giovagnoli et al. 1996) is the occurrence of artefacts on the EEG traces. Giovagnoli et al (1996) found that artefacts could be split into two categories: technical and biological. Technical artefacts included the disconnection of an electrode, the pulling of electrode leads and the drying out of the conductive paste. Biological artefacts included muscle activity (from the eye muscles –blinking and movements, from the jaw muscles when eating) and whole animal movements. This

study also recorded the simultaneous ECG and found that when the horses moved around their stables, the ECG was obscured by artefact. Other recording devices also suffer from artefact or noise; the activity recorder developed for use in dairy cattle by Müller and Schrader (2003) was so sensitive that even small movements made when cows were lying down were registered, creating a 'noisy picture' of activity levels.

2.2.5.4 Do remote recording devices affect the behaviour of the experimental animals?

One assumption made by researchers using remote recording devices is that the animals wearing the device would not be aversely affected, or in any way disrupted by wearing it. To take the assumption to its limit, it is expected that animals wearing remote recording devices would behave no differently from those that are not.

Most researchers would probably acknowledge that the attachment process of fitting remote recording devices to an animal can be stressful for the animal (especially if the animal is not used to human contact) and in many studies using remote devices, the equipment has been fitted to the animal under general anaesthetic or other drug-based immobilisation techniques (e.g. Van Oort et al. 2004). In most studies, a certain time period is not recorded immediately after fitting equipment to allow the animal to become accustomed to the device. However, the assumption is that after the acclimatisation period, the animal will not behave any differently from animals without devices (e.g. 14h acclimatisation period. Müller and Schrader, 2003).

The animal wearing the remote recording device may show similar overall behaviour patterns (for example, following normal migration patterns, Ferguson and Elkie, 2004), but may show more subtle changes in behaviour. Cypher (1997) found that San Joaquin kit foxes which were wearing radiocollars for ethological research had decreased activity levels and decreased survival compared with those without collars.

The factor commonly taken into account when designing remote recording devices is the mass of the device compared with the body mass of the animals. Cochran (1980) states that, for wildlife telemetry studies, most animals are able to cope with devices if they weigh less than 4% of the animal's body mass; Cuthill (1991) states that devices must be less than 5% of the animal's body mass. However,

this is based on research with small mammals, and therefore the weight of remote devices on larger animals may not be such an issue. Hulbert et al (1998) found that sheep fitted with Global Positioning System collars (that were approximately 2.2% of the animals' body mass) showed no differences in their feeding and ruminating behaviour compared with sheep not wearing collars.

Therefore, when carrying out experiments on the behavioural and electrophysiological characterisation of sleep in sheep, it is important to minimise stress during equipment attachment procedures; to understand that behaviour may be altered by equipment and to minimise such behaviour alterations by improvements to equipment wherever possible.

2.2.6 Sleep stages and automatic sleep scoring

Visual scoring is subjective and may lead to variability within and between studies (Hoffman and Jeakins, 1987). Therefore, automatic, quantitative methods have been developed to overcome subjectivity. The majority of Automated Sleep Score programmes designed for use in humans are based on the criteria for categorising the stages of sleep in humans set down by Rechtschaffen and Kales (1968). The stages of sleep are reviewed in full in chapter 1; however, a summary of electroencephalographic frequencies and associated sleep states is shown in table 2.3 of this chapter. Automated EEG analysis provides a readily obtainable quantitative 'amount' of various frequencies that comprise the EEG. The computer programmes that carry out automated sleep scores analyse one or more EEG channels simultaneously, and perform a frequency analysis on each trace at intervals (e.g. every 30 s). Each of these intervals is known as an epoch. The frequency analysis of each epoch leads to a decision making process using probabilities based on the amount of each sleep power band present in the epoch, to decide on the sleep stage of that epoch. The resulting sleep stage for each epoch is stacked with each successive epoch to provide a picture of the sleep stages over time. There are differences in the sophistication level of the automatic sleep score programmes. For example, some use information from other electrophysiological recordings to assist in the decision making process (such as a measure of rapid eye-movements to recognise REM sleep) (Kemp et al. 1997).

There have been developments of automatic sleep scoring programmes for non-human animals such as rats (e.g. Louis et al. 2004). These programmes have simpler rules for deciding on sleep stages. In the rat model, a sleep score was produced from electrophysiological recordings of the EEG and EMG using the following rules: wake = low amplitude, mixed frequency EEG + sustained EMG activity; Non-REM Sleep = high amplitude, low frequency EEG + low-level EMG activity; REM Sleep = Sawtooth-pattern EEG + flat (atonal) EMG (Louis et al. 2004). The computer scoring system correctly scored the three stages on approximately 85% of occasions compared with experienced visual scorers. However, only EEG that was deemed by visual scorers as unequivocal was scored by the computer, as artefact on the EEG reduced the accuracy of the scoring (Louis et al. 2004).

Grant et al (1995) developed an automatic sleep score for the neonatal lamb, based on the sleep/wake criteria of human neonates. As neonatal mammals spend a significant time in REM sleep, the computer programme was sensitive to the difference between wake and REM sleep. The transition from wakefulness to Non-REM sleep was more difficult to score. One difficulty in the automatic sleep scoring of the neonatal lamb was that during REM sleep there were many phasic twitches. The twitches raised the amplitude of the EMG recordings, and if the threshold was set too low, the programme would respond as if the sheep was awake. To overcome this, the threshold for EMG tone had to be set high. The authors suggest that neonatal sheep REM sleep was indistinguishable from wakefulness when scoring using the EEG alone: therefore, EMG and EOG must be scored in conjunction with the EEG to make sense of the data. The concordance between the visual and the automated sleep score was approximately 85% for the sleep states and over 90% for wakefulness.

There are several problems with automatic sleep score programmes that traditional visual scoring could easily overcome - the main problem being artefacts. The automatic sleep score programme cannot choose good epochs to score. If good epochs are not inputted manually, the decision making process used to sleep score can make the wrong decisions based on artefact-containing epochs (Gasser, 1988). A further difficulty of using automated sleep score programmes that are based on Rechtschaffen and Kales (1968), is that 30 s epochs make brief events difficult to deal with, which may be even more important when one is scoring the sleep of an

animal with short sleep episodes (Himanen and Hasan, 2000). An example of a brief event is an arousal from sleep (Feinsilver, 1998), as arousals of around 3 seconds or longer may or may not be associated with a change in sleep stage.

2.2.7 Validation of sleep scoring

There are a number of behavioural characteristics that are shared among mammals and birds from which sleep can be recognised (for a more detailed review of mammalian sleep, see chapter 1). One of these is a general reduction in activity followed by a relaxation of the body. Most mammals have species-specific characteristic body postures that they adopt during sleep (Tobler and Schwierin, 1996). Furthermore, these postures can be different between REM and Non-REM sleep. For example, horses can stand during Non-REM sleep, but lie down usually laterally, with their head resting on the ground for REM sleep. Mammals often close their eyes during sleep, but can also do this during sleep onset and rest, so eye closing alone is not indicative of sleep (Tobler, 1995). REM sleep is accompanied by movements of the eyes and twitching of the face and limbs but, these movements are not present throughout all of REM sleep, as these movements are present in bursts and are not present in REM sleep onset (Siegel, 1995).

The difficulty with using behaviour alone to recognise sleep in animals is that many changes in posture and muscle tension are subtle, and although these variables can be seen when watching the animal directly, they are hard to differentiate when observing using video recordings. This is especially the case with time-lapse video recordings (as less frames are recorded per second and subtle movements can be lost) and night filming, where areas of the animal's enclosure may be in darkness. Both time-lapse video and night filming are commonly used for observing sleep in animals. However, another common characteristic of sleep in animals is that it is more difficult to evoke a behavioural response when an animal is sleeping than when it is resting. There is a higher level of vigilance in the resting animal than the sleeping animal (Dimond and Lazarus, 1974).

The change in brain activity between the sleep and wakefulness states correlates with behavioural arousal in mammals (Storch et al. 2004). Therefore a new method of recording the EEG in an animal can be validated by using a known method

to arouse an animal from what behaviourally would be termed sleep to wakefulness. Rechtschaffen et al. (1966) have found a difference in frequency of the post-arousal EEG after using an arousal stimulus during either Non-REM or REM sleep in humans. In humans, it has been shown that the arousal threshold during sleep can be used to indicate differences between sleep stages (Rechtschaffen et al. 1966) with the 'deeper' stages of stages III and IV requiring the largest auditory stimulus to promote wakefulness. Neckelmann and Ursin (1993) found differences in the arousal threshold between Non-REM and REM sleep in the rat. In Non-REM sleep, those periods with a highest percentage of delta waves (0.1Hz to 4Hz) had the highest auditory arousal threshold. However, this was found not to be indicative of the depth of Non-REM sleep, as the deepest stage of sleep in the rat did not necessarily contain the highest percentage of delta waves (Neckelmann and Ursin, 1993; Dillon and Webb, 1965). It must be noted that an arousal stimulus may not always result in a change in behavioural state, even if there are electrophysiological changes post-stimulus. Bauer et al (2001) found no changes in the behavioural state of foetal sheep during and after auditory stimuli played both in Non-REM and REM sleep. Stimuli played to foetal sheep during Non-REM resulted in a decrease in heart rate, an increase in heart rate variability; and a reduction, lasting 40s post-stimulus, in the percentage of EEG in the delta frequency band. Auditory stimuli during REM sleep resulted in an increase in heart rate, an increase in heart rate variability; and a slight reduction in overall spectral power of the EEG trace. There were no differences seen in the delta power (Bauer et al. 2001). In all previous studies, the main changes in the EEG occur when the subject moves from sleep to wakefulness after an arousal stimulus (Rechtschaffen et al. 1966).

2.2.8 Recording the EEG from sheep

The EEG was first recorded in humans in 1929, and recorded in non-human animals soon after (Kleitmann, 1939). As discussed in chapter 1, ruminants were thought not to undergo sleep when early time budget research was being carried out (e.g. Brownlee, 1950; Balch, 1955). The first EEG experiments on ruminants carried out in 1960, were undertaken in goats and agreed with the sleeplessness argument (Bell, 1960). Bell used four monopolar electrodes, implanted onto the surface of the

cortex and held in place with screws drilled into the parietal bone. Each goat was placed in a metabolic cage during recordings. Bell successfully recorded the EEG in alert goats and when they became drowsy. The EEG had higher amplitude, lower frequency waves when the goats were drowsy as compared with when they were alert. Bell also showed that the drowsy state was seen during rumination, including the gaps between chewing.

First Ruckebusch (1962) and then Klemm (1966) found that ruminants did indeed sleep. Klemm recorded the EEG, the EOG, the EMG and the ECG from goats for five days. The electrodes were needle type, and were implanted subcutaneously over the frontal and occipital areas of the skull. Klemm (1966) rejected the more invasive methodology used by Bell (1960) and Ruckebusch (1962), as this method still showed jaw muscle artefact on the EEG during rumination: therefore he chose the less invasive EEG technique. The goats used in this experiment were housed singly, with limited movement, in metabolic cages, so were unlikely to damage the electrodes. Klemm (1966) used monopolar electrode derivations and had a reference electrode over the nasal bone.

Ruckebusch (1972) researched sleep in farm animals using the EEG, which was recorded from electrodes implanted onto the surface of the brain. This allowed for long term circadian rhythm experiments to be carried out where recordings were made over a month. When Ruckebusch recorded the EEG from sheep or goats, he used two channels to record the EEG from monopolar electrodes and always recorded other electrophysiological measures in conjunction with the EEG. Some authors used only one monopolar EEG channel (e.g. Tindal et al. 1978) and other authors used two bipolar EEG channels (e.g. Toutain et al. 1983) to record sleep in sheep or goats.

The EEG has been used to record the activity of the brain of sheep for other reasons than sleep research. Strain et al (1986) used the EEG to record the brain activity of sheep that were infected with scrapie. These authors used a modified 10-20 system to place their five pairs of electrodes on the head of the sheep. They opted for subcutaneous needle electrodes, which allowed for the maximum number of electrodes in a small space. In addition, a reference electrode was placed on the nasal bone. One sheep dislodged the needle electrodes during a scrapie seizure (Strain et al. 1986). However, repeated recordings were made over a period of two weeks with five

other sheep. Needle electrodes are often used to record the EEG from sheep before during and after stunning for research on slaughter (e.g. Blackmore et al. 1995) as the amount of time needed to record the EEG is short. Ong et al (1997) and Morris et al (1997) used the EEG to record pain evoked potentials in sheep. These authors experimented with non-invasive electrode techniques, but found them unreliable for their experiments and used electrodes implanted onto the surface of the dura instead.

More recently, the majority of sheep EEG research has been carried out on foetal sheep (used as a model for human neonates). The electrodes used in foetal sheep sleep recordings are usually the screw type - screwed into a drilled hole and rested on the surface of the dura when the pregnant ewe is under general anaesthetic (e.g. Schmidt et al. 2004). The number of channels used to record the EEG from foetal sheep varies from one (e.g. Schmidt et al. 2004) to three (e.g. Morrison et al. 1997). Most studies also implanted screw electrodes above the orbit to record the EOG and needle electrodes into the neck muscles to record the EMG (Szeto and Hinman, 1985; Morrison et al. 2001; Schmidt et al. 2004). All of these studies implanted the EEG electrodes bilaterally over the cortex, those with multiple channels implanting from the parietal to the frontal areas of the brain.

2.2.9 The present investigations

The techniques used throughout this series of investigations were originally developed and designed for use in humans. It was essential, when using the equipment for sheep that a simple method to recognise and score sleep in was designed. In addition, it was necessary to design validation exercises to show that the methodology for recording the EEG was workable; that the EEG was actually being recorded and the scoring of sleep in sheep was as accurate as possible.

The relationships between the recognition of sleep in sheep using behavioural observation of posture and that identified using a spectral analysis of the electroencephalogram were examined. The hypothesis tested was: there are no differences in the properties of the EEG 10 seconds before and after an auditory arousal stimulus that caused the sheep to change from a 'characteristic sleep posture' to an 'awake posture'.

Following on from this, three further validation experiments were carried out:

An investigation of the electrical resistance across a section of sheep skull at two different thicknesses was carried out in order to assess the magnitude and type of attenuation (by the skull) of the EEG in sheep.

An investigation was also undertaken to compare the amplitude and frequency recorded by electrodes placed on the scalp with electrodes placed on the surface of the brain, from an electrical signal artificially generated in the brain of a euthanased ewe's decapitated head.

An investigation was undertaken to compare the amplitude and frequency of the EEG from sheep under three depths of general anaesthesia, recorded by electrodes placed on the scalp with electrodes placed on the surface of the brain.

2.3 Behavioural and electrophysiological characterisation of sleep in sheep

2.3.1 General Methodology

2.3.1.1 Animals and husbandry

Adult (at least two years old), Dorset and Dorset X Finn ewes that had been kept at pasture were used for these investigations. The sheep formed part of the breeding flock of the Moredun Research Institute, Midlothian, Scotland, UK. The Dorset (polled) breed was chosen for their large size (>45 kg), calm temperament and flat broad head morphology (without the extensive sinuses of the roman-nosed breeds).

Sheep were brought into inside penning at least two weeks before they were used in the experiment to allow for habituation to the surroundings and the handling procedures. Two sheep were housed in individual 1.8 X 1.8 m pens bedded with straw lit with artificial lights on a 16hL:8hD cycle. Sheep were offered approximately 250g/d/sheep concentrated feed (Pentland lamb finisher, Seafield Mill, UK) from a bucket and *ad-libitum* hay and water.

The sheep were habituated to the attachment of a harness and fibre-glass helmet (see below), and to handling of the body and head. Sheep were offered small amounts of concentrated feed by hand while the experimenter was sitting in the pen. Over a period of days, the human contact was increased until sheep could be touched on the body and head without flinching or need of restraint.

2.3.1.2 Harness and helmets

A harness was developed that was capable of securely carrying the Embla data-logger in its waterproof box (see below) on the back of a sheep for over 24-h. In the preliminary study, the harnesses were 'ram harnesses' (Net-Tex, Meopham, Kent, UK) made of leather with metal buckles, which had a clipping mechanism to affix the waterproof box. The clipping mechanism was a Perspex block 120 X 40 X 20 mm, with two holes corresponding to metal pins on the waterproof box. The harnesses were worn 'upside down' on the ewes. The clip was on the back behind the withers. The straps wrapped around the posterior abdomen and around the brisket. When the waterproof box had been clipped in place, two elasticated surcingles (Cam

Equestrian, UK) were placed around the body of the sheep and over the box to hold it firmly in place. The harness and surcingles were secured with duct tape (Duck Tape$_{TM}$ Henkel UK, Winsford, UK) (Figure 2.1).

Figure 2.1 Left side photo: sheep wearing the original harness design and fibre glass helmet. Right side photo: sheep wearing the canvass harness. Also showing the placement of the waterproof box containing the Embla data-logger.

After the preliminary study, a new harness was developed to provide more comfort to the sheep and to help prevent slippage. This harness was made of a 400 X 400 mm section of heavy canvass material (as used in horse rugs). The clipping mechanism from the previous harness design was stitched into the centre of the canvass. Alongside the clipping mechanism, there were two padded areas supporting the waterproof box and maintaining an even weight across the back of the sheep. An elasticated strap and buckle added further support to the waterproof box. Cotton webbing straps of 20mm width were used to attach the harness to the sheep. An 'H' shaped strap was used to reduce harness slippage around the body of the sheep. The front part of the 'H' strap was fitted between the front legs over the brisket and was fixed at the front of the canvass at the shoulder. The back part of the 'H' strap was fitted around the abdomen and fixed to the middle of the canvass. Two further straps were used to tighten the harness: one at the rear of the canvass and one at the front.

Helmets were used to protect electrodes worn on the head and face of the sheep. Leather halters (Peasridge, Rye, UK) were used as the basis for the helmets. Helmets were made individually for each sheep by attaching Duct tape to the halter over the cheek area; posterior to the eyes and over the top of the head to use as a template. The halter was removed and the areas of Duct tape were covered in fibre-

glass (Plastic Padding Fibre Glass Kits, Loctite UK Ltd, Welwyn Garden City, UK). Once set, the fibre-glass was sanded to remove rough edges and covered completely in Duct tape. The helmets were big enough to accommodate the electrodes and a Vet-rap$_{TM}$ bandage (3M, St. Paul, USA) and allow airflow underneath to minimise sweating.

2.3.1.3 Wool removal and skin cleansing

Wool was removed to allow the skin surface electrodes to be attached to the skin. Livestock hair clippers were used to remove the majority of the wool on the electrode sites. The sites were: the top of the head, the jaw, behind the eyes, on the neck, on the chest, on the hind leg and on the rump (see Table 2.1 below for more details on the electrode sites). The sites were further cleared of wool using Veet Sensitive Skin Hair Removal Cream (Reckitt Benckiser, Hull, UK) on the day before electrophysiological recordings. The cream was placed on the skin for 1 min, and then removed using a swab (Vernaid gauze swabs, Vernon Carus Ltd, Preston, UK) soaked in 100% ethanol. If wool was still remaining, hair removal cream was reapplied for a further minute and cleaned; this was repeated until the site was free from wool. The skin was cleaned using a swab soaked in 100% ethanol. If any skin irritation occurred, the site was dried using a dry swab and covered in Dermisol cream (Pfizer Animal Health, Sandwich, UK).

2.3.1.4 Electrophysiological hardware

Two, 16-multipurpose-channel, electrophysiological data loggers - the Embla systems (Flaga hf. Medical Devices, Iceland) - were used throughout these investigations and are hereby known as 'the Embla(s)'. The Embla worked in conjunction with specialist sleep recording software 'Somnologica 2.1' that is detailed in section 2.3.1.5. The Embla works on a digital acquisition, digital filtering and digital storage basis: digitalising data directly after it has been picked-up from the sensoring device (in this case skin surface electrodes), filtering digitally reducing the need for high sample rates and being able to store directly to a digital device (in this case PCMCIA cards). The 16 channels were multipurpose in as much as they could be used to measure many different physiological variables with flexibility as to the numbers of channels used for each type of variable. The most common channel make

up used in these investigations was 2 to 3 channels EEG, 3 channels EMG, 1 channel EOG and 1 channel ECG. The channels were sampled simultaneously, as opposed to being sampled in sequence, thus ensuring no difference in time between physiological variables. The channels were bipolar. Therefore, for each channel there was a pair of electrodes, forming a circuit with the electrical generating device (e.g. the neurones in the brain of a sheep) and the signal acquisition system. This means that between each pair of electrodes there would be a voltage differential (positive and negative) and this differential would eventually form the trace to be analysed.

The beginning of the acquisition, filtering and storage process required the voltage differential between electrodes in a channel to be amplified and digitised. A low-gain amplifier amplified the voltage differential from the source and passed this now-amplified differential to an Analogue/Digital converter. This sampled the analogue waveforms at fixed intervals and converted the samples to digital values.

The digital signal was then sent to a digital signal processor for processing, which reduced the amplified signal to its original level. The signal was sampled at 100 or 200Hz depending on the type of biological data being sampled (100Hz for the EEG and the EOG and 200Hz for the EMG and ECG) with a bandwidth of 500Hz. The sampling rate agreed with the principle of Nyquist frequency (where the data is sampled at least twice the highest expected frequency of the signal) (Tyner et al. 1983). The data was processed with a 32-bit resolution and stored at 16-bits in order to store the maximum amount of data on the storage device. As the expected spectral frequency of the EEG in sheep was expected to be less than 50Hz, a notch-line filter rejected the 50Hz (± 1Hz) frequency from the signal as this is the frequency produced by many electrical devices. Electrical devices may have an effect on the data picked up by the Embla, as biological electrical signals, especially from skin surface electrodes, are small.

The filtered signal was then passed to the device control. The device control stored the digital data in the internal memory buffer. When the buffer was full, the device control switched access to the PCMCIA card and moved all the data from the buffer to the card. Once the data moving process was completed, the power was turned off from the card (saving battery power) and new data was saved in the internal memory buffer again. The device control also operated the LED signals on

the outside of the Embla; these gave an indication of the status of the Embla (e.g. ready, recording, recording finished and so on). The Embla sampled all channels simultaneously; and up to 16 signals were picked up, digitised, filtered and stored at the same time.

The Embla was used in ambulatory mode for most of the investigations (except the validation experiments 2.6.2 and 2.6.3, where it was used in the 'online' mode). When recording electrophysiological data in the ambulatory mode, the Embla was powered by two 7.2V Lithium-ion camcorder batteries (NP-F750 SONY, Japan). These were attached to the power port on the Embla with a connector on a battery holder. The data recorded by the Embla was stored during the recording process onto a 250 MB PCMCIA card (Move IT, Calluna Technology Ltd (UK) Glenrothes, UK). However, to save battery power, the data were not recorded onto the card in a continuous manner. Instead, the data were stored temporarily to an internal storage buffer with a maximum of 5 min data storage capacity and every few minutes the data would be transferred to the card. After the recording had finished, the card was retrieved and downloaded onto a desktop or laptop computer via a type II slot. The card was then wiped for reuse.

As the Embla had to be carried on the back of the sheep, it required to have more protection than the leather pouch with which it was supplied. A waterproof, dirt-proof, shockproof box was constructed to house the Embla whilst it was in use. The box was made of aluminium and Perspex. The base of the box had a metal spring clipping mechanism for attaching to the harness. The lid was made of transparent Perspex allowing the lights on the Embla to be viewed without opening the box. The lid had a rubber seal that was waterproof when the lid was screwed on tight. The Embla, the camcorder batteries and the battery holder were held in place inside the box with foam blocks, underneath, around the side and with a foam slice on the top of the Embla. The box was constructed in such a way in that, once the PCMCIA card and the batteries were in place, the lid could be screwed on and all interaction with the Embla could take place from outwith the box. In order to achieve this, connecting wires were used from the COM (Embla-PC) port to Lemo-connector Electrode ports on the Embla. These went through the sides of the box and led to connecting plug sockets, sealed with rubber, on the outside of the box (723-series Binder,

Neckarsulm, Germany). There were four 8-pin, Lemo-connector sockets, each capable of acquiring 4 bipolar signals on the outside of the waterproof box. The connectors were labelled 1-4, 5-8, 9-12, 13-16, each set of four numbers corresponding to four bipolar channels. In these investigations, only channels 1-12 were used. When not in use each connector socket was covered with a protective plastic cap. The Embla itself was 45mm x 83mm x 133mm and weighed 475g; when in the waterproof box also containing the batteries (117g each) the total weight was 2300g.

The electrodes were a combination of 9mm and 6mm silver/silver chloride cup electrodes with a 2.5mm deep cup with central hole (S.L.E. Diagnostics, Croydon, UK) (See Table 2.1 for the configuration of the electrodes on the sheep). These electrodes had a 'cup' made of silver that was chlorinated before use (see below). The chloride layer allows for a greater conductivity between the metal and the skin than silver alone. Each electrode had a plastic seal at the end of the lead to protect the internal wires from damage. At the point of wiring up the electrodes, a + or – label was put onto each electrode (except for ground electrodes) to indicate whether it was the positive or negative electrode of the pair. Each pair of electrodes had a different colour electrode lead to aid identification when attaching to the sheep. Each lead was 700mm long for the head electrodes and 900mm long for the body electrodes. The electrode leads were wired into a 'male' 12-pin connector plug (723-series Binder, Neckarsulm, Germany): they were wired in such a way, as each pair of electrodes would occupy a separate channel when connected to the Embla data logger. Therefore, there were four pairs of electrodes wired to each connector. The electrodes were wired in sets, so that one connector carried all the electrodes for the head and the other all those for the body. In addition, a reference electrode was wired in with each set. Before the initial, and each subsequent use, the electrodes were chlorinated by immersing the disks in <5% Chlorine based bleaching agent (Milton's sterilising liquid, Procter & Gamble, Weighbridge, UK) for up to 16h.

If any breakage occurred on the electrode lead, repairs were made. The lead was cut at the breakage point, and the insulation was pared back for 15mm, exposing the wires underneath. A section of heat shrink plastic insulation was threaded onto one of the halves of the lead. The two sections of wire were twisted together, and this

section was further bonded by soldering the wire. When the solder had set, the heat shrink plastic was positioned around the soldered area and for 20 mm along the lead on either side. The plastic cover was shrunk in a flame, and once cooled it was tested for strength. The electrode was tested for conductivity before being used on the sheep. New electrodes were used at the start of each investigation to avoid using worn wires.

2.3.1.5 Electrophysiological software

The human sleep analysis programme, Somnologica 2.1 (Flaga hf. Medical Devices, Iceland) was used throughout these investigations. Somnologica is a Windows NT (Microsoft, USA) programme that allows the recording and viewing of many different types of electrophysiological data, either 'online' to be analysed in real time, or stored onto a PCMCIA/ PC that can then be analysed later. All of the electrophysiological traces (from up to 16 electrophysiological channels) can be viewed simultaneously on a computer screen for comparison and scoring. Behavioural and sleep states and events can be recorded onto the working analysis pane and these can then be subjected to descriptive statistics within Somnologica.

The programme works on an integral basis with the Embla data logger, in that it is necessary to initialise a recording with the Embla and to read the resulting data collection. Somnologica can perform spectral analysis on any physiological variable and has a variety of tools useful for classifying variables. In these investigations, the 'Automatic Sleep Score' was the main tool used. For more information on the setting up of recordings and analysis of data, see below.

2.3.1.6 Behavioural recording set-up

The room in which the indoor pens were was set up to record 24-h video using time-lapse video equipment (Figure 2.2). The room was lit using artificial lighting on a 16hL:8hD cycle, controlled by a timer. During the 24-h recordings, supplementary lighting was provided by infrared lamps (Microlight 12V-50W medium, 715nM, Dennard Ltd, Fleet, UK), equipped with pass filters (30.5 mm, Dennard Ltd, Fleet, UK) to allow video recording in the dark. Two infrared lamps were attached to the ceiling and angled obliquely to create the largest lit area possible when in darkness.

Two black-and-white CCTV cameras (WV-BP124, Panasonic, Germany) were attached to the ceiling approximately 1.5m in front of the pens, and two additional cameras were attached to the ceiling to the side of the pens. All cameras were equipped with infrared pass filters and were powered from the mains and connected to a 16 channel digital multiplexer (Sprite dx, Dedicated Micros, Manchester, UK). This enabled all camera views to be recorded onto one videotape. A time-lapse videocassette recorder (CTR-3024, Computar, UK) was used on 24-h mode, allowing 24-h of video to be recorded onto a single 180 min tape.

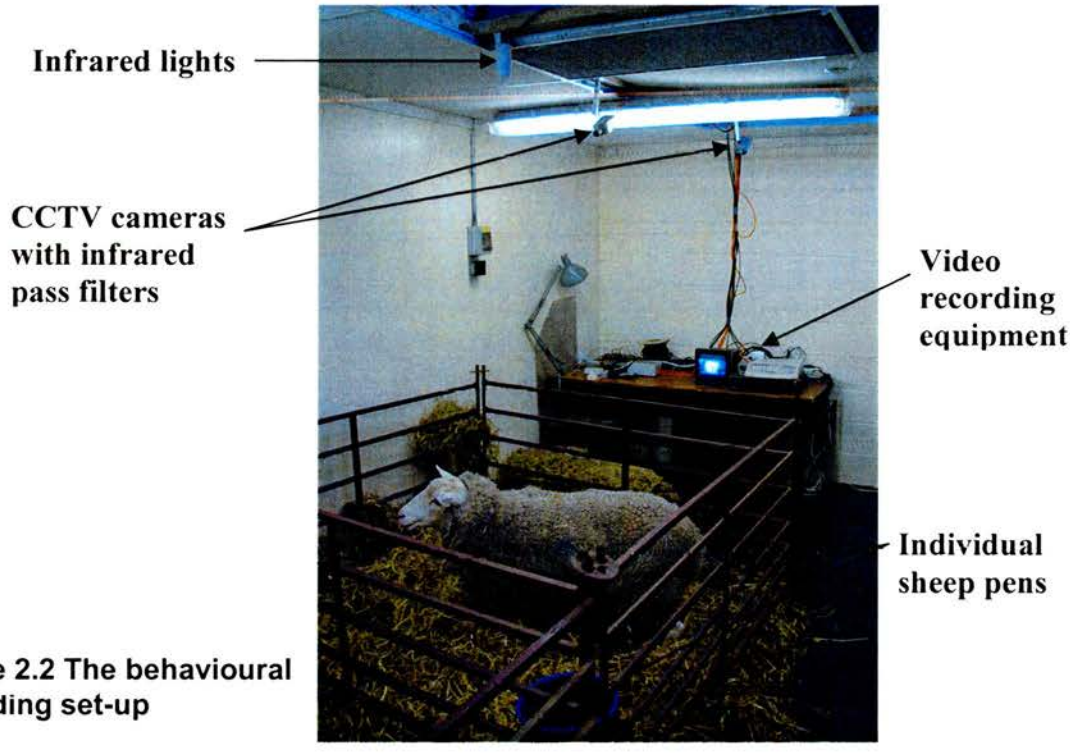


Figure 2.2 The behavioural recording set-up

2.3.2 preliminary study

2.3.2.1 Animals

Six, adult Dorset and Dorset X ewes were housed in pairs in indoor pens as described above (2.3.1.1). 24-h recordings were made from each animal up to twice per week until three satisfactory recordings had been made from each animal.

2.3.2.2 Electrode Attachment

The day before a recording commenced, the wool was removed from the electrode sites using clippers and hair removal cream. The electrode sites on the sheep's head are shown in Table 2.1

Table 2.1 Types of electrophysiological recordings, diameters and sites of skin surface silver/silver chloride cup electrodes

Type of recording	Site	Size of cup electrodes	Application
Electroencephalogram (EEG)	2 pairs over cranium See Figure 2.3 for positioning	6mm	Detection of electrical activity from the brain and recognition of sleep and wakefulness
Electro-oculogram (EOG)	1 pair over *orbicularis* muscle of each eye	6mm	Detection of eye muscle activity and recognition of rapid eye movement sleep
Jaw Electromyogram (EMG)	1 pair over *masseter* muscle	9mm	Detection of jaw muscle activity and recognition of rumination and eating
Neck Electromyogram (EMG)	1 pair over *braciocephalicus* muscle	9mm	Detection of neck relaxation and recognition of lying posture
Hind-Leg Electromyogram (EMG)	1 pair over off side *biceps femoris*	9mm	Detection of leg muscle activity and recognition of postural changes (lying/ standing)
Electrocardiogram (ECG)	1 pair on chest caudal to front legs	9mm	Detection of heart rate from activity of cardiac muscle
Ground for body electrodes	1 over the sacrum	9mm	Reference electrode
Ground for head electrodes	1 over the *occipital* bone	6mm	Reference electrode

Before the recording could take place, a recording template was set up in Somnologica 2.1. The recording template was used to define the channels used in the recording, their sensor type (e.g. EEG), name (e.g. EEG 1) and position (e.g. channel 1). The Somnologica programme supplied several templates. However as these investigations were recording from sheep rather than humans, a custom template was produced. The template used in the preliminary experiment was as follows:

8 channels

1) EEG-1
2) EEG-2
3) EOG
4) EMG-Jaw

} Head Set

5) ECG
6) EMG-Neck
7) EMG-Leg
8) EEG-3 (optional)

} Body Set

On the recording day, a PCMCIA card was fitted into the Embla. The camcorder batteries were fitted into the power supply port, ensuring the orange power-on light was blinking. The Embla was secured into the waterproof box using foam, and the watertight lid was screwed down. The harness was attached to the sheep and the waterproof box containing the Embla data logger was attached to the harness. The surcingles were placed around the body of the sheep.

When the harness was attached, the electrode sites were cleaned using a swab which was damp with 100% ethanol, to ensure a skin resistance, lower than 5kΩ. The connector plug containing the body electrodes was attached to the connector socket on the outside of the waterproof box. Each pair of electrodes was attached in succession, so that the remaining pairs were held out of the way on the back of the sheep. To obtain optimal signal quality and to reduce artefact noise a ground electrode was used for each 'set' of electrodes. The ground electrode for the body-set of electrodes was attached to the sheep first. The electrode lead was passed behind the harness straps and along the sheep's back. Each electrode lead had a small piece of Duct tape added, approximately 60mm from the electrode cup, which was used to fix the lead into the correct position at the edge of the cleared area of skin on the rump of the sheep. The leads were fixed in place with plastic clips (Henley's Medical Tube Clips, Welwyn Garden City, UK) which were clipped into the surrounding fleece; care was taken not catch the lead in the clip. The electrode cup was filled with electrode conductive gel (Electrode Gel, Dracard, London, UK) using a syringe and blunt ended needle. Superglue gel (Loctite Superglue Gel, Loctite Corporation, Ireland) was placed in discrete blobs around the brim of the electrode cup. The electrode was positioned in the middle of the cleared site and gently held in place for approximately 10 s, allowing the glue to bond. A strip of electrode adhesive (Aston University, Birmingham, UK) of 10 x 30 mm was attached over the electrode and on to the skin at either side. Finally, a plastic clip was used to secure the fleece from the surrounding area over the electrode site.

Each pair of body electrodes was attached in a similar manner to the ground electrode. When the electrodes were in pairs, each electrode was carefully positioned at least 20mm away from the other to minimise electrode cross-talk across the skin surface. The ECG electrodes were positioned on either side of the chest, directly

posterior to the front legs; the '+ve' electrode was on the off side of the sheep, the '-ve' electrode was on the near side of the sheep. The ECG electrode sites were covered in Duct tape for protection once the electrodes had been attached, as the wool in this region was not long enough to cover the site.

The connector plug containing the head electrodes was attached to the connector socket on the outside of the waterproof box. Each pair of electrodes was attached in succession, so that the remaining pairs were held out of the way on the back of the sheep. The ground electrode for the head-set of electrodes was attached to the sheep first. The electrode lead was clipped in place, using plastic clips to secure the fleece around the lead, along the sheep's neck leaving enough 'slack' for the sheep to fully extend its' neck. The lead was clipped at the edge of the cleared site at the base of the skull. The electrode cup was filled with electrode conductive gel and superglue gel was placed around the brim of the electrode. The electrode was positioned in the centre of the cleared site and gently held in place for 10 seconds. A strip of electrode adhesive of 10 x 30 mm was attached over each electrode and on to the skin at either side.

The EEG pairs of electrodes were added next. Figure 2.3 shows the position of the head electrodes on the sheep. The first pair added were those used to record EEG trace 1. Each electrode was filled with conductive gel and the brim covered in superglue gel and positioned carefully at least 10mm from the midline of the scalp, ensuring that the electrodes were at least 20mm from each other. A strip of electrode adhesive of 10 x 25 mm was attached over each electrode and on to the skin at either side. Then the pair of electrodes used to record EEG trace 2 were positioned. These were at least 20 mm caudal to the EEG 1 pair and were placed 15mm at either side of the midline. Finally, if the sheep had a big enough head, a 3rd pair of EEG electrodes was added, caudal to the 2nd. The pair were always placed so that the +ve electrode was on the off side and the –ve electrode was on the near side of the sheep. Care was taken not to fix the EEG electrodes over horn processes or over the mobile, wrinkled part of the scalp. Once the EEG 2 pair of electrodes was attached, the entire cleared area was gently covered in Duct tape for protection.

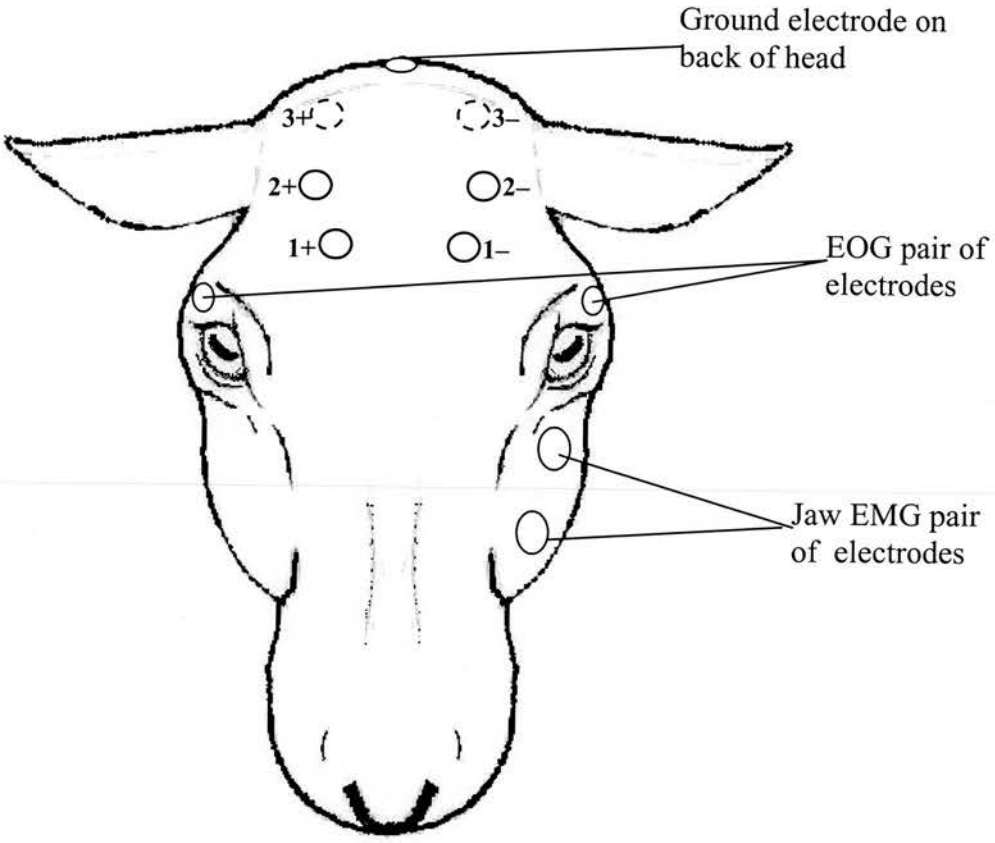


Figure 2.3 The approximate positions of electrodes on the head of a sheep (not to scale). The numbers refer to the pair of EEG electrodes. The EEG 3 was only used on sheep that had large, flat heads. All electrodes were 6mm in diameter except for the Jaw EMG electrodes which were 9mm in diameter.

The EOG pair of electrode leads was then clipped into place behind the ears; one on either side of the head. This allowed plenty of lead for head movement. Each electrode was attached to an eye site, posterior to the eyes. After attachment, each EOG site was covered in a piece of Duct tape (approximately 15 x 15 mm) to protect the electrodes. The jaw EMG pair of electrode leads was clipped into place behind the ears on the near side of the sheep (the near side of the sheep was used for the jaw EMG electrodes as this was opposite to the side where the buckles of the halter fastened). Each electrode was attached onto the cleared jaw site, at least 20mm from each other. Once the electrodes had been positioned and attached, the site was covered in Duct tape.

At this point, the electrode impedance was checked to measure the conductance of the electrodes and ensure correct attachment. The 4m Embla-PC cable was connected to the Com port at the rear of the waterproof box, and also connected to the laptop computer. In the Somnologica 2.1 programme an impedance check of each of the electrodes was taken. The EMG electrodes had to be within 1-20 kOhm, the ECG electrodes within 1-20 kOhm, the EOG electrodes within 1-10 kOhm and the EEG electrodes within 1-10 kOhm. When the electrodes were within these values a green signal appeared within the window on the screen for each pair and the impedance was given. If a pair was out of the ideal values by less than 15% in either direction, a yellow signal appeared at the pair. If the impedance was more than 15% outside of the ideal values in either direction, a red signal appeared next to the pair. The yellow signal usually appeared directly after electrode attachment and seemed to be indicative of the glue still being moist (decreasing the impedance). The red signal indicated that electrodes were not properly attached or that the glue had dried over the whole electrode base, reducing conductivity and thereby increasing impedance. Electrode pairs exhibiting a yellow signal on the laptop were checked and were frequently left as they were. Electrode pairs exhibiting a red signal were removed, cleaned with 100% ethanol and reattached using the procedure outline above. When all pairs exhibited at least a yellow signal (and the EEG electrodes had to exhibit a green signal before commencement), the Embla-PC cable was removed from the waterproof box.

To protect the electrodes on the head of the sheep, a Vet-rap bandage was loosely wrapped around the head, behind and in front of the ears and secured using Duct tape. The individually made, fibreglass helmet was eased over the head (so as not to dislodge the electrodes). The straps fastened from behind the ears and over the nose of the sheep, on the off side. Any loose sections of electrode lead from the head or the body electrodes were clipped into the fleece using plastic clips. At this point, the sheep was ready for a recording to be started.

2.3.2.3 Electrophysiological recording commencement

To start a recording, the Embla was reattached to the laptop PC via the isolation unit. In the Somnologica programme an ambulatory recording type was

chosen. The sheep ID, the recording template ID and the recording start and stop time and/or recording duration were entered. At this point, a second electrode impedance check was made to ensure that all electrodes were within the correct impedance limits (if not, the Embla would be disconnected from the PC and the electrode removed, cleaned and reapplied). The impedance of each electrode channel was noted. The duration of the recording was 24h 30min, allowing 30 minutes to leave the room and for the sheep to settle into 'normal' behaviour. The recording was then initialised, whereupon Somnologica checked the PCMCIA card for storage capacity. If successful, the green LED on the Embla would begin to flash, signalling the commencement of a recording and the Embla could be disconnected from the PC.

2.3.2.4 Removal and cleaning of equipment

At the end of the 24-h period, the electrophysiological recording automatically finished. The green LED on the Embla went out and the orange LED came on steadily. Shortly afterwards the equipment was removed from the sheep. The Embla was taken to the laboratory inside the waterproof box to avoid contamination. Each of the electrode leads was carefully unclipped from the fleece of the sheep, the electrode eased from the skin surface and the electrode sets were taken to the laboratory for cleaning. After the equipment had been removed from the sheep, all of the electrode sites were cleaned using warm water removing glue and conductive gel residues. Sites with any sign of irritation (such as reddened skin) were covered in dermisol cream. When three satisfactory recordings had been made, the sheep was checked by a veterinary surgeon and released back to the flock.

In the laboratory, the waterproof box was opened and the camcorder batteries were removed from the Embla power port and recharged. The PCICMA card was ejected from the card slot in the Embla and the files were downloaded onto a PC. The cards were then wiped of the files and defragmented for reuse. The data was saved on the hard drive of the PC and written to CD for back up. The electrodes were cleaned of wool and glue debris and immersed for at least 2h in a 3% solution of the decontamination agent Neutracon (Decon Laboratories Limited, Hove, UK). After cleaning, the electrodes were re-chlorinated by immersing the electrode cups in <5%

Chlorine based bleaching agent (Milton's sterilising liquid, Procter & Gamble, Weighbridge, UK) for 16h, taking care not to immerse the electrode leads.

2.3.2.5 The Ethogram

A simple ethogram was used when observing behaviour from the video to assign posture and ingestive behaviour to the sheep as shown in Table 2.2. The observations were simultaneously scored with the electrophysiological data.

Table 2.2 Ethogram

Behavioural State	Behavioural Description
Standing	Upright posture, bearing weight on all four legs or in locomotion
Lying Head Up	Recumbent posture with the body in contact with the floor and head raised above the withers. See Figure 2.4
Lying Head Down	Recumbent posture with the body in contact with the floor and head below the withers, resting on the bedding.
Eating	Mouthing at foodstuff or bedding followed by jaw movements and swallowing. Can be in Standing or Lying Head Up postures.
Rumination	Rhythmically chewing regurgitated cud. Can be in Standing or Lying Head Up postures.

Figure 2.4 The left side photograph shows a sheep lying down with head up. The right side photograph shows a sheep lying down head down. Note the sheep has her eyes closed in both postures

2.3.2.6 Electrophysiological and behavioural analysis

The postures and ingestive behaviour of the sheep were scored continuously for the 24-h recording to create an activity time budget. The 24-h time-lapse video recording was played back on a monitor. As the electrophysiological recording had been synchronised with the time code on the video at the time of recording, the events on the video and changes in electrical activity were simultaneous. As the video was watched, the electrophysiological data were observed and analysed on a PC as a workpad file in Somnologica 2.1. The workpad contained the visual settings, the information on how the data were recorded; and how the channels were to be viewed on the screen. In the workpad, the traces were moved around for ease of analysis. Here also the time scale and the amplitude scale was altered to suit the type of analysis. The raw data remained unchanged and stored and only the workpad file would change according to scoring and analysis. The workpad allowed the observer to add a score onto the electrophysiological data at the time point when the postural state started and continue it until the sheep changed posture. The change in posture could be marked onto the workpad by means of entering an *event* (the term event is used in the Somnologica software and used here in italics as the postures were behavioural states rather than behavioural events). Behaviours not found in humans (e.g. rumination), were added to the Somnologica *event* palette and were then chosen and added to the workpad at the appropriate times. *Events* were then resized to match the changes in the electrophysiological traces (see below) and the observations of the video.

Most changes in posture were easy to see on the video (for example changing from Standing to Lying Head Up) and the corresponding electrical activity changes in the Hind-leg EMGs were also characteristic (Figure 2.5 a and b). The transition from Standing to Lying Head Up was characterised on the Hind-leg EMG by an initial increase in amplitude of the EMG followed by an almost complete reduction of amplitude when the animal lay down with the hind legs relaxed. The wavelength frequency of the EEG appeared to remain stable throughout.

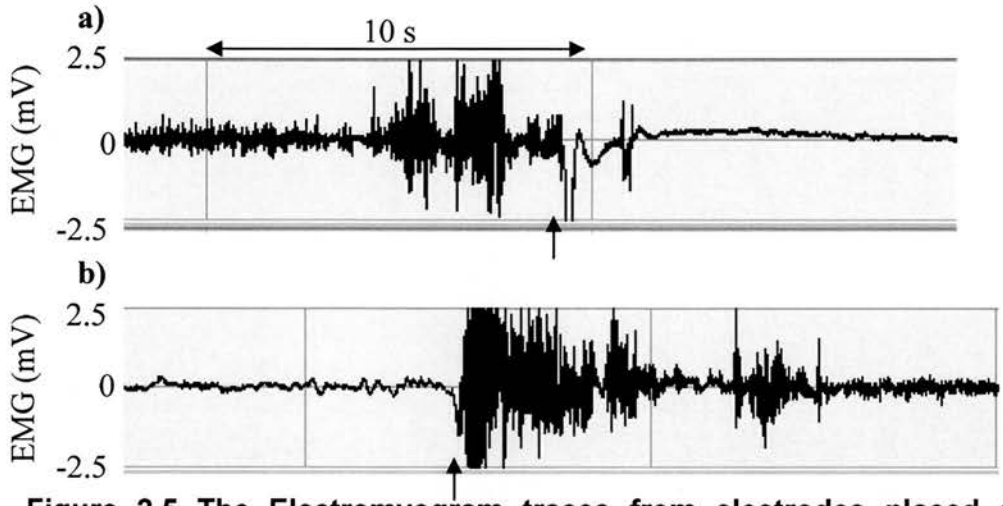


Figure 2.5 The Electromyogram traces from electrodes placed above the *biceps femoris* muscle of a sheep during a) the transition from Standing to Lying Head Up and b) the transition from Lying Head Up to Standing. The arrows indicate where the transition in posture would be scored.

Other changes in posture were harder to observe using behaviour alone. For example, when the sheep changed from Lying Head Up to Lying Head Down, it was sometimes difficult to ascertain exactly when the animal relaxed the neck muscles. Here, the observer was able to use the EMG recording from the neck to recognise when the change in posture happened (Figure 2.6 a and b). It can be seen from the trace that the Lying Head Up-Head Down transition was a gradual process. A gradual reduction in amplitude is seen on the Neck-EMG trace. Here the wavelength frequency appears to be reduced as the transition progresses.

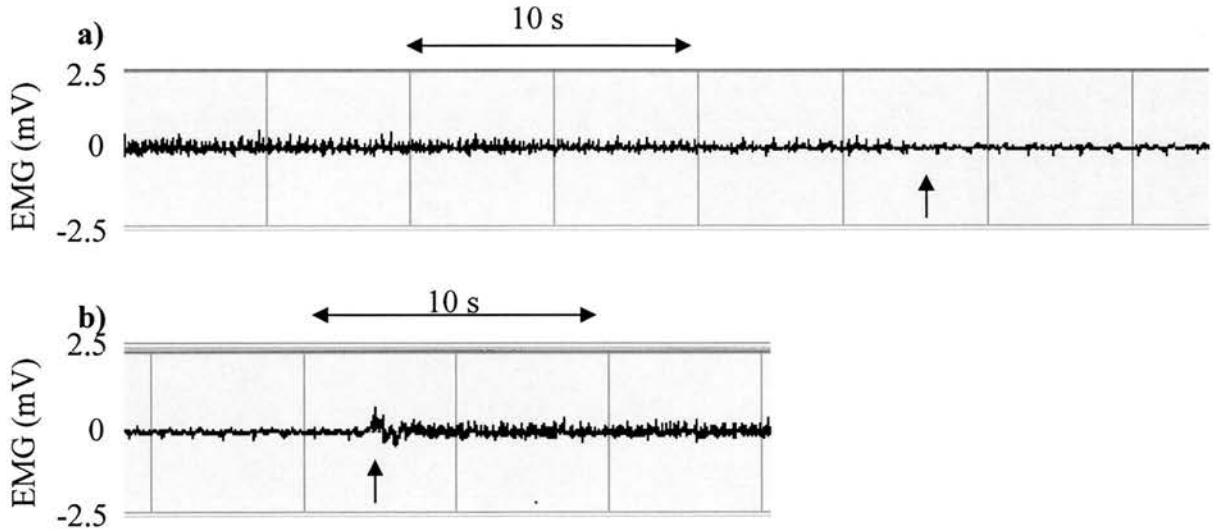


Figure 2.6 The Electromyogram traces from electrodes placed above the *brachiocephalicus* muscle of a sheep during a) the transition from Lying Head Up to Lying Head Down and b) the transition from Lying Head Down to Lying Head Up. The arrows indicate where the transition in posture would be scored.

The ingestive behaviour was analysed in the same way, correlating the behaviour seen on the video with the changes in electrical activity seen on the Jaw EMG trace. Both Eating behaviour and ruminating behaviour had characteristic EMG trace patterns (Figure 2.7 a and b). Eating behaviour was characterised by high amplitude, irregular waves (and artefacts on the other electrophysiological traces). Conversely, rumination was characterised by very regular, high amplitude, low frequency waves with low amplitude wave gaps of 2 to 5 s duration.

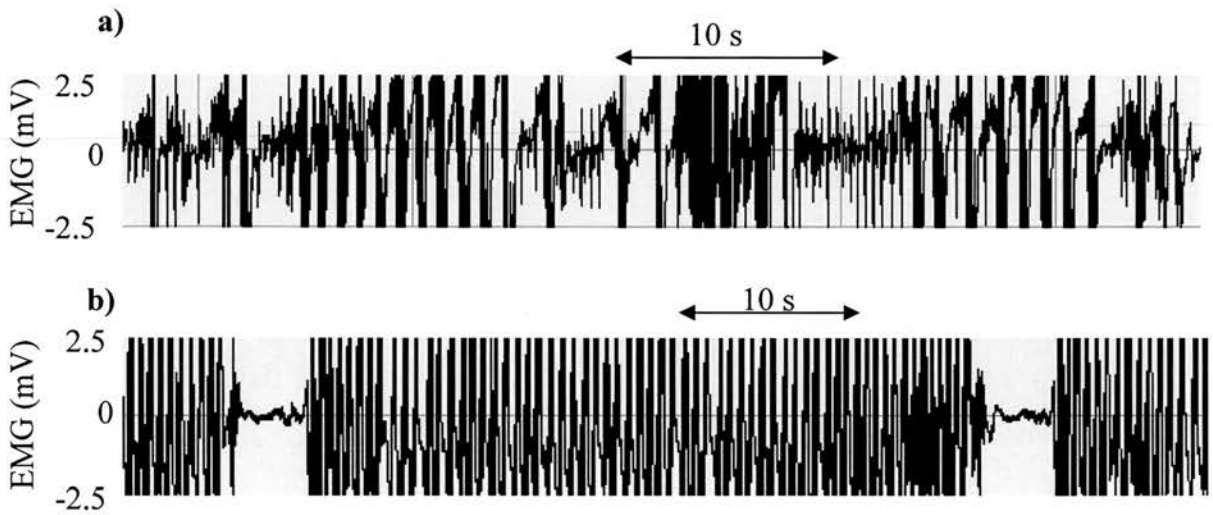


Figure 2.7 The Electromyogram traces from electrodes placed above the *masseter* muscle of a sheep during a) Eating behaviour and b) Rumination

The entire 24-h electrophysiological trace was analysed for posture and ingestive behaviour. When completed, an *Event Report* was produced in Somnologica 2.1 (see the appendix for an example) which provided time budget descriptive statistics on each type of *event*.

2.3.2.7 Spectral analysis of the EEG

In order to analyse the EEG, an Automatic Sleep Score was carried out in the Somnologica workpad. The Automatic Sleep Scoring Assistant used the criteria laid down by Rechtschaffen and Kales (1968) to score the EEG into different stages of sleep and wakefulness (see Table 2.3 for a summary) The EEG was scored in 30s epochs and the average of the whole epoch was used to determine the appropriate

score (e.g. if 30% of the 30 s epoch was considered stage I and 70% of the epoch stage II, an overall score for that epoch would be stage II).

An Analysis Start and an Analysis Stop *event* were added to the workpad at the points where the automatic scoring system was to start and finish. The Automatic Sleep Scoring Assistant rated each 30 s epoch of the 24-h electrophysiological trace and inserted a stage marker describing each epoch in terms of the different sleep stages outlined above. The automatic score took about 3 minutes to complete.

Table 2.3 A summary of the Sleep Stages as proposed by Rechtschaffen and Kales (1968)

Stage	Description
Stage W (wakefulness)	The EEG contains a high percentage of Alpha activity and the waveform has a low voltage and a medium to high frequency
Stage I	The EEG consists of a slightly higher voltage than wakefulness and a mixed frequency without rapid eye movements.
Stage II	Similar to stage I but with sleep spindles and K-complexes
Stage III	The EEG consists of high amplitude, low frequency waves
Stage IV	The EEG contains a higher percentage of high amplitude low frequency waves than stage III, called Delta activity.
Stage REM	The EEG contains low voltage, medium to high frequency waves, simultaneously there are rapid eye movements and a loss of muscle tone.

The Automatic Sleep Scoring Assistant consisted of five modules, which were used to help determine the sleep stage of each epoch. The modules were: the Delta-frequency module, the sleep spindle detection module, the eye movement detection module, the EMG tone detection module and the Sleep Staging module. Each module was 'consulted' to determine the sleep stage. If some of the traces were not of a satisfactory condition, (e.g. the EMG trace used to determine muscle tone during sleep was of poor quality) then the corresponding module could be manually left out of the Automatic Sleep Scoring Assistant process (five out of 18 recordings).

The Delta-frequency module evaluated the frequency of the EEG trace(s), in order to establish the percentage of the epoch that contained Delta frequency waves and the 'centre' frequency of the epoch. All EEG traces were added into the Sleep Scoring Assistant if they were satisfactory for the whole 24-h; any which flat-lined or

had any major artefact problem were excluded from the analysis. This module calculated the frequencies over 5 s epochs, before relating the results to the 30s epoch to be scored and compares the actual power of the Delta band and the 'centre' frequency with those set automatically in Somnologica. The automatic comparison 'centre' frequency was set at 4.4Hz (the centre frequency of a healthy adult human in stages I and II).

The Sleep Spindle module would detect the rhythmic bursts of 11-15Hz and up to 2 s that are the common characteristic of human sleep spindles. This module calculated the EEG in epochs of 2 s, before relating the results to the 30s epoch to be scored. As spindle density increases, then the probability of Stage II sleep increases.

The Eye Movement module detected the high amplitude activity contained on the EOG trace. Individual eye movements were not included in the analysis. However a 5 s epoch containing more than 5 eye movements was included. Increased eye movement activity increased the probability of scoring the epoch as either wakefulness or Stage REM sleep.

The EMG tone detection module was used to detect the level of muscle tone in the EMG (selected manually, the Neck EMG was chosen if it was satisfactory for the whole 24-h period). If the amplitude of the chosen EMG trace fell below a threshold level, then total muscle relaxation was occurring. Total muscle relaxation occurs during REM sleep, so the detection of very low tone increased the probability of REM sleep.

The Sleep Staging module brought together all the other modules to make the final decision about the score of the epoch. So for example, if rapid eye movements and very low muscle tone were detected, then a score of Stage REM was given. Similarly, if Delta power was high and the 'centre' frequency was lower than average, and if no eye movements were present and muscle tone was low, then a score of Stage III or IV were given (depending on the power of Delta frequency).

When the Automatic Sleep Scoring Assistant had completely scored the EEG trace(s), then a Sleep Report could be produced. This contained a hypnogram of the scored EEG trace and descriptive statistics of the sleep profile. However, in these investigations, the hypnogram and automatic score could not be used to determine the sleep/wake profile of the sheep. A number of problems were encountered, for

example that rumination (even when the sheep was standing –see below) was often scored as Stage IV sleep, so a manual score was produced using the following criteria (capital letters indicate the manual score added):

- If the sheep was Standing it was always scored as AWAKE
- If the sheep was Lying Head Up it was always scored as AWAKE
- When the sheep was Lying Head Down the Automatic Sleep Score was consulted
- If during Lying Head Down the Automatic Sleep Score had given a score of Wake to an epoch, it was manually scored as AWAKE
- In general, any score given by the Automatic Sleep Score during Lying Head Down was also given manually (e.g. Automatic Sleep Score gives Stage I, manual scorer gives STAGE I).
- On occasion, the sheep made jaw movements during the early part of an episode of Non-REM sleep. This produced artefacts on the EOG trace, which was scored as Stage REM by the Automatic Sleep Scoring Assistant. This was given a manual score of JAW MOVEMENT, as visually, it was easy to see the difference between jaw-related artefact and true rapid eye movement.
- If, in the middle of an episode of sleep, the sheep lifted its head for 2 s or less, the sheep was assumed to still be asleep. If the period of head lifting exceeded 2 seconds the sheep was scored as being awake and adjusting its position.

The scoring of the different stages of Slow Wave or Non-REM sleep by the Automatic Sleep Scoring Assistant seemed inconsistent, with no scores of Stage IV and very few of Stage III. There are differences between human sleep and sheep sleep (the main difference being the length of each sleep episode being much shorter in sheep sleep than humans sleep); 30s epochs may be too long to be able to score deeper slow wave Non-REM sleep. For this reason, all of the scores, STAGE I, II, III and IV and JAW MOVEMNET were classified as Non-REM sleep for later analysis and statistics.

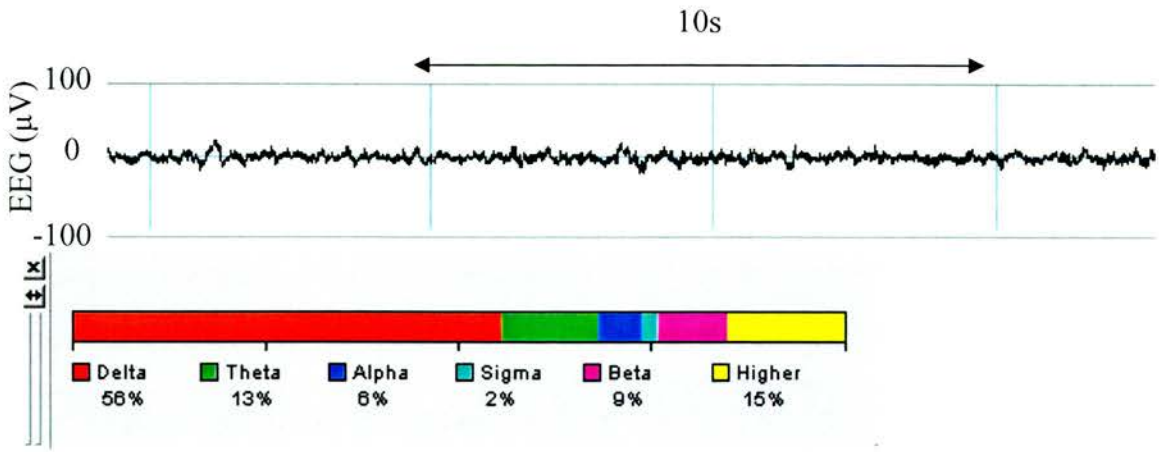


Figure 2.8 The Electroencephalogram from electrodes placed above the cranium when scored by Somnologica 2.1 as Sleep Stage III, manually scored as Non-REM Sleep. Accompanying Sleep Power Band analysis taken from the 10s shown by the arrow.

A section of the EEG trace could be selected in order to determine the relative power in each frequency band by viewing the Sleep Power Band analysis. This would show an estimated percentage of the waves that were in each frequency band category (e.g. the percentage of the EEG waves that were in the Delta band 0.5-4Hz and so on). This piece of analysis was used to record in more detail the spectral frequencies of the EEGs in Non-REM sleep in sheep. An example of Non-REM sleep EEG and its associated sleep power band analysis is shown in Figure 2.8.

Further spectral analysis of the EEG was undertaken in Somnologica to give more details on the spectral qualities of the EEG. These included the Fast Fourier Transform (FFT) and the Power Spectrum.

The FFT calculated the average frequency components of the EEG signal, where the average power (the amplitude is displayed as amplitude squared and is known as the power) of each frequency was plotted against frequency. A spectrum is a representation of the data based on the frequency distribution of its component sine waves. The output was the frequency with the maximum power amplitude (Hz) and the maximum power (mV/Hz).

The Power Spectrum analysis was used to smooth the FFT over larger sections of EEG trace (for example, in order to carry out spectral analysis over more than two 30 s epochs). The Power Spectrum calculated the average frequency power of a succession of (overlapped) FFT analyses, thus averaging the FFTs over a given

section of the trace. The automatic settings in Somnologica were an FFT sample size of 512, with an overlap of subsequent FFTs by 50%. This spectral analysis tool was not used very often, as many sheep Non-REM or REM sleep episodes were shorter than 90 s in length.

The Jaw EMG trace was used to recognise artefacts on the EEG trace. Somnologica (a human sleep analysis programme) automatically sleep-scores the EEG as 'Stage IV' sleep when the sheep were awake and ruminating. The artefacts of the muscle movement of rumination were at a frequency similar to the frequency of human Slow wave, Stage IV sleep (Figure 2.9). As the sheep were most frequently in the Lying Head Up posture (and occasionally, Standing) during rumination, a manual 'AWAKE' score was always given.

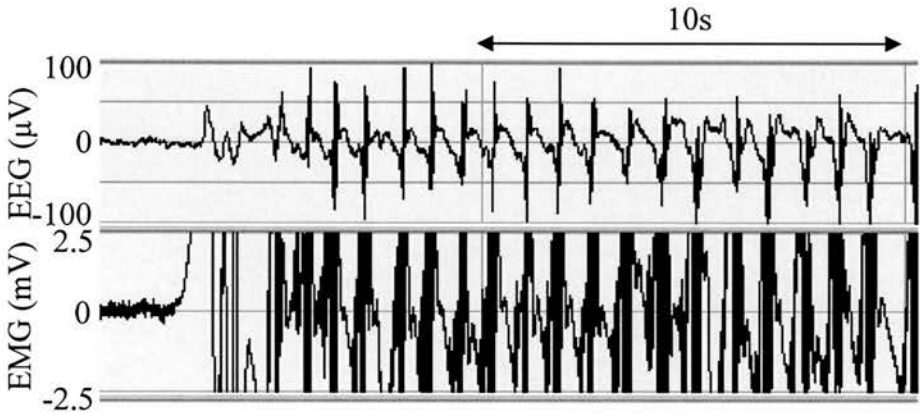


Figure 2.9 The Electroencephalogram (top trace) from electrodes placed above the cranium and the electromyogram (lower) trace from electrodes placed above the *masseter* muscle of a sheep during rumination. The muscle artefacts on the EEG trace come from the muscle activity of the jaw.

The EOG trace was also used to manually score REM sleep, as the eye movements (and therefore eye movement activity on the EOG trace) always accompanied REM sleep. Figure 2.11 shows the characteristic eye muscle activity before (high frequency, low amplitude waves) and during REM sleep (high frequency, high amplitude waves) (Figure 2.10).

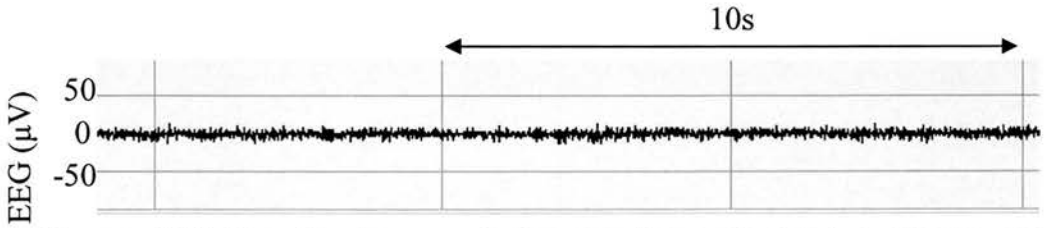


Figure 2.10 The Electroencephalogram from electrodes placed above the cranium when scored by Somnologica 2.1 as Stage REM and manually scored as REM sleep

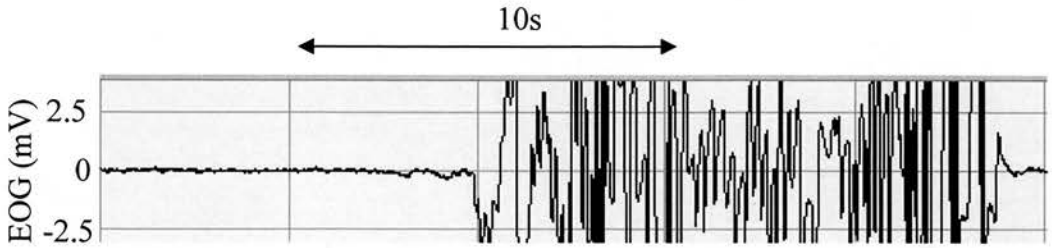


Figure 2.11 The Electro-oculogram from electrodes placed above the *orbicularis* muscle of a sheep during REM sleep. The muscle activity shows the characteristic eye movements of REM sleep in sheep.

The flow chart shown in Figure 2.12 was used to aid visual scoring of the electrophysiological traces.

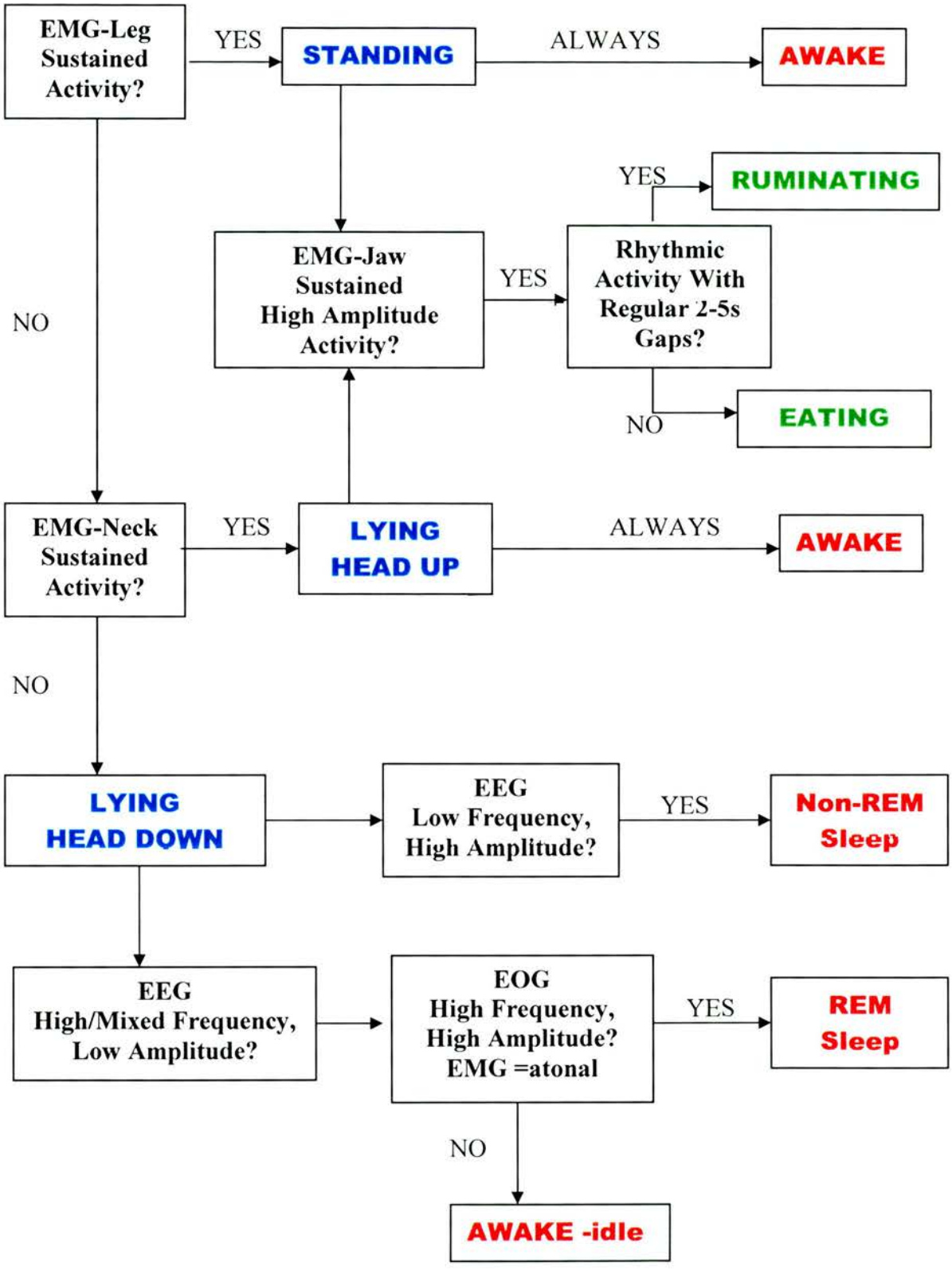


Figure 2.12 Schematic representation of the decisions made in order to score the electrophysiological traces.

2.3.2.8 Other physiological measures

The ECG was recorded from the sheep for 24-h. Somnologica 2.1 was used to determine the heart rate during sleep. Somnologica would take the ECG trace and locate the pulse in the trace by identifying heart rate markers, the Q-R-S parts of the waveform. The overall amplitude of the Q-R-S waves had to be 0.1mV for Somnologica to automatically detect a pulse. This lowest amplitude could be reduced further, but with a corresponding reduction in accuracy. The heart rate was displayed in beats per minute and the portion of trace (time) from which the calculation was made could be determined by the observer. Figure 2.13 shows the ECG of a sheep at rest.

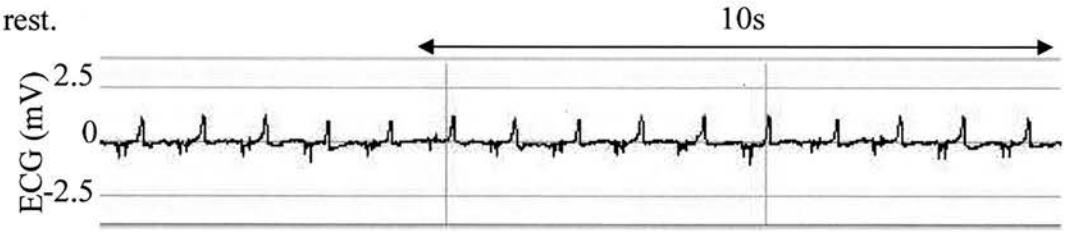


Figure 2.13 The Electrocardiogram from electrodes placed caudal to the front legs on the chest of a sheep. The ECG was taken from a sheep in the Lying Head Up posture.

2.3.2.9 Data analysis and Statistics

The manual scoring of the EEG (scoring each epoch using an *event* labelled AWAKE, STAGE I-IV, REM and JAW MOVEMENT) allowed the sleep score descriptive statistics for each recording to be produced in the *Event* Report in Somnologica. These data were analysed and descriptive and inferential statistics for all six sheep were carried out in Minitab 13. When analysing mean bout lengths over a period of time, there are problems with the fact that some bouts may fall at the start or end of the recording period and a true picture of the length of such bouts may not be obtained. In this preliminary experiment (and carried on into the three main experiments) the main focus was on the sleep of sheep. All electrophysiological recordings were started directly from a laptop computer (i.e. there was no time delay to start the recordings). This meant that sheep always started a recording when they were awake, so the bout lengths of sleep were not affected at the start. Out of the 18 usable 24-h recordings, one ended on a bout of Non-REM sleep. However, the video recording showed that the sheep was Lying with Head Up (and thus awake) within 30s of the recording end: therefore the bout was left in the analysis. In the three main

investigations the analysis was carried out using a residual maximum likelihood estimate method, which has been shown to be useful when splitting behaviour data into bouts (Langton et al. 1995).

A repeatability analysis was carried out using the method adopted by the British Standards Institution and proposed by Altman and Bland (1983). This method of analysis is a within subject calculation of the variation in repeated measurement data. The percentage of time in total sleep from the first two recordings from each sheep was used for the repeatability analysis. The difference between the two recordings was taken for each sheep, and the data were deemed to be repeatable if 95% of all the data points fell between ± two standard deviations of the mean of all of the recordings.

In addition, the data were transferred to the 'Observer 4.1' (Noldus Information Technology, Wageningen, The Netherlands) behaviour analysis programme to carry out a basic analysis of the temporal structure of the data using the 'Lag Sequential' statistical tool. In order to export the data from Somnologica to the Observer the *Event* score was saved as a text only file, containing the name of the *event* and the time from when it was scored in Somnologica. The text file was manipulated in Microsoft Word 2000 (Microsoft Corporation, 1983-1999. USA) so that it was in the same format as an ODF file, the text file that the Observer creates when carrying out a behavioural observation. The text files could then be read by the Observer and the statistical tools within that programme could be used.

The 'Lag Sequential' analysis records the percentage of times that one behavioural state follows another. It calculates the frequencies of transitions between pairs of behavioural states or events within a certain lag in time. The first event or state of each pair is called the *criterion event*, and the second event the *target event*. This enables one to ask, for example: "How many times is the (criterion) event 'Lying Head Down' followed by the (target) event 'Non-REM sleep'?" The output is the percentage of transitions, such as: when x% of the times behaviour a (criterion event) occurs, it is followed by behaviour b (target event).

All 18 recordings (3 recordings from each of six sheep) were analysed together to produce a matrix of total transitions of selected behaviours. The behaviours used in the analysis were REM sleep; Non-REM sleep; Awake; lying

head up; lying head down; and rumination. Each behavioural state was analysed as both a criterion event and a target event, in order to determine whether there were any sequences in these behaviours. The percentage of time a criterion event (C-event) was followed by a target event (T-event) was calculated using the following equation:

$$\frac{\text{Number of occurrences T-event followed C-event for all recordings}}{\text{Total number of occurrences of C-event for all recordings}} \times 100$$

2.4 Preliminary study results

The behavioural and the simultaneous EMG analysis from three recordings from six sheep show that the sheep spent (mean ± standard error) 43 ± 7.2% of a 24-h period standing (which includes movement), 31 ± 4.3% lying down with their head raised from the bedding and 26 ± 5.2% lying down with the neck relaxed, and the head resting on the bedding. The sheep adopted a characteristic posture: their legs folded underneath the body; their head resting on the bedding in front of their body, which took on a more flattened profile (indicative of muscle relaxation) during sleep episodes.

Non-REM sleep was scored when high amplitude low frequency waves were shown in the EEG trace. This EEG profile was accompanied with low muscle tone in the EMG traces from the jaw and the neck and occasional eye blinks shown on the EOG trace. REM sleep was scored when the EEG trace contained waves of high frequency and low amplitude, together with characteristic high frequency and high amplitude wave on the EOG recording. During REM sleep, the jaw and neck EMG traces showed a further reduction in muscle tone interspersed with occasional phasic twitches. Ear and body twitches could also be observed on the video during REM sleep, simultaneously, muscle artefacts would be visible on the EEG trace. In total, over eighteen 24-h recordings, sleep occupied on average 17.3 ± 2.45 % of a 24-h period.

On average, Non-REM sleep occupied 14.5 ± 2.38% of the 24-h period. There were 11 to 42 episodes of Non-REM per 24-h (a mean of 27 ± 11 episodes). In total the Non-REM episodes accounted for 83.8 ± 2.8% of the total sleep time and 56.7 ± 2.8 % of the total time spent lying down in the characteristic sleep posture (lying with

head down). The episodes of Non-REM sleep lasted between 30 s (1 scoring epoch) and 31 min (mean 7.51 ± 3.5 min).

Included in the Non-REM classification were periods of time when the EEG epochs were difficult to score because of Jaw EMG artefacts. These epochs were scored as Jaw Movement and classified as Non-REM sleep as the neck EMG remained at a constant low tonal level. Jaw movement occupied 7.2 ± 1.9% of 24-h period. This accounted for 46 ± 0.7% of the total sleeping time and 28 ± 0.7% of the total time spent lying down in the characteristic sleep posture.

REM sleep occurred during 2.8 ± 0.21% of a 24-h period. There were between 4 and 15 episodes of REM per 24-h (mean 9 ± 2.2) which accounted for 16.2 ± 1.3% of the total time spent sleeping and 10.7 ± 1.3 % of the total time spent lying down in the characteristic sleep posture. The episodes of REM lasted between 30 s and 5.4 min (mean 268 ± 20.1 s). Table 2.4 puts the results of the present study into context with those carried out in previous studies using more invasive methods.

Table 2.4 A comparison between the mean (% of 24h) Awake, Non-REM and REM sleep found in these preliminary investigations using non-invasive methods and those found in previous studies using electrodes implanted onto the brain surface. N = 6

Awake		Non-REM Sleep		REM Sleep		Reference
Mean	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**	
82.7	*2.45*	*14.5*	*2.38*	*2.8*	*0.21*	*Present study*
84.0		13.6		2.4		Ruckebusch, 1972
80.6		18.0	1.98	1.4	0.21	Ruckebusch and Gaujoux, 1976
82.4	4.29	17.3	1.95	1.1	0.36	Laurentie et al. 1989

A repeatability analysis was carried out using the within-subject repeated measures method (Altman and Bland, 1983). One hundred percent of the differences between the first two recordings (% of 24h spent in total sleep) were within ± 2 standard deviations of the mean and therefore the methodology was deemed repeatable (Figure 2.14).

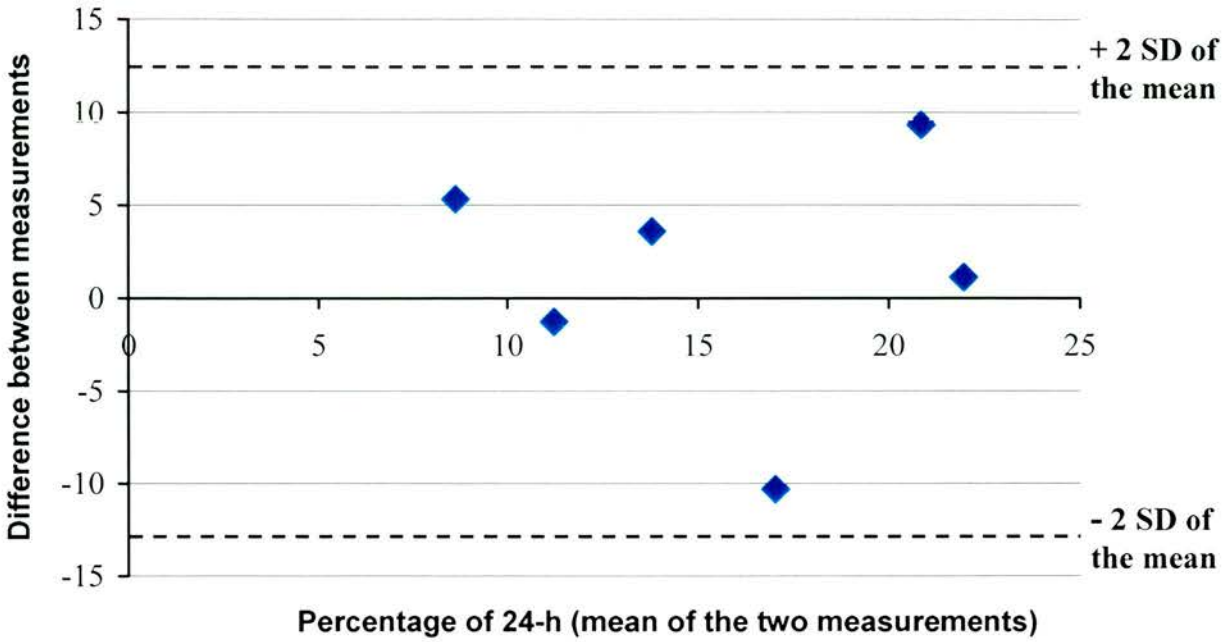


Figure 2.14 Repeatability of sleep recordings from sheep. Difference between the percentage of the 24-h period that was recorded as total sleep from the first two recordings on each of 6 sheep. All the points lie between ± 2 Standard Deviations of the mean. ◆ The difference between the mean total sleep time for the first two recordings in 6 sheep.

Sleep and wakefulness were distributed approximately equally between light (08:00-20:00) and dark (20:00-08:00) periods for all 6 sheep (Fig 2.15). The percentages of sleep data during the light and dark periods were normally distributed (the percentage of each type of sleep in either the light period or the dark period). There was no significant difference in the duration of REM sleep (mean ± s.e. % of REM sleep at night 56 ± 4.3; % of REM sleep during the day = 44 ± 4.3), Non-REM sleep (mean ± s.e. % of Non-REM sleep at night = 50 ± 5.3; % of Non-REM sleep during the day = 50 ± 5.3) and total sleep (mean ± s.e. % of total sleep during the night = 53 ± 4.3;% of total sleep during the day = 48 ± 4.3) between the night and day periods (2-sample t-test $P>0.05$).

When the average of three 24-h recordings for six sheep was divided into 1-h periods, there was no clear pattern of lying, or sleep during a 24-h period: both were distributed throughout the 24h. The distribution of total sleep was similar to that of lying head down posture. In the example recording shown in Figure 2.15 the sheep spent 20% of the 24h period sleeping (approximately 4h 44 minutes) and 26% of the time lying with head down (approximately 6h 10 minutes).

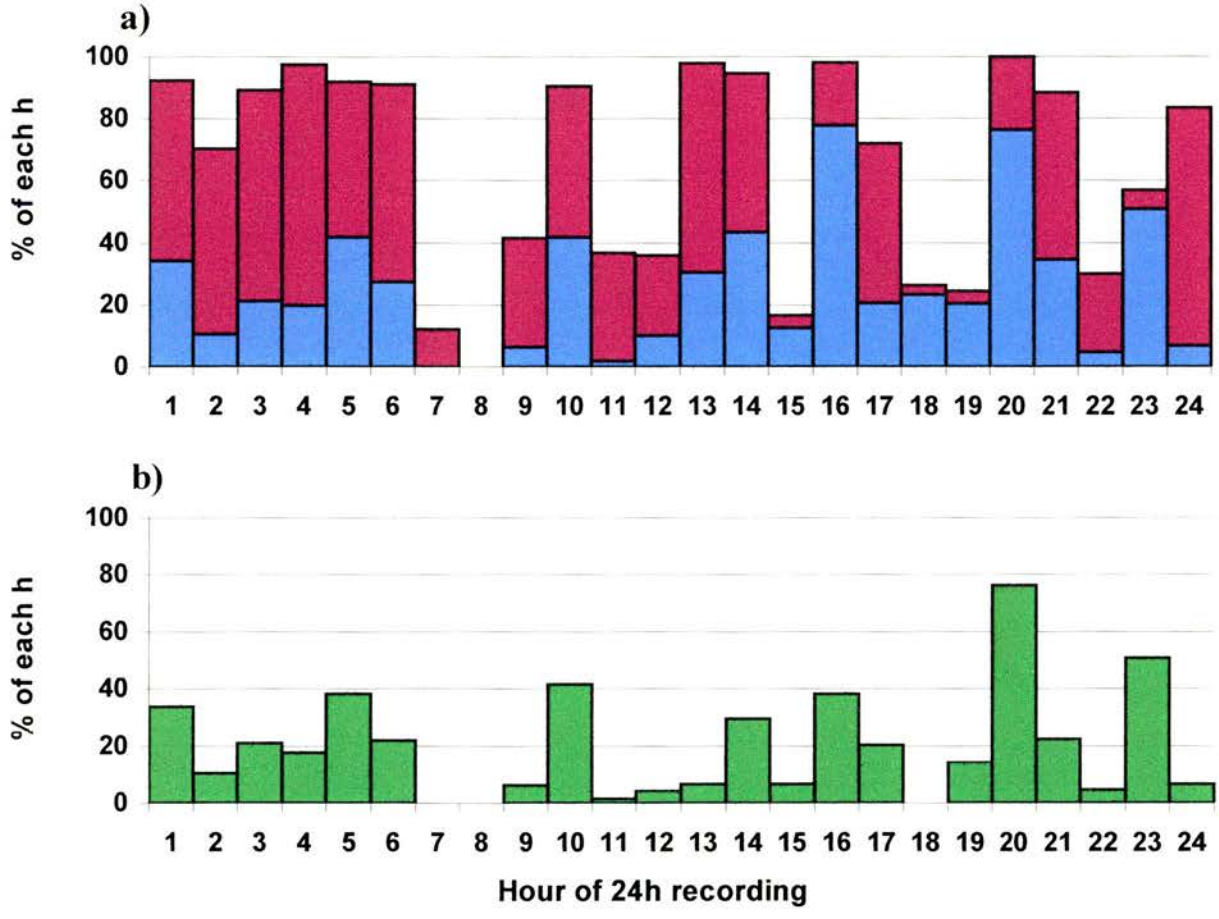


Figure 2.15 The distribution of a) Lying behaviour and b) Total sleep over the 24h recording period for Sheep 3 on the second recording. The recording start time was approximately 1000h.

Figure 2.16 shows the lag sequential analysis of the transitions in posture and sleep/wakefulness. When sheep lay down with head up, 73% of these occasions were followed by the characteristic sleep posture (lying with head down). When lying head down, 72% of the time the sheep were then scored as sleeping. When the sheep returned to wakefulness, 71% of the time they raised their head off the ground, but on 29% of occasions they remained in the characteristic sleep posture. On 56% of the occasions that a sheep was lying down and ruminating, this was followed by lying with the head down.

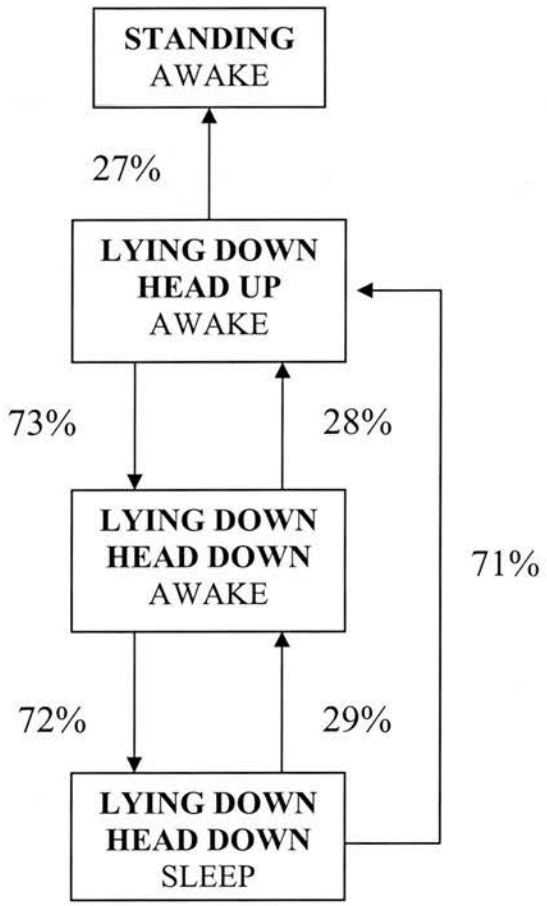


Figure 2.16 The lag sequential patterns of posture, sleep and wakefulness over three 24-h recording periods. The percentages are based on the number of times the target behaviour (after the arrow) follows the criterion behaviour (before the arrow) divided by the number of occurrences of the criterion behaviour in 24h. N = 6

Lag sequential analysis was also used to examine the transitions between sleep stages (Figure 2.17) REM sleep followed 20% of the episodes of Non-REM sleep. Waking followed 67% of the REM sleep episodes.

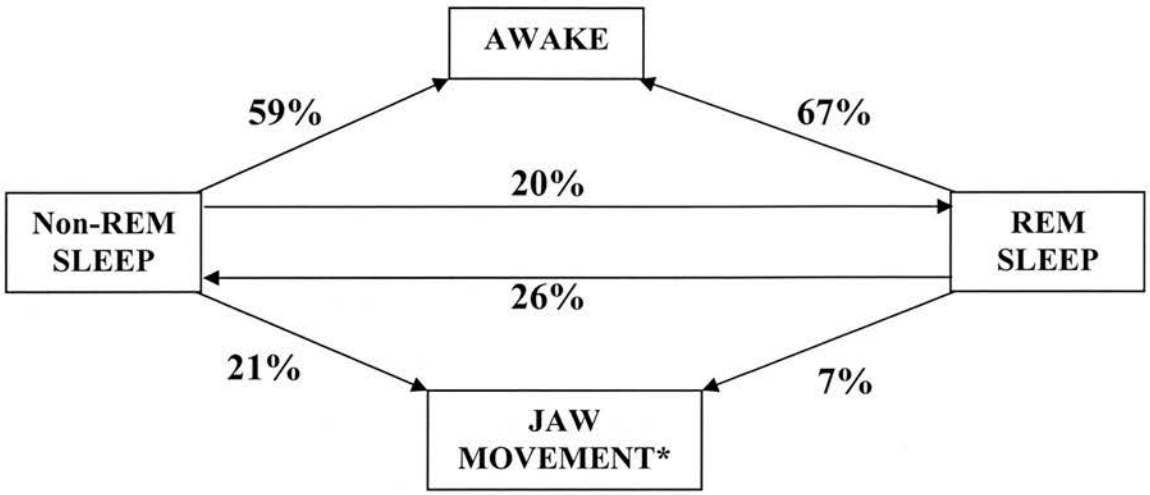


Figure 2.17 The lag sequential patterns of sleep stages over three 24-h recordings from six sheep. The percentages are based on the number of times the target behaviour (after the arrow) follows the criterion behaviour (before the arrow) divided by the number of occurrences of the criterion behaviour in 24-h. *Jaw movement was included within Non-REM for all other statistics. N = 6

2.5 Discussion

2.5.1The methodology.

One of the main reasons why more sleep research in large animals is not carried out using non-invasive electrode techniques is the unreliability of the skin surface electrodes over time (e.g. Ong et al. 1997). In the present investigations, approximately 3.5 24-h recordings were made on the six sheep during the preliminary study. Therefore, most recordings were successful enough to produce recordings of sufficient quality for subsequent analysis. A recording was deemed successful enough if at least one EEG channel, the EOG channel and the Neck EMG channel had remained attached and working throughout the 24-h (similar criteria were used by Giovagnoli et al. 1996). Some electrode sites were more successful than others. For example, the ECG electrode was always vulnerable to dislodgement in the position caudal to the front legs. The electrode attachment success varied with the size of the sheep. Large sheep were chosen for the study as they were most able to carry the

weight of the Embla in the waterproof box. However, this meant that the electrode leads of the Hind-leg EMG in particular were stretched, making it more likely that the EMG electrodes would be detached during the recording. Some sheep had a shorter face, which made the protection (using the fibre-glass helmet) of the Jaw EMG difficult. The Jaw EMG was an important part of the electrophysiological data, and recordings where this had become completely detached were rejected. However, some Jaw EMG recordings with movement artefact were accepted. Jaw related muscle movement was also picked up on the EOG trace, but during lying with head down posture, it became impossible to ascertain - if using the EOG alone - whether a movement on the EOG originated from the eyes or the jaw.

The EEG electrodes could also become detached. Some sheep had a small head and the area of scalp to place the electrodes was limited. It was important not to place the electrodes on the areas of the scalp that would be affected by ear muscle movement. Electrodes that were placed too far forward would be attached over the horn buds, where the bone thickness was much greater than that of the cranium (and the risk of amplitude attenuation was high). Some sheep had wrinkly scalps, with deep ridges, and this made it difficult to place the electrodes. In these cases, the electrodes were placed on the top of a ridge in an attempt to keep the electrodes attached for as long as possible. The level of electrode protection on the top of the head was high: each electrode was covered by adhesive tape; then covered in duct tape; loosely covered in crepe bandage and protected by the fibre-glass helmet. Unless the sheep was particularly irritated by the helmet and spent a lot of time rubbing their head, the EEG electrodes were likely to remain in place (even if they become unstuck). One sheep managed to remove her helmet and this recording was rejected.

The electrode placement in this investigation was similar to those used previously in sheep with invasive electrode techniques. The bipolar type of recording was used so that biological artefacts could be kept to a minimum as the difference between each electrode was measured. There are difficulties with using this method: if the areas of the brain under each electrode are exactly synchronous with each other, then there will be little registered on the EEG. However, in the non-invasive EEG recordings from sheep the biggest problem to overcome was that of muscular and

movement artefacts; the bipolar system overcomes this by removing the signals on the EEG that are common with the reference electrode (Fehmi and Sundor, 1989). Monopolar recording is possible in humans using non-invasive techniques as the reference can be placed somewhere relatively neutral - such as the ear, but as sheep have very mobile ears this is not possible. Other authors used monopolar type recordings where each electrode potential was measured against a neutral reference electrode. However, as they were using electrodes that were implanted onto the surface of the brain, most artefacts would be reduced (as compared with recording from the skin surface) (Bell, 1960; Ruckebusch, 1962).

Nonetheless, as Klemm (1966) suggested, when recording from ruminants the EEG will always be obscured during rumination, no matter where the EEG electrodes are attached. Therefore, it is impossible to record an artefact-free EEG during the chewing action of rumination. Both Klemm (1966) and Bell (1960) recorded the EEG from goats during the gaps between swallowing and regurgitation; Klemm showing the relaxed waking pattern of the EEG, Bell showing slow waves present. Bell (1960) claimed that ruminants were in a stage of sleep during rumination; Klemm refuted this. In the present investigations, the EEG was not analysed in any detail when the sheep were scored manually as awake; and the sheep were always scored as awake unless the sheep were in the lying head down posture. When the sheep were particularly quiet during the rumination gaps, the EEG was high frequency and low amplitude. However, muscle artefact was still present from ear and eye movement. This evidence suggests that the sheep in this study were restful but awake during rumination when lying down, perhaps being in a state described by Ruckebusch (1972) as drowsy.

Similarly, from this experiment no conclusion can be drawn on whether sheep can sleep while standing. Horses can undergo Non-REM sleep while standing (although they must lie down to experience REM sleep), and cattle have been shown to be able to get short periods of Non-REM sleep while standing (if restricted from lying down) (Ruckebusch, 1974). If cattle are allowed to lie they are not observed sleeping while standing. The sheep in this project were unrestrained and free to rest in any posture, so it is unlikely that they slept while standing. However, using the non-

invasive techniques to record the EEG to record sleep in sheep might be problematic if the animal is prevented from lying down.

Using the Somnologica 2.1 programme to score the electromyographic data was easy during this investigation. The differences between standing/moving and lying down were profound on the hind leg EMG trace (as shown in figure 2.5), so much so that the video was superfluous for basic posture analysis in the majority of cases. It remains to be seen whether the differences in frequency and amplitude seen on the leg EMG could be analysed in such a way as to give more information than just basic posture information. Giovagnoli et al (2002) used the EMG from the legs of horses to investigate balance preservation during transport.

Analysing the difference between the postures lying head up and lying head down was more difficult. This was mainly because sheep often changed between these postures in a gradual fashion (especially in the direction head up to head down) and the exact cut off between one posture and the other was sometimes difficult to place. After a bout of sleep, the sheep usually returned to the head up posture (on 71% of sleep bouts) and they usually did this in a sudden movement. A similar 'sudden awakening' is shown by Klemm (1966) in goats and by Ruckebusch (1972) in cattle. This meant that the head down to head up posture transition was easier to score. The neck EMG contained ECG artefact on eight of the 18 24-h recordings, and this further obscured the EMG. It was unclear why some recordings were affected and others were not. Filtering the Neck EMG traces with a 2Hz low pass filter was helpful in reducing ECG artefacts. Tong, et al (2001) used a more sophisticated filtering process to remove ECG artefact from the EEG. However, the EEGs in the present experiment seemed unaffected by ECG artefact.

Rutter et al (1997) found that programmes could be written to effectively separate rumination, eating and other jaw movement from digitally recorded jaw movement data. In terms of discriminating between different ingestion behaviour in the present investigation, the EMG trace made this very easy. The difference between the waveform of rumination and eating was profound; and in the case of rumination, it was fundamentally different from any other form of movement and artefact; rumination could easily be recognised by analysing the Jaw EMG alone. Eating

behaviour (in the case of hay and straw) also had a characteristic waveform, but may not be recognised using the Jaw EMG alone; instead one must analyse the other EMG traces alongside that of the Jaw. The Neck EMG showed spikes at approximately 3-5s intervals, where the sheep could be seen on the video, tearing at hay followed by the rapid chewing motion.

The success level of ECG recording was highly variable. Twelve out of the 18 24-h recordings did not contain a successful ECG trace throughout the 24-h. Eight out of the twelve were detached within 6h of the start of the recording. In addition, of those that were successfully attached throughout the 24-h, many included movement noise when the animal was standing and were only readable when the animal was lying down. Furthermore, the R peak was weakly recorded during this study, although visually the ECG could be scored for heart rate, often the Somnologica programme would fail to give a heart rate score.

Sleep was scored automatically by the Automated Sleep Score within the Somnologica sleep programme. The programme scored the whole EEG trace according to the rules set by Rechtschaffen and Kales (1968), using information from other available electrophysiological data, such as the neck EMG and the EOG. The programme carried out spectral analyses on 30s epochs of EEG and scored wake, stages I-IV and stage REM. While the sheep was observed on the video (and correlating leg and neck EMG) as standing or lying head up a manual score of AWAKE was added. This was necessary as there were a number of occasions during the 24-h recordings where the automatic score would give a score of sleep when the sheep was awake. The most common of these errors was during rumination, the automatic sleep score would give stage III or IV as the jaw-related artefact was in the delta frequency. In agreement with Klemm (1966), a manual score of awake was added as the sheep required muscle tone during rumination and had an EEG trace indicative of wakefulness in the gaps between swallowing and regurgitating.

When the sheep were sleeping, the automatic sleep score gave a score of stage I or II in the majority of cases (except during REM sleep); four of the sheep had no sleep scored as sleep stage III or IV. In addition, much of the sleeping EEG was scored automatically as stage REM, when it was obvious to the observer that the sheep was not experiencing REM sleep (no eye movements, no loss of muscle tone,

higher amplitude EEG). This error seemed to be caused by fast jaw movements that resulted in artefacts on the EOG trace: that were then scored as rapid eye-movements. A manual category of jaw-movement was created for this state. It was decided for analysis purposes, that all the slow wave sleep stages scored by the automatic score and the manual score of jaw movement would be added together as Non-REM sleep. In humans, the four slow wave stages and REM sleep are used as standard. However, in non-human animals, only other primates also have their Non-REM sleep split into four separate stages (Crofts et al. 2001). Rats are often given two Non-REM stages (light and deep –e.g. Bjorvatn et al. 1998), but most frequently only Non-REM sleep is considered in rats and other rodents (e.g. Meerlo et al. 2001; Lancel et al. 2003).

A difficulty that is common to both visual and automatic sleep scoring is what to score when the subject awakens (or moves their head) for a very short while. These are termed microarousals and in humans, microarousals can occur in up to 50% of the occurrences of the sleep cycle during a monophasic sleep bout (Terzanno et al. 1988). Some authors (e.g. Haba-Rubio et al. 2004) suggest that multiple microarousals are a sign of poor sleep in humans. However, sheep sleep in polyphasic bouts during 24h. Is it right to use the same microarousal criteria as for humans, when each microarousal is a bigger part of the sleep bout within which it occurs? The best response to this question is one of consistency: it is possible to have a 'movement arousal' and see very little change on an EEG trace (Halász et al. 2004), especially if the movement lasts for 1s or less. In the present investigations, movement of 2s or less has been defined as a movement arousal as opposed to an awakening and continued to score the EEG in the sleep stage that was present before the movement. The exceptions occur when the sleep stage was altered post the movement as compared with pre-movement, here the arousal was scored as awake. The problem may be a consequence of the epoch length of the scoring system. The Somnologica programme automatic sleep score uses 30s epochs as a default. However, as sheep sleep for short bouts, perhaps 30s epochs are too long. This may lead to underscoring all stages of sleep. Several other animal studies use epochs of a shorter length, such as 10s for rats (Kant et al. 1995).

REM sleep was straightforward to score visually (necessary to distinguish actual REM sleep from stage REM scored during jaw movement). The complete lack

of muscle tone on the neck and jaw EMG traces coupled with characteristic eye movements allowed REM sleep to be scored, even if the EEG was partially obscured by eye-related artefacts. In addition, phasic twitching could be seen on the video and in the EMG traces. It is likely that REM sleep was underscored using these techniques, as the transition between Non-REM and REM sleep occurs 1-2s prior to the complete loss of muscle tone. In this investigation, videotape was used to record behaviour using a 24-h time-lapse VCR. This meant that instead of a relative recording speed of 24 frames a second, the time-lapse video was recorded at a relative speed of eight frames a second. Therefore, some subtle behaviours that may have assisted in the visual scoring of sleep were not visible on the video (such as ear flicks and phasic twitching).

2.5.2 preliminary study results

These preliminary results demonstrate that it is possible to use non-invasive electrophysiological techniques to measure sleep in sheep. Behavioural observation in conjunction with the non-invasive, EEG, EOG and EMG recordings can give results for sleep durations which are comparable to those found in sheep using invasive techniques (Ruckebusch 1972). The electrophysiological recordings provide more information and give a more accurate representation of both the quantity of sleep and the quality (i.e. whether the sheep is in the Non-REM or REM stages) of sleep and wakefulness in sheep than using behaviour alone. It can be seen that, by only using behaviour, sleep may have been scored when sheep were in the characteristic sleep posture (Lying with Head Down), but brain activity as shown on the EEG, was still indicative of wakefulness approximately 33% of the time spent in the Lying with Head Down posture. In addition, without direct observation, the recognition of eye movements seen in REM was difficult from video observation and therefore the REM : Non-REM ratio may not be accurately recorded using behaviour alone. However, better quality video recording (e.g. without using time-lapse functions or multiplexers, both of which reduce video quality) may prove useful in the visual observations of REM versus Non-REM in sheep in situations where the electrophysiological equipment might be difficult to use (such as in neonatal lambs).

Interestingly, although the results of sleep and wakefulness are similar to those of Ruckebusch (1972), there were differences in the posture/behaviour patterns between sheep in this study and that of Ruckebusch. On average, sheep lay down for 57 % of the 24-h in the present study, while Ruckebusch (1972) observed sheep lying down for only 30 % of a 24-h period. These differences may be due to the confinement of sheep to metabolic crates. Such crates restrict movement, impede turning and reduce the space for lying postures, and in the case of Ruckebusch's study, were not bedded, and may not have been as comfortable for lying postures as straw bedded pens. However, the amount of sleep seen in both studies was similar, which suggests that sleep in sheep may be a relatively inelastic behaviour and is likely to be carried out even if the sheep is uncomfortable. The 57 % amount of lying in a 24-h period from the present study was more comparable to other behaviour research that was carried out with sheep in large, straw bedded pens (Done-Currie et al. 1984) or when sheep had access to pasture (Tobler et al. 1991)

The present research showed that adult sheep sleep on average for 17 % of 24-h. This is only just over half the total percentage of 24-h that adult humans sleep (approximately 30%) (Aserinsky and Kleitman, 1955) and about a quarter of the time that a cat spends sleeping (approximately 65%, 50% Non-REM, 15% REM)(Jouvet, 1967). This difference was expected as comparative behavioural ecology studies have shown that omnivores and carnivores sleep for more of the time and for longer bouts than obligate herbivores, such as ruminants (Allison and Cicchetti, 1976; Tobler, 1995). Herbivores, especially ungulates are prey species and the lower amount of sleep may be due to the need to remain vigilant (Dimond & Lazarus 1974). Sheep are naturally especially vulnerable to predation. They have few defence systems open to them and therefore they must remain vigilant. However, they live within a flock, this means that it is possible for them to sleep at some periods during the day as there are always other individuals vigilant for predators (Geist, 1971).

The lag sequential analysis showed that sheep usually woke up and lifted their head after a bout of sleep (71% of the time that sleep occurred). However, this means that 29% of the sleep bouts ended with the sheep waking but not changing their posture. If the behaviour was being scored without the electrophysiological data then it is possible that sleep would be over scored. When animals were lying with their

head up, the lying with head down posture usually followed (73% of lying head up bouts). Sixty-four % of rumination bouts overall (71% of ruminating lying down) ended with the sheep lying with head down. Both Non-REM and REM sleep were most frequently followed by wakefulness (67% of REM bouts, 59% of Non-REM bouts). The behaviour of the sheep appeared generally to follow a cycle of activity, in which the sheep would stand and eat; followed by lying down with rumination and most bouts of rumination would be followed by lying down head down.

The sleep of the sheep in this study occurred in short bouts, evenly dispersed throughout the 24-h period; a polyphasic pattern, different from the monophasic, night-time pattern of human sleep. However, previous studies of wild sheep and domesticated sheep at pasture have shown that the majority of sleep and a general reduction of activity occur at night (Geist, 1971; Tobler et al. 1991; Champion et al. 1997; Hulbert et al. 1998; Das, 2001). There are several possible explanations for the difference in behaviour between housed sheep and those at pasture. Housed sheep do not have to be active to forage for food: in the present study hay was provided *ad-libitum*, so less of the 24-h is taken up looking for food; more time can be used to rest. In the relatively unchanging environment of the sheep barn, (in the preliminary experiment containing only two individuals at any one time) vigilance during the day may be less important than out at pasture. During a 24h recording, the sheep would only experience human disturbance twice, when being fed and watered, the room had no windows so the sheep would be free from visual contact of humans for the majority of the day. The other rooms in the barn system were in use periodically, but most of the day the environment for the sheep would be quiet. Done-Currie et al (1984) found that sheep in an animal house would be the most active when people were in visual contact.

Moreover, there may be a lack of the appropriate environmental cues (changes in temperature, gradual changes in light) in the sheep barn as the only environmental change occurred at lights on and lights off, leading to a possible change in circadian rhythms of sheep behaviour. Finally, there is a possibility that sheep housed for a long period may become 'bored' with their surroundings and adopt more passive behaviours throughout the 24-hours as a result (Wemelsfelder and Farish, 2001).

Nevertheless, some aspects of this study should be taken with caution. All 18 of the behavioural recordings were analysed, recording the behaviour of the sheep wearing the electrophysiological equipment and comparing this with the behaviour of the companion sheep without equipment (Hollingsworth, 2001 –zoology BSc Thesis). It was clear that some of the behaviours, notably the time spent ruminating and the time spent lying with head down, were different between the sheep wearing the equipment and the sheep without equipment. From the video, it seemed that the main difficulty was that the harness would often slip round the side of the sheep unbalancing them (although this was not quantified). These results lead to a redesign of the harness that attached the Embla to the sheep. The new harness was developed in order to minimise the likelihood of the equipment slipping round either side of the sheep by increasing the number of straps and changing the positioning of straps. The new harness also provided padding under the Embla so there was no direct pressure from the Embla along the spine of the sheep. Finally, the straps were made of a soft webbing material which was designed to increase comfort for the sheep compared with the leather straps from the old harness design. The auditory arousal experiment was undertaken using the new harness design and completed without harness slippage.

2.6 Validation studies

2.6.1 validation study I: auditory arousal

2.6.1.1 Animals and husbandry

Fifteen-hour electrophysiological and behavioural recordings were carried out on six adult, Dorset an Dorset X ewes until three satisfactory arousal recordings had been observed and recorded from each sheep.

Sheep housing, video monitoring and electrode attachment followed the procedures outlined in the general methodology section (2.3.1). A 12hL:12hD light cycle was maintained with artificial lights and infrared lights for dark period recording. However, for this experiment to be carried out within working hours , the dark phase started at 1300h and the lights came back on at 0100h. Natural light from translucent ceiling tiles was excluded by covering them in black plastic. The sheep were gradually habituated to the change in lighting pattern by having the lights on

and lights off 1 h earlier than the proceeding day, each day for the week leading up to experimentation.

2.6.1.2 Electrophysiological recordings

The Embla and silver/silver chloride electrodes were attached to the sheep towards the end of the light phase. The sheep was exposed to the arousal stimulus (see below) when awake and 15 h ambulatory electrophysiological and video recordings commenced. The sheep was then left undisturbed and their behaviour was observed from a monitor in the next room.

2.6.1.3 Arousal protocol

After the sheep had been observed adopting the 'lying with head down' posture for two continuous minutes, the auditory arousal stimulus was played once every 40 s. The auditory arousal stimulus was an approximately 1.5 s audio recording of a dog bark. The dog bark had been recorded to compact disc so that the bark was replayed every 40 s increasing by 5 relative-dB on each play. The sound level was always started at the same point (which was only just audible to the human ear and did not elicit a behavioural reaction from the sheep when it was awake) and increased stepwise by 5 relative dB every 40 s. The audio speakers were situated 2.5 m from the sheep pens, 0.75 m off the ground. The exact sound pressure at the level of the sheep was not measured. Behavioural reactions to the arousal stimulus and the display on the compact disc player that produced the stimulus were simultaneously recorded on a video recorder.

The 'dog bark' playback was stopped when a behavioural orientation was made (i.e. when the sheep raised its head from the bedding and orientated towards the speakers). If the sheep regained the 'sleep posture', an interval of 5 min was left before the arousal stimulus was used again. The 'dog bark' playback was abandoned if any background noise was judged by the observer to have caused a behavioural orientation by the sheep. The 'dog bark' playback was repeated on each occasion that the sheep adopted the appropriate 'sleep posture' for up to eight hours during the dark phase.

2.6.1.4 Electrophysiological analysis

The posture was scored using the ethogram outlined in the general methodology section. An 'automated sleep score' was carried out on the EEG traces using the Somnologica programme as described in the above section 2.3.2.6. As the time of the behavioural orientation and the arousal stimuli were recorded simultaneously, it was possible to find the exact point on the EEG trace when the orientations and stimuli occurred. Spectral analysis of the EEG was carried out on 10 s epochs before and after the penultimate arousal stimulus (e.g. if 12 'dog barks' had been played during an arousal and an orientation response was seen on the 12^{th} 'bark' then the EEG before and after the 11^{th} 'bark' would be used). The penultimate stimulus was chosen as muscular movement during orientation caused electrical artefacts on the EEG trace. All arousal events that included rapid eye movements during the penultimate stimulus were discounted as the eye movements caused muscular artefacts on the EEG traces. Only 'artefact-free' arousal events were analysed from each sheep. Using the Somnologica sleep analysis programme, a mean was calculated of the frequency at maximum power (using the fast Fourier transform) and the percentage of delta waves (using sleep power band analysis) in both the pre- and post-stimulus EEG from all six sheep.

2.6.1.5 Statistics

Descriptive statistics and paired t-tests comparing before and after the penultimate arousal stimulus were carried out using Minitab 13 (Minitab Inc, USA).

2.6.1.6 Results of the auditory arousal study

Each sheep had at least two arousal events which were appropriate to use in analysis (Less than 2s of muscle artefact, no extraneous noise, electrodes still attached). The median (Q1-Q3) number of arousal stimuli played to the sheep (within one arousal event) until the orientation response was seen, was 8.5 (6.5 – 11.5). The median number of arousal stimuli corresponded to an increase of 37.5 relative dB from the first play of the dog bark.

The 30 s epochs of EEG directly prior to the penultimate stimulus of each artefact-free arousal used in the analysis were scored by the 'Somnologica automated

sleep score' as either sleep stage II or stage III sleep. In all arousals used in the analysis, the 30 s epochs directly after the behavioural orientation were automatically scored as 'wake' by the Somnologica software. The mean frequency of the EEG significantly increased from 1.1 ± 0.2 Hz (mean ± s.e) pre stimulus to 21 ± 3.9 Hz post stimulus (paired t test, $t = 3.1$ $p<0.05$, $n=6$). The median (Q1 – Q3) percentage of delta waves (0.5 to 4 Hz) significantly decreased from 38 % (34.8 – 44.3) pre stimulus to 14 %(10 – 15.8) post stimulus (Wilcoxon sign test, $w = 21$ $p<0.05$, $n=6$). Figure 2.18 gives an example of the EEG trace taken before and after the penultimate arousal stimulus of an arousal event, the change in wave pattern can be seen visually as well as being shown in the spectral analysis (for this example: % delta waves pre = 32.4, % delta waves post = 17.1). Over all the arousal events for each sheep there was no significant difference in the amplitude (pre 2390 ± 1098 mV; post 624 ± 69mV), no correlation between the depth of sleep (both frequency level and % of delta waves); and the number of arousal stimuli needed for the orientation response to be seen.

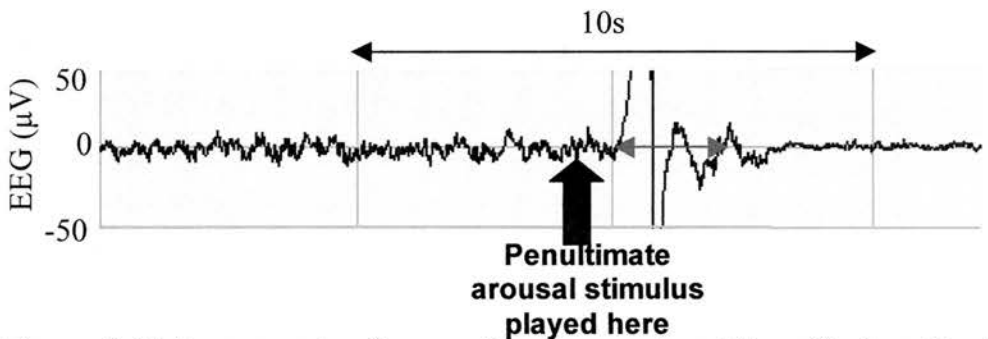


Figure 2.18 An example of an auditory arousal and the effect on the EEG. The EEG from sheep 11 showing the sections of the trace before and after the Penultimate arousal stimulus. There is a small amount of muscle artefact (blue arrow) directly following the arousal stimulus caused by a jaw movement.

2.6.1.7 Discussion of the auditory arousal study

A decrease in the lowest frequency of the EEG (i.e. delta power) results from a desynchronisation of rhythmic, cortical, high voltage activity (Steriade et al. 1990). This change in frequency is indicative of a change from slow wave sleep (characterised by a high percentage of low frequency delta waves) to wakefulness (characterised by a high percentage of higher frequency waves). The results shown in the auditory arousal study suggest that the EEG recorded from surface electrodes is capable of detecting a change from sleep to wakefulness in sheep. It also seems that

the waking, as seen from the EEG, occurs before the behavioural sign of waking (e.g. lifting the head). However, the methodology used to evoke arousal can be criticised in a number of ways. The sound pressure (dB) at the start and the frequencies of the dog bark recording were unknown. Ames and Arehart (1972) showed that sheep had different responses to arousal stimuli of different frequencies: arousal thresholds lowering from a stimuli of 100Hz to a minimum threshold at 7000Hz; then the threshold rising again as frequency of the stimulus increased beyond 7000Hz. Therefore, the present experiment could be improved by measuring sound pressure and frequency spectrum at each 'bark level'. In addition, each sheep was played the stimulus several times, in order to obtain an artefact-free section of EEG trace to analyse. Sheep may have habituated to the sound and shown increased arousal thresholds as a consequence. However, Ames and Arehart (1972) found that sheep did acclimatise to auditory arousals but that the acclimatisation was only seen over 12 days. In the present study, each sheep was tested on a maximum of five separate occasions, so habituation was unlikely. Finally, as artefact-free traces were needed, arousals during REM sleep were excluded due to eye movement artefact. Therefore, only arousals during Non-REM sleep were used. This reduces the amount of information on the arousal thresholds during REM sleep. Rechtschaffen et al (1966) found that in humans, arousal thresholds were highest during REM sleep. Bauer et al (2001) found that foetal sheep were not awakened by a stimulus, and that the EEG changed very little during REM sleep. This is compared with a stimulus played during Non-REM sleep where there were changes across the whole of the power spectrum. There are obvious differences in the EOG and EMG traces between REM sleep and wakefulness (decreased rapid eye movements and increased muscle tone). However, as the present method of measuring the EEG in sheep can only ever pick up an 'average' of the brain activity that is found across the cortex, it may be difficult to distinguish between REM sleep and wakefulness before and after a stimulus using the EEG alone. More sophisticated imaging and/or the EEG taken from electrodes implanted within the brain structures are able to distinguish differences in spectral power and changes in activation site between REM sleep and wakefulness. For example, the pons is more active in REM sleep than in wakefulness, and activity in the prefrontal areas is lower in REM sleep than wakefulness (Halász et al. 2004).

2.6.2 validation study II: post-mortem study

2.6.2.1 Post-mortem material

An 80 x 30 mm section of bone from the cranium on one side of the midline was removed from an adult 'greyface' ewe *post-mortem*. This area approximately corresponded to the area which pairs of electrodes were placed to record the skin surface EEG in live sheep and partially covered the cerebral cortex. The bone had a depth of 8mm at the narrowest point and 20 mm at the widest. The bone was cleaned of tissue and blood using 100 % alcohol and dried around the edges. A 9 mm diameter silver chloride cup electrode, filled with electrode conductive gel, was attached using superglue to the inner surface of the cranium. A second electrode was attached in the same manner to the outer surface of the cranium in line with the inner electrode. The bone was placed on an electrically isolated board.

One electrode was connected to a signal generator. The second electrode completed the circuit via a switch box (see Figure 19). A signal of 10Volts peak to peak, at a frequency of 10Hz was applied across the bone and the resulting peak-to-peak voltage generated across the resistor R_s was measured on an oscilloscope.

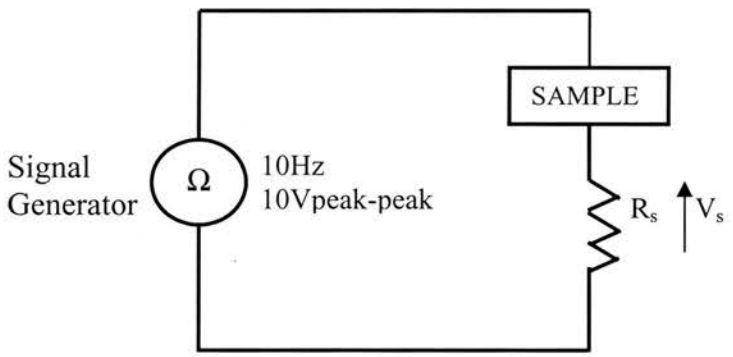


Figure 2.19 A schematic representation of the electrical circuit within which the electrical resistance across the bone was measured.

The following formula was used to calculate the resistance across the bone:

$$R_b = R_s (10 - V_s)/V_s$$

Where R_b = bone resistance (ohm), R_s = known resistor switch box setting ($10^2\Omega$ to 10^6 Ω) and V_s is the peak-to-peak reading from the oscilloscope (Volts).

2.6.2.2 Generation of an electrical signal

An adult, cull, *greyface* sheep was euthanased and the head of the sheep was removed and placed on an electrically isolated board. As shown in Figure 2.20, two 6mm holes were drilled through the cranium, 10 mm either side of the midline along a line between the caudal edges of each eye to expose the brain surface. One 6 mm silver chloride electrode were placed through each of the holes and rested on the surface of the dura. The electrode leads were brought out through rubber bungs and plugged into each hole, to reduce possible current flow from the brain to the skin surface. An area of scalp, 20 mm caudal to the holes drilled into the cranium, was prepared as described previously and a pair of 6 mm silver chloride electrodes was attached to the scalp with superglue. A ground electrode was attached to the back of the head in the same fashion.

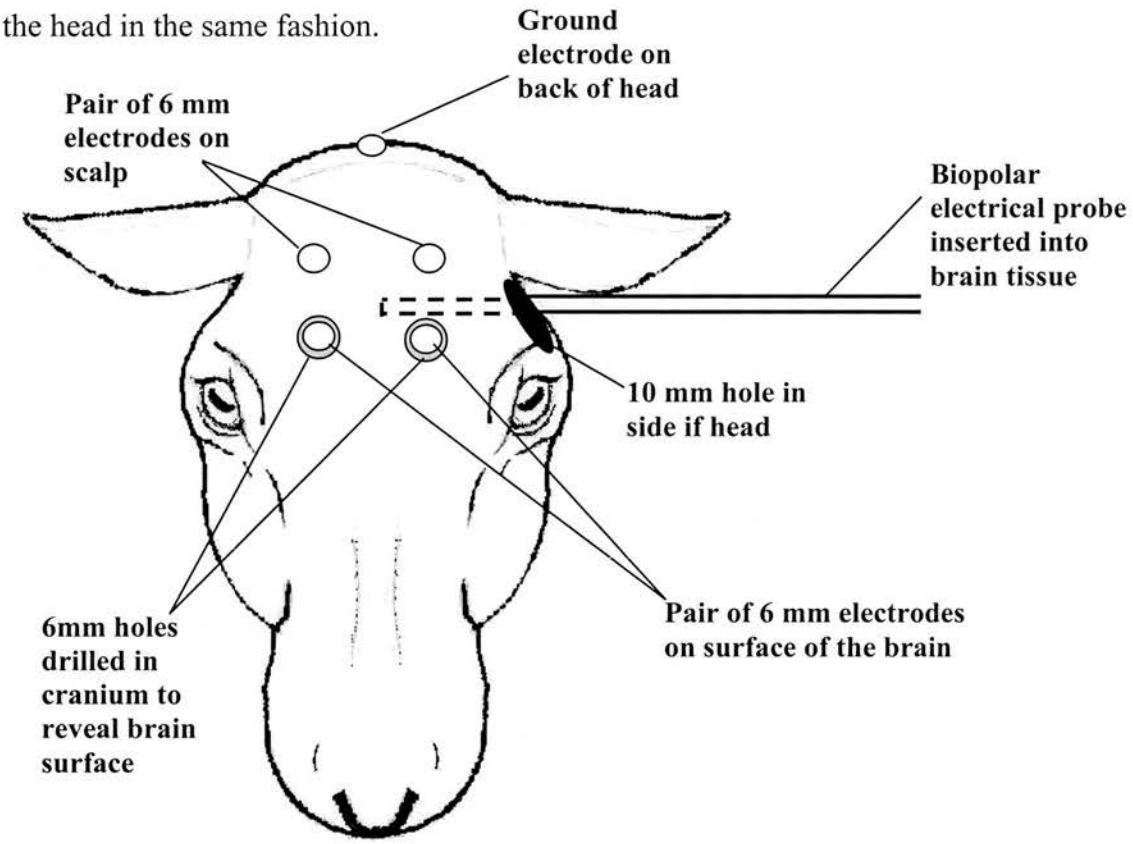


Figure 2.20 The approximate positions of the scalp and brain surface electrodes on the head of a sheep *post-mortem*. The dashed lines represent the probe position in the brain tissue. The rubber bungs used to isolate the electrodes and the current are not shown on the diagram.

A bipolar electrical probe was inserted into the brain through a hole drilled into the side of the head. The probe had a diameter of 8mm and, at the inner end, had

two aluminium electrode rings, each 4mm wide and separated by 10mm. The probe was inserted so that the electrode pair was as near to the centre of the brain tissue as possible. An electrical current was passed between the bipolar electrode pair into the brain tissue for 2 minutes at three frequencies (1 Hz, 10 Hz and 100 Hz) and at five amplitudes from 0.01mV to 10mV. Each frequency and amplitude combination was repeated twice.

2.6.2.3 Electrophysiological analysis

The frequency and amplitude of each trace (scalp surface and brain surface) was analysed using the FFT over 4 x 30s epochs for each combination of frequency and amplitude (2 min generation).

2.6.2.4 Statistics

Descriptive statistics were undertaken in Minitab 13.

2.6.2.5 Results -across the skull

The electrical resistance across the cranium was 0.98 Mohm over a bone thickness of 8 mm and 1.2 Mohm over a bone thickness of 12 mm.

2.6.2.6 Results -generation of signal

The electrodes on the surface of the brain recorded the same amplitude and frequency as the electrical signal generated in the brain. The skin surface electrodes recorded a mean reduction in amplitude of the electrical signal of 31 ± 4.5 % across the range of frequencies and at all of the amplitudes tested. There was a 2 % reduction in frequency spectrum recorded from a signal input of 1 Hz; no reduction of frequency spectrum recorded from an input of 10 Hz and a 7 % reduction of frequency spectrum recorded from an input of 100 Hz.

2.6.2.7 Discussion -across the skull

The cranium presents a high resistance barrier to electrical activity flowing from the brain to scalp electrodes. The consequence of high resistance of the bone is a reduction in amplitude of the electrical signal from the brain received by scalp as compared with electrodes placed on the surface of the brain. This may lead to the

scalp surface EEG being more affected by electrical muscular artefacts and movement artefacts from the stretching of the skin under the electrodes, than the EEG measured with electrodes on the surface of the brain (Haueisen et al. 1997). In deciding where to place electrodes on the scalp of a live sheep, post-mortem studies of the particular age and breed of sheep may be required: to ascertain where the thinnest part of the skull over the cortex may be, as resistance was lower across the thinner section of bone than the thicker section.

2.6.2.8 Discussion -generation of electrical signal

The lower frequency waves were less affected by noise than the higher frequency waves (less change in the frequency spectrum at low frequency input as compared with higher frequency input). As the frequency of the EEG during sleep includes a high percentage of low frequency waves, these results suggest that scalp electrodes would be suitable to record an EEG that would be useful to differentiate between sleep and wakefulness. Furthermore, the frequency of the EEG during the change from sleep to alert wakefulness has a high percentage of waves between 8 to 14 Hz; these results suggest that scalp electrodes would be less affected by noise at these frequencies and that they would correctly record the EEG. The largest reduction in frequency spectrum recorded by the scalp electrodes as compared with that generated in the brain occurred at 100 Hz. EEG traces with waves above 25 Hz are classified as awake and it is very likely that waves above 40 Hz recorded by scalp electrodes would originate from sources other than the brain. Therefore, a potential reduction in the recording of high frequencies by the scalp electrodes should not affect the recognition of sleep. However, as this experiment was carried out using an artificial technique with dead tissue, there were no electrical artefacts from muscles and movement and the electrical signal generated in the brain did not exactly mimic the signal produced in the live brain. In human studies, the reported decrease in amplitude between potentials recorded from brain electrodes and those recorded from scalp electrodes is between 2 and 58 times (Fisch, 1999). The reduction in amplitude depends on many factors such as, the depth of the source of electrical activity (Okada et al. 1999); the size of the area of cortex that is participating in electrical activity. The larger the area of participating cortex, and the higher the level of neural

synchrony, the smaller the difference recorded by scalp electrodes compared with brain electrodes (Abraham, 1958). This may be because a larger number of neurones are involved -which could increase amplitude- or that with the increase in area of the signal there is an increased likelihood of electrical current travelling through the structures of the cranium. The bipolar probe used in this experiment produced a non-uniform electrical signal that extended throughout the whole volume of the brain. However, the placement of the electrodes (bipolar and measurement) meant that the electrical field generated in the region of the measurement electrodes was sufficiently uniform for the purposes of these tests.

The measured attenuation of the signals between the brain and scalp electrodes was less than has been reported elsewhere. This may be due to the specific nature of the signals used, which was quite different from that generated by a live brain. It may also be to the placement of the bipolar probe at the centre of the brain. In a study with a model of a human head, Haueisen et al (1997) found that electrical surface potentials were not only sensitive to the changes in resistivity of tissues at the source, but also to resistivity at the electrode sites. The attenuation of brain electrical activity caused by tissues and bone is difficult to predict because there are many individual differences in tissue depth and skull thickness (Haueisen et al. 1997). Further validation of the methodology by making EEG recordings from implanted brain electrodes compared with scalp electrodes was considered necessary.

2.6.3 validation study III: anaesthesia

This study was carried out under a Home Office Licence after gaining approval from the University of Edinburgh and Royal (Dick) School of Veterinary Studies ethical committees. The anaesthetic regime was controlled by a qualified veterinary anaesthetist and the surgery was carried out by a human-brain surgeon. This study was funded by a SHEFC grant and was not funded or supported by UFAW.

2.6.3.1 Animals

Six healthy, adult, Dorset, cull ewes were fasted for 12 h prior to anaesthesia.

2.6.3.2 Anaesthesia regimen

Anaesthesia was introduced with intra-venous Edominate (0.05mg/kg) and Medazolam (0.05mg/kg). The trachea was entubated with an endo-tracheal tube. Anaesthesia was maintained with halothane delivered from a calibrated vaporiser (Fluotec Mk III Cyprane, Keighley, UK) and carried in an oxygen:nitrous oxide mixture at a 1:2 ratio. The lungs were mechanically ventilated (Manly Pulmovent MPP, Manly, South Africa) so that entidal carbon dioxide concentrations were maintained at 40mm/Hg.

After implantation of electrodes (see below) the sheep was maintained at a constant (delivered) concentration of 1.5 % halothane for 15 minutes, after which simultaneous recordings were made from the implanted and skin electrodes for 5 minutes. This procedure was repeated after the halothane level had been increased first to 2.5 % and maintained for 15 minutes, and then to 3.5 % and maintained for 15 minutes. After the final 5 minute recording at 3.5 % halothane the sheep were euthanased with Pentabarbitol. Simultaneous EEG recordings from the brain and the scalp were taken during euthanasia.

2.6.3.3 Insertion of electrodes

Two 7.5 mm holes were drilled through the cranium 10 mm apart, on one side of the midline until the dura was visible (Figure 2.21). Two silver/ silver-chloride ball electrodes were placed in each hole and positioned on the surface of the dura, ensuring that each electrode did not touch the other. The holes were then filled with dental cement. On the opposite side of the midline, two pairs of electrodes were attached to the intact, cleaned, scalp. Electrodes were position to record the EOG and ECG as described in the section 2.3.2.4. All electrodes were connected to the Embla to enable data to be stored and subsequently analysed using the Somnologica software.

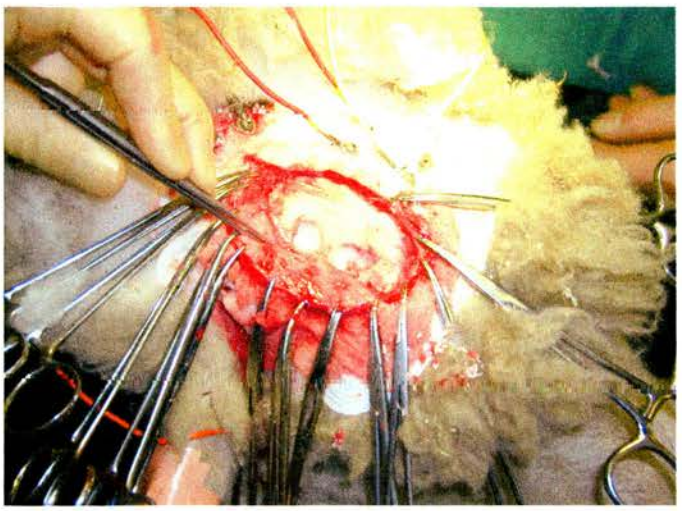


Figure 2.21 the placement of electrodes during general anaesthesia. On one side of the midline, two pairs of skin surface electrodes were attached. Also visible, the two holes drilled into the cranium after the removal of outer tissue, showing the surface of the dura. Each hole received two ball electrodes that were placed on the dura surface.

2.6.3.4 Electrophysiological recordings

An online electrophysiological recording was made, starting as soon as the electrodes were fixed in position. This meant that the traces could be viewed in real time while the anaesthesia regime was undertaken. After the periods of anaesthesia stability (each 15 mins) the time on the Somnologica PC was noted and 5 mins of stable recording were carried out. After the final 5 min recording (at 3.5% halothane), the sheep was euthanased, during which time the electrophysiological recording continued. After death, the recording was stopped and saved appropriately.

2.6.3.5 Electrophysiological analysis

Spectral analysis of five 30-s epochs of the EEG recorded by each electrode (four brain and two scalp) was undertaken for each of the three anaesthetic levels (one epoch per minute of the five minute stable anaesthetic period). Mean frequencies and amplitudes from the five 30-s epochs for each electrode during an anaesthetic concentration were calculated. Then the mean frequencies and amplitudes from the scalp and from the brain electrodes for each anaesthetic concentration were calculated for each sheep.

2.6.3.6 Statistics

Recordings made from scalp and brain electrodes were compared within each anaesthetic concentration and the changes that occurred between anaesthetic concentrations were then compared. The data were not normally distributed. The non-parametric comparisons were carried out in Minitab 13.

2.6.3.7 Results -anaesthesia

The amplitude of the EEG recordings from the brain electrodes was considerably higher than that recorded from the scalp as can be seen in Figure 2.22; the brain electrodes were recording an amplitude measured in mV, whereas the scalp electrodes were recording amplitudes of μV. The frequency spectrum of the EEG recorded by both scalp and brain electrodes was within the same order of magnitude. On average the scalp electrodes recorded 4-5 % more delta (waves of 0.1 to 4 Hz) waves than the brain electrodes. The percentage of delta waves recorded by both the scalp and brain EEG tended to increase with increasing halothane concentration, but this was not significant due to high variation and low numbers of animals.

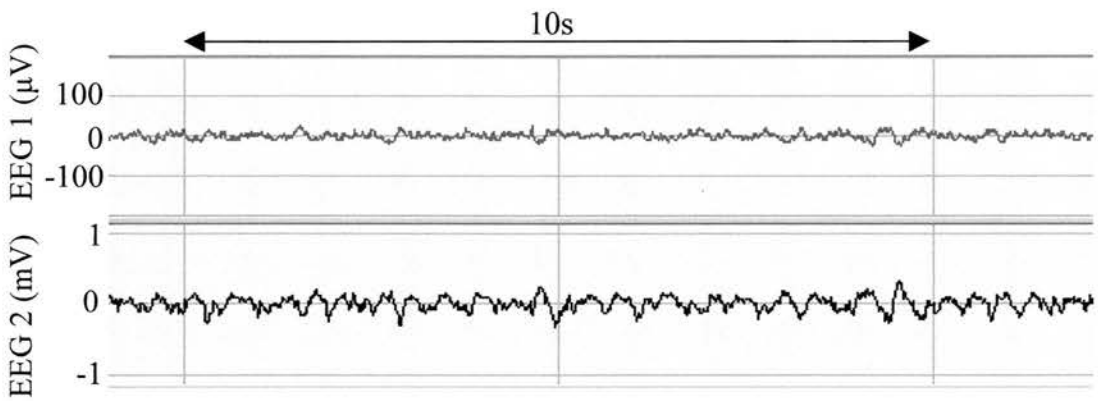


Figure 2.22 The EEG (top trace) from the skin surface electrodes and the EEG (lower trace) from the electrodes placed on the surface of the dura from sheep C. The anaesthesia level was 2.5% Halothane. Note the difference in amplitude scale.

2.6.3.8 Discussion -anaesthesia

The results in this experiment were highly variable. Some of this high variation may be accounted for by a large difference in the thickness of the cranium across the six sheep (range 3 mm to 12 mm). This would lead to a large difference in

electrical resistance provided by the skulls of the six animals. In addition, the results for all six sheep were not usable as during one sheep there were technical problems with the equipment and one sheep died under anaesthetic.

These results suggest that it is possible to record the EEG using skin surface electrodes in sheep. However, this recording method would give less specific, more generalised data than the EEG recorded by electrodes implanted on the surface of the brain and would be more prone to error in interpretation. It may only be possible to record the EEG from scalp electrodes in sheep when there are few muscle artefacts, e.g. during sleep. In addition, the sleep EEG may have advantages over the waking EEG in ease of use of scalp electrodes. Cooper et al. (1965) found that during Non-REM sleep recordings of the EEG using scalp electrodes (generally a period of synchronised activity which occurs over a wide area of the cortex, (Fisch, 1999)) suffered less attenuation in amplitude compared with recordings of EEG using implanted electrodes than that occurring during wakefulness.

2.7 General Conclusions

The preliminary investigation and validation experiments have shown that it is possible to record sleep in sheep using non-invasive electrophysiological techniques. The technique used brings problems: the EEG was difficult to record when muscle and movement artefact was present, meaning that when the sheep was standing/moving and when it was lying down with the head up, for the majority of this time the EEG was obscured. Therefore, some of the information regarding the boundaries between wakefulness and sleep will be lost using this method. The 'drowsy' state investigated in detail in ruminants by Ruckebusch (1972) may be difficult to determine using non-invasive techniques. However, the results from the preliminary study are very similar to those found using invasive EEG techniques in sheep (see table 2.4). Consequently, the method used for these investigations was successful in determining differences between wakefulness, Non-REM sleep and REM sleep. The basic information gained on sleep in sheep using the non-invasive electrophysiological techniques allows us to understand a sheep's circadian rhythms in greater detail than using behavioural observation alone.

The auditory arousal validation experiments lend further weight to this conclusion, as they showed a marked change in spectral power before and after the playing of the stimuli. Nevertheless, caution should be used if trying to determine more information (such as sleep stages I-IV) from the EEG during sleep using these methods. Although post-mortem and anaesthetic studies have shown that electrical activity is attenuated by the bone and tissues between the brain surface and the scalp, which can cause differences, in amplitude, and in average frequency power, the effects were unlikely to have affected the ability of the methodology to differentiate between sleep and wakefulness.

Chapter 3. Effects of movement from pasture to a novel indoor environment on sleep in sheep

3.1 Abstract

In humans, sleep can be disturbed by novelty and aversive experiences. This experiment investigated whether the quantity, quality and distribution of sleep in sheep was affected by the potentially aversive experience of movement from pasture to novel inside penning. Simultaneous, non-invasive 24-h EEG, EOG, EMG and ECG recordings were made from 10 pairs of control (remained at pasture) and treatment (moved to inside penning) ewes, during Period 1) while both were at pasture, Period 2) immediately after treatment sheep were moved to inside penning and Period 3) 7 days post-movement. Light/dark periods and the availability of water, hay and concentrate feed were similar at both sites.

The data were analysed using repeated measures mixed models analysis of covariance, with the baseline recording at pasture as the covariate. Bout frequency data and heart-rate data were analysed using G.L.M.M. procedure comparing like time periods. During the first 24 hours post-movement, there was no treatment effect on the percentage of time sheep spent asleep (8.8 ± 0.9 %), but the percentage time spent by treatment sheep in rapid eye movement (REM) sleep (2.8 ± 0.2 %) was greater than controls (1.8 ± 0.2) (the difference between control and treatment sheep in recording Period 2 was different from that in recording Period 1, $P<0.05$). There was no significant difference in the duration of REM sleep bouts, but there was a tendency for more REM sleep bouts in treatment sheep (10 ± 1.9) than controls (7 ± 2.1). The latency to sleep was shorter in treatment sheep (138 ± 25 minutes) than controls (280 ± 35 minutes) compared with the difference between control and treatment sheep in the baseline recording (Period 1)($P<0.05$).

There were no significant treatment effects on the percentage of total time spent, bout lengths or number of bouts of non-rapid eye movement (Non-REM) sleep. However, the percentage of delta waves during Non-REM sleep was higher in treatment sheep (49 ± 2.1 %) than controls (37 ± 4.3 %) in the first 24 hours post-movement.

Other than a change in the distribution of sleep in the penned sheep, where there were more sleep bouts during the morning and afternoon/evening, compared

with sheep at pasture (where most sleep was in the middle of the night), no significant treatment effects on sleep during the first 24-h post-movement were still present 7 days post-movement. There was only one treatment effect on sleep that occurred 7 days post-movement and was not present in the first 24 hours post-movement: the number of eye movements in 10 second epochs of REM sleep ('eye-movement density') were higher in treatment sheep (79 ± 9) than controls (54 ± 13).

Treatment sheep lay down more than controls (compared with the difference in the baseline recording, $P<0.01$) and spent less time eating (compared with the difference in the baseline recording, $P<0.01$), but there was no treatment effect on the duration, bout length, or number of bouts of rumination. Behaviour was probably influenced by factors, such as a change in diet and a reduction in size and complexity of the environment, however, there were subtle treatment effects on sleep that were consistent with a psychological effect followed by adaptation within 7 days.

3.2 Introduction

3.2.1 Aims

To determine the quantity, type and distribution of sleep in sheep at pasture.

To investigate the effect of moving sheep from familiar pasture to a novel inside environment on the distribution of sleep and wakefulness and the quantity and type of sleep 24-h and 7d post movement.

To test the hypothesis that potentially aversive waking experiences could affect the subsequent sleep and rest behaviour of sheep in both the short-term and the long-term.

3.2.2 Movement of sheep from pasture to penning

Sheep are one of the few species in modern agriculture that are usually farmed in an extensive manner (as low as 1-2 ewes/hectare, Waterhouse, 1996) allowing them to exhibit most natural behaviours and interact within a social group (Deag, 1996). However, there are times when sheep are moved to novel inside penning, for example lambing, at market, in lairage prior to slaughter and for research. In addition, sheep are often winter-housed for feeding and protection in very cold climates (Berge, 1997). The movement to inside penning may not only

cause sheep stress from the novelty of the change in environment, but also results in many other alterations to the environment, such as a reduction in the size and complexity of the physical space, changes in social interactions and changes in diet and these factors may alter and perhaps limit the behaviour of sheep. For example, sheep at pasture graze for approximately 8-h a day but, in housed conditions, the time budget of the sheep substantially changes, they may have access to hay, or be offered concentrated feed, and this reduces the time needed to forage and consume nutrients to 4-5 h a day (for hay)(Lynch et al. 1992). In addition, for sheep that have had little contact with humans, movement to inside penning can increase contact and this may constitute an additional stressor (Goddard et al. 1998).

3.2.3 Sleep and rest behaviour of sheep in the wild and at pasture

The sleep and resting behaviour of any animal is related to its behavioural ecology. Sheep, like all ungulates are prey animals and must look out for predators, creating a trade-off in the amount of time they can safely spend sleeping as sleep requires the reduction in consciousness (Elgar, 1988). Prey animals such as sheep, tend to live in social groups where at least one individual can remain vigilant while others sleep (Frid, 1997). In addition, large prey animals tend to sleep less than similar sized predators (Allison and Cicchetti, 1976).

Sheep kept on hills (or wild/semi-wild sheep in mountainous country) seek out high ground for sleeping and resting at night (Bowns, 1970), usually free from trees and shrubs, giving a good view of potential predators. Mountain sheep tend to rest as a group, some animals ruminating, others lying alert and others lying with their head down on the ground (Geist, 1971). During daylight, mountain sheep spend approximately 26 % of the time resting, including lying ruminating (Bowns, 1970), some (unquantified) of this time is spent lying with the head resting on the ground and therefore the sheep may have been sleeping. Bueno and Ruckebusch (1979) found that sheep at pasture rested for approximately 40 % of 24 hours mainly either at night or between the two major grazing periods of the day (that occur at sunrise and again towards sunset).

All studies of wild sheep (e.g. Geist, 1971; Langbein et al. 1996), hill sheep (e.g. Arnold, 1984) and sheep on lowland pasture (e.g. Bueno and Ruckebusch,

1979; Tobler et al. 1991) note the same major pattern in behaviour. Sheep graze at sunrise, rest and ruminate during the late morning interspersed with grazing, have a major grazing period in the afternoon and evening and are much more inactive after sunset (although with some grazing, Langbein et al. 1996). In hot climates, sheep may spend more of the day inactive (standing idle) and more of the night active (grazing) than in cooler climates (Squires, 1971). Tobler et al (1991) found that a drop in activity of sheep often occurred between 1200-1500h on warm days.

Very little research has been published on sleep in sheep at pasture. Tobler et al. (1991) measured activity levels in sheep and could indirectly record REM sleep episodes by the complete cessation of measurable movement (corroborated by video evidence). They found that sheep at pasture only went into REM sleep of bout lengths of 2-6 minutes and only at night.

3.2.4 Circadian rhythms and sleep

As can be seen from the studies on sheep activity and resting behaviours above, the behaviour and physiology of mammals is affected by day-length (photoperiod), and as day-length alters over the year, there are seasonal changes in behaviour (Geist, 1971) and physiology (Sumová et al. 2002). Light appears to act as an external zeitgeber ('time giver') to the animal. Day-length influences melatonin production in the pineal gland and the melatonin cycle acts as an internal zeitgeber to the body, a hormone that tells the body of a time of environmental darkness (Turek and Gillette, 2004). Melatonin secretion is thought to have some phase maintaining regulatory function in the circadian rhythm of sleep. In humans, there is evidence to suggest that giving participants exogenous melatonin can increase sleepiness and lead to a lower latency to sleep after administration (Cajochen et al. 2003). Although, endogenous melatonin is not necessary for sleep in humans, as patients that have had their pineal gland removed can experience a normal sleep/wake cycle (Turek and Gillette, 2004).

In sheep, activity and feeding at pasture seem to be entrained by photoperiod (Mohr and Krzywanek, 1995). Rumination does not seem to be entrained by day-length, but is more affected by diet type (Dutilleul et al. 2000). Although melatonin has been shown to be important in seasonal changes of behaviour, it is not clear to

what extent, melatonin is involved in the circadian rhythm of sleep in sheep. (Lincoln et al. 2003) (For a more detailed review of circadian rhythms and sleep, please see chapter 1 of this thesis.)

3.2.5 Sleep and rest behaviour of sheep when housed

There have been many studies investigating the behavioural and physiological effects of different housing on sheep, however, most of this research focuses on changes in active behaviours between housing types and alterations in physiology, with less attention paid to sleep and rest in different housing types.

Tobler et al (1991), recorded activity of ewes with an actometer in three different housing conditions, group inside penning, groups of ewes at pasture during the day and housed at night, and an extensive hill paddock. The authors noted that in all conditions, there was a reduction in activity (defined as counts on the actometer) during the night, however the day/night difference was lowest in the penned condition. The lowest activity values (activity count of 97 per 7.5-min episode in the pens compared with 120 per 7.5 min episode in the field) and the highest rest values (0.98 rest episodes per hour in the pens compared with 0.35 rest episodes in the field) were in the penned animals. The onset of activity in the penned conditions was abrupt and induced by human activity, rather than by photoperiod. In addition, rest onset, in the penned conditions, was determined by 'lights-off', which coincided with a reduction in human activity. The authors suggest that penned sheep had their rest times determined by human activity, sheep in pens further from human activity lay down more than sheep nearer to human activity (Tobler et al. 1991). REM sleep, measured indirectly by cessation of movement, was recorded only at night in all three conditions, for periods of 2 to 6 minutes. With this type of recording method some sleep bouts may be missed due to movements during sleep and between stages of sleep and therefore more detailed identification of sleep/wake distribution using electrophysiological methods would be beneficial.

Das (2001) recorded inactive behaviours (standing idle 'loafing', resting recumbent without rumination and sleeping) of 100 sheep that were maintained in a shed with an open paddock (without grass) with access to *ad-libitum* feed (lucerne/maize) and water. Adult sheep were inactive for approximately 10.5 hours

during 24-h, although there were differences between ewes and rams and pregnant and non-pregnant ewes. Time spent resting was higher between 0900-1200h (daylight) and 0000-0300h (night) than at other times. The onset of resting was abrupt and occurred after 'lights-off'. The author identified sleep by posture, REM sleep was recognised by lateral lying (seen in sheep in hot climates) and eye-movements, Non-REM sleep by lying with the head resting on the bedding. Sheep slept for approximately 56 minutes a day. Das (2001) did not find any significant diurnal variation in sleep. Seventy-one percent of sleeping bouts were less than 16 minutes, 10 percent were more than 30 minutes.

Casamassima et al (2001), compared group-housed ewes with ewes kept at pasture. Sheep were given similar foods in both conditions as the pasture contained little grass. The authors recorded that time spent walking was higher in the sheep at pasture and that idling (standing inactive) was higher in the penned sheep. There was no difference between groups in the time spent lying. The authors suggest that the animals in the field walked more as they had more space and a more complex environment to explore, and that conversely, indoor ewes may idle more due to a reduction in the range of stimuli in the environment.

3.2.6 Effects of a novel environment on sheep

3.2.6.1 The novel environment as a stressor

Exposure to a novel environment is a common 'natural stressor' in animal experiments (especially rodents) to determine anxiety level (e.g. McQuade and Sanford, 2001; Thiel et al. 1999; Steiger et al. 2000). In order to survive in the wild, a prey animal must continually screen their environment for novel and biologically relevant stimuli such as a predator. Rats and mice have a natural aversion to open spaces, so an open novel environment is used to induce anxiety (Tang et al. 2004). Rodents bred for different anxiety levels show different behaviour when exposed to the novel environment (Thiel et al. 1999). McQuade and Sanford (2001) found that there was an increase in concentration of noradrenaline in the frontal cortex and the hypothalamus of rats when put into a novel environment that was indicative of a stress response. Abbott et al (1986) found that putting rats into a novel environment stopped them reacting to an exogenous formalin pain. The authors suggest that the

stress of the unfamiliar environment attenuates the behavioural response to the pain, that it is more important for the rats to escape the unfamiliar situation than to respond to a painful injury (Abbott et al. 1986).

In order to investigate the effect that the suprachiasmatic nucleus and its relationship with the circadian rhythm of mammals has on the secretion of ACTH in rats, Buijs et al (1997) subjected intact and suprachiasmatic-nuclei-lesioned animals to a novel environment at different points during the light/dark cycle. They found that after exposure to the novel environment plasma corticosterone increased in all animals to a peak at 30 minutes. Intact animals returned to baseline activity levels within 15 minutes, whereas lesioned animals took longer. In intact animals, ACTH secretion patterns depended on the point in the circadian cycle that the animals were introduced to the novel environment; in the dark, there was a decrease in ACTH followed by an increase in corticosterone; in the light, there was a rapid increase in ACTH. In lesioned animals, a novel environment results in a rapid increase in corticosterone and ACTH at any point in the circadian cycle (Buijs et al. 1997).

3.2.6.2 Responses of sheep to stressors such as a novel environment

The exposure to a novel environment or an open field test is often used as a stressor to produce a response from the animal indicative of the negative emotions of fear and anxiety. Prey animals, such as sheep show a variety of anti-predator fearful-responses, which may be the main effect on the emotional state of a sheep experiencing a novel environment (Hansen et al. 2001). It is the novelty of the environment that provides the threat to the animal as potential locations of danger are not known and animals would need more time to seek escape routes in case of threat (Welp et al. 2004). Negative emotional states, such as feelings of fear can be brought about by a sheep's perception of actual (fear) or possible (anxiety) threats (Boissy, 1997). When moving sheep to a novel environment, the transport to the environment may cause a state of fear (an actual threat as far as the sheep is concerned) and the novelty of the environment may cause a state of anxiety (a possible/unknown threat). Novelty is a threat on a cognitive level, because recognition of any stimulus (an environment, or an object) as novel requires a cognitive comparison between the present and the past. The fear reactions on

exposure to an environment decrease with repeated or prolonged exposure as it becomes familiar (Boissy, 1995).

Anxiety related behaviours of sheep exposed to a novel environment include escape behaviour (attempting to jump/climb out of the environment) and immobility; anxiety also reduces feeding/ruminating behaviour and exploratoration (Vandenheede et al. 1998; Romeyer and Bouissou, 1992). There are individual differences in the strength of fear behaviours shown by sheep to novelty, but sheep that showed more fear reactions to a novel object also showed less exploratory behaviours when the novel object was present (Vandenheede et al. 1998). Sheep exposed to a novel environment spent less time eating and consumed less feed than when exposed to a familiar environment (Romeyer and Bouissou, 1992). The authors showed that there were breed and early experience differences in how sheep reacted to a novel environment, however this experiment was flawed as the sheep were tested individually so isolation stress is a confounding variable. Boissy and Bouissou (1995) exposed cattle to a novel environment and observed fear behaviour including immobility and standing with head in an upright position (possibly a position of increased vigilance), again the results were confounded by isolation stress. Animals would be expected to decrease their vigilance when in a group as compared with isolation, so animals should show less fear behaviour in a group than on their own (Beauchamp, 2001). Veissier and Le Neindre (1992), carried out a novel environment experiment with isolated or groups of four cattle and they found that isolated animals and grouped animals behaved in a different manner to the same stimulus. Groups of cattle showed more immobility and less exploration (more fear?), whereas isolated individuals explored the novel environment more (less fear?). The authors suggest that this apparent reversal of the isolation stress hypothesis may actually be the isolated animals attempting to escape to return to their herd (Veissier and Le Neindre, 1992).

Alterations in levels of vigilance may be a good way of determining fear in prey animals. Welp et al (2004), found a decrease in vigilance in three minute tests, on repeated exposure of dairy cattle to a novel environment. Cattle increased vigilance levels once again when, after habituation to the environment, a person or a dog was introduced to the environment. Deep sleep (slow wave Non-REM sleep)

and REM sleep require a reduction in the level of consciousness of an animal, and therefore require the animal to relinquish vigilance. It would therefore be interesting to compare the profile of vigilance found in a series of 3 minute tests (as in the Welp et al. 2004 study) with the profile of wakefulness, sleep and resting behaviour during the first 24-h of exposure to a novel environment (e.g. the present study).

3.2.6.3 Behavioural and physiological effects of moving sheep to novel environments

Many authors have recorded a behavioural and/or physiological adaptation over time to novel housing in sheep. Done-Currie et al (1984) moved sheep from pasture to novel inside penning. A number of sheep were group housed, others housed individually, and these were compared with sheep that had been either group, or individually housed for 6 months prior to the start of the experiment. Behaviour was recorded twice weekly, once on a weekday and once on a weekend. Sheep that were moved to novel individual penning spent less time eating (and consumed less foodstuffs –lucerne and oats) over the first week than sheep that had been previously housed. All sheep in a novel environment spent more time ruminating during the 8 hours of observations than the sheep in a familiar environment. The sheep which had been moved to the novel housing, showed more lying behaviour and other non-alert behaviour than sheep familiar with the environment, except in the presence of environmental stimuli (stockmen) where the sheep in the novel environment reacted more than the sheep in the familiar environment. The authors suggested that the reduced activity was a period of withdrawal that reduced over time due to adaptation to the penning environment (the active behaviours increased after three weeks of confinement in the sheep moved to a novel environment) (Done-Currie et al. 1984). Although interesting, this experiment could have been improved in a number of ways, including observing the sheep for whole 24h periods (rather than 8) as this would give a clearer idea of the activity/inactivity relationship following movement of sheep to a novel environment.

Fordham, et al (1991), also gave evidence to suggest that there may be 'withdrawal' behaviour period when sheep are adapting to a novel environment. In this study, six sheep were taken from pasture and put into inside crates (commonly used for metabolic studies of sheep) and provided with *ad-libitum* water and 1kg of

concentrated food per day. The crates were large enough for the sheep to stand up and lie down, but not large enough for them to turn around. The sheep were in visual contact with others. Behaviour was sampled for two 1-h periods per day for 9 weeks. The percentage of time spent resting (lying recumbent, including rumination) increased from approximately 72 % on day one to approximately 85 % by weeks 2 and 3 before reducing (to around 65 % by week 9). Whether the withdrawal period (increase in resting overall) was an accurate conclusion and not just a change in when resting occurred, is difficult to tell, as only 2 hours were sampled from each day. Conversely, active behaviours increased after week 3, with the percentage of abnormal active behaviours increasing every week from week 3. The authors speculate that the withdrawal period was an indicator of a psychological adaptation to the stress of the novel environment, whereas the increase in active/abnormal behaviour after week 3 could be seen as a way for the sheep to alleviate boredom (Fordham et al. 1991).

The above study also recorded physiological variables, blood was sampled via a catheter every hour for the first 24h post-movement, and then on day 5, 30 and 60 (Fordham et al. 1991). The blood was assayed for plasma cortisol and β-endorphin concentrations. The average plasma cortisol concentration was unchanged between day one and day 30 at approximately 25μg/l and had dropped to 5μg/l in males and 11μg/l in females by day 60. The cortisol concentrations on days 1 to 30 were similar to those found in sheep after handling. As the concentrations had dropped to those found in undisturbed sheep by day 60, the authors suggest that movement to a novel environment is a mild stressor and that it took sheep two months to acclimatise to the novel environment (Fordham et al. 1991).

Bowers et al (1993) recorded physiological and behavioural parameters from 8 week-old lambs that had been kept on pasture and were moved to metabolic crates. Blood samples were taken (via jugular catheter) 7 days prior to movement, and 2 and 9 days after movement. Plasma cortisol concentrations and the cortisol response to exogenous ACTH given via catheter were recorded, as were plasma concentrations of tri-iodothyronine and thyroxine. The adrenal response to ACTH were significantly highest in the animals that had been confined for 2 days, then the animals confined for 9 days and lowest in the baseline measures (Bowers, et al,

1993). Thyroxine concentrations were higher in confined animals than the baseline concentrations. Both physiological changes may be a response to the confinement stressor. The authors suggest that the increase in thyroid function may be as a result of chronic stress as previous studies (e.g. Falconer and Marchant, 1970) had shown that thyroid function increases under chronic environmental conditions such as cold stressors. In addition, an open field test was used to assess the motivation of the lambs to move. The test was carried out before movement to metabolic crates and on day 9 post movement. Lambs were more active after movement to confinement than before movement (Bowers et al. 1993). Comparable results were found in sheep that were brought from pasture to housing and tested in an open field at week 0, after 1 week of housing, after 4 weeks or after 12. Sheep tested in an open field after they had been housed, moved more than those that had been at pasture (Goddard et al. 1998).

In a similar experiment, Kapp et al (1997) recorded physiological and behavioural parameters from 8-month old lambs that had been kept at pasture, previously handled, tamed and 4 months prior to the start of the experiment had spent 5 days in metabolic crates. The sheep were put into four treatment groups: metabolic crates, group housed in a pen on restricted diet, group housed with *ad-lib* food and out at pasture. Plasma cortisol concentrations remained at baseline levels for all sheep except those in the metabolic crates, where concentrations were higher on the first two sampling days (2 and 4 post-movement) and returned to baseline by day 6. This suggests that the lambs were responding to the stress of confinement, with adaptation after six days. Lambs confined to the metabolic crates were more active in an open field test than those from the group pen who were in turn more active than the pasture lambs. The authors suggest that the lambs had an increased motivation to move after close confinement compared with those that had more space. Perhaps the absence of a plasma cortisol response to movement from pasture to housing in this study as compared with that found by Fordham et al (1991) was because the sheep were very tame and had been previously confined to crates so that the environment was no longer novel (Kapp et al. 1997).

To investigate the effect of changes in the environment on the 24 h sleep profile of farm animals, Ruckebusch (1975b), used electrodes implanted on the brain

surface of sheep, cattle and horses to measure the EEG over a maximum of three months Three horses were moved from familiar inside stalls to unfamiliar outside tethering. In the stall they lay down at night and REM sleep was recorded during this time. On the first night outside none of the horses lay down, recumbency was reduced by 40% over the subsequent three nights and remained low for a month and total sleep time was similarly reduced. REM sleep was more affected than Non-REM as horses can gain light Non-REM sleep while standing (Ruckebusch, 1975).

Three cows were moved from pasture to inside penning for a month and offered grass, then their diet was changed to hay and concentrate for a month and finally they were prevented from lying for 14h daily for one month. The amount of REM and Non-REM sleep increased after the first day of penning and was constant after 7 days. After 1 week of penning, cattle started to exhibit REM sleep during the day. When recumbency was prevented cows were able to perform light Non-REM sleep when standing. REM sleep was not observed when the animal was prevented from lying down. This meant that, over time more REM was seen in the daylight when recumbency was allowed. The alteration in the circadian rhythm took 5 to 6 days to stabilize (Ruckebusch, 1975).

Three sheep were housed in metabolic crates and offered hay for one month and then offered concentrated food for 1 month, which had the effect of reducing the feeding and rumination time by 70%. Sheep offered hay slept 'randomly' mostly at night. After the change in diet, rumination was greatly reduced, but there were no major changes in the total duration of REM or Non-REM sleep. The number of Non-REM bouts increased after the diet change. When animals reverted to hay the sleep profile returned to normal within 48h. Ruckebusch (1975) also noted that at the weekend, when human activity was much reduced, episodes of Non-REM and REM were recorded more frequently at midday in both cattle and sheep, and the total sleep time was increased by about 10%.

Lambs have also been shown to be more neophobic of food when in a novel environment and more likely to consume familiar, but aversive food in a novel environment than a familiar one (Burritt and Provenza, 1997). Scott et al (1996), suggests that social facilitation can override food preferences in a novel environment.

3.2.6.4 Effects of movement to a novel environment in humans: the 'first night effect'.

In humans, sleep can be disturbed by aversive experiences, such as painful clinical conditions and changing work shifts (e.g. Raymond et al. 2001; Åkerstedt et al. 2002; Hohagen et al. 1994 respectively, for greater detail please see chapter 1 of this thesis). Movement to a novel environment (specifically a sleep laboratory) can affect sleep in humans. Agnew et al. (1966), were the first to describe what was coined as the 'first night effect' in humans. They showed that on the first night in a sleep laboratory, healthy human subjects exhibited more periods of wakefulness, less Non-REM sleep in the stages II to IV and less REM sleep compared with subsequent nights. There was a longer latency to enter deep sleep (stage IV) and REM sleep. In addition, the sleep exhibited was more changeable between the stages than on subsequent nights (Agnew et al. 1966).

One 'effect' that persisted into the second night and was no longer present during night four, was the mean number of changes between stages. The amount of REM was constant by the second night (Agnew et al. 1966). This phenomenon, although being an irritant for the sleep medicine clinic (as recordings have to be disregarded for the first night), gives us an insight into the psychological adaptation over time to a stressful environment and its effects on sleep.

Le Bon et al (2001) suggested that humans may take longer to habituate to a novel laboratory environment than just one night. The authors also argue that the novelty in such experiments is multi-faceted, in these studies the equipment as well as the environment is novel and there are possible psychological consequences of being watched (Le Bon et al. 2001). Their study of the 'first night effect' took place in their subject's home environment (thus removing the environment stress). These results suggest that some measures of REM (particularly latency) were not stable until the third night of recording. In addition, Le Bon et al (2001) showed a greater intensity of Non-REM sleep (a higher proportion of slow waves seen in Non-REM) on the second night as compared with both the first night and nights three to four. The authors suggest that this phenomenon occurs because of sleep rebound from the disturbed sleep on the first night, similar to that shown in partial sleep deprivation studies (e.g. Tobler et al. 1983). This was due to an almost two-fold increase in

wakefulness seen on the first night. Furthermore, this experiment showed that it is not only the novel environment that causes the 'first night effect', but also the novel situation of electrophysiological recordings. However, Browman and Cartwright (1980) carried out experiments with electrophysiological recordings of humans in the home and the sleep laboratory and found that the 'first night effect' was more pronounced in the novel sleep laboratory than the familiar home environment. Similarly, Kingshott and Douglas (2000) showed that there was no difference in the total sleep time in participants recorded in their own home or at a sleep laboratory, however, participants showed a greater percentage of REM sleep at home than in the laboratory. Lorenzo and Barbanoj (2002), show that the 'first night effect' is still present even when sleep recordings are taken in a novel environment of maximal comfort where participants have been habituated to the recording equipment, and that here, REM sleep parameters are the most affected on the first night.

Most researchers in this area agree that multiple nights of recordings need to be carried out in order to ensure stability (e.g. Scholle et al. 2003; Lorenzo and Barbanoj, 2002; Le Bon et al. 2001).

3.2.6.5 Effect of a novel environment on sleep in non-human animals.

There has been little research into the 'first night effect' in non-human animals. As most experimental animals are confined to the experimental laboratory/home cage when sleep recordings take place, the first 'night' of recordings is often used as the environment is not novel to the animal (e.g. Ambrosini et al. 1994). Animals with electrodes implanted onto the surface of their brain can habituate to the recording equipment before recording takes place.

Irmiš (1971) moved rats to a novel experimental environment and recorded the EEG for 7 hours during the rats' normal sleep period (during the daylight, 'night') from the first 24h and the second 24h post-movement. The majority of rats had reduced amounts of total sleep and showed a higher number of changes between stages of sleep and wakefulness on 'night' one as compared with 'night' two. However, some rats fell asleep sooner and slept more in the novel environment during 'night' one as compared with 'night' two (Irmiš, 1971), a phenomenon not reported in the human literature.

During their experiments on chronically implanted cats, Wallach et al. (1976) often had to ignore the first night of recordings due to differences in sleep on subsequent nights. These observations lead to a study of the 'first night effect' in cats. The cats used had been electrophysiologically recorded from before the experiment, minimising any equipment effect, to ensure any change in sleep could be attributed to the movement to the novel environment alone. The total duration of REM sleep was significantly higher on the first recording day post-movement as compared with subsequent days, due to an increased number of REM bouts. This provides more evidence that non-human animals may have a different 'first night effect' to humans (Wallach et al. 1976).

Tang et al (2004), suggest that investigating the period after exposure to a novel environment would provide information on the emotional and learning processes that take place during and after the novel environment. An anxious animal may be expected to show a decrease in REM and an animal that explored the novel environment more may be expected to show an increase in REM/Non-REM associated with learning after exposure to a novel environment (Tang et al. 2004). Tang et al (2004), found differences in the REM and Non-REM sleep in mice after exposure to a novel environment compared with baselines and these differences were no longer present after 24h post-exposure. Mice were put, individually into a novel enclosure for 30 minutes and then returned to their home cages and during the subsequent 48h, the EEG was recorded. There were significant reductions in REM in the first 2 to 8-h of the light period (when mice mainly sleep) depending on strain of mouse (the 'high anxiety' mice had reductions in REM for longest). In the least 'anxious strains' of mice this was followed by significant increases in REM during the dark period, but the increase in REM was not seen in the most 'anxious strains' (Tang et al. 2004). A reduction in Non-REM was also seen in the light period of the most 'anxious strains'. The authors suggest that the most anxious strains of mice showed a reduction in REM and Non-REM sleep due to the anxious waking experience of the novel environment. The least 'anxious strains' of mice were seen to explore the environment more and showed a shorter reduction in REM sleep followed by an increase in REM sleep in the dark period. The authors suggest this increase in REM sleep could be related to exploration and learning as it has been

previously theorised that learning and REM sleep are connected (see chapter 1 of this thesis)(Tang et al. 2004). The same laboratory also investigated a conditioned fear response and the effect it had on sleep in mice (Sanford et al. 2003). Here, mice were subjected to a foot shock, which was associated by training to a non-noxious cue. The results show that fearful cues can reduce REM and Non-REM sleep in the hours immediately after testing, and even a single foot shock was aversive enough to reduce REM sleep significantly in the first 2 h post testing (Sanford et al. 2003).

The main difficulty with interpreting the 'first night effect' in humans and other animals is that there is a lack of pre-movement baselines. It is assumed that the first night is different from 'normal' and that the sleep returns to 'normal' on subsequent nights as the individual habituates to the environment and recording procedure. However, sleep in the sleep laboratory, for example, could be fundamentally different from 'normal'. In addition, there are the problems in attributing any changes in sleep shown on the first night to the novel environment as opposed to the novel recording situation (Le Bon et al. 2001).

3.3 Materials and Methods

3.3.1 Animals

Twenty-six, adult, polled, Dorset cross, ewes that had been kept outside on pasture for at least one year, were randomly paired. Within each pair, sheep were randomly assigned to either a control or a treatment group.

3.3.2 Husbandry details

From late February until the end of September 2002, groups of four sheep were brought, from the main flock, into an experimental paddock (0.14 hectares, grazing) for at least two weeks before their use in the experiment. As the experiment used pairs of sheep on a continuous basis, there were never less than eight sheep on the paddock during the experiment.

The paddock incorporated a semi-covered, hard floor area (4m x 6m), which included a 1m x 1.5m pen used for handling sheep and attachment of electrophysiological equipment. The sheep had free access to the handling area throughout the experiment and were habituated to the researcher herding them into the area four times a week. The sheep were offered approximately 250g/d/sheep concentrated feed (Pentland lamb finisher, Seafield Mill, UK) from a trough and *ad-libitum* hay from a rack in the handling area. The sheep were also habituated to the attachment of a harness and fibre-glass helmet (as described in the general methodology section, chapter 2).

3.3.3 Experimental treatment schedule

Twenty-four-hour electrophysiological recordings were made from a control and treatment sheep as shown in table 3.1. Seven days after the baseline recording the sheep assigned to the treatment group was moved from the paddock to an inside pen. The control sheep remained outside in the paddock. Twenty-four-hour electrophysiological recordings were made from the pair on the day of movement and seven days after movement.

Table 3.1. The experimental treatment schedule

24-h Recording Number	24-h Recording Name	Day	Treatment Group	Control Group
1	Baseline	-7	Outside in Paddock	Outside in Paddock
2	Movement day	0	Movement to Inside Pen	Outside in Paddock
3	7d post-movement	+7	Inside Pen	Outside in Paddock

3.3.4 Electrophysiological recordings

On the morning of the baseline and movement day recordings, a pair of sheep (the order of which, was chosen randomly) were brought in from the paddock to the handling and attachment pen using the concentrated feed as a food reward. For the 7d post-movement recording, the control sheep was brought into the attachment pen with a companion sheep, and the treatment sheep remained in the inside pen.

The electrode attachment and removal was carried out as outlined in the general methodology section (chapter 2). However, as sheep had to wear the electrophysiological equipment outside at pasture in the novel environment experiment, a number of extra precautions were taken in an attempt to ensure electrode adhesion for each 24h period. Firstly, after the electrodes had been attached to the sites, all the electrode sites on the body were covered in duct tape to keep the electrode sites dry. The duct tape was glued to the fleece using all purpose glue (Bostik All Purpose Adhesive, Bostik Findley Ltd, Stafford, UK). On removal, the duct tape was carefully cut from the fleece avoiding both the sheep's skin and the electrode leads. As the experimental sheep were to be in a paddock with conspecifics, the electrode leads had to be protected from possible damage caused by sheep biting them. As mentioned in the general methodology, the electrode leads were clipped into the fleece, in addition, tubular, elasticated netting (Netelast$_{TM}$ Seton Health Care Group Plc, Oldham, UK) was placed around the neck and secured by the plastic clips. This meant that even if the electrode leads were to come out from under the fleece, after the sheep was released in the paddock, the leads would still be protected from harm by the netting.

A method of recording the respiration rates of the sheep had been developed and was carried out during the novel environment experiment. Temperature differential electrodes (thermocouples) were used to record the temperature of

inhaled and exhaled breath from the nostrils of the sheep. Four thermocouples were connected by 700 mm leads to the 13-16 connection port of the Embla (via the connectors on the waterproof box). A change of helmet design was made to incorporate the thermocouples. A 20mm wide nose-piece was added, that joined to the top of the helmet and lay on the front of the face of the sheep. The lower end of the nose-piece followed the contours of the nose of the sheep, placing two thermocouples by each nostril. The thermocouple leads were also clipped into the fleece and covered by the elasticated netting. The electrophysiological trace of respiration was analysed in Somnologica in conjunction with the other electrophysiological information. The temperature difference between inhaled and exhaled air created the differential, the properties of which could then be analysed using spectral analysis. Figure 3.1 shows the sheep in the experimental pasture wearing the electrophysiological equipment.

Figure 3.1 A sheep wearing the electrophysiological equipment in the experimental pasture.

Both treatment and control sheep were returned to the paddock for the baseline recording. On the movement recording day, the treatment sheep was moved, with a non-experimental companion sheep, in a 1.5m x 2m trailer from the attachment pen, approximately 300m to an inside single pen. The pen (2m x 2m), described in detail in the general methodology section was adjacent to the companion sheep. The control sheep was released into the paddock. The treatment sheep were also offered 250g/d/sheep concentrated feed and had *ad-libitum* access to hay and water in the inside pen.

On the 7d post-movement recording day, the attachment procedure and recordings of the treatment sheep took place in the inside pen. After completion of three, successful electrophysiological recordings, the sheep were inspected by a veterinarian and returned to the main flock. This procedure was repeated until satisfactory electrophysiological recordings had been made from ten pairs of sheep.

3.3.5 Circadian environment

The length of natural daylight varied during the experimental period from 11h 39mins at the start to a maximum of 18h 12mins, through to 11h 28mins at the end of the experiment (NASA sunrise and sunset times, 2002). To maintain approximately similar circadian daylight patterns for the control and treatment sheep, artificial lighting was used for the inside penning, on a 12hL:12hD cycle, with a light period from 0700h to 1900h. To allow for natural day length changes, the inside pen was equipped with translucent ceiling panels letting light into the room during the 12hD artificial light cycle. The outside, daytime, maximum temperature on experimental days ranged from 9°C to 21°C (as recorded for the east Pentland region of Scotland by The Meteorological Office, UK). The temperature (measured on a thermometer and recorded on each experimental day when the recordings began) in the inside pen remained within approximately 5°C of the outside temperature.

3.3.6 Electrophysiological and behavioural analysis

The behavioural and electrophysiological recordings were analysed as detailed in the general methodology section (chapter 2). The analysis of sheep posture during the baseline and control recordings was undertaken from the electrophysiological recordings alone without video recordings.

In addition to the analysis carried out in the general methodology, a basic analysis of respiration was undertaken. The temperature differences of inhaled and exhaled air were picked up by the thermocouple electrodes at the nostrils. This produced a sine wave trace when analysed using Somnologica. The number of wavelengths in ten second epochs were calculated in Somnologica as a method of recording the rate of respiration.

A method of determining potential differences during REM sleep was used during the novel environment experiment (as developed by Douglass et al. 1992). The density of rapid eye movements in REM sleep was calculated. REM sleep episodes were split into 10 s epochs and the total number of complete sine waves from the EOG trace were recorded. The first 10 s epoch of REM sleep was disregarded as there were often few eye movements in this period and this may be a transitory phase. The mean density for each episode was calculated from the 10 s epochs (except the first). Then a mean eye movement density was calculated for all episodes in a given time period.

3.3.7 Statistical analysis

Descriptive statistics were carried out in Minitab 13 (Minitab, USA).

The data for the novel environment experiment was transferred from Somnologica 2.1 to the 'Observer 4' behaviour analysis programme to carry out a thorough analysis of the elementary statistics relating to distribution of bouts of behaviour. In addition, the Observer allowed the total recording (24h) to be split into intervals. In order to export the data from Somnologica to the Observer the *Event* score was saved as a text only file, containing the name of the *event* and the time from when it was scored in Somnologica. The text file was manipulated in Microsoft Word so that it was in the same format as an ODF file, the text file that the Observer creates when carrying out a behavioural observation. The text files could then be read by the Observer and the statistical tools within that programme could be used.

The time distribution and bout data were sorted by the Observer programme and the data exported as an Excel file (Microsoft Office, USA) The Excel file was then read through the SAS 8.2 programme (SAS for Windows, SAS Institute Inc, North Carolina, USA). A mixed model (using the PROC MIXED procedure in SAS) analysis of covariance with repeated measures was used for the majority of comparisons, assuming a normal distribution. Mixed models are regression analyses that take into account any correlations between observations in the data (i.e. the mixed model does not assume that all observations are independent from one another) (Brown and Prescott, 1996).

The following procedure was run for each posture, ingestive behaviour and sleep stage:

PROC MIXED;
CLASS <period> <pair>;
MODEL <behaviour A> = <period> <difference in A between treatment conditions in baseline>
/DDFM = SATTERTH;
REPEATED <period> / SUBJECT = <pair> TYPE = CS R RCORR;
LSMEANS <period> / DIFF PDIFF CL;
RUN;

Estimation method = Residual maximum likelihood (REML)(not entered into the statement as it is the default estimation method for PROC MIXED in SAS).

Degrees of freedom method = Satterthwaite

REPEATED = repeated measures for chosen fixed effects in this case 'recording period'

Covariance Parameter estimate = Compound Symmetry (CS), covariances are equal.

LSMEANS = the calculation of the least squares mean estimates of the fixed effects, which in this case is the 'recording period'.

DIFF = shows differences between each pair of least squares means
PDIFF = shows the significance value for the differences in least squares means.
CL = shows the confidence intervals for the least squares means.

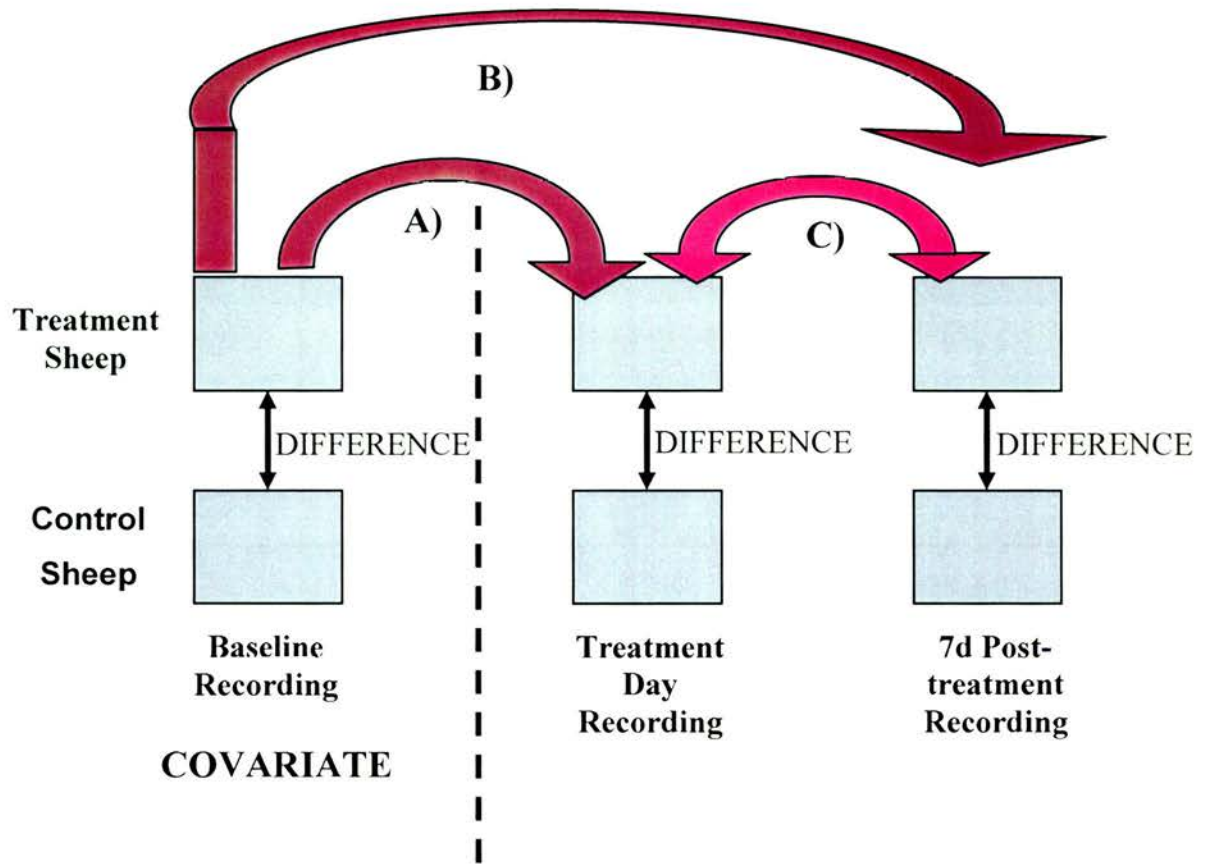
The experiment had two experimental conditions (treatment and control) so the data was always measured in a pair-wise fashion, each sheep had a partner in the opposite condition and the differences between the partners were analysed in the mixed models. The repeated measures were applied to the factor 'recording period'. The mixed model was chosen as a method of analysis as it is powerful, and is still able to be carried out when there are missing values in the data set (as long as the missing values occur by random, i.e. they are not related to the fixed effects) (Brown and Prescott, 1996). The mixed model is able to analyse the data with both fixed and random effects. The percentage of time spent in each posture and sleep stage was measured in all three main experiments. The data was considered 'normal enough' (Prescott, personal communication) to carry out the mixed model assuming a normal distribution.

When comparing within each condition (treatment or control), the PROC MIXED with repeated measures was used without the covariance statement. Instead, each time period was taken as a separate group and mean comparisons were carried out using an ESTIMATE procedure in SAS, 1) baseline vs first 24-h post-movement, 2) baseline vs 7d post-movement and 3) first 24-h post-movement vs 7d post movement.

Similarly, to analyse sleep of sheep at pasture (from the baseline recording) the data from the control and treatment groups were amalgamated and the 24-h recordings were split into four 6-h time blocks. The bout lengths from each time block were compared using the PROC MIXED with repeated measures for the following ESTIMATES: 1500-2100 vs 2100-0300; 1500-2100 vs 0300-0900; 1500-2100 vs 0900-1500; 2100-0300 vs 0300-0900; 2100-0300 vs 0900-1500; 0300-0900 vs 0900-1500.

A generalised linear mixed model was used for Bout Frequency, Sleep Latency and Heart-rate analysis assuming the Poisson distribution (DIST = P) as neither data set were normally distributed (the GLMM was used instead of the GLM as it is able to analyse covariance patterns). The PROC GEN MIXEDMOD with REPEATED statement procedure was used, with other aspects remaining the same as the mixed model (e.g. the use of REML and CS). The significance test statistic was the Wald F-test. Heart rate data were analysed with the GLMM, even though the data were normally distributed as there were many missing values and the groups were therefore unbalanced (Wolfinger and Chang, 2005).

In both methods of analysis, the difference between sheep from each experimental condition (e.g. control and treatment) during the baseline 24h recording period was used as the covariate. As there should be no difference between sheep from the experimental conditions during the baseline period, the difference would have had a mean/median around zero. Figure 3.2 outlines the analysis profile of the mixed model analysis of covariance.



A) Are the differences between control and treatment sheep during the treatment recording different from the differences between control and treatment sheep in the baseline recording?

B) Are the differences between control and treatment sheep during the 7d post-treatment recording different from the differences between control and treatment sheep in the baseline recording?

C) Are the differences between control and treatment sheep during the treatment and 7d post-treatment recordings different from one another, with the baseline as a covariate?

Figure 3.2 Schematic representation of the analysis profile of the mixed model analysis of covariance carried out in SAS 8.2. The differences between the control and treatment sheep were taken for each recording day. The difference between the control and the treatment sheep on the baseline recording day was used as the covariate in the mixed model.

3.4 Results

3.4.1 Sleep in sheep at pasture

In this section, results are taken from the baseline recording of both the control and treatment sheep (N = 20). The control and the treatment groups were not significantly different (two sample t-test) from each other in the baseline recordings in any of the measured factors in terms of posture, ingestion behaviours or sleep. The mean percentage total and the mean bout length for each behaviour are shown in table 3.2. Sheep were asleep for approximately 63 % of the time that the spectral analysis of the electromyogram recordings indicated that the sheep were lying down with their head resting on the ground. The mean (± s.e.) total duration within 24h that sheep spent sleeping at pasture was 107 ± 7.2 minutes, consisting 28 ± 2.9 minutes of REM sleep and 79 ± 5.8 minutes of Non-REM sleep On average throughout the 24h, each bout of sleep (total sleep) lasted for approximately 6 minutes. There were 22 ± 3 bouts of total sleep (consisting of 22 ± 3 bouts of Non-REM and 7 ± 2 bouts of REM Sleep).

The distribution of Total Sleep bout lengths for sheep at pasture is shown in Figure 3.3, which shows that sleep bouts were longest between 2100h-0300h and shortest between 0900h-2100h (Mixed model $P<0.01$). In addition, 59 ± 4.3 % of sleep occurred during the darkest period of the night (2100h-0300h), 27 ± 4.1 % during the early morning (0300-0900h), 6 ± 2.2 % during the middle of the day (0900-1500h) and 8 ± 2.6 % in the afternoon/evening (1500-2100h).

Table 3.2. Behaviour and sleep of sheep at pasture (mean ± standard error) during the baseline recording for both treatment and control groups N=20)

	Posture			Ingestion		Sleep		
	Standing	Lying Head Up	Lying Head Down	Eating	Ruminating	Total Sleep	REM Sleep	Non-REM Sleep
% of 24h	47 ± 1.9	41±2.3	12 ± 1.4	35 ±1.9	20 ±1.4	7.4 ±0.5	1.9 ±0.2	5.5 ±0.4
Bout length	28. ± 3 mins	12 ± 1.1 mins	6 ± 0.4 mins	26 ± 2.2 mins	12 ± 1 mins	368 ± 17 s	183 ± 9 s	218 ± 20 s

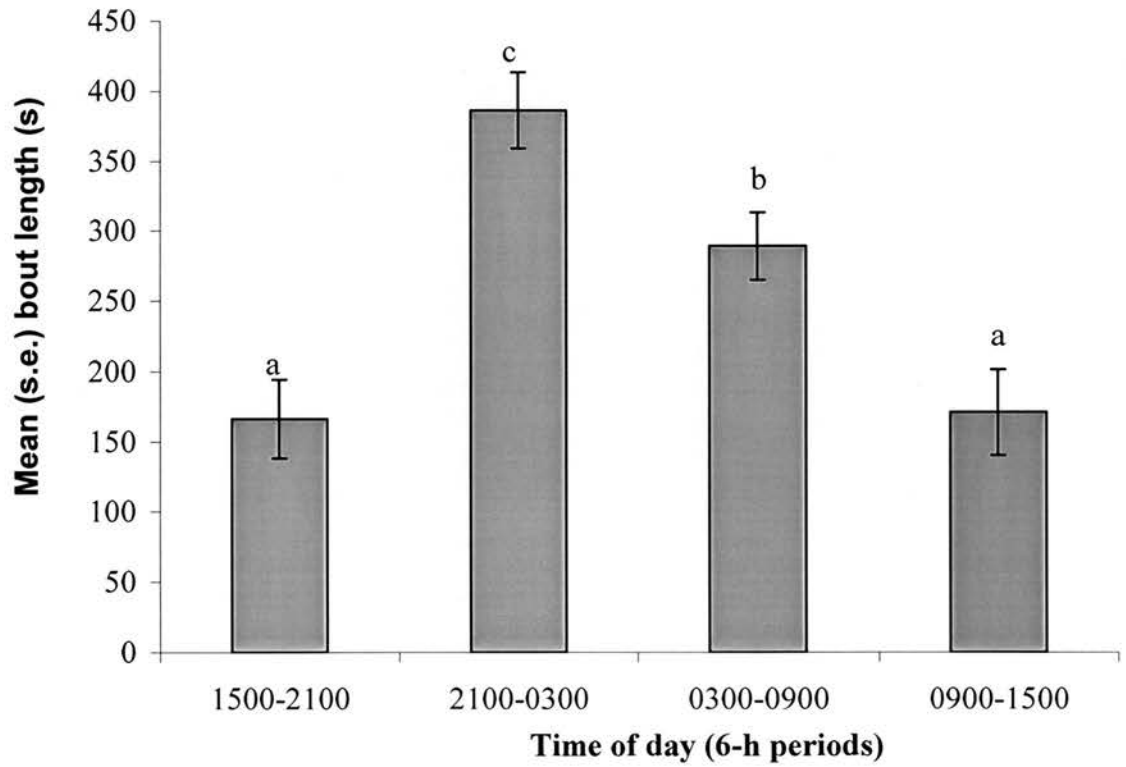


Fig 3.3. The mean bout duration (s) of total sleep (REM + Non-REM sleep) from sheep at pasture in each 6-h time period during 24-h. Different letters indicate means differ from each other (Mixed model analysis with repeated measures P<0.01) N = 20.

3.4.2 Effects of movement to a novel environment on sheep

The effects of movement of sheep to a novel environment on their behaviour are summarised in table 3.3. and 3.4 . The effects of movement of sheep to a novel environment on their sleep are summarised in table 3.5. There were no differences in behaviour or sleep of the control sheep left out on the pasture between the three recording periods.

Table 3.3 Effects of movement to novel indoor penning from pasture on posture (mean ± s.e.) before, for 24-h directly post-movement and 7d post movement (n = 10 pairs)

Measurement		24-h Baseline Recording		24-h Post-movement Recording		7d Post-movement Recording		Difference between baseline and 24-h post-movement recording[†]	Statistical significance of the difference between baseline and 24h post-movement [†]	Difference between baseline and 7d post-movement recording[‡]	Statistical significance of the difference between baseline and 7d post-movement[‡]
		Control	Treatment	Control	Treatment	Control	Treatment				
Standing	% 24h	49 ± 1.8	48 ± 1.2	45 ± 1.5	26 ± 5.3	45 ± 1.6	33 ± 6.6	-22.8 ± 5.6	**	16.9 ± 5.6	*
	Bout length (s)	1865 ±267	1802 ±298	1910 ± 255	1138 ± 297	1907 ± 301	841 ± 264	961 ± 447	NS[††]	1026 ± 447	*
	No of Bouts	43 ± 5	42 ± 4.7	43 ± 4.2	38 ± 3.9	44 ± 4.1	38 ± 4.1	-5.4 ± 4.2	NS	-5.6 ± 4.2	NS
Lying Head Up	% 24h	39 ±2.2	40 ± 2.1	41 ± 1.7	53 ± 4.1	41 ± 2.8	48 ± 6.4	-13.1 ± 5.9	*	-9.0 ± 5.9	NS
	Bout length (s)	745 ±81	752 ± 79	739 ± 81	629 ± 96	748 ± 90	603 ± 127	171 ± 127	NS	98 ± 127	NS
	No of Bouts	45 ± 5.2	45 ± 5.1	46 ± 6.3	50 ± 3.4	45 ± 4.9	49 ± 5	4.8 ± 5.2	NS	4.3 ± 5.2	NS
Lying Head Down	% 24h	12 ±1.3	12 ± 1.5	14 ± 2.1	21± 3.1	14 ± 2.3	19 ± 3.8	-9.8 ± 3.7	*	-7.9 ± 3.7	NS[††]
	Bout length (s)	396 ±29	386 ± 21	378 ± 25	304 ± 19	384 ± 29	371 ± 44	8.5 ± 50.4	NS	-42 ± 50.4	NS
	No of Bouts	19 ± 3.6	18 ± 3.4	18 ± 3.3	24 ± 6.2	19 ± 2.9	22 ± 5.7	7.1 ± 5.4	NS	4.4 ± 5.4	NS

Using GLMM for the number of bouts of each posture and mixed model analysis of covariance with repeated measures for other measurements

[†] Differences between baseline recording and 24-h post-movement recording in the differences between pairs of sheep from the control and treatment groups within a 24-h time period.

[‡] Differences between baseline recording and the 7d post-movement recording in the differences between pairs of sheep from the control and treatment groups within a 24-h time period.

[††] $P > 0.05$, but < 0.1

Table 3.4 Effects of movement to novel indoor penning from pasture on eating and ruminating behaviour (mean ± s.e.) before, for 24-h directly post-movement and 7d post movement (n = 10 pairs)

Measurement		24-h Baseline Recording		24-h Post-movement Recording		7d Post-movement Recording		Difference between baseline and 24-h post-movement recording[†]	Statistical significance of the difference between baseline and 24h post-movement[†]	Difference between baseline and 7d post-movement recording[‡]	Statistical significance of the difference between baseline and 7d post-movement [‡]
		Control	Treatment	Control	Treatment	Control	Treatment				
Eating	% 24h	36 ±2.2	38 ± 2.3	37 ± 2.8	9 ± 1.6	40 ± 2.7	16 ± 2.2	31.1 ± 2.5	***	25.0 ± 2.5	***
	Bout length (s)	1581 ±166	1397 ± 201	1413 ± 199	293 ± 46	1563 ± 192	543 ± 90	1642 ± 203	***	1504 ± 203	***
	No of Bouts	45 ± 3.7	46 ± 3.9	46 ± 4.1	14 ± 4.9	44 ± 3.8	22 ± 5.6	35.2 ± 9.6	**	23.4 ± 9.6	**
Ruminating	% 24h	21 ±1.7	21 ± 1.9	20 ± 1.4	22 ± 1.9	23 ± 2.3	27 ± 3.8	1.5 ± 3.7	NS	-6.2 ± 3.7	NS
	Bout length (s)	701 ± 61	711 ± 52	695 ± 66	635 ±73	712 ± 82	854 ± 114	126 ± 105	NS	31 ± 105	NS
	No of Bouts	23 ± 2.2	26 ± 3.4	24 ± 2.8	27 ± 3.2	24 ± 3	27 ± 3.9	-1.4 ± 2.2	NS	-1.8 ± 2.2	NS

Using mixed model analysis of covariance with repeated measures

[†] Differences between baseline recording and 24-h post-movement recording in the differences between pairs of sheep from the control and treatment groups within a 24-h time period.

[‡] Differences between baseline recording and the 7d post-movement recording in the differences between pairs of sheep from the control and treatment groups within a 24-h time period

Table 3.5 Effects of movement to novel indoor penning from pasture on sleep (mean ± s.e.) before, for 24-h directly post-movement and 7d post movement (n = 10 pairs)

Measurement		24-h Baseline Recording		24-h Post-movement Recording		7d Post-movement Recording		Difference between baseline and 24-h post-movement recording[†]	Statistical significance of the difference between baseline and 24h post-movement [†]	Difference between baseline and 7d post-movement recording[‡]	Statistical significance of the difference between baseline and 7d post-movement[‡]
		Control	Treatment	Control	Treatment	Control	Treatment				
Total Sleep	% 24h	7.3 ± 0.8	7.2 ± 0.7	7.3 ± 0.7	8.8 ± 0.9	7.4 ± 0.8	8.1 ± 0.9	-2.7 ± 1.4	NS[††]	-1.2 ± 1.4	NS
	Bout length	345 ± 26	336 ± 34	347 ± 34	352 ± 38	344 ± 29	351 ± 37	-16 ± 18	NS	-19.1 ± 18	NS
	No bouts	22 ± 3	22 ± 2.8	22 ± 2.1	26 ± 2.1	22 ± 3	25 ± 3.1	-4.1 ± 2.8	NS	1.8 ± 2.8	NS
Non-REM Sleep	% 24h	5.4 ± 0.4	5.3 ± 0.6	5.4 ± 0.4	6.5 ± 0.7	5.5 ± 0.6	6.2 ± 1.1	-1.9 ± 1.2	NS	-1.1 ± 1.2	NS
	Bout length	227 ± 22	221 ± 21	235 ± 21	238 ± 20	229 ± 24	239 ± 26	-4.5 ± 14	NS	-9.4 ± 14	NS
	No bouts	20 ± 3.1	21 ± 2.5	21 ± 2	26 ± 1.9	22 ± 2.9	25 ± 2.5	-4.3 ± 2.6	NS	1.7 ± 2.6	NS
REM Sleep	% 24h	1.9 ± 0.2	1.9 ± 0.3	1.8 ± 0.2	2.8 ± 0.2	1.9 ± 0.3	2.1 ± 0.1	-1.8 ± 0.3	*	-0.1 ± 0.3	NS
	Bout length	234 ± 17	230 ± 15	226 ± 19	238 ± 22	230 ± 19	240 ± 23	12.3 ± 17	NS	11 ± 17	NS
	No bouts	7 ± 2.1	7 ± 1.8	8 ± 2.1	10 ± 1.9	7 ± 2.1	8 ± 1.8	-2.6 ± 1.2	NS[††]	1 ± 1.2	NS
Sleep latency (mins)		280 ± 35	274 ± 33	275 ± 31	138 ± 25	270 ± 30	231 ± 36	141.9 ± 62.2	*	65.1 ± 62.2	NS
Eye-movement density in 10 s epochs of REM		54 ± 13	60 ± 11	59 ± 14	60 ± 10	60 ± 14	79 ± 9	-3.6 ± 3.4	NS	-10 ± 3.4	*
% delta waves in Non-REM Sleep (10 s epochs)		37 ± 4.3	35 ± 5	35 ± 4.8	49 ± 2.1	36 ± 4.9	40 ± 3.4	-9.5 ± 2.2	*	-3.4 ± 2.2	NS

Using GLMM for the number of bouts and sleep latency and mixed model analysis of covariance with repeated measures for other measurements

[†] Differences between baseline recording and 24-h post-movement recording in the differences between pairs of sheep from the control and treatment groups within a 24-h time period.

[‡] Differences between baseline recording and the 7d post-movement recording in the differences between pairs of sheep from the control and treatment groups within a 24-h time period.

[††] $P > 0.05$, but < 0.1

3.4.2.1 Effects of movement to a novel environment on posture

There were differences in posture between treatment sheep and control sheep post-movement as compared with the baseline.

Treatment sheep spent less time standing than controls in the first 24-h post-movement as compared with the baseline. This difference was still present 7d post-movement. There were no differences in the number of bouts of 'Standing' post-movement. However, there was a tendency for the mean bout length of 'Standing' in treatment sheep to be shorter than the baseline (Mixed Model, estimate -724 ± 308, t = -2.1 $P<0.07$). Seven days post-movement the difference in the bout lengths of 'Standing' between control and treatment sheep was different from that of the baseline, treatment sheep showed shorter bouts of standing compared with baseline (Mixed Model, estimate –934 ± 312, t = -3.6, $P<0.05$).

The treatment sheep spent more time 'Lying Head Up' than the control sheep in the first 24-h post-movement compared with the baseline. There was no difference in the total % time spent Lying head up 7d post-movement. There were no significant treatment effects on the mean bout length, or the number of bouts of Lying Head Up in either post-movement recording.

Treatment sheep spent more time in the 'Lying Head Down' posture than control sheep in the first 24-h post-movement compared with the baseline. There was a tendency for this treatment effect on the percentage time spent 'Lying Head Down' to be present in 7d post-movement ($P<0.06$). There were no significant treatment effects on the mean bout length, or the number of bouts of Lying Head Down in either post-movement recording.

3.4.2.2 Effects of movement to a novel environment on ingestive behaviour

There were no treatment effects on the total percentage, mean bout duration, or the frequency of 'rumination' in either the first 24h post-movement or the 7d post-movement recordings as compared with the baseline.

On the other hand, there were significant treatment effects seen between control and treatment sheep in the total time spent 'eating' during both the first 24-h post movement and the 7d post-movement as compared with that of the baseline. Treatment sheep spent less time eating than control sheep in the 24h post-movement

and 7d post-movement, compared with the baseline. The bout lengths of 'eating' in treatment sheep were shorter in the first 24h post movement than baseline and this was still shown 7d post-movement. Treatment sheep had fewer bouts of eating than the control sheep in both post-movement periods as compared with the baseline.

3.4.2.3 Effects of movement to a novel environment on sleep

Sheep moved to a novel environment had a tendency to lie down more quickly in the first 24h post-movement than controls when compared with the baseline (GLMM, estimate 136.2 ± 75.7, $F = 2.2$, $P<0.08$). There was no significant difference in the latency to lie down between treatment conditions by 7d post-movement. Furthermore, as shown in table 3.5 the treatment sheep also showed a reduced latency to sleep (Non-REM and REM) in the first 24h post-movement than controls as compared with baseline recordings, treatment sheep sleeping 142 ± 25 minutes earlier than their control partner did.

There was a tendency for an increase in 'Total Sleep' from the baseline to the first 24-h post-movement in treatment sheep as compared with the control sheep, which was no longer present 7d post-movement. There were no other significant differences in 'Total sleep' between treatment conditions.

Treatment sheep spent more time in REM sleep than control sheep in the first 24-h post-movement as compared with the baseline. There was a 30% increase in the mean of REM sleep during the first treatment period in treatment sheep as compared with the baseline (Mixed model, estimate -1.9 ± 0.3, $t = -2.6$, $P<0.05$). An increase of this magnitude in REM sleep was seen in eight out of 10 treatment sheep (one was unchanged and one showed a decrease in REM sleep as compared with baseline). Control sheep, on the other hand differed in the total percentage of REM by 9.5 ± 1.2 %, four sheep increased, six decreased. The difference in REM sleep shown by treatment sheep in the first 24h post-movement is no longer present 7d post-movement. There was no difference in the bout lengths of REM between control and treatment sheep, however the number of REM sleep bouts had a tendency to be higher in treatment sheep 24h post-movement than in control sheep as compared with the baseline.

The only treatment effect on REM sleep that was seen 7d post-movement was in the density of rapid-eye-movements. Treatment sheep showed a greater density of eye-movements during REM episodes than controls compared with baseline.

There were no significant differences between control and treatment sheep in the time spent, the bout lengths or the number of bouts of Non-REM sleep in either post-movement recording period as compared with the baseline. However, the spectral properties of Non-REM sleep were affected by the treatment condition. There was a difference in the percentage of delta waves (0.1 –4 Hz) in 10s epochs of Non-REM sleep between control and treatment sheep as compared with that of the baseline. Treatment sheep showed a higher percentage of delta waves in Non-REM sleep post-movement than controls as compared with the baseline, which was no longer present 7d post-movement.

The distribution of sleep in sheep over 24h was affected by movement to a novel environment. At pasture, sheep sleep mainly during the darkest part of the night. As shown in Figure 3.4, during the first 24h post-movement, treatment sheep spend less time asleep during the darkest time block (2100-0300h) (Mixed model, estimate, 1044 ± 210, $t = 3.6$, $P<0.05$) and an increase in the duration of sleep during the light time blocks (0900-1500h: Mixed model, estimate -1340 ± 218 $t = 4.0$, $P<0.01$; 1500-2100h: Mixed model, estimate -986 ± 357, $t = 2.8$ $P<0.05$) as compared with baseline and controls. This results in the flattening of the profile of sleep duration over the first 24h post-movement. The majority of this flattening effect is still present 7d post-movement in treatment sheep (with a tendency for sleep to be reduced in the darkest time block, Mixed model, estimate -565 ± 210, $t = 2.4$, $P=0.06$).

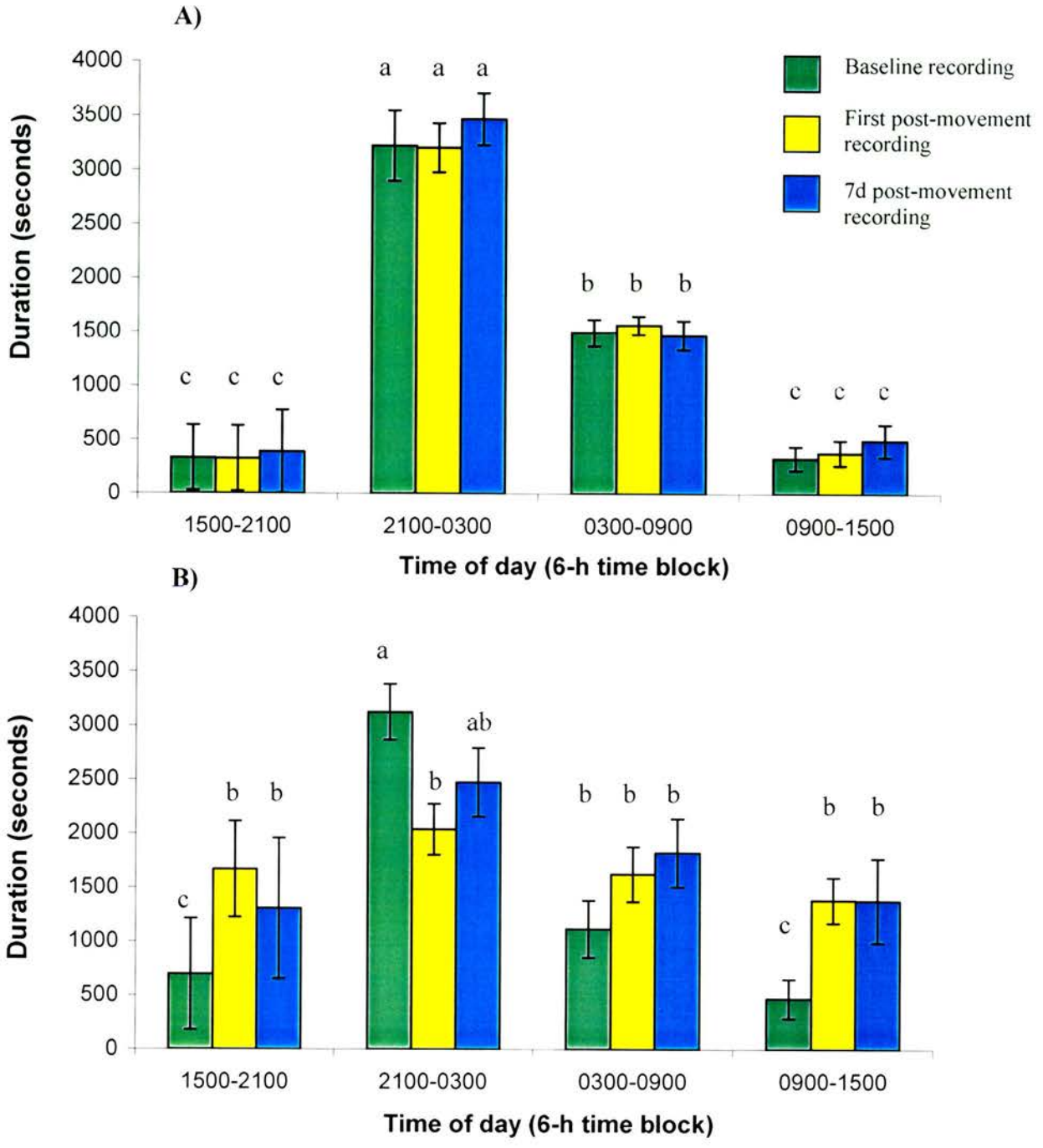


Figure 3.4. The mean (s.e.) duration of 'Total Sleep' per 6-h period during a 24-h recording period for A) Control sheep and B) Treatment sheep during the baseline recording, 1st post-movement recording and 7d post-movement recording. Means within a 6-h time period with a common letter do not differ significantly (P>0.05). (ab column had a tendency to be different P<0.06.) There is no significant difference between the baseline recordings of control and treatment sheep. (Mixed model analysis of variance with repeated measures) N = 10 for each group.

3.4.2.4 Effects of movement to a novel environment on heart rate and respiration rate

Unfortunately, only three usable respiration recordings were made from treatment sheep and one usable respiration recording was made from a control sheep from the post-movement recordings. Therefore, results are not presented on the respiration rate.

Table 3.6 shows the effect of movement and of a novel environment on the heart rate of sheep. During the 24-h post-movement recording, the heart rate between 30s and 120s coincided with the period of transit, where the treatment sheep was in a trailer being moved to the inside penning. The heart rate was high in sheep immediately after release to the pasture, movement to the inside pen and the start of the recording in the inside pen 7d post-movement. The heart rate appears to slow between 'release' and 30mins post-release in both treatment conditions for all recording periods. There were differences in heart rate between treatment and control sheep at 30s, 60s and 10mins post-movement compared with the same time points post-release in the baseline recording.

There was also a treatment effect on heart rate seen 7d post-movement as treatment sheep had a lower heart rate than controls at 90s, 120s, 20mins and 30mins after the start of the recording as compared with equivalent time points from the baseline recording.

Table 3.6 Effects of movement to novel indoor penning from pasture on heart rate (beats per 10s epoch ± s.e.) during the baseline, during the first 24-h post-movement and during the 7d post movement recordings. 10s of artefact free heart rate taken at times post release back to pasture (baseline and controls) and during and post movement (treatment sheep). N = 10 pairs unless otherwise shown

Time after start of recording	24-h Baseline Recording		24-h Post-movement Recording		7d Post-movement Recording		Difference between baseline and 24-h post-movement recording[†]	Statistical significance of the difference between baseline and 24h post-movement[†]	Difference between baseline and 7d post-movement recording[‡]	Statistical significance of the difference between baseline and 7d post-movement [‡]
	Control	Treatment	Control	Treatment	Control	Treatment				
30s	18 ± 0.7	18 ± 0.7	17 ± 0.5	20 ± 0.8	18 ± 0.8	17 ± 0.7	-3.4 ± 0.8	**	1.5 ± 0.9	NS
60s	16 ± 0.7	18 ± 0.7	17 ± 0.5	20 ± 0.8	17 ± 1.0	16 ± 1.0	-2.9 ± 1.1	*	1.9 ± 1.1	NS
90s	17 ± 0.3	17 ± 0.5	17 ± 0.7	18 ± 1.0	17 ± 1.0	14 ± 0.8	-2.4 ± 0.9	NS	3.8 ± 1.1	**
120s	16 ± 0.3	16 ± 0.5	16 ± 0.5	17 ± 0.8[††]	17 ± 0.8	13 ± 0.7	-1.8 ± 1.0	NS	3.1 ± 1.0	*
10mins	14 ± 0.5	14 ± 0.3	14±0.5[††]	17 ± 0.7[††]	14 ± 0.5[††]	12 ± 0.5	-2.1 ± 0.7	*	1.2 ± 0.7	NS
20mins	14 ±0.3[††]	14 ± 0.5	14±0.5[††]	15 ± 0.7[††]	13 ± 0.5[‡‡]	11 ± 0.8[††]	-0.7 ± 0.5	NS	2.4 ± 0.5	*
30mins	12 ±0.3[††]	12 ± 0.3[††]	13±0.3[††]	13 ± 0.5[††]	12 ± 0.3[‡‡]	11 ± 0.5[††]	0.2 ± 0.3	NS	1.3 ± 0.4	*

Using GLMM with repeated measures. Measurements taken between 30 and 120s after the start of the recording were taken while the sheep was in transit to the inside penning during the treatment recording.

[†] Differences between baseline recording and 24-h post-movement recording in the differences between pairs of sheep from the control and treatment groups within a 24-h time period.

[‡] Differences between baseline recording and the 7d post-movement recording in the differences between pairs of sheep from the control and treatment groups within a 24-h time period

[††] N = 8, [‡‡] N = 7

3.5 Discussion

3.5.1. Recording sleep in sheep at pasture

This was the first study to use non-invasive electroencephalographic techniques to record sleep and wakefulness in sheep at pasture. Other studies have monitored activity levels and behaviour of free-ranging sheep (e.g. Geist, 1971; Bueno and Ruckebusch, 1979; and Tobler et al. 1991), and they found that sheep had a mainly bimodal activity pattern: of grazing from dawn to late morning and again from early afternoon until dusk. All of the studies report that sheep undertake restful behaviour in between the grazing periods and are mainly inactive at night. Sleep, indirectly measured by lack of activity (Tobler et al. 1991), or assumed by postural observation (Geist, 1971) was described as occurring at night. The results from the present study corroborate these previous studies, in as much as 86% of sleep scored from the EEG traces of sheep at pasture (n = 20) occurred between 2100h and 0900h. In addition, sleep bouts were longest at night. However, although previous studies indicated that sheep rest during the middle of the day, these results show that sheep do indeed sleep, (albeit for shorter periods than at night) during the daylight hours. Tobler et al (1991) found that sheep underwent REM sleep during the night for bouts of 2-6mins, the present results also show that sheep had bouts of REM sleep lasting approximately 3mins.

Using the Somnologica automatic sleep scoring assistant, sheep were shown to be asleep (Non-REM and REM) for approximately 63% of the time that the EMG from the hind leg and the neck indicated that the sheep was lying in the 'head down' posture. This suggests that the previous research that relies on body posture to ascertain sleep quantity in sheep may overestimate the period of time spent sleeping. However, one must be cautious with these results, as there was no direct observation or video of behaviour during the 24-h recordings of sheep at pasture. On the other hand, in the preliminary and validation studies (chapter 2), there was concordance between the behaviour observed from the video and the electromyographic traces. The score given to the hind leg EMG (i.e. standing or lying) was always the same as that given to the behaviour from video observation (and occurred at the same time). With observer practice, the two lying postures (lying 'head up' or 'head down') were also consistently scored as compared to the video. The exception was that of the exact

point of scoring the transition between postures could vary (especially going from lying head up to lying head down) by 1-2s from the score on the video to the score on the EMG trace. However, the variation in scoring was not consistently under or over and the maximum discrepancy would be less than 60s in a 24h recording. Therefore, the technique, although not perfect, was deemed good enough to be used with the sheep in the pasture without video back-up.

As discussed in chapter 2, other authors have found that non-invasive, skin surface electrode techniques were too unreliable for use in free-ranging animals (e.g. Baldock et al. 1987). The present study also experienced difficulties with electrode unreliability, especially with certain sites, only one sheep at pasture (out of forty 24-h pasture recordings) had an ECG trace that maintained high enough quality to be analysed throughout the 24-h. Similarly, the thermocouple electrodes, designed to record the temperature differences in inhaled and exhaled air (and thus record respiration rate), had a high level of unreliability as it seemed they were damaged when sheep were grazing. Fortunately, the measures taken in an attempt to keep the electrode sites waterproof (e.g. affixing duct tape with adhesive to the fleece around the electrode sites), seemed effective and most recordings were successful enough to be used in the study.

3.5.2. Effects of movement to a novel environment on sheep

After sheep were moved from grazing at pasture to a novel environment, their behaviour and sleep was altered. During the first 24-h post-movement, treatment sheep lay down 'head up' more than sheep at pasture. In addition, treatment sheep lay down 'head down' more than sheep at pasture. Although rumination was not affected by the movement to a novel environment, the time spent eating, the number of eating bouts and the mean length of eating bouts were lower in the treatment sheep than the sheep at pasture.

The hypothesis at the start of this study was that a potentially aversive experience, such as movement to a novel environment would have an effect on the quantity and distribution of sleep in sheep. There was indeed an effect on sleep post-movement, but the change in sleep post-movement was subtle, seen in eight out of ten sheep, that showed an increase of approximately 30% in the total time spent in REM

sleep. There was no corresponding increase in the mean length of REM bouts, but there was a tendency for the number of bouts to increase post-movement as compared to baselines at pasture. There was no difference in the time spent in Non-REM Sleep, the number of bouts or the mean length of Non-REM bouts post movement. However, the spectral properties of Non-REM sleep did alter in the novel environment as the % of delta waves in 30s epochs of Non-REM was higher post-movement than at pasture. In addition, the distribution of sleep throughout the 24-h became flattened post-movement, with more sleep being present in the daylight and less at night and this change in the profile of sleep remained different to that of sheep at pasture when the 7d post-movement recording was made.

Most of the above changes were no longer observed in the treatment sheep 7d post-movement, There were no longer any differences in quantity of REM sleep as compared to sheep at pasture. However, the density of the rapid-eye-movements in REM sleep was higher in the treatment sheep 7d post-movement compared to sheep at pasture. The difference in spectral properties of Non-REM that had been seen in the first 24-h post-movement was no longer present. The reduction in eating time and bouts seen in the first 24-h post-movement was still shown in the 7d post-movement recording.

Fearful experiences, in rats, while awake increase the latency to sleep and decrease the amount of REM and Non-REM during the first few hours after the experience (Tang et al. 2004). Yet, a stressor that does not induce fear (such as chronic mild stress regimes, e.g. Cheeta et al. 1997) can increase REM sleep duration (for a full review of sleep and stress see chapter 1 of this thesis). Perhaps the increased duration of REM sleep is a response to the stress of movement as opposed to fear caused by the novel environment. Most previous experiments that use a novel environment as a stressor expose the animal to it in isolation, whereas sheep in the current experiment were exposed to the environment in familiar pairs, perhaps reducing a very fearful situation to a mildly stressful one. Carbajal and Orihuela (2001) isolated ewes and measured plasma cortisol concentrations; they found that the addition of only a second ewe returned cortisol concentrations back to baseline levels. The authors suggest that sheep in pairs no longer consider themselves isolated. In addition, in the current experiment, the companion sheep had been exposed to the

inside penning environment on a number of occasions. The behaviour of the companion was not recorded during this experiment but, it is possible that, as she was habituated to the environment, she may not have exhibited stress/fear reactions after movement. If this was the case then the relatively relaxed behaviour of the companion sheep may have reduced the behavioural response of the experimental sheep. (Carbajal and Orihuela, 2001) It is also possible that the sheep in this experiment were not stressed by movement to a novel environment, but that they were experiencing 'luxury sleep' (more than they require) due to being in a comfortable environment as suggested in housed cattle by Ruckebusch (1975). However, it is unlikely that this effect would be seen on the first night after movement (the cattle had been housed for 3 weeks before showing luxury sleep) and the increase in sleep was no longer seen 7 days post-movement.

There is evidence to suggest that mild to moderate acute stressors can promote sleep. For a more detailed review of the effects of different stressors on sleep, please see chapter 1 of this thesis. An immobilisation stressor of 1 to 2 h in rats can produce up to a 50 % increase in REM sleep in the dark period as compared to baseline values (Rampin et al. 1991; Cespuglio et al. 1995; Dewasmes et al. 2004). Furthermore, Koehl et al (2002), showed that the increase in REM sleep was seen in the dark phase whether the stressor was applied in the dark phase or the light phase. 'Non-stressful' novelty can also increase REM sleep in rats, possibly due to learning and habituation to the novel item (Schiffelholz and Aldenhoff, 2002). It is interesting to note that in all of the above experiments on acute stress in rats, the experiments on the effects of fear and anxiety on sleep in mice, and the present experiment on the effects of movement to a novel environment on sleep in sheep, there is a common finding: the mean durations of the bouts of REM sleep are not affected, it is always the number of bouts of REM sleep that increase or decrease. It seems that REM sleep bout lengths under these conditions have an inelastic quality.

A more severe stressor can have a different effect in rats, rather than an increase in REM sleep there can be changes seen in Non-REM sleep. Meerlo et al (1997, 2001) have undertaken a series of experiments investigating the effects of the intensity of wakeful experience on the waveform of subsequent Non-REM sleep in rats. They found that a severe social defeat would increase the percentage of delta

waves (0.1 to 4 Hz) in Non-REM for the first half of the sleep period in rats. In the present experiment, sheep were also found to exhibit an increase in the percentage of delta waves in the Non-REM sleep from the first 24h post-movement. However, care must be taken in assessing this result as Meerlo et al (1997) also found an increase in delta waves in rats that had been handled gently for 1h (although this increase was smaller than that from the social defeat). Nevertheless, this result does provide more evidence to suggest that the sheep perceived the movement to a novel environment as a stressful waking experience (as the increase in % of delta waves does not seem to be associated with sleep deprivation in this case), but not a fearful experience (as there was no corresponding decrease in REM Sleep).

The increase in time spent inactive (lying) seen in the present study are similar to the description of a withdrawal period (a reduction in activity seen after movement to novel housing) given by Done-Currie, et al (1984). There are a number of explanations for the changes seen in behaviour post-movement. Firstly, the environment available to the sheep was reduced in size and complexity, there was less room to move around and as there was no need to forage for food, sheep no longer had to move between feeding areas. Secondly, giving sheep hay from a hay-rack allows sheep to eat in a lying down posture, seen less frequently at pasture. In addition, the inside penning environment itself was out of visual contact with other sheep (except for the companion animal) and was undisturbed by humans except for feeding and watering. On the other hand, the pasture was in view of human activity from the surrounding buildings and sheep were in visual and physical contact with other conspecifics. Tobler et al (1991) found that sheep that were disturbed by humans had their circadian rhythms dictated by humans presence or absence. Perhaps the inside penning provides a secure environment in which sheep can remain lying for longer than at pasture.

It is possible that the increase in lying seen during the first 24h post-movement was associated with the experience of human contact (during loading onto the trailer), movement in the trailer and placement into a novel environment. These experiences all have the potential to be aversive to sheep, and could induce a stress response that required recovery, encouraging the sheep to remain in the restful lying posture for longer than the controls returned to pasture. Other stressors have been shown to induce

resting in sheep, Hunter and Milner (1963) noted that after every time that hill sheep had been herded together by a shepherd and dog, sheep would disperse and rest for up to 2.5 h (although this was confounded by the exercise of being herded). The heart rate results from the present study seem to show that the movement itself was stressful, as treatment sheep (less active) had higher heart rates when transported to the novel environment, than control sheep (more active) that were released to the pasture. Heart rates were high in all sheep when the electrophysiological recordings started indicating that the attachment procedure in itself was stressful (even though the sheep had been 'habituated' to the procedure, Hargreaves and Hutson, 1990). Interestingly, the treatment sheep that had been housed for 7d recovered 'normal' heart rates quicker than control sheep. This may indicate that they were no longer experiencing a stress response due to the environment (once the attachment procedure was finished), but also may be due to the lack of space in which to move (unlike the control sheep, most of whom where seen to engage in grazing immediately post-release to pasture).

The time spent feeding was reduced in the sheep moved from grazing to inside penning, as sheep did not need to forage and may be able to eat more dry matter in a shorter period of time than when eating grass. Interestingly, there was no difference in the total time spent ruminating, or the number of bouts, nor the mean bout length of rumination. The amount of concentrates offered were the same between the controls and the treatment sheep (and therefore the protein content of the diet would be similar) however, the change from grass to forage might have altered the rumination time (Pearce, 1965). Future research may use the EMG information as a method to analyse the number of chews made in each rumination cycle, to assess differences in rumination associated with the change in forage (Kaske et al. 2002).

It is likely that changes in sleep after movement to inside penning could be due to changes in light duration and intensity (particularly at either end of the daylight period when the artificial lights were switched on and off), especially as light seems to be such an important zeitgeber in sheep at pasture (Lincoln et al. 2003, Tobler et al. 1991). However, the changes that occur in the suprachiasmatic nucleus and the pineal gland as a result of changes in day-length and light quality may take time to take effect. Sumová et al. (2002), suggest that the circadian clock of rats has 'memory'. If sheep have a similar mechanism, any differences seen in sleep of sheep during the first

24-h post-movement were unlikely to be caused by changes in light intensity or photoperiod. On the other hand, in a study investigating the circadian rhythms of pineal melatonin secretion in pigs after abrupt lighting changes, Tast et al (2001) found that pigs could respond immediately (within 24-h) to some changes (lighting changed from long-day to short-day) and within 1wk to other changes (short-day to long-day). In the present study, the photoperiod was not dramatically altered (as in the above pig study) but subtle differences especially around dawn and dusk would have been present. What effects these subtle differences in light would have on the sheep circadian rhythms are unclear.

So, why is it that sheep, cats (Wallach et al. 1976) and to a lesser extent rats (IIrmiš, 1971) show the opposite reaction in terms of sleep to humans on the first night after movement to a novel environment? It is possible that humans are fundamentally different from other mammals in terms of the relationship between sleep and stress (further research on non-human primates may reveal further details). Moreover, humans do seem unique in being able to worry about abstract thoughts and future events and this can have an effect on sleep (e.g. Åkerstedt et al. 2002), perhaps this is why the first night of a sleep recording in the laboratory or at home is so different to subsequent nights, with night two experiencing more REM and Non-REM sleep as a result of partial sleep deprivation. Sheep may show fear and anxiety when exposed to novelty, but there is no evidence that they have anxiety about future events (far future). The reactions of sheep may be due to the stressors from the recent past and the anxiety of the present novel situation. However, there does seem to be psychological assessment of the environment by the sheep, the changes in sleep that were present in the first 24-h post-movement are no longer present in the 7d post-movement (apart from the distribution changes). Therefore, these alterations in sleep (increase in REM sleep and changes in the spectral properties of Non-REM) cannot be due to the physical difference in the environment alone, as the environment was the same 7d post-movement. There is evidence that learning can affect subsequent sleep in humans and in rats (e.g. Maquet, 2001). It is possible that some of the changes seen in sleep in the first 24-h post movement were associated with learning about the novel situation and habituation to it. There may be no further learning after 7d, which may account

for the difference between the 7d post-movement recording and the 24-h post-movement recording.

Different strains of mice exposed to a novel environment exhibit different changes in REM sleep. Those strains of mice that are most 'anxious' (exhibit less locomotion and more defaecation in novel environments) have a greater reduction in REM sleep in the hours after exposure to a novel environment than less anxious strains (Tang et al. 2004). Breed has been shown to have an effect on the amount of fear reactions sheep exhibit in a novel environment, with heavier (lowland) breeds reacting less than lighter (hill sheep) breeds (Romeyer and Bouissou, 1992). The current experiment used Dorset and Dorset cross ewes, which are heavy, lowland sheep. The breed was used partly because of its head morphology (wide flat heads, for ease of electrode attachment) and partly as the breed is docile, and easy to habituate to human contact (for ease of electrode attachment). It is likely that a lighter hill sheep breed, such as the Scottish Blackface would find movement to a novel inside environment more aversive and the stress response in terms of changes to the quality and quantity of sleep may have been quite different.

Finally, it is interesting to find an increase in eye movement density in REM episodes 7 days post movement as compared to baselines and 24h post-movement. Very little work has been done in this area in non-humans, but in humans, there is evidence to suggest that an increase in eye movements is seen in subjects who are suffering from chronic stress, or who are clinically depressed (Douglass et al. 1992). More research is needed to determine the factors that affect eye movement density in sheep.

3.6 Conclusion

Sheep showed an increase in the total time spent in REM sleep and an increase in the intensity of Non-REM sleep after movement to a novel environment. This difference was not just due to the reduction in size and complexity of the environment as these alterations in sleep were no longer present 7 days post-movement. The increase in REM sleep and increase in slow-wave activity in Non-REM sleep that occur in sheep during the 24 hours post-movement indicate a response to the aversive experience of movement and the novel environment. As this response is no longer

present 7 days post-movement, the changes in REM and Non-REM are indicative of a return to baseline levels after a period of psychological adaptation. As sheep exhibit an increase in REM sleep, rather than a reduction post-movement, the results suggest that a movement to novel inside penning with a companion is a mild stressor and not a particularly fearful experience for adult sheep. The increase in REM sleep may also be partly due to the learning processes associated with the sheep habituating to the novel environment.

However, there were long-term changes in the sleep distribution, still present after 7 days post-movement, which may be indicative of environment mediated changes to the circadian rhythm of sheep. Further research is necessary to ascertain whether the distribution of sleep in sheep is permanently altered by movement to small, simple physical environments such as, inside penning. If the distribution of sleep is different in penning as compared to pasture, experimental sleep data that is taken from sheep housed in inside penning may not be able to be extrapolated to sheep at pasture.

Chapter 4. Effects of an 8h road journey on sleep in sheep

4.1 Abstract

In humans, sleep can be disturbed after stressful experiences and tiredness and fatigue can affect sleep quality and quantity. This experiment investigated whether the quantity, quality and distribution of sleep in sheep were affected by an 8h road journey. Simultaneous, behavioural recordings and non-invasive 24-h EEG, EOG, EMG and ECG recordings were made from 10 pairs of ewes while in inside pens as a baseline. One-week later, simultaneous behavioural and electrophysiological recordings were made of control (remained in indoor penning) and treatment ewes (loaded, transported by road for 8h, unloaded and returned to penning for a further 16h). The journey consisted of two similar 140-mile loops on a variety of single and dual carriageway roads, separated by a break in the journey of 22mins. Post-transport the treatment sheep were returned to the pens and recorded for a further 16h.

Each 24h recording was split into three 8-h periods, the transport period and two 8h post-transport periods. The data were analysed using mixed models analysis of the difference between control and treatment sheep comparing like time-periods from the baseline and the treatment recording days. Bout frequency data were analysed using G.L.M.M. procedure comparing like time periods. Depending on data distribution, Paired t-tests or Wilcoxon signed rank test were used to analyse the behaviour of sheep during the two transport loops.

Treatment sheep did not sleep during the 8h transport. They stood for longer during transport (72 ± 7.4%) than during the baseline recording (34 ± 2.8%) ($t = -6.4$, $P<0.001$) and stood more in the first journey loop (78 ± 8.5%) than in the second (66 ± 2%) ($t = 2.73$, $P<0.05$). Rumination and the relaxed 'lying head down' posture were observed infrequently during transit. Control sheep also stood for longer during the transport period (38 ± 5.1%) as compared with baseline (31 ± 3.1%) ($t = -2.6$, $P<0.05$). The controls had fewer bouts of Non-REM sleep during the transport period (3.2 ± 0.6) than during the baseline (6.3 ± 0.8) ($t = 2.3$, $P<0.05$).

After unloading from the vehicle, treatment sheep lay down within 2mins and began eating. After their first eating bout (18 ± 3mins) sheep switched to rumination (32 ± 1mins), this pattern was repeated for the first 2h post-transport. Post-transport,

treatment sheep had fewer (8.6 ± 0.8 to 11.7 ± 1, F = 2, P<0.05) and shorter bouts (251 ± 23s to 288 ± 27, t = 2.4, P<0.05) of Non-REM sleep compared with controls. Treatment sheep had a higher percentage of Delta waves in post-transport Non-REM sleep (42 ± 4.5%) than during baseline recordings (25 ± 2.1%)(t = 5.6, P<0.001). There were no differences between control and treatment sheep in REM sleep post-transport as compared with the baseline recordings. Treatment sheep had fewer bouts of REM post-transport (5.3 ± 0.7) than during the baseline (7.1 ± 0.5) (F = 3.4, P<0.01). Treatment sheep had longer bouts of 'lying head up' (1022 ± 97 s compared with 843 ± 41 s, t = -3.9 P<0.05) and had fewer bouts of 'lying head down' (8.6 ± 1.1 compared with 12.6 ± 1.3, F = 3.1 P<0.01) in the first 8h post-transport than in the equivalent baseline period.

An 8h road transport journey affected the sleep of sheep in the first 8h post-transport, by reducing the amount of Non-REM sleep, and increasing the percentage of slow-waves (0.1-4Hz) during Non-REM sleep. Most differences in sleep between control and treatment sheep were no longer present by the second 8h post-transport period.

4.2 Introduction

4.2.1 Aims

To determine whether sheep sleep during road transport.

To investigate the effect of an 8h road journey on the distribution of sleep and wakefulness and the quantity and quality of sleep for 16h post-transport, to try and answer two main questions:

1. does the extra physical exertion required during transport result in sheep resting or sleeping more post-transport,
2. does the potential stress associated with the 8-h transport disrupt sleep post-transport.

To investigate the distribution of resting behaviour during transport to help determine if there is a relationship between resting and fatigue.

4.2.2 The transport of sheep

Sheep are transported in the UK and Europe for a variety of reasons: to and from auction markets, as breeding animals, sold on to finishers and for slaughter. Large numbers of animals bred in the UK are transported to continental Europe for slaughter. In 2003, 229848 sheep were transported from the UK to continental Europe (DEFRA, 2004). Road (and ferry) transport from the UK to the continent often requires long journey times, especially when sheep are destined for southern Europe. In the UK, the majority of journey times are shorter, with the average period of transit of 4.7h for sheep sent from farm to slaughter (Warriss et al. 1990), however time spent in transit can become considerably increased when sheep are sent to market prior to slaughter (Warriss, 1996). In 1995, 19.3 million sheep were slaughtered in the UK and 65% of those were sold via livestock auction markets (Murray et al. 2000)

Under current European legislation, maximum journey times for sheep are 8h, though this may be extended if transporting vehicles meet further requirements, to 14h of transport. After the 14h, sheep must have a rest period of at least 1h sufficient to provide water and if necessary feed, however, the sheep can remain on the vehicle. A further 14h of transport can follow the break, after which the sheep are required to be unloaded and rested with feed and water for 24h (Council Directive 98/290/EEC, The Protection of Animals during Transport).

There is public concern about the welfare of sheep during transport. There are many factors in the transport of sheep that they might find aversive, stressful or may otherwise affect their welfare. These include: initial rounding up and handling; loading and unloading (Parrott et al. 1998b); mixing of social groups; reduced space allowances leading to lying deprivation (Cockram et al. 1996; Knowles et al. 1998); the novelty, noise and vibration of the vehicle; temperature, humidity and ventilation extremes (Randall, 1993); the time spent in auction markets (Knowles et al. 1994b); the length of the journey and the physical exertion of maintaining balance (and possible traumatic events) which may lead to fatigue (Cockram et al. 2004). Which parts of transport a sheep finds most stressful may depend on how that animal perceives and reacts to them, which could relate to previous experience, age, sex and breed of the animal (Manteca and Ruiz de la Torre, 1996). Many studies have been

carried out in an attempt to discover the effects of transport on the behaviour and physiology of sheep (see below).

A fatigued animal is not considered fit for transport (Welfare of Animals (Transport) Order, MAFF, 1997), however there is not a fixed definition of what fatigue is in farm animals, nor enough information on the indictors of fatigue for inspectors of animals to reliably assess an animal's fitness for transport. In humans, fatigue is considered a multidimensional concept: including being physically fatigued (e.g. bodily aching, less able to carry out physical tasks etc.) mentally fatigued (feeling tired and becoming less able to carry out cognitive tasks) and having increased sleepiness (an increased tendency to fall asleep); and is often measured by self-report. It is possible that these categories of fatigue can be used in some way to determine fatigue in farm animals in order to assess their fitness for transport.

4.2.3 Physiological indications of stress during transport

There have been many studies carried out to investigate the physiological stress responses of sheep and other animals to transport. There is evidence to suggest that sheep find transport stressful. Sheep show a plasma cortisol response to the early stages of transport (e.g. Cockram et al. 1996). Parrott et al (1994), found that simulated transport had the highest associated plasma cortisol, prolactin and adrenaline concentrations after 30min in sheep than other stressful experiences (including isolation and standing in water).

The loading of sheep onto the vehicle could cause a stress response. However Cockram et al (1997) showed that the stress response was due to the actual transport not just to the loading onto the vehicle. They measured plasma cortisol concentrations from sheep during continuous 24h transport and 24h confined to a stationary vehicle. The study found that cortisol concentrations were higher in sheep transported for 24h than those confined to a stationary vehicle throughout the 24h period. Broom et al (1996) came to a similar conclusion as they found that plasma cortisol concentrations from the sheep were higher when transported than when stationary.

Heart rate changes are often used as a measurement of stress during transport. Kent (1997) reports an increase in heart rate of sheep seen during loading and the first 3 hours of road transport, which appears higher than that from sheep confined on a

stationary vehicle. After the first 3 hours, the heart rate decreases and remains stable except for sudden peaks until increasing again during unloading.

4.2.4 Physiology and fatigue and recovery of physiological stress post-transport

Changes within the blood plasma can be measured to investigate not only the physiological stress responses of a sheep to transport, but also reflect the changes in the amount of work and damage of muscle tissue during and after transport. High plasma activities during or post-transport of the cellular enzymes creatine kinase and lactic dehydrogenase, for example, can be an indication that transport may be associated with muscle activity/fatigue and also be indicative of muscle injury (e.g. Boyd, 1988).

In an investigation of the effects of transport and lairage durations on lambs, Knowles et al (1996) found that plasma creatine kinase activity was high during transport and they decreased rapidly during 12h of lairage. Plasma creatine kinase activity continued to fall during the subsequent 36h of lairage, bringing them down towards probable pre-transport activity levels (no pre-handling levels were collected so no absolute baseline was available). Knowles et al (1998) found increased creatine kinase activity in lambs that had been transported for 12h at high stocking densities as compared with lower stocking densities. Here, it is possible (although not quantified) that sheep did not lay down as frequently at high densities compared with low densities and were therefore more fatigued, showing muscle damage. However, many studies do not find an increase in plasma creatine kinase activity during or post-transport in sheep (Cockram et al. 1996; Cockram et al. 1997; Broom et al. 1996)

An issue relating to the physiological stress of transport is that of recovery post-transport. The length of recovery may give some indication of how stressful the transport procedure was. In most studies in which sheep have been transported for 30h or less, blood constituents return to baseline levels/concentrations within 24-h post-transport (e.g. Cockram et al. 1996). In one of the most extreme studies of long-distance transport, Sutton and Van den Heever (1968) investigated the effects of 2 to 5 days unbroken rail transport on sheep (without food and water). Half the sheep from each journey group were slaughtered at the end of the journey, the other half

were put into lairage with food and water for 24h before slaughter and a group of sheep were slaughtered without prior transport as controls. The sheep had exhausted their liver glycogen energy stores and had mobilised other energy resources as ketone levels were raised post-transport. They concluded that sheep were physiologically abnormal, even after 24h rest with food and water, when transported for 3 days or more, and therefore 24h is not long enough to recover from the effects of 3 days rail transport (Sutton and Van den Heever, 1968).

4.2.5 The behaviour of sheep during transport

Researchers often use behaviour in an attempt to ascertain fatigue in transported animals and will state e.g. 'Simple observations of resting behaviour...can indicate the levels of fatigue'(Warriss, 1996) without being able to distinguish between a fatigued animal and an animal comfortable with, or habituated to its surroundings. Hall et al (1998) suggest that 'lying in a relaxed position [during transport] might be seen as an indicator of good welfare', however, as the authors point out, this would be impossible in the majority of commercial conditions as there would be too high a stocking density to observe lying down with the head down. Few authors have noted the quality (relaxed or vigilant) of the lying behaviour of sheep during or after transport (however this has been done with dairy calves, e.g. Kent, 1977). More research should be carried out on the quality of the resting behaviour, of animals during transport.

The type of road can affect the behaviour of sheep, as road type will influence the level of physical exertion required to remain balanced while standing and the likelihood of the sheep lying down. For example when driven on minor roads, sheep are often required to make changes in posture to maintain balance, using energy, however when driven on motorways the journey is smooth enough allowing the sheep to lie down to rest and ruminate (Cockram et al. 2004). If a sheep has to make many adjustments in posture to remain standing on a windy road, it is probable that minor roads would be more tiring than travelling on motorways. On motorways, there are less driving events that affect the sheep, and they can lie down more frequently and for longer bouts than on minor roads (Cockram et al. 2004).

Sheep behaviour during transport may also be affected by the quality of the driving; Cockram et al (2004) found differences between drivers in the amount of lying and rumination that occurred in the sheep when transported by different drivers.

Different forms of transport may also affect an animal's ability to rest during transport. Horses have been shown to be able to rest during air transport when the plane is in level flight, and may be able to go into light sleep (Stewart et al. 2003). Observations of adult sheep during rail transport for up to 5 days without food and water found that sheep rarely lay down during transit (Sutton and Van den Heever, 1968).

The longer a road journey, the more likely sheep are to lie down (Cockram et al. 2004). After first 3h of a 12h road journey, Cockram et al (1996) found that sheep at high stocking densities lay down less than sheep with more space.

Hall et al (1998) found that hardly any lying (0.01 of the time) was seen during an 8h road journey at a relatively high space allowance (0.59m^2 per sheep). Knowles et al (1993) found that more non-transported sheep penned at a space-allowance used during transport lay down at any one time than transported sheep and that sheep transported for 14h rarely lay down during the first 5h of transport.

4.2.6 The behaviour of sheep after transport

Lairage is often used in slaughterhouses, partly to ensure slaughtering efficiency, but also to allow the animals to rest, drink and eat after a journey. In addition, lairage is required by law during long journeys, for example if a journey is to take longer than 29 hours, sheep must be unloaded and a lairage period of 24 hours is required (Council Directive 98/290/EEC, The Protection of Animals during Transport). The duration of lairage on arrival at a slaughterhouse can be varied, Warriss et al (1990b) found that in one plant, 66% of sheep were killed within 10h of arrival (mean 8.8h lairage), whereas in another plant 37% of sheep were killed within 10h and 30% of sheep spent over 20h in lairage (mean 17.9h lairage). Jarvis and Cockram (1995) describe the behaviour of groups of sheep for the first 3 hours of lairage post-transport. They found that at least one sheep in a group would lie down within the first 30 minutes followed by other individuals. Sheep spent 17% of the first 3 hours of lairage lying down and this was not affected by transport duration, arrival

time or group size (although it should be noted that the transport durations only differed by 40 mins and arrival time differed by 2h 35 mins) (Jarvis and Cockram, 1995).

Parrott et al (1998a) recorded behaviour and food and water intake during a 1h period of lairage from fleeced and shorn sheep that had been transported for 14h. The authors found that all sheep spent the majority of the lairage hour eating. Some of the fleeced sheep lay down, but only for the last 10mins of the lairage period; none of the shorn sheep lay down during the lairage period.

In an experiment that investigated the effects of duration of lairage on the behaviour and physiology of sheep during lairage, during post-lairage transport and post-transport, Cockram et al (1997) found that after sheep had been transported for 12h, eating was a priority. Rumination was only observed in the third hour of a 3h period of lairage and in the 12h post-transport, sheep were seen to ruminate for approximately 30% of the time. Sheep also spent the first 2h of lairage standing and were seen to lie down during the third hour (Cockram et al. 1997). Sheep lay down less in first few hours after transport than in the corresponding time pre-transport. The decrease in lying lasted for 4h in sheep that had not been given access to food or water during transport and 3h or 2h in sheep that had been offered food for 3 h during transport or 12h during transport respectively. This indicated that the priority of sheep post-transport was to eat rather than to rest. It should be noted that the space allowance during this experiment enabled the sheep to lie down during transport.

Similar observations were made by Knowles et al (1994a) from lambs after 18 and 24h road and ferry journeys. Lambs were observed to eat first, then drink and finally to rest during the first few hours of lairage, however there are no statistics to back up these observations. In a further experiment, Knowles et al (1998) observed sheep after 12h road transport. They found that there was more standing, eating and drinking in sheep during the first 5h post-transport than in pre-transport baselines, however between 5 and 24h post-transport there was a decrease in standing compared with the baselines. This may indicate that the first priority of sheep post-transport is to replenish food and water and once satiated the next priority is to rest.

Observations of sheep after unbroken rail journeys of between 2 and 5 days showed that sheep changed their priorities from food to water as the length of the

journeys increased, with sheep unloaded from a 5-day rail journey only feeding after drinking (Sutton and Van den Heever, 1968), unfortunately resting behaviour was not observed after transport in this study. In order to determine whether drinking and resting would become priorities for sheep in post-transport lairage in the absence of food, Cockram et al (1999) observed the behaviour of sheep in lairage for 12h subsequent to 15h of transport (and non transport controls), either with or without access to hay. The authors found that sheep without hay lay down sooner than sheep with feed (hay), but there was no difference in the time spent lying down in the groups that had or had not been transported. Sheep in lairage without food spent more time foraging and investigating the environment than sheep with hay. The authors suggest that sheep may only rest post-transport after the possibility of food has been explored (which is the priority of sheep after a period of starvation) (Cockram et al. 1999).

4.2.7 Increased sleepiness in relation to physical fatigue and transport

During transport it is possible that sheep can get mentally tired (i.e. have increased sleepiness) as well as physically tired, e.g. the motivation to rest and sleep may increase over the journey time as sheep get more tired. There are to date no published reports looking at mental tiredness in farm animals during and after transport. Stressful situations have been shown to affect subsequent sleep in a variety of ways (see chapter 1 for a review) (e.g. Cespuglio et al. 1995; Dewasmes et al. 2004; Meerlo et al. 2001). Moreover, changes in sleep pattern have been shown to affect how animals react to subsequent stressors (e.g. Brock et al. 1994). It is important to know how long sheep need to recover from aversive experiences to minimise compromises to welfare, especially during long-distance transportation.

There is research on the links between physical exertion, mental fatigue and increased sleepiness in humans. In situations where humans are undergoing poor sleep patterns, such as shift work, jet lag and sleep deprivation experiments, exercise increases sleepiness (e.g. Åkerstedt et al. 2002 and Young et al. 1998, respectively) In rats too, light physical exercise has been shown to increase sleepiness and increase the length of subsequent Non-REM and REM sleep (Gambelunghe et al. 2001). For people who have normal sleep patterns however, there is conflicting evidence that

regular light exercise affects sleep, some research shows that it does not (Youngstedt et al. 2003), others that it does (Sasazawa et al. 1997). The difficulty here is deciding when physical exercise becomes physically fatiguing, potentially leading to mental fatigue and/or sleepiness, especially if an animal is unable to rest (e.g. due to long distance transport at low space allowances).

It is important that, in humans, mental fatigue and increased sleepiness should not be confused. In a multi-factorial study on mental fatigue, work and sleep, Åkerstedt et al (2004) found that physical exercise and active jobs did not increase mental fatigue, but did increase sleepiness –leading to a perception of a better night's sleep. Whereas, psychological stress from work or home life did increase mental fatigue (leading to a perceived reduction in ability to do cognitive tasks) and was correlated with disturbed sleep.

In relation to transport, or in human terms, driving or being a passenger, there has been a lot of research into the effect of driving on mental fatigue and sleepiness. Driving for long distances has been shown to have an effect on mental task performance, indicating mental fatigue (Phillip et al. 2003). There is evidence to suggest that driving between midnight and 0600h can be more mentally fatiguing than at other times (Feyer and Williamson, 1995).

Considering that it is illegal to transport a fatigued animal in the UK, it is perhaps surprising that more research has not been carried out on the factors which affect fatigue in transported animals, the amount of time needed to recover from fatigue and the indicators of fatigue in animals. The current experiment will be the first to explore using a combination of behaviour and electrophysiological recordings, whether sheep sleep and rest during an 8h road transport journey and will investigate whether sleep is disturbed in sheep post-transport in an attempt to start to answer some questions on fatigue, tiredness and sheep transportation.

4.3 Materials and Methods

4.3.1 Animals

Twenty-four, adult, polled, Dorset cross, ewes that had been kept outside on pasture, were randomly paired. Within each pair, sheep were randomly assigned to either a control or a treatment group.

4.3.2 Husbandry details

The experiment was carried out between March 2003 and September 2003. Sheep were brought into inside penning at least two weeks before they were used in the experiment to allow for habituation to the surroundings and the handling procedures. Four sheep were housed in individual 1.2 X 1.8 m pens bedded with straw lit with artificial lights on a 16hL:8hD cycle. Pairs of sheep were brought into the experimental barn on a staggered basis so that there were always one pair on experiment and one pair habituating to the environment. The habituating sheep would act as companions for the control sheep on the transport day. Sheep were offered approximately 250g/d/sheep concentrated feed (Pentland lamb finisher, Seafield Mill, UK) from a bucket and *ad-libitum* hay and water. The sheep were habituated to the attachment of a harness and fibre-glass helmet (as described in the general methodology section).

4.3.3 Experimental treatment schedule

Twenty-four-hour electrophysiological recordings were made from a control and treatment sheep during a baseline and a treatment day as shown in Table 4.1. Seven days after the baseline recording the sheep assigned to the treatment group was transported on a road journey for approximately 8h and then returned to the experimental barn. The control sheep remained in the experimental barn.

Table 4.1. The experimental treatment schedule

24-h Recording Number	24-h Recording Name	Day	Treatment Group	Control Group
1	Baseline	-7	Inside Pen	Inside Pen
2	Transport day	0	8h road transport and returned to inside pen	Inside Pen

4.3.4 Electrophysiological recordings

On the morning of the baseline recording, both the treatment and control sheep were fitted with electrodes and 24h electrophysiological recordings of the EEG, the EOG, EMG from the jaw, neck and hind-leg and the ECG were made while the sheep remained in their home pens. In addition, 24-h time-lapse video recordings were made of both sheep. The electrode attachment, removal, and electrophysiological techniques were carried out as outlined in the general methodology section (chapter 2).

On the transport recording day both treatment and control sheep were fitted with electrodes and 24h electrophysiological recordings were started, the treatment sheep was loaded around 0900h, with a non-experimental companion sheep, into a trailer (see below for details) and driven on a standard road journey for approximately 8 hours. The treatment sheep did not have access to food or water during transit, although sheep could consume the straw bedding. When transport was completed, the treatment sheep was unloaded and returned to its home pen for the remainder of the 24h. The control sheep remained in the inside pen (alongside two other sheep) with access to hay and water. Time-lapse video recordings were made of the treatment sheep during transport and of the control sheep and treatment sheep while penned.

After completion of two, successful, electrophysiological recordings, the sheep were inspected by a veterinary surgeon and returned to the main flock. This procedure was repeated until satisfactory electrophysiological recordings had been made from ten pairs of sheep.

Figure 4.1 The experimental sheep wearing electrophysiological equipment in the trailer. Companion sheep in adjacent pen (just seen)

4.3.5 Vehicle equipment details

A single deck, metal, rear loading trailer (Ifor Williams, UK) towed by a Landrover Defender (LandRover, UK), was used for each journey. The trailer was divided into three sections with metal hurdles. The rear sections enabled each sheep (treatment and companion sheep) to be in adjacent single pens (0.9 X 1.85m), which were bedded with straw.

The front section contained the recording equipment and batteries. Two black and white CCTV cameras (Panasonic WV-BPL24, UK) were fitted laterally and in front of the treatment sheep pen to record the behaviour of the treatment sheep during transit. Two lamps were fitted adjacent to the cameras to provide light during transit. Another CCTV camera recorded the visual display of a *G*-force meter (AC22 Performance meter, Race Technology Ltd, Nottingham, UK) that was fixed at the front of the trailer at sheep body height (45 cm from the trailer base) to record the approximate force experienced by the sheep during cornering events. A further CCTV camera was fixed to the dashboard of the Landrover to record the road type and driving events during transit. All four cameras were attached to a digital multiplexer (Sprite DX dedicated micros, Manchester, UK), and a time-lapse video cassette recorder (12V, TLSS8000, S-VHS, Timelapse Sanyo, Japan). The video time was synchronised with the electrophysiological data-logger before the sheep were loaded. A monitor (powered by the Landrover battery) was carried during transit to check for video recording problems. All electrical equipment in the trailer was powered by four 12V leisure batteries (110 amp hours, Fulmen Giant, Fulmen UK), with the direct current being transformed to alternating current by a mains converter to power the cameras and the multiplexer.

4.3.6 Road journey details

Four experienced livestock drivers were used. Driver 1 drove three first half journeys and four second half journeys, driver 2 drove three first half and three second half journeys, driver 3 drove three first half and three second half journeys, and driver 4 drove one first half journey. Transport-recording days always took place on the same day at a similar time to minimise traffic differences. Transport started from the Moredun Research Institute (Midlothian, Scotland, UK) between 0900 and

0920h (except for one recording which started at 1010h), travelling on B-graded roads for 8.6 miles and A-graded roads for 54.7 miles (including 34.8 miles of dual carriageway) to Berwick-upon-Tweed (Northumbria, England, UK) and back along A-graded roads for 59.7 miles to the farm. The vehicle remained stationary for 20-25 minutes and driver was changed over, allowing for the sheep and electrical equipment to be checked. A similar journey was then carried out. On the final return to the farm, the treatment sheep was unloaded and returned to its inside pen.

The mean (± s.e.m.) duration of the 10 transport journeys from when loading of the experimental sheep started, including both journey loops and the driver break, until unloading of the sheep finished was 7h 45mins ± 13mins. The break between the two loops was, on average, 22 ± 3 mins in duration. The journey (by duration) was made up of 77 % A listed roads (which included 28 % dual carriageways) and 17% B listed roads, the remaining 6% of the journey was on unclassified roads on the farm. There were 22 roundabouts per loop. Using data supplied by the *G*-force meter (analysed for 6 of the journeys), on average there were 4.3 ± 1.5 corners above 0.3*G*, 25 ± 9.3 corners between 0.2 and 0.3*G* and 44.8 ± 5.2 corners between 0.1 and 0.2*G* per loop.

4.3.7 Electrophysiological and behavioural analysis

The behavioural and electrophysiological recordings were analysed as detailed in the general methodology section (including the REM sleep eye movement density analysis detailed in the Novel Environment chapter 3).

4.3.8 Statistical analysis

Descriptive statistics were carried out in Minitab 13 (Minitab, USA). Data were tested using the Anderson-Darling test for normality to confirm the distribution of the data. Paired Student's t-tests were used to compare differences of behaviour during transport. Where the during-transport data were not normally distributed, Wilcoxon signed rank tests were used instead of the paired t-test.

All data comparing controls and treatment sheep, except for bout frequency and sleep pattern analysis were carried out using the mixed model analysis of variance with repeated measures on the difference between control and treatment

sheep comparing like time periods from transport and baseline recording days in SAS 8.2 (The SAS Institute, California, USA). Bout frequencies were analysed using general linearised mixed models comparing like time periods in SAS 8.2. The method for carrying out these analyses is described in chapter 3. The sequences of behaviour and sleep post-transport were analysed using lag-sequential analysis in Observer 4.1 (as described in the general methodology section chapter 2).

However, as the post-treatment period was only 16h (compared with 24h in the Novel Environment experiment) each 24-h recording (both baseline and treatment) was split into 3 subsequent 8-h time periods. Recordings were split into: period 1 (approximately 0900-1700h), period 2 (approx 1700-0100h) and period 3 (approx 0100-0900h). Treatment day recordings were split into: period 1 (approximately 0900-1700h, which always included the transport journey time from loading to unloading of the treatment sheep), period 2 (Post unloading-0100h) and period 3 (0100-0900h) as shown in Table 4.2. Driver was added as a factor in the model.

The mixed model and G.L.M.M. estimate statements were: period 1 baseline v period 1 treatment; period 2 baseline v period 2 treatment; period 3 baseline v period 3 treatment; and (period 2 + period 3 baseline) v (period 2 + period 3 treatment).

Table 4.2. The 8-h time periods from the 24-h recording days

Recording Day	0900-1700h	1700-0100h	0100-0900h
Baseline	Period 1	Period 2	Period 3
Treatment	Period 1	Period 2	Period 3

4.4 Results

4.4.1 Active behaviour during transport

The reaction of the sheep to events during the journey was analysed for six of the sheep. There were three falls in total during the six journeys. On average sheep lost their balance on 8 ± 2.8 occasions per journey. Sheep were always standing when the vehicle was going round roundabouts and corners that registered 0.1*G* and above on the *G*-force meter. This meant that even if sheep were lying down directly before the roundabout or corner they were always standing after the event.

4.4.2 Rest and sleep during transport

Sheep were never assessed as asleep during transport (N=10) or during the driver break. Sheep behaved differently regarding their posture depending on whether they were on the first loop of transport or the second loop. The comparisons of posture during each loop of the journey (excluding the driver break) are shown in Table 4.3.

Table 4.3. Comparison between journey loops of the 'lying head up' posture in treatment sheep (n = 10).

Lying with head up	1st Transport Loop mean ± s.e	2nd Transport Loop mean ± s.e	Statistical significance of difference
% loop spent Lying Head Up	22 ± 8.4	33 ± 7.1	*
Duration of bouts (s)	510 ± 141	546 ± 139	NS
Latency to lie down after start of loop (s)	1602 (205 – 5683)†	420 (10 – 1421)†	*

Statistical probability level, * = P<0.05, ** = P<0.01 and NS = P≥0.05. Paired t-test used for normally distributed data. †median (Q_1-Q_3), Wilcoxon signed rank test used for non-parametric data.

The average time spent lying head up during each hour in transit is shown by Figure 4.2.

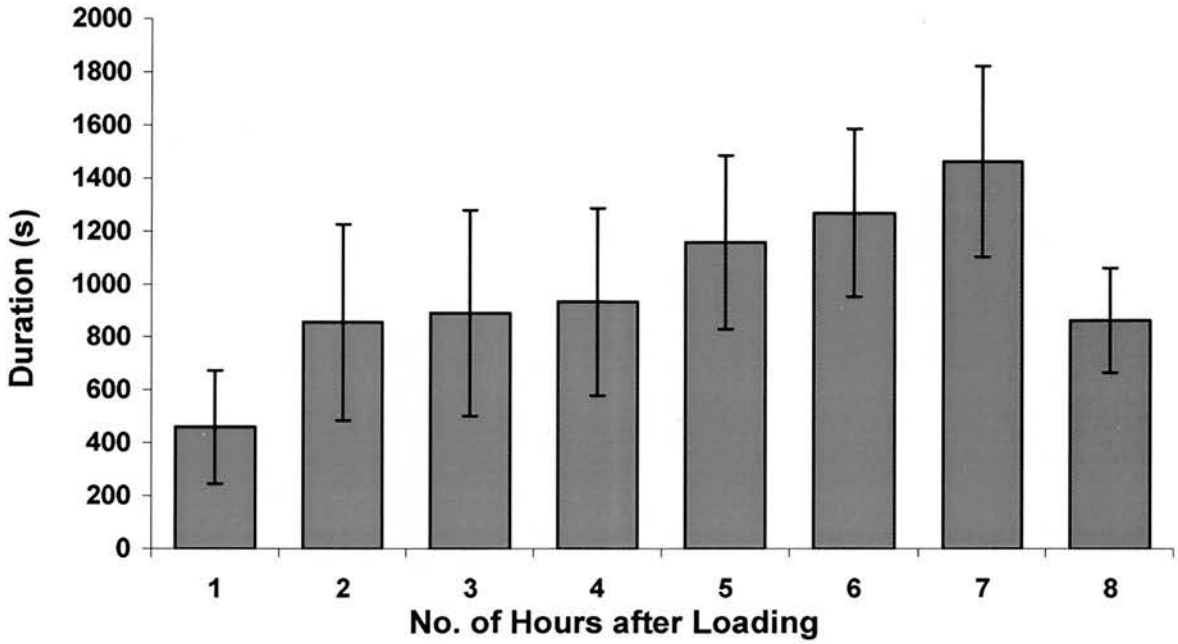


Figure 4.2. Mean duration per hour that the treatment sheep (n=10) spent Lying with Head Up during transport (vertical bars indicate s.e.).

Two of the 10 treatment sheep were never observed to lie down during transit or in the driver break. Lying down with the head resting on the bedding, the characteristic sleep posture of sheep, was seen very infrequently during the transport. Two sheep were recorded in the lying head down posture on the first loop of the journeys, both on one occasion, for a maximum bout duration of 211 seconds. Four sheep were recorded in the lying head down posture on the second loop of the journey, all on one occasion (the shortest bout length was 36s and the longest bout length was 488s). All occurrences of lying head down were recorded on straight dual carriageways. The 'lying head down' posture during transport was accompanied by an EEG trace with low voltage, high frequency waves, indicative of, and scored as 'awake' by the Somnologica sleep programme.

Four sheep ruminated during the first loop of the journey and five sheep ruminated on the second loop. On the first loop of the journey, the total duration of rumination ranged from 126s to 844s, on the second, the total duration ranged from 316s to 2338s. On the first loop of the journeys all sheep that ruminated did so on one occasion, on the second loop up to three bouts of rumination were observed.

During the break period, six of the 10 sheep remained standing, the four that lay down all lay down within 10mins of the engine being switched off. Five sheep were seen to ruminate during the break period, including all four that lay down and one of the sheep that remained standing.

4.4.3 Effects of transport on sleep

4.4.3.1 Non-REM sleep

Table 4.4 shows the effects of transport on Non-REM sleep.

There were no differences in the latency (from start of the period) to Non-REM sleep between treatment and control sheep, post-transport as compared with the equivalent baseline periods. Treatment sheep had fewer bouts of Non-REM sleep than controls during the 1st 8h post-transport (G.L.M.M., estimate 3.1 ± 1.2, F = 2.0, P<0.05) but not during the 2nd 8h post-transport as compared with the equivalent baseline. Control sheep had fewer bouts of Non-REM sleep during the transport period than during the equivalent baseline period (G.L.M.M. estimate 3.5 ± 1.4, F = 2.3, P<0.05).

Treatment sheep had shorter bouts of Non-REM sleep than controls during the 1st 8h post-transport (Mixed model, estimate 102 ± 55, t = 2.4, P<0.05).

The total duration of Non-REM sleep (as a percentage of the 8h interval) in the 1st 8h post-transport as compared with the equivalent baseline period differed between treatment and control sheep and was no longer different in the 2nd 8h post-transport compared with baseline. Treatment sheep had a significantly smaller percentage of Non-REM sleep in the 1st 8h post-transport than controls (Mixed model, estimate –5.7 ± 1.4, t = 3.1, P<0.01) and this difference was also apparent when the difference between control and treatment sheep were compared during this period and the equivalent baseline period.

The percentage of Delta waves in the second 30s epoch of each Non-REM bout of controls minus treatment sheep was different in 1st 8h post-transport as compared with the equivalent baseline period. There was a tendency for this difference to remain in the 2nd 8h post-transport as compared with baseline. Treatment sheep had a higher percentage of Delta waves during the second 30s

epochs of Non-REM bouts during the 1st 8h post-transport than during the equivalent baseline (Mixed model, estimate 17.2 ± 3.9, t = 5.6, P<0.001).

4.4.3.2 REM sleep

Table 4.5 shows the effects of transport on REM sleep.

There were no differences in the latency (from start of the 1st post-transport period) to REM sleep between treatment and control but treatment sheep had a higher latency to REM sleep in the 1st 8h post-transport than in the equivalent baseline period (Mixed model, estimate –4656 ± 2200, t = -2.12, P<0.05).

There were no differences between controls and treatment sheep in the number of bouts of REM sleep post-transport however, there was a significant decrease in the number of bouts of REM sleep in treatment sheep between baseline and 1st 8h post-transport (G.L.M.M, estimate 3.8 ± 0.9 F = 4.4, P<0.01).

4.4.3.3 Total sleep

Table 4.6 shows the effects of transport on Total sleep (Non-REM + REM sleep). There were no significant differences in total % duration of the 8h periods and no differences in the mean duration of sleep bouts between controls and treatment sheep. However, there was a tendency for treatment sheep to decrease the total time spent sleeping in the 1st 8h post-transport (Mixed model -5.8 ± 2.9, estimate, t = -2.0, P<0.06) and a tendency for a reduction in the number of bouts of sleep in treatment sheep in the 1st 8h post-transport (G.L.M.M, estimate -2.6 ± 0.9, F = -1.9, P<0.06) compared with the equivalent baseline period. In addition, control sheep had fewer bouts of sleep during the transport period than in the equivalent baseline period (G.L.M.M, estimate –4.0 ± 1.7, F = -2.6, P<0.05).

Table 4.4. Non-REM sleep (mean ± s.e) before, during and after an 8h road journey (n = 10 pairs)

Measurement	Time period‡	Baseline Recording Period		Treatment Recording Period		Difference between baseline and treatment period††	Statistical significance of the difference between baseline and treatment periods††
		Control	Treatment	Control	Treatment		
No. of bouts	1	6.3 ± 0.8	7.5 ± 0.8	3.2 ± 0.6	0 ± 0	n/a	n/a
	2	9.8 ± 1.2	11.7 ± 1	10 ± 1.1	8.6 ± 0.8	-4.3 ± 1.8	*
	3	7.9 ± 1	7.8 ± 0.8	7.4 ± 1.3	7.7 ± 1	0.4 ± 1.8	NS
Duration of bout (s)	1	270 ± 37	283 ± 22	295 ± 68	0 ± 0	n/a	n/a
	2	278 ± 19	278 ± 27	301 ± 52	251 ± 23	-259 ± 114	*
	3	269 ± 43	283 ± 29	300 ± 52	277 ± 33	-188 ± 85	NS†
% of time period	1	5.1 ± 0.8	6.5 ± 1.1	3.9 ± 1.2	0 ± 0	n/a	n/a
	2	8.8 ± 1.5	9.9 ± 1.7	9.3 ± 1.4	7.2 ± 0.2	-7.2 ± 2.1	**
	3	6.2 ± 1	6.8 ± 0.9	7.3 ± 1.6	6.8 ± 1.2	-1.1 ± 2.1	NS
Latency (s)	1	6730 ± 1567	5402 ± 1182	7644 ± 1558	n/a	n/a	n/a
	2	n/a	n/a	3822 ± 901	6512 ± 1518	n/a	n/a
% Delta waves in 30 s bouts	1	30 ± 2.4	29 ± 2.1	31 ± 2.9	n/a	n/a	n/a
	2	25 ± 1.9	25 ± 2.1	25 ± 1.8	42 ± 4.5	17 ± 3.3	**
	3	22 ± 1.7	24 ± 1.5	23 ± 1.8	28 ± 3	5 ± 1.7	NS†

The test used for the number of bouts was a GLMM, all other measurements were tested using the mixed model analysis with repeated measures.

†A statistical probability of P>0.05 but, <0.1

‡Time period 1 was equivalent to the transport period, time period 2 equivalent to the 1st 8h post-transport and time period 3was equivalent to the 2nd 8h post-transport.

††Differences between the equivalent 8h time periods before (baseline recording), during and after (treatment recording) in the differences between pairs of control and treatment sheep within an 8h time period.

Table 4.5. REM sleep (mean ± s.e) before, during and after an 8h road journey (n = 10 pairs)

Measurement	Time period[‡]	Baseline Recording Period		Treatment Recording Period		Difference between baseline and treatment period[††]	Statistical significance of the difference between baseline and treatment periods[††]
		Control	Treatment	Control	Treatment		
No. of bouts (s)	1	3.5 ± 0.8	4.5 ± 0.7	2.2 ± 0.3	0 ± 0	n/a	n/a
	2	7.4 ± 0.9	7.1 ± 0.5	5.9 ± 0.8	5.3 ± 0.7	-2.3 ± 1.4	NS
	3	5.5 ± 0.9	5.3 ± 0.8	4.3 ± 0.7	4.8 ± 0.6	0.7 ± 1.4	NS
Duration of bout (s)	1	207 ± 20	232 ± 37	263 ± 47	0 ± 0	n/a	n/a
	2	234 ± 25	227 ± 17	236 ± 21	289 ± 45	59.2 ± 54	NS
	3	247 ± 40	222 ± 27	249 ± 31	238 ± 22	14.4 ± 54	NS
% of time period	1	2.5 ± 0.6	3.3 ± 0.6	2.2 ± 0.4	0 ± 0	n/a	n/a
	2	6.9 ± 1.1	7.2 ± 0.8	4.7 ± 0.7	5.1 ± 1	-0.9 ± 1.3	NS
	3	3.9 ± 0.5	3.6 ± 0.6	3.7 ± 0.6	3.9 ± 0.7	0.5 ± 1.3	NS
Latency (s)	1	8856 ± 1972	7056 ± 1407	8521 ± 1636	n/a	n/a	n/a
	2	n/a	n/a	3838 ± 857	7447 ± 2298	4365 ± 2803	n/a
Eye-movement Density in 10s epochs	1	49 ± 17	53 ± 11	46 ± 18	n/a	n/a	n/a
	2	58 ± 11	55 ± 8	57 ± 7	59 ± 13	3.5 ± 7	NS
	3	46 ± 8	50 ± 10	51 ± 10	52 ± 11	-5.4 ± 7	NS

The test used for the number of bouts was a GLMM, all other measurements were tested using the mixed model analysis with repeated measures.

[†]A statistical probability of P>0.05 but, <0.1

[‡]Time period 1 was equivalent to the transport period, time period 2 equivalent to the 1st 8h post-transport and time period 3was equivalent to the 2nd 8h post-transport.

[††]Differences between the equivalent 8h time periods before (baseline recording), during and after (treatment recording) in the differences between pairs of control and treatment sheep within an 8h time period.

Table 4.6. Total sleep (Non-REM + REM sleep) (mean ± s.e) before, during and after an 8h road journey (n = 10 pairs)

Measurement	Time period[‡]	Baseline Recording Period		Treatment Recording Period		Difference between baseline and treatment period[††]	Statistical significance of the difference between baseline and treatment periods[††]
		Control	Treatment	Control	Treatment		
No. of bouts (s)	1	6.8 ± 0.6	7.2 ± 0.5	3.2 ± 0.7	0 ± 0	n/a	n/a
	2	9 ± 1.7	11.1 ± 1.1	10.8 ± 1.2	8.9 ± 0.7	2.6 ± 1.3	NS
	3	8.2 ± 0.6	7.7 ± 0.5	7 ± 1.1	7.2 ± 0.9	0.5 ± 0.4	NS
Duration of bout (s)	1	507 ± 49	530 ± 52	558 ± 79	0 ± 0	n/a	n/a
	2	532 ± 39	535 ± 57	557 ± 72	541 ± 42	-23.8 ± 17	NS
	3	536 ± 63	535 ± 59	544 ± 69	530 ± 52	-14.1 ± 8	NS
% of time period	1	7.6 ± 1.4	9.8 ± 1.7	6.1 ± 1.6	0 ± 0	n/a	n/a
	2	15.7 ± 2.6	17.1 ± 2.5	14 ± 2.1	12.3 ± 1.2	4.6 ± 2.5	NS
	3	10.1 ± 1.5	10.4 ± 1.5	11 ± 2.2	11.7 ± 1.9	-1.4 ± 1.2	NS

The latency to total sleep is the same as for Non-REM

The test used for the number of bouts was a GLMM, all other measurements were tested using the mixed model analysis with repeated measures.

[†]A statistical probability of P>0.05 but, <0.1

[‡]Time period 1 was equivalent to the transport period, time period 2 equivalent to the 1st 8h post-transport and time period 3was equivalent to the 2nd 8h post-transport.

[††]Differences between the equivalent 8h time periods before (baseline recording), during and after (treatment recording) in the differences between pairs of control and treatment sheep within an 8h time period.

As can been seen from Figure 4.3, treatment sheep do not make up the sleep lost during transport during the 16h post transport. The cumulative amount of sleep was significantly lower both at the end of the 1st 8h post transport (2-sample t-test t = 2.7, P<0.01) and the 2nd 8h post-transport in treatment sheep than in control sheep (2-sample t-test, t = 2.3, P<0.05). The cumulative amount of sleep was also significantly lower for treatment sheep at the end of the transport recording day as compared with the end of the baseline recording day (Paired t-test, t = 3.63, P<0.01). There was no difference between the control sheep baseline and transport recordings.

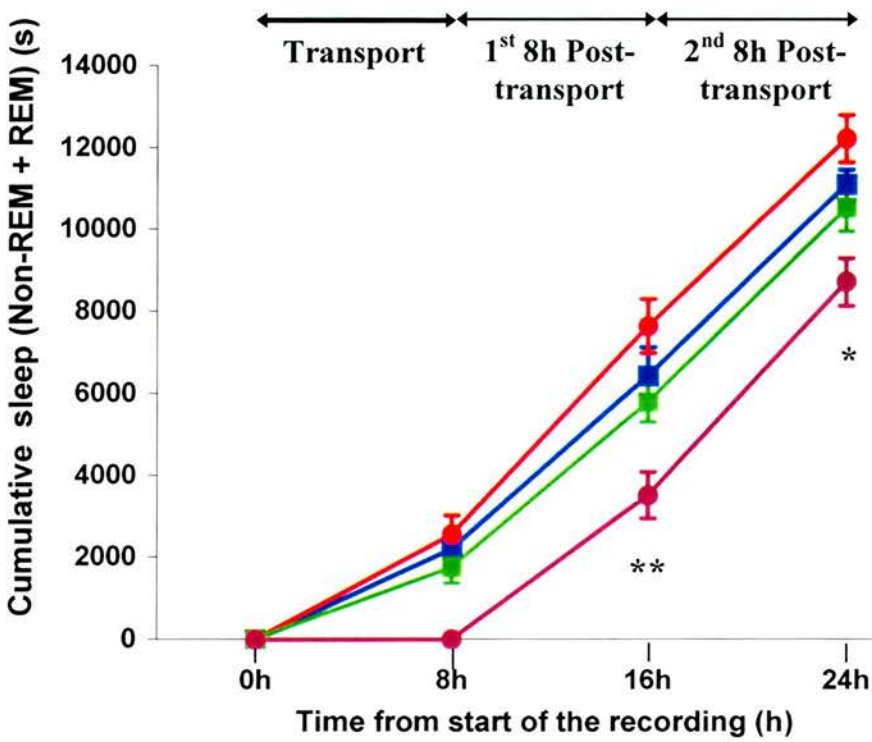


Figure 4.3. The cumulative amount of sleep (Non-REM + REM sleep) over a 24h recording period. Asterisks indicate a significance difference (2-sample t-tests) between treatment and controls on the transport day * = P<0.05, ** = P<0.01.

Baseline-treatment Baseline-control

Transport-treatment Transport-control

4.4.3.4 Posture comparisons

Tables 4.7 (a-d) show the effects of transport on Standing, 'Lying head up' and 'Lying head down' postures.

In treatment sheep, the total duration of standing increased during transport compared with the equivalent baseline period 1 (Mixed model, estimate –33.7 ± 5.8

%, t = -6.4, P<0.001). Furthermore, a similar increase was recorded in control sheep during the transport 8h as compared with baseline period (Mixed model, estimate –7.1 ± 2.8% t = -2.6, P<0.05). There was a tendency for a reduction in the percentage of standing by treatment sheep in the 2nd 8h post-transport as compared with the equivalent baseline period (Mixed model, estimate –8.4 ± 5.8%, t = 3.6, P<0.1).

The treatment sheep had longer bouts of standing during transport than at baseline (Mixed model, estimate –1432 ± 372, t = -3.8, P<0.01). In addition, control sheep had longer bouts of standing in the 8h of transport compared with the equivalent 8h of baseline (Mixed model, estimate –278 ± 135, t = -2.1, P<0.05). There were no differences in the duration of standing bouts between controls and treatment post transport as compared with baselines.

Treatment sheep spent less of transport lying head up than in the baseline period (Mixed model, estimate 21.9 ± 5.4 t = 4, P<0.01, n = 8). Control sheep had a tendency to spend less time lying with their heads raised during the transport 8h than in the baseline period (Mixed model, estimate 4.9 ± 2.6, t = 1.9, P<0.07).

There was a tendency for the total duration (%) of 'lying head up' to be different between the 1st 8h post transport and the baseline. Treatment sheep spent significantly more of the 1st 8h post transport lying head up than in the equivalent baseline period (Mixed model, estimate –12.4 ± 5.4 t = -2.3, P<0.05). There were no differences in the 2nd 8h post transport between control and treatment sheep.

There was a tendency for a difference in the mean bout length of 'lying head up' during transport as compared with the equivalent baseline period (Table 4.7 b). Treatment sheep had longer bouts of 'head up' in the 1st 8h post transport in comparison to the equivalent baseline period (Mixed model, estimate –479 ± 123, t = -3.9, P<0.01).

There were no significant differences in the number of bouts of 'lying head up' between control and treatment sheep during transport as compared with the baseline (Table 4.7 c). However, there was a tendency for both treatment and control sheep to have fewer bouts of 'lying head up' during the transport period than the baseline period (G.L.M.M. estimate 4.9 ± 2.9, F =1.96, P<0.06, n = 8 for treatment sheep; estimate 4.2 ± 2.9, t = 1.94, P<0.07, n = 10 for control sheep).

Table 4.7. 'Standing', 'lying head up' and 'lying head down' postures before, during and after transport (n = 10 pairs)

a) Total (percentage of the 8h period) duration

Measurement	Time period‡	Baseline Recording Period		Treatment Recording Period		Difference between baseline and treatment period††	Statistical significance of the difference between baseline and treatment periods††
		Control	Treatment	Control	Treatment		
% 'Standing'	1	30.9 ± 3.1	33.9 ± 2.8	38 ± 5.1	71.7 ± 7.4	30.6 ± 6.9	**
	2	16.7 ± 2.8	16.4 ± 2.8	18.2 ± 2.9	19.7 ± 2.3	1.9 ± 6.9	NS
	3	25.1 ± 3.3	28.6 ± 3.7	21.2 ± 2.7	19.1 ± 2.2	-5.6 ± 6.9	NS
% 'Lying Head Up'	1	57.3 ± 4.4	54.9 ± 2.4	52.4 ± 5	27.9 ± 7.2[a]	-17.1 ± 6.6	*
	2	64.2 ± 4.2	59.8 ± 2.4	60.7 ± 4.2	64.2 ± 3.2	14.9 ± 6.6	NS†
	3	57.1 ± 4.1	52.8 ± 4.2	57.9 ± 4.3	59 ± 3.1	6.4 ± 6.6	NS
% 'Lying Head Down'	1	11.9 ± 1.8	11.1 ± 2.2	9.7 ± 1.8	0.4 ± 0.2[b]	n/a	n/a
	2	19.1 ± 3.3	23.7 ± 4.1	22.2 ± 3.9	16 ± 2.3	-18.8 ± 4.4	**
	3	17.8 ± 2.3	18.6 ± 2.3	20.4 ± 3	18.5 ± 3.2	-2.7 ± 4.4	NS

N = 10 pairs of treatment and control sheep except, [a] where n=8 as two treatment sheep did not lie head up and [b] where n=4 as six treatment sheep did not lie head down the transport period. Mixed model analysis with repeated measures. †A statistical probability of P<0.1 ‡Time period 1 was equivalent to the transport period, time period 2 equivalent to the 1st 8h post-transport and time period 3was equivalent to the 2nd 8h post-transport. ††Differences between the equivalent 8h time periods before (baseline recording), during and after (treatment recording) in the differences between pairs of control and treatment sheep within an 8h time period.

b) Mean duration of bouts

Measurement	Time period‡	Baseline Recording Period		Treatment Recording Period		Difference between baseline and treatment period††	Statistical significance of the difference between baseline and treatment periods††
		Control	Treatment	Control	Treatment		
Duration Standing bouts (s)	1	865 ± 168	816 ± 74	1143 ± 231	2249 ± 610	1154 ± 424	**
	2	523 ± 163	421 ± 64	632 ± 165	615 ± 125	113 ± 424	NS
	3	874 ± 172	654 ± 121	749 ± 146	545 ± 65	118 ± 424	NS
Duration Lying Head Up bouts (s)	1	843 ± 112	735 ± 46	956 ± 107	519 ± 108[a]	-229 ± 152	NS
	2	904 ± 97	843 ± 41	878 ± 38	1022 ± 97	504 ± 152	**
	3	745 ± 84	696 ± 43	831 ± 74	870 ± 144	187 ± 152	NS
Duration Lying Head Down bouts (s)	1	365 ± 30	425 ± 51	680 ± 211	54 ± 23[b]	n/a	n/a
	2	504 ± 72	522 ± 56	704 ± 128	637 ± 110	-85 ± 150	NS
	3	469 ± 60	461 ± 34	605 ± 80	499 ± 61	-98 ± 150	NS

N = 10 pairs of treatment and control sheep except, [a] where n=8 as two treatment sheep did not lie head up and [b] where n=4 as six treatment sheep did not lie head down during the transport period. Mixed model analysis with repeated measures. . †A statistical probability of P<0.1 ‡Time period 1 was equivalent to the transport period, time period 2 equivalent to the 1st 8h post-transport and time period 3was equivalent to the 2nd 8h post-transport. ††Differences between the equivalent 8h time periods before (baseline recording), during and after (treatment recording) in the differences between pairs of control and treatment sheep within an 8h time period.

c) Number of bouts

Measurement	Time period‡	Baseline Recording Period		Treatment Recording Period		Difference between baseline and treatment period††	Statistical significance of the difference between baseline and treatment periods††
		Control	Treatment	Control	Treatment		
No of Standing bouts	1	12.4 ± 1.4	12.6 ± 0.7	12.5 ± 1.8	15.5 ± 3.4	2.8 ± 2.9	NS
	2	11.3 ± 1	11.4 ± 0.7	10.6 ± 1.4	11.4 ± 1.6	0.7 ± 2.9	NS
	3	9.4 ± 0.9	11.6 ± 0.9	10 ± 1.2	11 ± 1.1	-1.2 ± 2.9	NS
No of Lying Head Up bouts	1	21.9 ± 1.9	23.8 ± 1.5	17.7 ± 2.5	16.9 ± 3.4[a]	-2.7 ± 3.8	NS
	2	22.3 ± 1.7	23.9 ± 1.6	20.1 ± 2.3	19.8 ± 2.8	-6.9 ± 3.8	NS†
	3	20.6 ± 1.1	22.9 ± 1.2	19.5 ± 1.9	21.2 ± 1.9	-0.6 ± 3.8	NS
No of Lying Head Down bouts	1	9.7 ± 1.2	11.5 ± 1.4	5.5 ± 0.9	1.6 ± 1.1[b]	n/a	n/a
	2	11.1 ± 0.9	12.6 ± 1.3	9.7 ± 1.2	8.6 ± 1.1	-7.6 ± 2	**
	3	11.7 ± 0.7	11.9 ± 1	10.2 ± 1.3	11.4 ± 1.7	1 ± 2	NS

N = 10 pairs of treatment and control sheep except, [a] where n=8 as two treatment sheep did not lie head up and [b] where n=4 as six treatment sheep did not lie head down during the transport period. GLMM. †A statistical probability of P<0.1 ‡Time period 1 was equivalent to the transport period, time period 2 equivalent to the 1st 8h post-transport and time period 3was equivalent to the 2nd 8h post-transport. ††Differences between the equivalent 8h time periods before (baseline recording), during and after (treatment recording) in the differences between pairs of control and treatment sheep within an 8h time period.

d)Latency from the start of the period

Measurement	Time period‡	Baseline Recording Period		Treatment Recording Period		Difference between baseline and treatment period††	Statistical significance of the difference between baseline and treatment periods††
		Control	Treatment	Control	Treatment		
Latency from start of period to Lie with Head Up (s)	1	634 ± 225	925 ± 504	1300 ± 475	4963 ± 1864[a]	9223 ± 2443	**
	2	n/a	n/a	610 ± 202	103 ± 72	n/a	n/a
Latency from start of period to Lie with Head Down (s)	1	4262 ± 1163	3415 ± 843	6644 ± 2285	15839 ± 4429[b]	n/a	n/a
	2	n/a	n/a	3163 ± 943	4422 ± 1323	n/a	n/a

N = 10 pairs of treatment and control sheep except, [a] where n=8 as two treatment sheep did not lie head up and [b] where n=4 as six treatment sheep did not lie head down during the transport period. Mixed model analysis with repeated measures. . †A statistical probability of P<0.1 ‡Time period 1 was equivalent to the transport period, time period 2 equivalent to the 1st 8h post-transport and time period 3was equivalent to the 2nd 8h post-transport. ††Differences between the equivalent 8h time periods before (baseline recording), during and after (treatment recording) in the differences between pairs of control and treatment sheep within an 8h time period.

There was a significant difference in the latency (from the start of the period) for sheep to 'lie down head up' during transport as compared with baseline (Table 4.7 d). Treatment sheep took longer to lie down during transport than in the first baseline period (Mixed model, estimate -3743 ± 938, t = -3.9, P<0.001, n = 8). There were no significant differences in the time taken to lie down between control and treatment sheep post-transport. However, control sheep took significantly longer to lie down during the 1st 8h post transport than in the equivalent baseline (Mixed model, estimate –666 ± 333 t = -2.2, P<0.05).

No mixed model comparisons of 'lying head down' posture were made during the transport period as only four treatment sheep were observed in this posture and the data was not normally distributed. Treatment sheep spent less of the 1st 8h post transport in the 'lying head down' posture than at baseline (Mixed model, estimate –12.4 ± 5.4, t = -2.28, P<0.05). Control sheep had longer durations of 'lying head down' bouts during the transport period compared with the baseline (Mixed model, estimate –314 ± 141, t = -2.22, P<0.05).

Control sheep had fewer bouts of 'lying head down' posture in the transport period than in the equivalent baseline period (G.L.M.M. estimate 4.2 ± 1.5, F = 2.9, P<0.01). Treatment sheep had fewer bouts of 'lying head down' posture in the 1st 8h post transport than in the baseline (G.L.M.M, estimate 9.1 ± 2.9, F = 3.16, P<0.01).

There were no significant differences between controls and treatment sheep in the latency to lie 'head down' post transport compared with baseline. However, treatment sheep had a tendency to take longer to lay down 'head down' in the 1st 8h post transport than in the baseline (Mixed model, estimate –3188 ± 1929, t = -1.65, P<0.08).

4.4.3.5 Ingestion behaviour comparisons

Table 4.8 shows the effects of 8h transport on rumination and eating. No comparisons of rumination or eating between control and treatment sheep during transport were made as only five treatment sheep were observed to ruminate and only three observed to eat.

There were no differences in the number of bouts of rumination between control and treatment sheep post-transport as compared with baselines. However, treatment sheep had more bouts of rumination in the 1st 8h post transport (G.L.M.M, estimate –1.9 ± 0.8, F = -2.1, P<0.05) and a tendency to have more bouts of rumination in the 2nd 8h post-transport than in the baseline periods (G.L.M.M. estimate –1.7 ± 0.8, F = -1.9, P<0.06).

There was a difference in the total duration (%) of rumination between control and treatment sheep in the 1st 8h post-transport compared with the baseline, this difference was no longer present by the 2nd 8h post transport. Treatment sheep ruminated for longer in the 1st 8h post-transport (Mixed model, estimate –11.9 ± 3.3, t = -3.6, P<0.01) and in the 2nd 8h post-transport (Mixed model, estimate –12.3 ± 3.3, t –3.7, P<0.01) than in the baseline. Control sheep also ruminated for longer in the 2nd 8h post transport than in the baseline (Mixed model, estimate –9.3 ± 3.3 t = -2.7, P<0.01).

There were no differences in the number of bouts, the mean bout length, or the total duration of eating behaviour post transport between control sheep and treatment sheep as compared with baseline periods. However, there was a tendency for treatment sheep to spend a higher percentage of time eating in the 1st 8h post-transport than in the baseline period (Mixed model, estimate –6.4 ± 3.2, t = -2, P<0.06).

Table 4.8 Rumination and Eating before, during and after transport (n = 10)

Measurement	Time period‡	Baseline Recording Period		Treatment Recording Period		Difference between baseline and treatment period††	Statistical significance of the difference between baseline and treatment periods††
		Control	Treatment	Control	Treatment		
No. of bouts of Rumination	1	9.8 ± 1.1	10.3 ± 0.8	11.2 ± 0.9	1.8 ± 0.5[a]	n/a	n/a
	2	9.3 ± 0.4	8.7 ± 0.6	9 ± 0.9	10.5 ± 0.7	2.1 ± 1.3	NS
	3	6.1 ± 0.9	6.4 ± 0.5	7.2 ± 0.6	8.1 ± 0.5	0.6 ± 1.3	NS
Duration of Rumination bouts (s)	1	1188 ± 167	971 ± 36	1020 ± 94	427 ± 144[a]	n/a	n/a
	2	1281 ± 79	1269 ± 107	1408 ± 97	1369 ± 75	-17.7 ± 178	NS
	3	1242 ± 72	1195 ± 85	1434 ± 96	1440 ± 102	53.1 ± 178	NS
% of time period Rumination	1	34.9 ± 3	34.1 ± 2.9	36.8 ± 1.9	3.8 ± 1.2[a]	n/a	n/a
	2	40.6 ± 2.9	36.5 ± 2.7	41 ± 2.4	48.4 ± 3.1	11.4 ± 4	**
	3	24.8 ± 3.4	26.6 ± 2.9	34.1 ± 2.6	38.9 ± 2.4	3 ± 4	NS
Latency to ruminate(s)	1	2061 ± 1138	2192 ± 1022	1808 ± 476	18385 ± 5642[a]	n/a	n/a
	2	n/a	n/a	1475 ± 662	759 ± 468	n/a	n/a
No. of bouts of Eating	1	11.2 ± 1.3	10.3 ± 0.6	12.1 ± 1.4	2.8 ± 0.7[b]	n/a	n/a
	2	7.6 ± 1.3	3.4 ± 0.8	7.1 ± 0.8	7.4 ± 0.6	2.5 ± 1.5	NS
	3	7.1 ± 1	7.8 ± 1.1	6.6 ± 0.7	6.9 ± 0.5	-0.4 ± 1.5	NS
Duration of Eating bouts (s)	1	789 ± 90	870 ± 63	814 ± 86	734 ± 205[b]	n/a	n/a
	2	931 ± 151	673 ± 120	781 ± 88	865 ± 115	342 ± 263	NS
	3	1347 ± 309	1022 ± 138	1005 ± 110	870 ± 82	189 ± 263	NS
% of time period Eating	1	27.7 ± 1.9	30.7 ± 2.9	30.9 ± 2.5	5.9 ± 1.1[b]	n/a	n/a
	2	19.5 ± 2.9	13.8 ± 2.8	18.4 ± 2.1	20.2 ± 1.6	7.9 ± 4.2	NS†
	3	23.6 ± 2.3	25.7 ± 3.9	21.4 ± 2	19.8 ± 1.4	-3.7 ± 4.2	NS
Latency to eat(s)	1	1053 ± 443	524 ± 344	1081 ± 385	22696 ± 2477[b]	n/a	n/a
	2	n/a	n/a	964 ± 395	161 ± 57	n/a	n/a

The test used for the number of bouts was a GLMM, all other measurements were tested using the mixed model analysis with repeated measures. N = 10 except [a] where n = 5 as five treatment sheep did not ruminate and [b] where N = 3 as seven treatment sheep did not eat during the transport period. . †A statistical probability of P> 0.05, but <0.1 ‡Time period 1 was equivalent to the transport period, time period 2 equivalent to the 1st 8h post-transport and time period 3was equivalent to the 2nd 8h post-transport. ††Differences between the equivalent 8h time periods before (baseline recording), during and after (treatment recording) in the differences between pairs of control and treatment sheep within an 8h time period.

4.4.4. Distribution of behaviour post-transport

Treatment sheep were unloaded from the vehicle and put back into their home pens, the harness and electrophysiological equipment was readjusted if necessary. The unloading process which took 135 ± 49 s from the time the ramp was opened on the vehicle to the time when the sheep was left in its home pen (n = 9, one sheep took 6 minutes to unload). All of the treatment sheep (n = 10) were lying down in the 'head up' posture within 2mins of unloading. Three of treatment sheep started eating before lying down, the other seven lay down first and then started to eat the hay. All of the treatment sheep were eating while lying 'head up' within 3mins of the end of the unloading process. All treatment sheep were observed to eat for at least 10mins during their first eating bout post-transport. The mean bout length of the first bout of eating was 18.4 ± 2.9mins.

One sheep stood up and drank water after the first post-transport bout of eating, the other nine treatment sheep began ruminating (without changing posture) within 5s of ending the first eating bout. The first post-transport bout of rumination was 32.2 ± 1.1 mins in length. Six sheep returned to eating after the first bout of rumination (while lying), two sheep had a period of lying with head up with no ingestion behaviour for >1min and two of the treatment sheep were standing after their first bout of rumination. All treatment sheep were eating (the second bout post-transport) within 10mins of the end of their first rumination bout. A pattern of alternation between eating (35% of bouts standing, 65% lying) and rumination (94% lying) was observed in treatment sheep at the start of the first post-transport 8h. This continued until the first bout of Non-REM occurred, 108 ± 24minutes after unloading (in 7 sheep, this was on the second bout of 'lying head down' posture, the other 3 sheep first slept during their 3rd bout of 'lying head down' posture).

Control sheep were disturbed when the treatment sheep re-entered the room post-transport and all were standing at the start of 8-h post transport period. All control sheep started to eat once treatment sheep were eating, however they remained standing during the first bout of eating 'post-transport'. Control sheep followed a similar pattern (20mins eating, 30mins ruminating) to treatment sheep, except that eating was carried out while standing in 80% of occasions. In addition, each period of rumination was followed by a period of 'lying head down' posture. In control sheep, the first 'post-transport' bout of Non-REM sleep occurred 63.7 ± 15mins after the treatment sheep re-entered the room

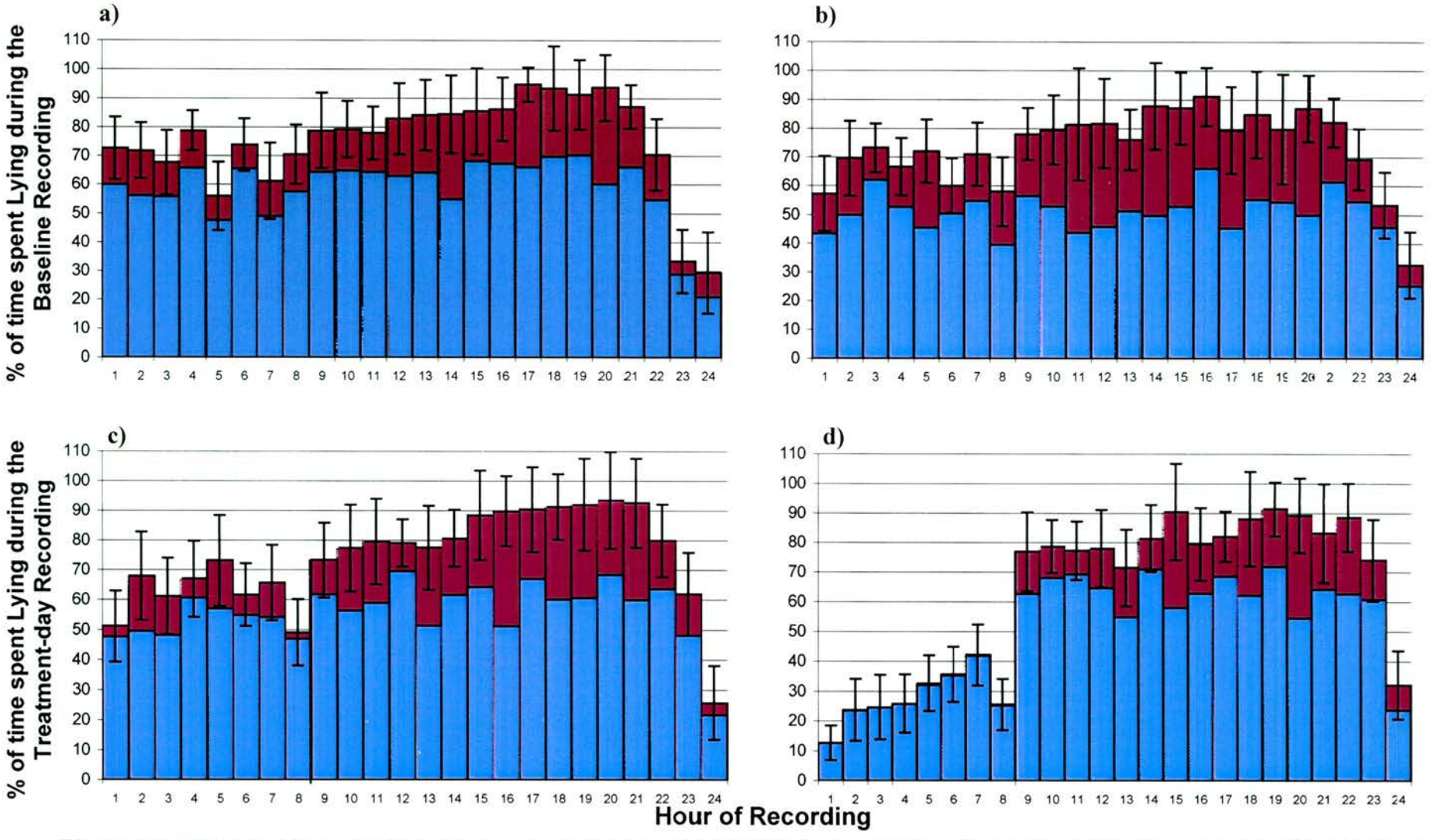


Figure 4.4. The mean (error bars indicate standard error of total lying) percentage of Lying Head Up (blue bars) and Lying Head Down (red bars) during each hour of 24h recordings. a) Control Baseline, b) Treatment Baseline c) Control Treatment-day d) Treatment Treatment-day. N = 10 sheep in each condition.

The hour-by-hour distribution of postures in the 16h post-transport was not significantly affected by transport as can be seen in Figure 4.4, between the baseline recording (b, for treatment sheep) and the transport recording (d for treatment sheep). Towards the end of the 16h post transport the pattern of the postures in each hour is similar to that of the baseline recording.

4.4.5 Distribution of sleep post-transport

In treatment sheep, the first Non-REM sleep bout (latency = 108 ± 24mins post-unloading) always occurred within 30 seconds of the end of a bout of rumination. Four of the sheep had a bout of REM sleep during their first bout of sleep post-transport, three sheep had their first REM sleep bout during the second sleep bout post-transport and three sheep during their 3rd sleep bout. The mean latency to REM sleep was 124.1 ± 38mins post unloading in treatment sheep. All sleep bouts of treatment sheep in the first 8h post-transport started with Non-REM sleep before approximately 75% progressed to REM sleep. No bouts of sleep started with REM sleep in this period.

In control sheep, the first Non-REM sleep bout (latency = 63.7 ± 15mins after the start of the period) occurred within 30s of rumination in 4 sheep and occurred more than 1min post rumination in the other 6 sheep. All control sheep had a bout of REM sleep during the first sleep bout of the period. All bouts of sleep started with Non-REM sleep before 60 % progressed to REM sleep. Both treatment and control sheep aroused to wakefulness after all bouts of REM sleep in the first 8h post-transport.

Figures 4.5 and 4.6 show the hour-by-hour distribution of Non-REM sleep and REM sleep, respectively. The baseline graphs have the typical peak in the amount of sleep in the darkest period of the night and less sleep during the day, especially around feeding times. After transport, the treatment sheep seem to show a slight phase delay in the occurrence of the peak in Non-REM sleep, with the hour containing the most Non-REM sleep being later in the night. The hour-by-hour distribution of REM sleep post-transport is similar to that of the baseline for treatment sheep.

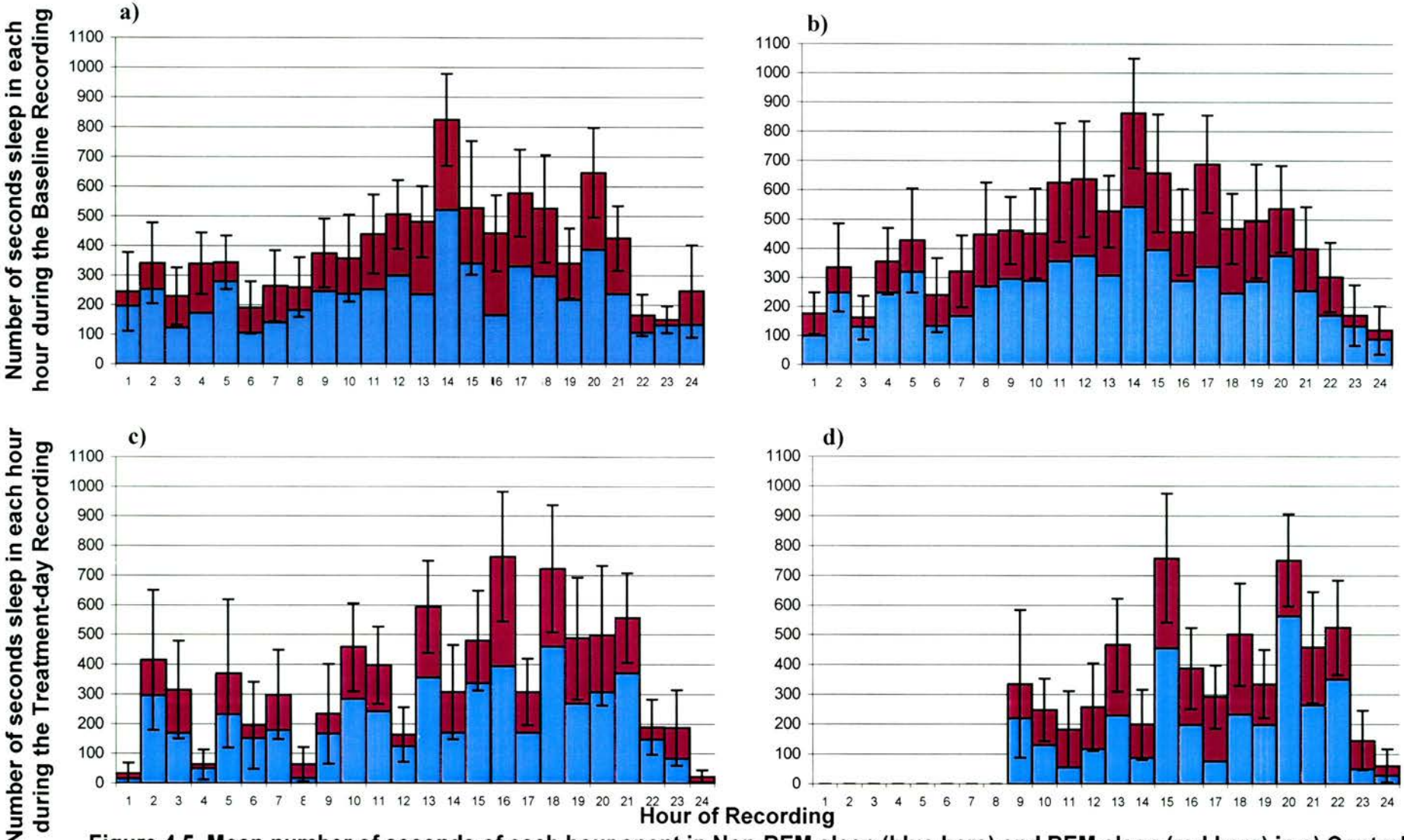


Figure 4.5. Mean number of seconds of each hour spent in Non-REM sleep (blue bars) and REM sleep (red bars) in a) Control sheep Baseline, b) Treatment sheep Baseline, c) Control sheep Treatment-day, and d) Treatment sheep Treatment-day N = 10 sheep in each condition. Non-REM REM

4.5 Discussion

4.5.1 Behaviour and sleep during transport

On this series of 8h road journeys, sheep did not sleep during the journey or the break in the journey. There was no evidence from the spectral analysis of the EEG traces that sheep were asleep even when they were lying down with their heads resting on the bedding in the characteristic sleep posture. During this study the conditions that the animals were being transported in, could be considered optimum, as sheep had room to lie down with no competition for space (in single, adjacent pens of 0.9 X 1.85m) and the trailer was bedded with clean, dry straw. It is likely that these conditions would be more suitable for sheep to be able to sleep in than commercial transport which could have high stocking densities, competition for space and wet, soiled or no bedding. Sheep did lie down in the journey and, especially on the second loop, some animals performed behaviours that have been associated with resting in sheep (i.e. lying ruminating and lying with the head resting on the floor) (Cockram et al. 1996). This may suggest that over time, when sheep have habituated to the novelty, movement, etc of transport, resting behaviour is possible. If one extrapolates from this, under these optimum conditions, sheep may be able to sleep on a longer journey. Nevertheless, it is worth noting that two sheep did not lie down during the whole journey time, even though there was space, clean bedding and no adverse social interactions. An alternative view of what is happening when sheep lie down during transport is that they become fatigued and then lie down if they are able to do so. This may suggest that the two sheep that did not lie down during the 8h journeys were not sufficiently fatigued to have to lie down.

One difficulty in recording non-invasive electrophysiological measures from sheep during transport is that the electrophysiological traces may have been affected by 'noise' from the constant vibrations of the vehicle. Because the leg EMG trace was profoundly different between standing and lying (see chapter 2), even when the traces were affected by vibration-related artefact, they were still easy to visually score. It was more difficult to visually score the neck EMG trace for transitions between the lying 'head up' and the lying 'head down' posture than it was in the previous studies. The video of the behaviour of the sheep in the vehicle was of sufficient quality to assist in the scoring of the neck EMG trace. Similarly, the EEG trace was affected by

vibration-related artefact therefore the automatic sleep score carried out on the EEG traces during transport must be treated with caution. However, the number of sheep that actually spent any time in the lying 'head down' posture was small, and the time spent in this posture was minimal. Therefore, even if the automatic sleep score was incorrect due to the vibration artefact, the most that can be said is that some sheep may be able to undergo light (as there was no evidence of a high percentage of slow-waves) Non-REM sleep for a short period of time on the smooth types of road.

The present study confirms that of previous work from other authors (e.g. Cockram et al. 1996 and Cockram et al. 2004) that lying was observed most frequently in the second half of the journey as compared with the first half. Where studies have looked at physiological indicators of stress, such as blood plasma cortisol concentrations, most have found that there is a peak in activity, and have therefore concluded a peak in stress response, during the first 3h of a road journey (e.g. Broom et al. 1996; Parrott et al. 1994; and Cockram et al. 1997). It is difficult to determine whether, or not, sheep were lying down more in the second half of the journey because they were habituated to their situation and therefore comfortable enough, or because they were becoming fatigued or tired due to the physical exertion and psychological stress of transport. Half of the treatment sheep ruminated and lay down during transit (especially in the second half of the journey), possibly more indicative of rest rather than fatigue. However, very little lying in the head down posture was observed, suggesting that full relaxation was difficult for sheep during transit. Here, further research into the use of the electromyogram to measure fatigue in transported animals would be useful. In this experiment, the EMG from the hind leg of sheep was used to determine the changes in posture, but information from electrophysiology could be used to determine and quantify muscle fatigue during balance preservation needed in transit (Giovagnoli et al. 2002).

In addition, the present study also confirms work done by Cockram et al (2004) showing that lying behaviour is more frequent on major roads rather than minor roads. In this experiment, sheep were able to lie down when driven on major dual carriageways as compared with windy roads. However, even on the major roads, sheep still stood up at every roundabout, so cornering is a disturbing event and should be kept to a minimum if possible. The results suggest that for optimum welfare of

sheep during road transport in relation to the ability of sheep to lie down, road hauliers should attempt to confine the majority of their journey to dual carriageways or motorways.

During transport, treatment sheep stood for longer bouts when compared with baselines. They also took longer to lie down than they had during the same time period during the baseline. In ideal conditions, sheep lie down to ruminate and this affords sheep a certain amount of rest (Ruckebusch, 1972), treatment sheep carried out these behaviours very infrequently during transport

4.5.2 The control sheep during the transport period

Interestingly, the control sheep seem to have been affected by the removal of the treatment sheep from the experimental room as they also stood up for longer bouts during the 'transport' period than during the equivalent 8h baseline period. In addition, control sheep had a longer latency to lying down and had fewer bouts of 'lying head down' posture during the period when the treatment sheep were transported. The control sheep also had fewer bouts of Non-REM during the transport period, giving more evidence to suggest that they were adversely affected by the removal of the treatment sheep from the experimental barn. It was possible, as there were two pens used to house sheep during their experimental period, that the position of the pen within which the control sheep lived might have had an effect on how the control sheep behaved in the absence of the treatment sheep. One pen (pen B) was situated adjacent to the other pair of sheep that were in the room, the other pen (pen A) was adjacent to pen B and approximately 1.3m away from the other sheep. Sheep were assigned to the pens in a random manner. There was no evidence to suggest that pen position (when used as a fixed effect in the mixed models analysis) accounted for any of the variation seen in behaviour of the control sheep during the transport period. As there were other sheep present in the barn it is unlikely that the change in the control sheep behaviour was due to an isolation affect (Carbajal and Orihuela, 2001).

4.5.3 The effects of an 8h road journey on behaviour and sleep post-transport

At the start of the study, two main questions were posed: a) did the physical exertion during transport result in sheep resting and/or sleeping more post-transport,

and b) did the potential stress associated with the transport disrupt sleep post transport. These are difficult questions to answer because both questions could have an answer which involves less or more sleep post-transport. Therefore, any result (change in the amount of sleep post-transport as compared with the baseline) could be result of either exertion, or stress responses.

The treatment animals stood for more of the time when transported than in the equivalent baseline period. However, the treatment sheep had to undergo more physical exertion than the controls as they were not just standing during transport, but they had to maintain balance and readjust posture around corners etc, control sheep were just standing in their home pens. Treatment sheep had longer bouts of lying down head up in the first 8h post-transport suggesting that they were resting after the physical exertion of transport. On the other hand, treatment sheep took longer to lie down in the relaxed 'head down' posture post-transport and had fewer bouts of lying head down during the first 8h post transport as compared with baselines. Maybe was this a recovery from physical tiredness (fatigue) rather than a need to reduce sleepiness? Humans report feeling physically fatigued (weary, with a depletion of energy) during exercise tasks and this requires rest, not sleep to recover (Johns, 2000).

Cockram et al (1997), Knowles et al (1994a) and Parrott et al (1998a) all suggest that the first priority of sheep post-transport is to eat, rather than to lie down and rest. The experimental sheep home-pen set up in the present study, was designed to reduce the amount of wear on the electrodes, electrode leads and harnesses. It was for this reason that each sheep had her own pen. However, this meant that sheep could easily lie down and feed from their hay, which was placed behind the hurdles of the pen. Unfortunately, it is difficult to ascertain the primary motivations of sheep after unloading in this experiment, as they could eat and lay down simultaneously, there was no competition for food, and sheep could gain rest whilst eating. However, it does seem that treatment sheep were more motivated to undertake ingestive behaviour than to sleep, directly post-transport. All treatment sheep spent the first hour and 30mins doing a combination of eating and ruminating. There was a tendency for treatment sheep to spend longer eating in the first post-transport period than the equivalent baseline and treatment sheep ruminated for more of the post-transport periods (both 2 and 3) than the baselines. Control sheep carried out most of their eating bouts while

standing (80%), treatment sheep only stood for 35% of their eating bouts, adding further evidence to the suggestion that treatment sheep were physically tired from transport and were motivated to rest and eat simultaneously.

As can be expected from the results regarding posture, treatment sheep had fewer, shorter bouts of Non-REM leading to a lower percentage of Non-REM sleep in the first 8h post-transport compared with both baselines and control sheep. There were no significant differences in the latency to Non-REM sleep between control and treatment sheep post transport as compared with baselines. However, this maybe due to the large variation in latency to sleep between individuals, the standard errors were over 15% of the means. The means suggest that treatment sheep did take longer to go into sleep compared with controls and baselines, here it would have been useful to have more sheep in the experiment to minimise variation due to individual differences.

Similar to Non-REM sleep, there were no significant differences in the latency to REM sleep post-transport. However, once again there were very high standard errors, and large amount of individual variation. There was a tendency for treatment sheep to take a longer time to reach REM sleep in treatment period 2 than in baseline period 2.

When comparing the differences between control and treatment sheep to find differences between post-transport periods and baselines, there was no difference in the number of REM bouts. However, this was due to a reduction in REM sleep bouts in both treatment and control sheep in the 8h directly post-transport. The reduction could, be due to the second week of recording, or an effect of transport and social interaction changes in the treatment and control sheep respectively.

REM bout lengths were not significantly different at any time period between control and treatment sheep. In this experiment, it seems that in sheep the length of REM bouts is an inelastic quality and any change in total REM duration is due to a change in the number of bouts rather than any increase in bout length. This has also been found to be the case in rats (Rampin et al. 1991; Cespuglio et al. 1995; Dewasmes et al. 2004). There were no significant differences in the percentage of total duration of REM sleep at any time post-transport as compared with baselines.

There were no differences in the density of rapid eye movements in 10s epochs of REM sleep post-transport as compared with baselines. Eye movements have been

shown to increase in density in humans suffering from chronic stress conditions (Douglass et al. 1992), so a change in sheep subjected to an 8h road journey was not expected.

Therefore, to answer the first question relating to rest and sleep post-transport, it seems that there is evidence that the sheep were physically tired post-transport as they spent more time lying down and had longer bouts of lying down compared with baselines. In addition, treatment sheep carried out most of their eating bouts lying down, whereas the controls remained standing to eat. However, there is no evidence from the amount of sleep (Non-REM or REM) post-transport that sheep needed to reduce sleepiness that had built up during the transport journey.

The, second question raised in the aims of this experiment is harder to answer. Did the potential stress of the transport affect the sleep in the treatment sheep post-transport? From the results, the amount of sleep post-transport did not appear to have been affected by any stress during transport. However, using electrophysiological techniques to record sleep in sheep, and not just relying on behavioural observation allows more information to be gathered on the qualities of sleep. Meerlo et al (1997 and 2001) have shown that Non-REM sleep differs in its quality as well as its quantity after stressful waking events. They found that rats subjected to a social stressor subsequently had Non-REM sleep that had a high percentage of Delta waves (0.1-4Hz) as compared with a rat that had been subjected to sleep deprivation for the same period of time. Non-REM is known to contain a higher percentage of the slowest waves the longer an animal is deprived from sleeping, but Meerlo et al (2001) showed that the intensity, not just the length, of the waking experience had an effect on the subsequent sleep. One of the best pieces of evidence that suggests the treatment sheep had been affected by their experience during transport was the large change in the intensity of the subsequent sleep, post-transport. A significant increase in the percentage of Delta-frequency waves in Non-REM sleep was seen in the 8h post-transport in the treatment animals compared with the controls and baselines and there was a tendency for this effect still to be present between 8 and 16h post transport. There was no similar increase in the control sheep, even with the evidence that their behaviour, rest and sleep had been affected during the 'transport' period. This suggests that the treatment sheep did find their transportation aversive or stressful. It would,

however, be useful to ascertain how much of the variation in the intensity of Non-REM sleep was affected by the 8h of sleep deprivation as opposed to the stressor of transport. Further research could attempt to determine this by subjecting sheep to a stressor and sleep depriving controls in as 'non-stressful' a manner as possible. Cockram et al (1999) found that there was no difference in lying down behaviour between sheep in 12h of lairage that had and had not been transported. However, this experiment suggests that it is the quality of the rest and sleep that is important in how sheep respond to 8h transport, not just the quantity.

Treatment sheep did not make up the sleep they 'lost' during transport in the 16h post-transport (see figure 4.2). It would be interesting to study sheep for a longer period post-transport to determine if subsequent sleep was affected. Furthermore, the majority of a sheep's sleep under 'natural' conditions is carried out during the night, it would be very interesting to determine if an overnight road journey would affect the sleep distribution more than a daytime journey.

It should be noted that sheep in this experiment were handled and gentled extensively before the baseline measurements took place. This was an attempt to make the harnessing and equipment fitting procedure as stress free as possible. However, this may have had an effect on the responses of sheep to transport. Hall et al (1998) found that in general, there was no difference in the cortisol response of sheep to transport after taming, however, there was evidence to suggest that individual sheep that had responded most to taming were less stressed during transport.

4.6 Conclusions

There was no evidence to suggest that sheep can sleep during an 8h road transport journey, even during optimum conditions. Sheep can rest during transport if the conditions in the vehicle allow and the road quality is sufficiently high without too many corners.

There was evidence to suggest that the subsequent sleep of sheep is affected by an 8h road transport journey. There were reductions in the amount of Non-REM sleep that may have more to do with changes in the priority of sheep after a period without food than the stress of 8h sleep deprivation. However, there were also changes in the quality of Non-REM sleep, in the increase in slow waves that could indicate a

psychological reaction to an aversive waking experience. In addition, this experiment shows that recording the EEG from sheep during and after an aversive experience, gives more information about how sheep respond to stressors than using behaviour alone.

Effects of a 29-h space restriction at space allowances used during transport on sleep in sheep

5.1 Abstract

Sleep-deprivation and broken sleep patterns can affect subsequent sleep quality and quantity both in humans and in rats. This experiment investigated whether the reduction of floor space caused by penning sheep at the space allowances used during road transport would reduce lying behaviour sufficiently to alter the post-treatment rest and sleep behaviour of sheep. Simultaneous, behavioural recordings and non-invasive 24-h EEG, EOG and EMG recordings were made for 24-h on pairs of ewes kept in single pens. One-week later, pairs of ewes were randomly allotted to either a minimum space allowance ($0.3m^2$/sheep) group or a maximum space allowance ($0.8m^2$/sheep) group. Two 14-h periods of restricted space allowance without food and water were produced by adding four sheep to each pen and reducing the dimensions of the minimum space allowance pen. After the first 14-h period, a 1-h period at $0.8m^2$/sheep with feed and water was provided. The second 14-h period was then started, and on completion, the extra four sheep were removed from each pen and feed and water were provided. The behaviour of the sheep was recorded during the 29-h treatment period and the behaviour and electrophysiology was recorded for 24-h during the post-treatment period. This treatment was repeated on new pairs of sheep until satisfactory recordings were obtained from 10 pairs of ewes.

The effect of space allowance on behaviour and the quantity, quality and distribution of sleep in sheep was examined using the mixed models analysis of covariance, by comparing the difference between the treatment groups during the baseline recordings with that during the post-treatment recordings. Bout frequency data were analysed using the GLMM procedure. Mixed models analysis of variance were used to compare the behaviour of sheep during the two 14-h periods of space restriction.

During the 29-h space restriction period, sheep from the minimum space allowance stood for longer, spent less time lying with their head up and less time lying with their head down than those from the maximum space allowance. In both

treatment conditions, the amount of time spent standing was higher, and the amount of time spent lying (head up and head down) was lower in the first 14h of space restriction compared to the second 14h.

Post-treatment, sheep from the maximum space allowance stood for longer than during the baseline and compared to the sheep from the minimum space allowance. Both treatment groups took longer to lie down (both with the head up and with the head down) post-treatment as compared to the baseline. Post-treatment, both treatment groups spent more time and had more bouts of eating than in the baseline period, with sheep from the maximum space allowance spending more time eating than sheep from the minimum space allowance.

There were no significant differences in the number, or duration of Non-REM sleep bouts post-treatment as compared to baselines for either treatment condition. However, sheep from both treatment conditions had an increased latency to sleep post-treatment in comparison to baselines. Post-treatment, sheep had a greater percentage of delta waves in 30s epochs of Non-REM sleep compared to baseline, with sheep from the minimum space allowance having a greater percentage of delta waves in Non-REM sleep than sheep from the maximum space allowance condition.

Sheep from the minimum space allowance had a greater number of bouts and spent more time in REM sleep compared to the baseline period, and this difference was seen during the first 6h of the post-treatment 24h. In addition, sheep from both treatment conditions showed an increase in eye-movement density during REM sleep post-treatment in comparison to the baseline 24h.

The space allowance affected the resting behaviour of sheep during a 29h period of space restriction. Post space-restriction, both resting behaviour and sleep were different from pre-treatment baselines. Sheep that had been penned at $0.3m^2$/sheep had more changes in sleep and rest than those penned at $0.8m^2$/sheep, especially in the first 6h post-treatment. The implications for the long-distance transportation and post-transport lairage conditions of sheep are: that as sheep are unlikely to get rest and sleep at low space allowances, for optimum welfare more space ought to be provided for sheep to rest during transport. In addition, welfare

will be improved if the lairage post-transport has sufficient space to allow all transported sheep to recover from partial rest/sleep deprivation.

5.2 Introduction

5.2.1 Aims

To investigate the effect of two different space allowances over the maximum permitted transport times on lying behaviour in sheep penned at the space allowances allowed for road transport.

To determine the effects of 29h at two different reduced space allowances on the distribution of sleep and wakefulness and the quantity and quality of sleep for 24h post-treatment, and answer two main questions:

1. does the potential lying deprivation and sleep deprivation associated with reduced space, result in sheep resting or sleeping more post-treatment,
2. does the potential stress associated with the 29-h at reduced space allowance without food and water disrupt sleep post-transport.

To investigate the distribution of lying behaviour during and after penning space allowances used during transport to help determine if there is a relationship between resting and fatigue.

To ascertain which, if either, space allowance condition allows the sheep to lie down and rest while sheep are being kept in pens and not exposed to the additional stimuli associated with transport that may impair or interfere with lying behaviour.

5.2.2 The long distance transport of sheep

Under current European legislation, maximum journey times for sheep are 8h, though this may be extended if transporting vehicles meet further requirements, to 14h of transport. After the 14h, sheep must have a rest period of at least 1h sufficient to provide water and if necessary feed, however, the sheep can remain on the vehicle. A further 14h of transport can follow the break, after which the sheep are required to be unloaded and rested with feed and water for 24h (Council Directive 98/290/EEC, The Protection of Animals during Transport). Road transport journeys have increased in time and distance in recent years, there were less than half the number of slaughterhouses in the UK in 1999, when compared to 1979 (FAWC,

2003) and therefore, sheep have to be transported further to be slaughtered (FAWC, 2003).

From an economic point of view, low space allowances for animals during transport makes sense, as more animals can be carried in a vehicle and costs per vehicle are similar regardless of animals carried. However, in a recent European Commission report it was stated that

> "...for journeys longer than four hours, all animals should be able to lie down...a space allowance of 0.8m^2/100kg...for journeys over 12 hours where sheep have to be fed and watered on the vehicle a space allowance of 0.44m^2/40kg animal should be provided"
>
> (Scientific Committee on Animal Health and Animal Welfare, 2002).

Warriss et al (2002) surveyed the space allowances of UK slaughter sheep, and found that the average space allowance was 0.65 m^2/100 kg, the average liveweight of slaughter sheep was 40kg. They found that 94% of sheep in the survey were transported between 0.3 and 0.9m^2/100 kg. One percent of slaughter sheep were transported at less than 0.3m^2/100 kg, and 5% were transported at more than 0.9m^2/100 kg (Warriss et al. 2002).

5.2.3 Space Allowance and lying behaviour

Animals occupy a certain amount of space according to their physical size, their shape and extra space is required for them to perform changes in posture such as lying down from a standing posture. In addition, animals, such as sheep have preferred lying traits, such as not facing an animal higher up the social hierarchy than themselves (Geist, 1971). Sheep are very social animals, but they tend to prefer open space around their heads and with enough space, will not lie touching another individual (except for ewes and their lambs) (Lynch et al. 1992). Cattle have been shown to prefer to orientate themselves alongside the pen sides of an enclosure when housed in open sided pens, and therefore not use the space in the middle of the pen (Stricklin et al. 1979). Hutson (1984) observed that when sheep were lying down in an enclosure, they lay down parallel to the sides, whereas there was no preference in space usage when sheep were standing in the same enclosures (Hutson, 1984). During transport, cattle prefer to orientate themselves either parallel, or perpendicular to the direction of travel, when standing (e.g. Tarrant et al. 1992,

Lambooy and Hulsegge, 1988). However, Cockram et al (1996) report no preference for orientation in sheep during transport. A group of sheep take up more ground-space when lying than they do when standing, so in order for all sheep to be able to lie down during transport more space is needed. However, that does not mean that sheep will feel comfortable enough to lie down during the journey. An increase in stocking density may bring increased threat of aggression, as it is more difficult for a sheep to move out of a threatening encounter.

It could also be claimed, that low space allowances during transport are beneficial as they protect sheep from injury. Here, as sheep are packed together more tightly, they are able to maintain balance more easily and are less likely to fall. However, a study investigating space allowance for transporting cattle found that interactions between cattle increased at lower space allowances and this accounted for more losses of balance than driving events alone (Tarrant et al. 1988). Tarrant et al (1988) also showed that at low space allowances, cattle that had fallen were less able to stand and were more likely to be injured by other individuals standing on them. For sheep, Cockram et al (1996) found more falls and losses of balance when transported at higher space allowances of 0.27 and 0.41m^2/sheep than at low space allowance of 0.22m^2/sheep, however the total number of events during transport were not different between the three space allowances and there was also no evidence of increased injuries (as measured by plasma creatine kinase activity) at any of the space allowances (Cockram et al. 1996).

In most studies, sheep have been observed to lie down less during transport than they do before transport (e.g. Cockram et al. 1997). During a 12h road journey, sheep in a space allowance of 0.22m^2/sheep stood more after the first 3h than sheep at a higher space allowance (0.41m^2/sheep). The authors suggest that the sheep transported at 0.41m^2/sheep were able to lie down, and once they were habituated to the conditions of transport, they did lie down (Cockram et al. 1996). The sheep transported at 0.22m^2/sheep were less able to lie down, even after habituation. The sheep may have laid down because they were physically tired rather than due to habituation, which would suggest that sheep transported at 0.22m^2/sheep were less able to lie down, even when they were tired.

5.2.4 Lying deprivation

It is possible, especially when transported in low space allowances, that sheep may be able to lie down a little or not at all during a journey. This may affect their behaviour post-transport.

In an experiment to assess the effects of lying deprivation, dairy cows were prevented from lying for two periods of 7h in a 24-h period for 8 weeks (Munksgaard and Simonsen, 1996). Cows that had been prevented from lying, lay down for the majority of the period that lying was allowed. The duration of lying bouts was not affected by treatment, but the frequency of lying bouts was increased in cows that had been prevented from lying compared to controls (Munksgaard and Simonsen, 1996). Fisher et al (2002) measured the plasma cortisol/plasma ACTH ratio in cows that were prevented from lying down for 16h per day and found that the ratio was increased after a corticotrophin releasing hormone challenge compared with feed restricted cows, suggesting that lying deprivation is stressful for cattle.

In a further experiment to measure the demand characteristics of dairy cattle that were prevented from lying, Jensen et al (2004) found that the longer the period of lying deprivation the more cows would work for the opportunity to lie down. In addition, the elasticity of the demand function for rest was reduced with increased lying deprivation (Jensen et al. 2004).

5.2.5 Physical exertion and the relationship with tiredness

Fatigue is not a simple phenomenon, there are many emotional, behavioural and cognitive factors which build up to the subjective feeling of fatigue (Dirnberger et al. 2004). There are short-term changes in electrical activity in the brain before and after voluntary movements, and this activity is altered by psychological factors such as fatigue (Dirnberger et al. 2004). In a study investigating the effects of fatigue on the EEG directly before, during and after a minor-physical task, human participants were given a questionnaire to self-report both physical and mental fatigue. Those participants which had reported the most fatigue had a reduced post-movement potential on their EEG recording. This type of fatigue is not the same as muscular fatigue, the type that occurs after a physically demanding task, but it is still affecting the brain activity. In a study investigating a more severe exercise in rats, there were changes (increases in theta activity) in the waking EEG post-exercise as

compared with sedentary rats for up to 70 minutes post-treatment (Arai et al. 2002). There were also increases in the amount of delta activity found in the waking EEG of rats that had been exercised 'to exhaustion' as compared to those that had undergone a lighter exercise regimen (Arai et al. 2002). It is important to note that the forced exercise of rats may be psychologically stressful, which could additionally affect the EEG, however, the authors also undertook a exercise-free stressor control and found no corresponding EEG changes, indicating, as the authors suggest, the changes are related to fatigue.

There is evidence from research on humans, to suggest that performing a fatiguing task, especially a task using the limbs, affects subsequent postural stability, i.e. that the body sways more when fatigued than it does when rested (Caron, 2004). Moreover, Caron (2003) reports that the body sway induced by strenuous exercise in humans requires more postural adjustments and greater muscular activity to maintain posture. It remains to be seen whether the exercise sheep undergo during transport would be fatiguing enough to affect the postural stability during the journey.

If the definition of sleepiness as the increased feeling and propensity to go to sleep is used, sleepiness in humans is affected by posture, and the amount of physical and mental activity being undertaken (Johns, 2000) (see chapter 1 for a review of sleepiness and fatigue). People report that they do not feel sleepy during a physical or mental task, especially if they have to maintain an upright posture during the task. This is different to fatigue, people report feeling physically (and mentally) fatigued (weary, weakened and a depletion of energy – Pigeon et al. 2003) during exercise tasks, and mentally fatigued during mental tasks and this requires rest, not sleep to recover (Johns, 2000). If people are in a recumbent posture while undertaking a mental task, then they are more likely to report increased sleepiness than when in an upright posture. There is a debate as to what the mechanisms are that reduce sleepiness with physical exercise and postural changes, one of the explanations is that both raise the body core temperature and this has an alerting effect (e.g. Matsumoto et al. 2002; Caldwell et al. 2000).

Exercise may decrease sleepiness while it is being carried out, but there is evidence to suggest that subsequently, sleep is increased after exercise. In a study investigating the effect of exercise on subsequent sleep in humans, Sasazawa et al

(1997) showed that the latency to sleep and sleep fragmentation is reduced after exercise. Most participants also showed an increased amount of the stage III and IV sleep, that containing the highest percentage of slow waves, during the first half of the night on exercise days as compared to non-exercise days. It is worth bearing in mind that in this study, participants were doing heavy exercise that they were accustomed to, animals being transported may not be as physically fit and may also find the experience psychologically stressful.

The effect of physical exercise and forced standing on fatigue in sheep is poorly understood. Sheep have been shown to stand for up to 70% of the 24h day, although this differs between studies, husbandry conditions (e.g. Ruckebusch, 1972 recorded three adult sheep in metabolic cages) and ages of the sheep (See chapter 2). Forced exercise and standing (for example being transported at low space allowances and requiring to maintain balance) for long periods may cause sheep to become physically fatigued. In addition, there is as yet no evidence to suggest that sheep can sleep standing up sleep, unlike horses, which can achieve Non-REM sleep while standing, and therefore, prolonged standing may cause sleep deprivation and sleepiness in sheep. Cattle have been shown to be able to have Non-REM sleep standing up when deprived from lying (Ruckebusch, 1974). Post-transport, or after forced standing periods, sheep may require a period of rest to recover from the physical exertion of balance maintenance and/or prolonged standing.

5.2.6 Sleep deprivation, post-deprivation sleep and stress

Reduced space allowance conditions may reduce the sheep's ability to lie down in a fully relaxed manner and, therefore affect their sleep during the space restriction period. There is evidence to suggest that when humans have to sleep at an angle other than horizontal (in a train seat, for example), even when all other environmental conditions are 'ideal', total sleep times and amounts of REM sleep are reduced and there are increased periods of wakefulness (Aeschbach et al. 1994).

In a study where humans were sleep deprived for 64h, the EEG was recorded during different postures, sitting and standing. Delta activity increased during the sleep deprivation time when the participants were seated, but was minimal, and unchanged over time when participants were standing (Caldwell et al. 2000). Theta

activity increased over time in both postures, but more so when seated as compared to standing, participants had 'microsleeps' when seated, but remained awake when standing.

Sheep (in a similar fashion to cattle, see below) may be able to undergo some light Non-REM sleep without lying down in a fully relaxed manner, but without space to lie and hence inadequate muscle relaxation, REM sleep may be prevented. Ruckebusch (1974) carried out an experiment to determine the effects of REM sleep deprivation on the following sleep periods in cattle. Recumbency, and therefore REM sleep, (cattle can exhibit Non-REM sleep while standing) was prevented for 14 h each day for four weeks, 20-h/day for two weeks and 22-h/day for two weeks. In the first four weeks the sleeping pattern of the cattle had adapted within five days so that a similar total amount of REM sleep to the baseline period (no lying restriction) was seen, but it occurred during the day when lying was permitted (Ruckebusch, 1974). In the final four weeks, REM sleep was much reduced (and absent in the 22 h/day deprivation weeks). There was an increase in Non-REM sleep during this time. The bouts of Non-REM decreased in length as compared to the baseline period. In the four day post deprivation, rebounds were seen in both Non-REM and REM sleep. Fragmentation of Non-REM was reduced (i.e. bouts increased in length) and REM sleep showed double the number of episodes compared to baseline values. This included sleep during the day, although by the fifth day post deprivation sleep only occurred at night (Ruckebusch, 1974). Interestingly, Ruckebusch notes that the cattle became more aggressive towards humans by the end of the experiment which may indicate psychological stress.

The typical effects of total sleep deprivation are: reduced sleep latency and an increase in slow frequency waves in Non-REM sleep in the subsequent recovery sleep (see chapter 1) (Tobler, 1995). REM sleep seems to be less affected after short amounts of sleep deprivation, with rebound of REM only occurring after 2 or 3 nights without sleep in humans (Brunner et al. 1990). However, when REM sleep is selectively deprived in humans, REM rebound can be seen in the first night of recovery sleep (Brunner et al. 1990). Therefore, it might be expected that if sheep can get some Non-REM sleep during a period of space restriction, but be mainly REM sleep deprived, post-space restriction REM rebound might be seen.

This experiment will investigate the link between space allowance and resting behaviour and will assess the effects of space restriction on sleep and rest post-treatment.

5.3 Materials and Methods

5.3.1 Animals

Twenty-two, adult, Dorset poll cross, ewes that had been kept outside on pasture, were randomly paired. Within each pair, sheep were randomly assigned to either a minimum or a maximum space allowance group (see below). In addition, eighteen, adult, mixed breed (mainly Scottish Blackface X Suffolk), ewes that had previously been housed were used as 'packing sheep' to simulate the stocking density of transport.

5.3.2 Husbandry details and experimental set-up

The experiment was carried out between February 2004 and June 2004. Sheep were penned at least two weeks before the start of the experiment. Four Dorset sheep were housed in individual 2 X 2 m pens bedded with straw and with artificial lighting on a 16hL:8hD cycle. Pairs of sheep were brought into the experimental barn on a staggered basis so that there were always one pair on experiment and one pair habituating to the environment and handling procedure. Sheep were offered approximately 250g/d/sheep concentrated feed (Pentland lamb finisher, Seafield Mill, UK) from a bucket and *ad-libitum* hay and water. The sheep were habituated to the attachment of a harness and fibre-glass helmet (as described in the general methodology section). Two black and white CCTV cameras with infrared pass filters were placed at right angles to each of the four pens and one infrared lamp was directed at a 45° angle to each pen. The cameras were attached, via a digital multiplexer and a time-code generator, to a 24-h time lapse video recorder.

The 'packing sheep' were housed in the same barn in 3 X 5.5m pens each containing eight sheep. The pens were bedded with straw and fenced with solid hurdles. Packing sheep were offered approximately 250g/d/sheep concentrated feed from a trough and *ad-libitum* hay and water. All sheep were weighed when they were brought into the barn and the 'packing sheep' were initially assigned to four groups of four sheep, balancing across groups for weight. After the first experimental day, two of the 'packing sheep' were considered too small (< 40 kg) and were exchanged for larger sheep.

5.3.3 Experimental treatment schedule

Twenty-four-hour electrophysiological and simultaneous time-lapse video recordings were made from pairs of experimental sheep during a baseline day starting at approximately 1330h. Seven days after the baseline recording, the pairs of experimental sheep were fitted with the equipment harnesses and 29-h electromyogram and simultaneous time-lapse video recordings were made at reduced space allowances. Directly after the 29-h recordings, the experimental sheep were refitted with electrophysiological equipment and 24-h electrophysiological and simultaneous time-lapse video recordings were made. The treatment schedule is shown in Table 5.1.

Table 5.1. The experimental treatment schedule

Recording					Group Size	Space Allowance		Food & water	Measur-ments taken
No.	Type	Start time	Day	Length		Minimum Group	Maximum Group		
1	Baseline	1330h	-7	24-h	1	$4m^2$/sheep	$4m^2$/sheep	Yes	EEG, EOG, EMG & B
2	Treatment	0800h	0	14-h	5	$0.3m^2$/sheep	$0.8m^2$/sheep	No	EMG & B
2	Treatment	2200h	0	1-h	5	$0.8m^2$/sheep	$0.8m^2$/sheep	Yes	EMG & B
2	Treatment	2300h	0	14-h	5	$0.3m^2$/sheep	$0.8m^2$/sheep	No	EMG & B
3	Post-Treatment	1400h	1	24-h	1	$4m^2$/sheep	$4m^2$/sheep	Yes	EEG, EOG, EMG & B

Start times are approximate. On three occasions, the baseline recording was 4 days prior to the treatment recording. B = behavioural measurements made from time-lapse video.

5.3.4 Electrophysiological recordings

On the morning of the baseline recording, both the minimum and the maximum group sheep were fitted with electrodes and 24h electrophysiological recordings of the EEG, the EOG, EMG from the jaw, neck and hind-leg were made while the sheep remained in their home pens. In addition, 24-h time-lapse video recordings were made of both sheep. The electrode attachment, removal, and electrophysiological techniques were carried out as outlined in the general methodology section (chapter 2).

Four to seven days after the baseline recording, the pairs of experimental sheep were fitted with the equipment harnesses and 29-h electromyogram and simultaneous time-lapse videos recordings were made. After the EMG equipment had been fitted, one group of four 'packing sheep' was added to each experimental

sheep pen and all food and water was removed. Opaque boards were placed between the experimental pens so each group became visually isolated from other sheep in the barn. The pen containing the sheep that had been assigned to the Maximum space allowance group remained at 2 X 2 m, giving a space allowance of approximately $0.8m^2$/sheep. The pen containing the sheep that had been assigned to the Minimum space allowance group was reduced in size using extra hurdles to 1.25 X 1.2 m, giving a space allowance of approximately $0.3m^2$/sheep (see Figure 5.1). The sheep were then left without human contact for 14h (although they were checked by means of a remote monitor).

Figure 5.1. The experimental pens showing the space restriction. Five sheep on the left are at a space allowance of $0.3m^2$/sheep. The five sheep on the right are at a space restriction of $0.8m^2$/sheep. The visual barrier between groups is also shown.

After 14h, the extra hurdles were removed from the Minimum space allowance group. Five full hay nets were attached to the front of each pen and four buckets of water were attached to the sides of each pen allowing the sheep easy access to both hay and water. At the end of 1h, the equipment on the experimental sheep was checked, the food and water removed and the extra hurdles added to the Minimum space allowance pen. The sheep were left for a further 14h.

During the final hour of the reduced space allowance treatment, the EMG equipment was removed from the experimental sheep and the electrode sites were cleaned using 100% ethanol. When 29h of reduced space allowance treatment had been completed, the extra hurdles were removed, the 'packing sheep' were removed, hay and water were added and fresh straw bedding was laid down. The experimental sheep were refitted with the electrophysiological equipment and post-treatment 24-h electrophysiological and simultaneous time-lapse video recordings were made from

the experimental sheep. The video recordings were started within 5 minutes of the removal of the packing sheep. However, the first hour of the post-treatment recording included the time taken for the experimenter to refit the electrodes for electrophysiological recording. A pilot space reduction was carried out before the main trial, and it was observed that all of the sheep spent at least the first hour standing and eating post-treatment. It was therefore assumed that the electrode attachment would have a minimal effect on behaviour as the sheep would not be affected and would continue to eat.

The experimental procedure was repeated until satisfactory electrophysiological recordings had been made from ten pairs of sheep. Experimental sheep were reused for the reduced space part of the experiment as a 'packing sheep', each sheep experiencing reduced space conditions no more than 5 times in total (including once as an experimental sheep). Sheep would be used in the minimum space allowance no more than twice (once as an experimental sheep and once as a packing sheep).

5.3.5 Electrophysiological and behavioural analysis

The behavioural and electrophysiological recordings were analysed as detailed in the general methodology section (chapter 2).

5.3.6 Statistical analysis

Descriptive statistics were carried out in Minitab 13 (Minitab, USA). Data were tested using the Anderson-Darling test for normality to confirm the distribution of the data. Where the data were normally distributed, the mixed model analysis of variance in SAS 8.2 (The SAS Institute, California, USA) was used to assess the differences in posture between the Minimum and Maximum space allowance sheep during the 29-h reduced space treatment. The generalised linear mixed model procedure in SAS 8.2 was used to analyse the latency and frequency of lying bouts (head up and head down) during the space restriction period.

All data comparing the baseline and the post-treatment recordings for the Minimum and Maximum space allowance sheep, except for bout frequency and sleep latency and sleep distribution analysis, were carried out using the mixed model

analysis of covariance with repeated measures in SAS 8.2 with pair of sheep as a fixed effect and time period as the repeated measure. This was to examine whether the sleep and resting behaviour of sheep was affected by the reduced space and if there was a difference in the behaviour between pairs of sheep from the minimum and maximum space allowances. The mixed models compared the differences between the pairs of sheep during the baseline recording period and compared these differences to those during the 24-h post treatment period. Bout frequencies were analysed using the general linear mixed model procedure comparing the baseline and the post-treatment 24h in SAS 8.2. (Please see chapter 3 methods section for full details on statistics used).

The sequences of behaviour and sleep post-treatment were analysed using lag-sequential analysis in Observer 4.1. Paired t-tests in Minitab compared changes in the sequences of sleep within treatment groups between the baseline and the post-treatment recording, where the distribution of the data allowed. Analysis of the distribution of Non-REM and REM sleep was undertaken by splitting the 24h post-treatment period into four 6-h time periods to examine whether there was a treatment effect on the distribution of sleep in the 24-h post space restriction. These data were analysed using the mixed models procedure with estimate statements in SAS 8.2 comparing the like 6-h time periods across treatment groups.

Further analysis independent of the treatment groups, was carried out by splitting the sheep into different groups according to the amount of time they spent lying with head down in the space restriction period. Sheep that lay down with head down for 3% or less of the 29h were placed in the 'low lying' group and sheep that lay down with head down for more than 8% were placed in the 'high lying' group, regardless of whether they were from the minimum or the maximum space allowance treatments. The post-treatment 24h was then analysed to compare the percentage of lying with the head down, Non-REM and REM sleep from 'low lying' and 'high lying' sheep using the mixed model analysis of variance in SAS 8.2.

5.4 Results

5.4.1 Behaviour of sheep during the restricted space allowance period

Table 5.2 (a-d) shows the behaviour of sheep in the two different space allowance categories during each of the 14h space restriction periods.

Social interactions were observed during the space restriction period, but not quantified. Aggressive interactions were observed in the maximum space allowance condition.

Maximum space allowance sheep stood for less of each 14h period and had more, but shorter bouts of standing than the minimum space allowance sheep. In addition, sheep from both minimum and maximum space allowances were standing for more of the first 14h period than the second 14h period (Mixed model 21 ± 6.8, t = 3.1, $P<0.05$ for minimum, 17.8 ± 1.6, t – 10.9, $P<0.001$ for maximum sheep). Maximum sheep had shorter bouts of standing in the second 14h period than the first (Mixed model 416 ± 62.5, t = 6.7, $P<0.01$) and minimum sheep showed a similar tendency (Mixed model 3776 ± 1902, t = 2, $P<0.07$).

Sheep from the maximum space allowance lay down 'head up' for longer in each 14h period and had more bouts of 'lying head up' than sheep from the minimum space allowance. Sheep from both the maximum and minimum space allowances spent more of the second 14h period 'lying head up' than they did in the first 14 h period (Mixed model –13.6 ± 2.1, t = -6.5, $P<0.01$ for maximum, -18.4 ± 6.7, t = -2.7, $P<0.05$ for minimum sheep). The bout lengths of lying head up were not affected by the treatment time period for sheep from either the maximum or minimum space allowances. Minimum sheep had more bouts of lying head up in the second 14h period as compared with the first (GLMM –6.2 ± 1.8, F = 3.4, $P<0.01$).

Sheep from the minimum space allowance took longer to lie down head up than sheep from the maximum space allowance in both treatment time periods. Moreover, both minimum and maximum sheep took longer to lie down head up in the first time period than the second (GLMM, 2470 ± 683, F = 3.6, $P<0.01$ for maximum sheep, 5492 ± 2504, F = 2.2, $P<0.05$ for minimum sheep).

Sheep from the maximum space allowance also spent more time and had more bouts of 'lying head down' posture than sheep from the minimum space allowance in both treatment time periods. Again, sheep from both the maximum and

minimum space allowances spent more time lying head down in the second 14h period than the first (Mixed model, -4.2 ± 1.5, t = -2.8, P<0.05 for maximum, -2.6 ± 1, t = -2.6, P<0.05 for minimum). Sheep from the maximum space allowance had a tendency to have longer bouts of lying with head down in the second 14h than the first (Mixed model, -80 ± 39, t = -2, P<0.07). Sheep from the minimum space allowance had more bouts of lying with head down in the second period than the first (GLMM –4.5 ± 1.2, F = -3.9, P<0.01).

Figure 5.2 shows the hour-by-hour distribution of the two lying postures, lying with head up and lying with head down during the 29h space restriction period. It illustrates the large variation between individual sheep in the amount of time spent lying with the head down in both conditions.

In both treatment time periods, sheep from the maximum space allowance were quicker to lie down with head down than sheep from the minimum space allowance. In addition, sheep from both the maximum and minimum space allowances were quicker to lie down with head down in the second 14h period than they were in the first 14h period (GLMM, 4881 ± 1971, F = 2.5, P<0.05 for maximum sheep, 20013 ± 4519, F = 4.4, P<0.01 for minimum sheep).

The majority of experimental sheep were standing and eating for the duration of the 1h 'rest-period'. Two sheep lay down during the 'rest-period', both from the maximum space allowance condition (one for 28% and the other for 51% of the 1h 'rest-period'). Both sheep seemed to have difficulty reaching the hay nets so were unable to spend any of the hour eating. Both sheep did not lie 'head down' and were therefore unlikely to have slept during the 'rest period'.

Table 5.2. The effect of reduced space allowance on 'Standing', 'Lying head up' and 'Lying head down' postures (mean ± s.e) during a 29-h space restriction period

a) Total duration (as a % of each 14-h recording period)

Measurement	1st 14h Space Restriction		P	2nd 14h Space Restriction		P
% of time	Minimum	Maximum		Minimum	Maximum	
Standing	68.2 ± 7.6	38.9 ± 2.0	**	47.2 ± 5.4	21.9 ± 1.9	**
Lying Head Up	30.1 ± 7.5	55.5 ± 2.0	**	48.5 ± 4.9	69.1 ± 2.8	**
Lying Head Down	1.7 ± 0.6	5.6 ± 1.1	*	4.4 ± 1.3	9.8 ± 1.8	*

N = 11 pairs of minimum and maximum group sheep. Mixed model analysis with repeated measures. The probability refers to the difference between Minimum and Maximum in each treatment time period. P= statistical probability, * = P<0.05, ** = P<0.01. † = P>0.05, but <0.1

b) Bout duration

Measurement	1st 14h Space Restriction		P	2nd 14h Space Restriction		P
Bout Duration (s)	Minimum	Maximum		Minimum	Maximum	
Standing	6048 ± 2336	946 ± 120	NS †	2271 ± 497	530 ± 94	**
Lying Head Up	1064 ± 211	919 ± 110	NS	1445 ± 204	1009 ± 127	NS †
Lying Head Down	229 ± 86	249 ± 35	NS	250 ± 57	329 ± 27	NS

N = 11 pairs of minimum and maximum group sheep. Mixed model analysis with repeated measures. P= statistical probability, * = P<0.05, ** = P<0.01. † = P>0.05, but <0.1

c) Number of bouts

Measurement	1st 14h Space Restriction		P	2nd 14h Space Restriction		P
No of Bouts	Minimum	Maximum		Minimum	Maximum	
Standing	11.6 ± 1.8	23.6 ± 2.1	**	12.8 ± 1.7	23.2 ± 2.2	**
Lying Head Up	13.5 ± 2.4	35.7 ± 4.9	**	19.6 ± 2.7	37.5 ± 3.8	**
Lying Head Down	3.0 ± 1.1	13.8 ± 3.8	*	7.5 ± 2.1	16.2 ± 3.2	*

N = 11 pairs of minimum and maximum group sheep. GLMM with repeated measures. . P= statistical probability, * = P<0.05, ** = P<0.01. † = P>0.05, but <0.1

d) Latency

Measurement	1st 14h Space Restriction		P	2nd 14h Space Restriction		P
Latency (s)	Minimum	Maximum		Minimum	Maximum	
Lying Head Up	9986 ± 2389	3491 ± 631	*	4494 ± 789	1021 ± 243	**
Lying Head Down	38933 ± 4391	10570 ± 1846	**	18920 ± 5459	5688 ± 540	*

N = 11 pairs of minimum and maximum group sheep. GLMM with repeated measures. SP= statistical probability, * = P<0.05, ** = P<0.01, † = P>0.05, but <0.1

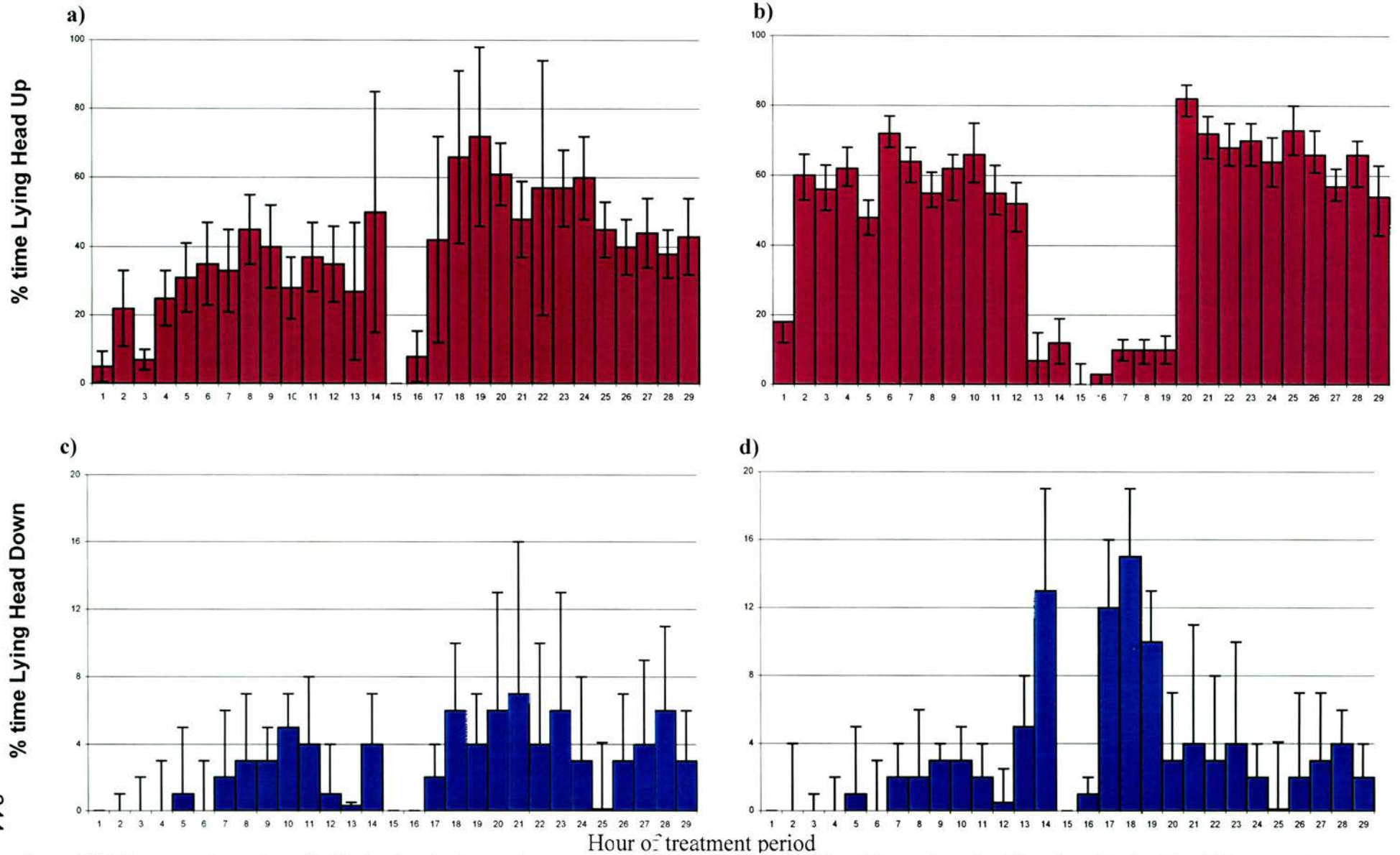


Figure 5.2 The mean (error bars indicate standard error) percentage of time spent Lying Head Up posture (red bars) and Lying Head Down posture (blue bars) during each hour of 29h space restriction periods. a) minimum space allowance, b) maximum space allowance for Lying Head Up. c) minimum space allowance, d) maximum space allowance for Lying Head Down. N = 11 sheep in each condition. Note: hour 15 was the feeding hour.

5.4.2 The effects of 29h restricted space allowance on sleep in sheep

5.4.2.1 Non-REM sleep

Table 5.3 shows the effects of 29-h restricted space allowance on Non-REM sleep as compared with pre-treatment baselines.

There were no significant differences in number or duration of Non-REM sleep bouts between the sheep from the minimum and maximum space allowances post-treatment as compared to baselines. There were also no significant differences in the number and the duration of Non-REM bouts within sheep from either the minimum or maximum space allowances post-treatment as compared to baselines. There was a tendency for sheep in the minimum space allowance group to spend less time in Non-REM sleep post-treatment when compared to the baseline (estimate 1.7 $\pm$ 0.9, t = 1.9 P<0.07).

Although there was no difference between sheep from the minimum and maximum space allowances in the latency to Non-REM sleep post-treatment as compared to baseline, both minimum and maximum groups showed a significant increase in latency post-treatment as compared with the baseline (GLMM, estimate –10964 $\pm$ 1680, t = -6.5, P<0.01 for minimum sheep and estimate –13149 $\pm$ 2055, t = -6.4, P<0.01 for maximum sheep).

The percentage of Delta waves in the second 30s epoch of each Non-REM bout of sheep from the minimum group was different from sheep from the maximum group in the post-treatment recording as compared to that from the baseline. Sheep from both the maximum and minimum space allowances had a higher percentage of Delta waves during the second 30s epochs of Non-REM bouts during the post-treatment than during the equivalent baseline (Mixed model, estimate 15.2 $\pm$ 3.2, t = 5.1, P<0.001 for maximum sheep, 19.5 $\pm$ 4.1, t = 5.9, P<0.001 for minimum sheep).

5.4.2.2 REM sleep

Table 5.4 shows the effects of 29-h restricted space allowance on REM sleep as compared with pre-treatment baselines.

There were no significant differences in the mean duration of REM sleep bouts post-treatment as compared to baselines. However, for both the number of bouts and the percentage of time spent in REM sleep the difference between the space

allowance groups was greater post-treatment than in baseline (GLMM –1.8 ± 0.6, F = -2.7, P<0.05 and Mixed model –0.8 ± 0.3, t = -2.4, P<0.05 respectively). In the sheep from the minimum space allowance, but not in the sheep from the maximum space allowance, the number of bouts and the percentage time spent in REM sleep increased post-treatment compared with baseline.

Although there was no difference between sheep from the minimum and maximum space allowances in the latency to REM sleep post-treatment as compared to baseline, both minimum and maximum groups showed a significant increase in latency post-treatment (GLMM, estimate –7593 ± 1296, t = -5.9, P<0.01 for minimum sheep and estimate –11714 ± 2340, t = -5.0, P<0.01 for maximum sheep).

There was no difference in eye movement density in 10-second epochs of REM sleep between sheep from the minimum and maximum space allowances post-treatment as compared to baseline. However, sheep from both the maximum and minimum space allowances tended to show an increase in the density of eye movements post-treatment as compared with the baseline.

5.4.2.3 Total sleep

Table 5.5 shows the effects of 29-h restricted space allowance on Total sleep as compared with pre-treatment baselines. There were no significant differences between maximum and minimum sheep in the total time (%) spent sleeping, or the number or the duration of sleep bouts post-treatment as compared to baselines.

The hour-by-hour distribution of Non-REM and REM sleep is shown in Figure 5.3. The 24-h post-treatment period was split into four 6-h time periods to determine where any differences in the time spent in Non-REM and REM sleep occurred post-treatment, between sheep from the two treatment groups. There were no differences in the time spent in Non-REM between treatment groups at any of the 6-h periods post-treatment. However, there were significant differences in the time spent in REM sleep between treatment groups in the first and second 6-h periods post-treatment. In the first 6-h post-treatment sheep from the minimum space allowance had 472 ± 114 s of REM sleep, compared to the sheep from the maximum space allowance, 179 ± 68 s of REM sleep (Mixed model, estimate 304 ± 72, t = 4.9, P<0.05). In the second 6-h post-treatment (6 to 12h post-treatment) sheep from the

minimum space allowance had 1477 ± 166 s of REM sleep, compared to the sheep from the maximum space allowance which had 933 ± 151 s of REM sleep (Mixed model, estimate 538 ± 97, $t = 5.9$, $P<0.05$). There were no differences in REM sleep between treatment groups after 12h post-treatment.

Table 5.3. Effects of a 29-h restricted space allowance on Non-REM sleep (mean ± s.e) (n = 11 pairs)

Measurement	24-h Baseline Recording Period		24-hPost-Treatment Recording Period		Difference between baseline and post-treatment period‡	Statistical significance of the difference between baseline and post-treatment periods‡
	Minimum	Maximum	Minimum	Maximum		
No. of bouts	16.6 ± 1.2	16.5 ± 1.6	17.4 ± 1.5	17.1 ± 3	-0.1 ± 2.5	NS
Duration of bout (s)	331 ± 36	261 ± 15	301 ± 41	243 ± 30	12.5 ± 41	NS
Total Duration (% of period)	7.1 ± 1.1	5.6 ± 0.8	5.4 ± 0.9	6.4 ± 1.6	2.5 ± 1.2	NS†
Latency (s)	6051 ± 1213	5810 ± 2007	17015 ± 1592	18960 ± 2464	2185 ± 1383	NS
% Delta waves in 30 s bouts	23 ± 2.1	24 ± 1.9	52 ± 4.5	42 ± 4.1	-14 ± 7.4	*

Using Proc GLMM for the no. of bouts and latency to Non-REM sleep and mixed model analysis with repeated measures for all other measures.

† =A statistical probability of $P \geq 0.05$, but < 0.1.

‡Differences between baseline recording and post-treatment recording in the differences between pairs of sheep from the minimum and maximum space allowances within a 24-h time period.

Table 5.4. Effects of a 29-h restricted space allowance on REM sleep (mean ± s.e) (n = 11 pairs)

Measurement	24-h Baseline Recording Period		24-h Post-Treatment Recording Period		Difference between baseline and post-treatment period‡	Statistical significance of the difference between baseline and post-treatment periods‡
	Minimum	Maximum	Minimum	Maximum		
No. of bouts	12.6 ± 0.8	13.6 ± 1.6	14.5 ± 0.9	12.9 ± 1.2	-2.5 ± 1.1	*
Duration of bout (s)	253 ± 23	212 ± 15	291 ± 16	236 ± 22	-14.2 ± 27	NS
% of time period	3.6 ± 0.4	3.4 ± 0.5	4.4 ± 0.3	3.1 ± 0.3	-1.2 ± 0.5	*
Latency (s)	8606 ± 1645	7801 ± 2264	16200 ± 1477	19515 ± 2369	4120 ± 2865	NS
Eye Movement Density in 10 s epochs	58 ± 12	54 ± 9.2	63 ± 10	60 ± 11	-2.4 ± 3.2	NS

Using Proc GLMM for the no. of bouts and the Latency to REM sleep and mixed model analysis with repeated measures for all other measures.
‡Differences between baseline recording and post-treatment recording in the differences between pairs of sheep from the minimum and maximum space allowances within a 24-h time period.

Table 5.5. Effects of a 29-h restricted space allowance on total sleep (Non-REM + REM sleep) (mean ± s.e) (n = 11 pairs)

Measurement	Baseline Recording Period		Post-Treatment Recording Period		Difference between baseline and post-treatment period‡	Statistical significance of the difference between baseline and post-treatment periods‡
	Minimum	Maximum	Minimum	Maximum		
No. of bouts	17.2 ± 1.3	17.2 ± 2.0	18.1 ± 1.4	17.7 ± 2.2	-0.4 ± 2.1	NS
Duration of bout (s)	541 ± 45	492 ± 23	561 ± 41	493 ± 30	22.5 ± 47	NS
% of time period	10.7 ± 1.5	9.0 ± 1.3	9.8 ± 1.2	9.5 ± 1.9	-1.1 ± 0.7	NS

Using Proc GLMM for the no. of bouts and mixed model analysis with repeated measures for all other measures.
‡Differences between baseline recording and post-treatment recording in the differences between pairs of sheep from the minimum and maximum space allowances within a 24-h time period.

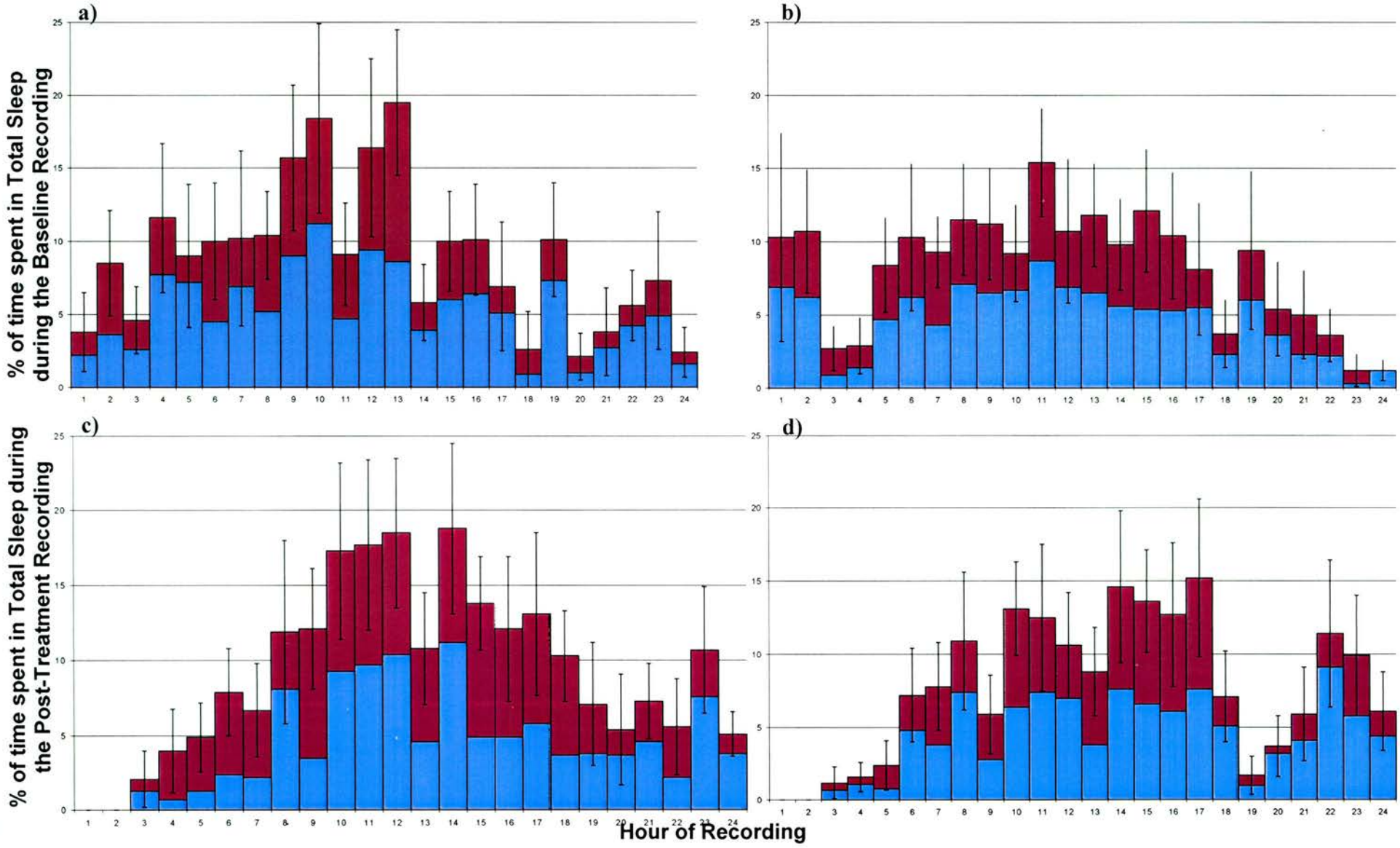


Figure 5.3 The mean (error bars indicate standard error of total sleep) percentage of Non-REM and REM Sleep during each hour of 24h recordings. a) minimum space allowance baseline, b) maximum space allowance baseline. c) minimum space allowance post-treatment, d) maximum space allowance post-treatment. N = 11 sheep in each condition.

5.4.3 The effects of 29h restricted space allowance on posture in sheep

5.4.3.1 Posture changes

Table 5.6 (a-d) shows the effects of a 29h restricted space allowance period on standing, lying with the head raised and lying with the head resting on the bedding as compared with pre-treatment baselines.

There were no significant differences in the total time (%) spent standing, the bout duration or the number of bouts of standing between sheep from the minimum and maximum space allowances post-treatment as compared to baselines. However, the sheep from the maximum space allowance stood for more of the post-treatment 24-h compared to the baseline (mixed model estimate –7.1 ± 2.0, t = -3.9 $P<0.01$) and had longer bouts of standing (mixed model estimate –476 ± 135, t = -3.4, $P<0.01$).

There were no significant differences in the total time (%) spent, the bout duration, or the number of bouts of 'lying head up' between sheep from the minimum and maximum space allowances post-treatment as compared to baselines. However, sheep from the maximum space allowance did spend significantly less time 'lying head up' post-treatment than in the baselines (Mixed model, estimate 8.5 ± 3.3, t = 3, $P<0.05$). Although there were no significant differences between sheep from the minimum and maximum space allowances in the latency to 'lying head up', both groups took longer to lie down post-treatment than baselines (GLMM estimates –7901 ± 1760, F = -4.5, $P<0.01$ for minimum sheep, -9204 ± 872, F = -10, $P<0.001$).

There were no significant differences in the total time (%) spent, the bout duration, or the number of bouts of 'lying head down' between sheep from the minimum and maximum space allowances post-treatment as compared to baselines. There was a tendency for the duration of lying head down bouts to be different between sheep from the minimum and maximum space allowances, this difference occurs in the baseline as two sheep in the minimum space allowance group had unusually long bouts of lying head down. When these sheep are treated as outliers and removed from the analysis, the tendency is no longer present.

There was also a tendency for sheep from the minimum space allowance to have more 'lying head down' bouts post-treatment as compared to baselines (GLMM, estimate –6.1 ± 3.1, F = -1.9, $P<0.06$). Similarly to the lying head up posture, there were no differences in 'lying head down' between sheep from the minimum and

maximum space allowances. However, sheep from the minimum group had a tendency to take longer and sheep from the maximum group took significantly longer to lie down head down post-treatment as compared to baselines (GLMM estimates – 8492 ± 5554, F = -1.82, P<0.07 for minimum sheep, -10462 ± 2336, F = -4.5, P<0.01 for maximum sheep).

The hour-by-hour distribution of Lying Head Up and Lying Head Down posture in the baseline and post-treatment periods is shown in Figure 5.4.

5.4.3.2 Distribution of behaviour post-treatment

The electrode reattachment process at the beginning of the 24-h post-treatment period, took, on average 4578 ± 949 s from the time the packing sheep were removed to the time when the sheep was left in its home pen.

All of the treatment sheep were standing during the electrode reattachment process. Eighteen sheep spent 80 ± 5.4% of the reattachment time eating the fresh hay that had been provided. The four remaining sheep spent 47 ± 9.8% of the reattachment time eating. Sheep from the maximum space allowance spent 73 ± 5.1% of the second hour, 63 ± 4.8% of the third hour and 61 ± 4.9 of the fourth hour post-treatment eating. The fifth hour saw a change in behaviour from the majority of the time spent eating to the majority of the time spent ruminating (52 ± 4.5%). Sheep from the minimum space allowance spent 64 ± 5.2% of the second hour, 55 ± 5.1 % of the third hour and 42 ± 5.0 % of the fourth hour post-treatment eating. Sheep from the minimum space allowance seemed to switch to rumination earlier than those from the maximum condition, spending 46 ± 4.2% of the fourth hour ruminating (compared to 31 ± 4.7% of the maximum sheep); although the switch to majority ruminating happened in the same hour for both groups of sheep (the fifth hour, 52 ± 5.1% rumination for sheep from the minimum space allowance).

In the baseline period, the percentage of sleep that started with REM sleep (without any epochs of Non-REM sleep first) was 3.4 ± 1.2 % during the first 6h of recording for both groups of sheep. After the first 6h, the percentage of sleep that was direct to REM was 5.2 ± 2.9 %. In sheep from the maximum space allowance condition this had significantly increased to 12 ± 5.2 % during the first 6h post treatment (Paired t-test, t = 3.4, P<0.01). In sheep from the minimum space allowance

condition the percentage of sleep straight to REM had significantly increased to 21.5 ± 8.5 % in the first 6h post treatment (Paired t test, $t = 5.8$, $P<0.001$). After the first 6h post treatment, the percentage of sleep that started with REM was 9.4 ± 6.0 and 7.4 ± 5.9% for the maximum and minimum space allowances respectively.

Table 5.6. Effects of a 29-h restricted space allowance on 'Standing', 'Lying head up' and 'Lying head down' postures (mean ± s.e)

a) Total duration (as a % of the 24-h recording period)

Measurement	Baseline Recording Period		Post-Treatment Recording Period		Difference between baseline and post-treatment period‡	Statistical significance of the difference between baseline and post-treatment periods‡
% of time	Minimum	Maximum	Minimum	Maximum		
Standing	35.9 ± 1.3	36.2 ± 2.3	39.9 ± 1.2	45.3 ± 1.6	4.5 ± 6.1	NS
Lying Head Up	48.9 ± 2.1	50.2 ± 1.7	46.3 ± 1.1	41.7 ± 2.4	-6.0 ± 5.8	NS
Lying Head Down	15.3 ± 2	15.6 ± 2.2	13.8 ± 1.2	12.9 ± 2.6	0.9 ± 2.4	NS

N = 11 pairs of sheep from the minimum and maximum space allowances. Mixed model analysis with repeated measures.
‡Differences between baseline recording, and the post-treatment recording in the differences between pairs of minimum and maximum sheep within a 24h recording period.

b) Bout duration

Measurement	Baseline Recording Period		Post-Treatment Recording Period		Difference between baseline and post-treatment period‡	Statistical significance of the difference between baseline and post-treatment periods‡
Bout Duration (s)	Minimum	Maximum	Minimum	Maximum		
Standing	1248 ± 251	1179 ± 191	1454 ± 111	1655 ± 217	271 ± 144	NS
Lying Head Up	863 ± 106	823 ± 67	834 ± 83	754 ± 67	-40 ± 154	NS
Lying Head Down	607 ± 80	425 ± 27	485 ± 66	436 ± 47	132 ± 71	NS†

N = 11 pairs of sheep from the minimum and maximum space allowances. Mixed model analysis with repeated measures. NS †A statistical probability of P≥0.05, but < 0.1
‡Differences between baseline recording, and the post-treatment recording in the differences between pairs of minimum and maximum sheep within a 24h recording period.

c) Number of bouts

Measurement	Baseline Recording Period		Post-Treatment Recording Period		Difference between baseline and post-treatment period‡	Statistical significance of the difference between baseline and post-treatment periods‡
No of Bouts	Minimum	Maximum	Minimum	Maximum		
Standing	30.1 ± 3.3	29.9 ± 2.5	27.3 ± 1.9	29.6 ± 3.1	2.5 ± 4	NS
Lying Head Up	53.6 ± 4.4	56.3 ± 4.9	56.8 ± 4.8	55.3 ± 4.9	-3.8 ± 8	NS
Lying Head Down	24 ± 2.5	26.8 ± 3.8	30.1 ± 3.6	26.8 ± 3.6	-6.1 ± 6.1	NS

N = 11 pairs of sheep from the minimum and maximum space allowances. GLMM with repeated measures.
‡Differences between baseline recording, and the post-treatment recording in the differences between pairs of minimum and maximum sheep within a 24h recording period.

d) Latency

Measurement	Baseline Recording Period		Post-Treatment Recording Period		Difference between baseline and post-treatment period‡	Statistical significance of the difference between baseline and post-treatment periods‡
Latency (s)	Minimum	Maximum	Minimum	Maximum		
Lying Head Up	1270 ± 476	1261 ± 331	9171 ± 433	10466 ± 694	1303 ± 1085	NS
Lying Head Down	5631 ± 1230	5137 ± 1465	14124 ± 1832	15599 ± 1701	2969 ± 2092	NS

N = 11 pairs of sheep from the minimum and maximum space allowances. GLMM with repeated measures.
‡Differences between baseline recording, and the post-treatment recording in the differences between pairs of minimum and maximum sheep within a 24h recording period.

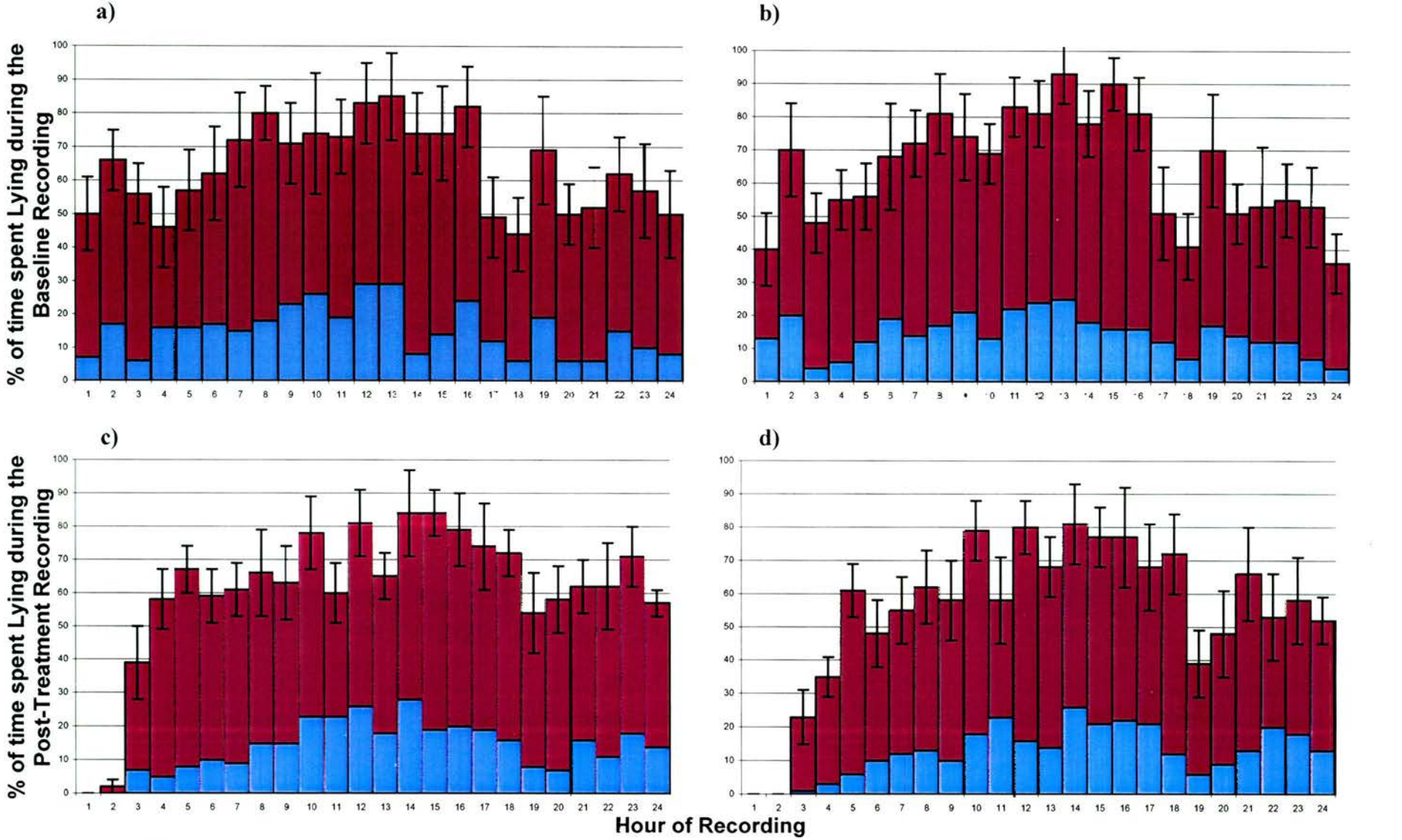


Figure 5.4. The mean (error bars indicate standard error of total lying) percentage of Lying Head Up and Lying Head Down during each hour of 24h recordings. a) minimum space allowance baseline, b) maximum space allowance baseline. c) minimum space allowance post-treatment, d) maximum space allowance post-treatment. N = 11 sheep in each condition.

5.4.3.3 Other Lying and Sleep Comparisons

When the data were split into two groups according to the amount of lying head down seen during the space restriction period, irrespective of treatment groups, there were no significant differences between the 'low' (3% or less lying head down) and 'high' (8% or more lying head down) lying groups for lying with head down or Non-REM sleep post-treatment. However, there was a significant difference in the percentage of the time spent in REM sleep during the 24-h post-treatment between 'low' and 'high' lying groups. Sheep that had spent less time lying in the space restriction period ('low' lying) had significantly more REM sleep post-treatment than sheep that had spent more time lying in the space restriction period ('high' lying) (Mixed model 1.4 ± 0.2, $t = 3.1$ $P<0.05$). Figure 5.5 shows the mean (± s.e.) of lying with head down, Non-REM and REM Sleep in each lying 'amount' group.

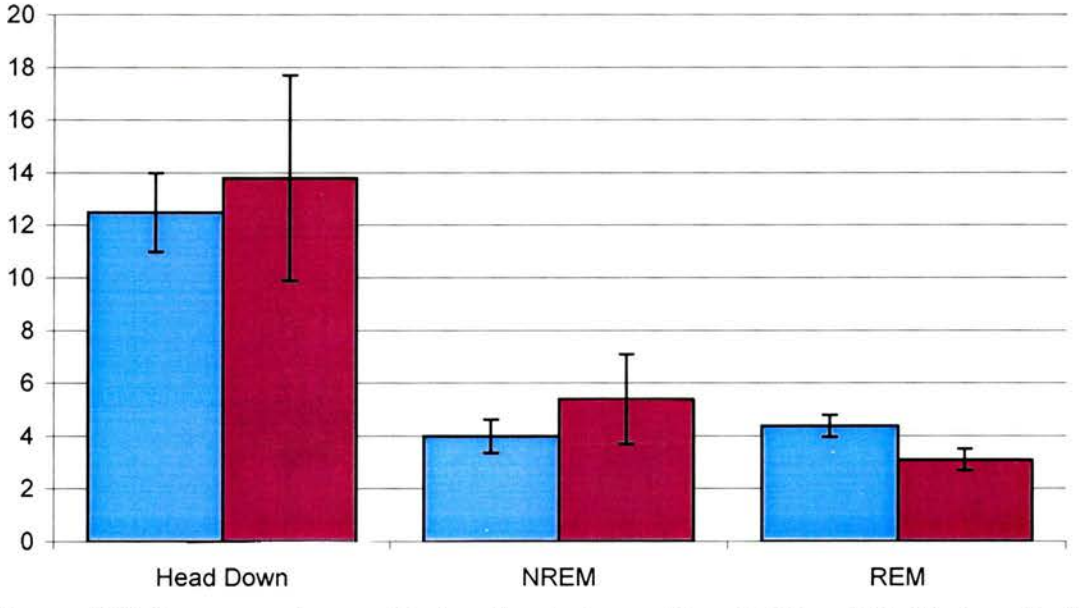


Figure 5.5 The percentage of Lying Head down, Non-REM and REM sleep in the post-treatment 24h between sheep split into groups according to how much they had been lying head down during the 29h space restriction period.
3% or less lying head down **More than 8% lying head down**
N = 8 in each group

5.4.4 The effects of 29h restricted space allowance on ingestive behaviour in sheep

Table 5.7 shows the effects of a 29h restricted space allowance period on ruminating, eating and drinking as compared with pre-treatment baselines.

There were no significant differences in the total time (%) spent in, the duration of bouts, or the number of bouts of rumination between minimum and maximum conditions. However, the sheep from the maximum space allowance spent significantly less time ruminating post-treatment than in the baselines (mixed model estimate 5.4 ± 1.6, t = 3.3 P<0.01). The time taken to begin to ruminate was not different between sheep from the minimum and maximum space allowances, but both groups took longer to start to ruminate post-treatment than in the baselines (GLMM estimate –7143 ± 818, F = -8.7, P<0.001 for minimum sheep, -7937 ± 998, F = -7.9, P<0.001 for maximum sheep). The hour-by-hour distribution of rumination in the baselines and the post-treatment recordings is shown in Figure 5.6.

The number of bouts of eating was not different between sheep from the minimum and maximum space allowances however, both groups had more bouts of eating post-treatment than baselines (GLMM, –6 ± 2.3, F = -2.6, P<0.05 for minimum sheep, -5.9 ± 2.7, F = -2.4, P<0.05 for maximum sheep). Similarly, there was no difference between sheep from the minimum and maximum space allowance in the duration of eating bouts, but sheep from the maximum space allowance had longer eating bouts post-treatment compared to baseline (mixed model –123 ± 36, t = -3.4, P<0.01).

The total time spent eating was different between sheep from the minimum and maximum space allowances post-treatment as compared to baseline. Both groups spent more time eating post-treatment as compared to baseline (mixed model –4.5 ± 1.3, t = -3.4, P<0.01 for minimum sheep, -9.2 ± 1.2, t = -7.5, P<0.001 for maximum sheep). The hour-by-hour distribution of eating in the baselines and the post-treatment recordings is shown in Figure 5.7.

The duration of drinking bouts and the total time spent drinking was not affected by treatment condition. However, sheep from the minimum space allowance had a tendency to spend more time drinking and sheep from the maximum space allowance spent significantly more time drinking post-treatment than during the baseline (mixed model –0.3 ± 0.1, t = -2, P<0.06 for minimum sheep, -0.1 ± 0.02, t = -3, P<0.05 for maximum sheep). The number of bouts of drinking increased post-treatment (GLMM, –5.3 ± 1.4, t = -3.9, P<0.01 for minimum sheep, -2.6 ± 0.6, t = -3, P<0.05 for maximum sheep).

Table 5.7 Effects of a 29-h restricted space allowance on Rumination, Eating and Drinking (mean ± s.e) (n = 11 pairs)

Measurement		Baseline Recording Period		Post-Treatment Recording Period		Difference between baseline and post-treatment period‡	Statistical significance of the difference between baseline and post-treatment periods‡
		Minimum	Maximum	Minimum	Maximum		
Rumination	No. of bouts	24.8 ± 2.2	26.6 ± 2	27.3 ± 1.7	26.8 ± 2.8	-2.2 ± 2.5	NS
	Bout duration (s)	1405 ± 128	1418 ± 116	1290 ± 61	1371 ± 137	68 ± 133	NS
	% of time	37.8 ± 1.0	41.2 ± 1.6	36.5 ± 1.5	35.8 ± 2.7	-4.2 ± 2.2	NS†
	Latency (s)	1751 ± 564	2148 ± 708	8895 ± 718	10086 ± 947	793 ± 1350	NS
Eating	No. of bouts	40 ± 3.8	40 ± 4.0	46 ± 2.6	46.4 ± 4.1	-0.2 ± 4	NS
	Bout duration (s)	668 ± 52	725 ± 133	728 ± 90	849 ± 108	64 ± 86	NS
	% of time	29.1 ± 1.5	29.2 ± 2.3	33.6 ± 1.7	38.4 ± 2.1	4.7 ± 2.1	*
Drinking	No of Bouts	4.9 ± 0.9	4.9 ± 0.6	10.2 ± 1.6	7.6 ± 0.9	-2.6 ± 1.2	*
	Bout duration (s)	32.7 ± 6	24.3 ± 3	36.2 ± 6	27.5 ± 4	-0.3 ± 5	NS
	% of time	0.15 ± 0.02	0.13 ± 0.01	0.45 ± 0.18	0.21 ± 0.03	-0.2 ± 0.15	NS

Using proc GLMM for the no. of bouts and the Latency to ruminate, and the mixed model analysis with repeated measures for all other measures. ‡Differences between baseline recording and post-treatment recording in the differences between pairs of minimum and maximum sheep within a 24-h time period.
†= a statistical probability of P≥0.05, but < 0.1

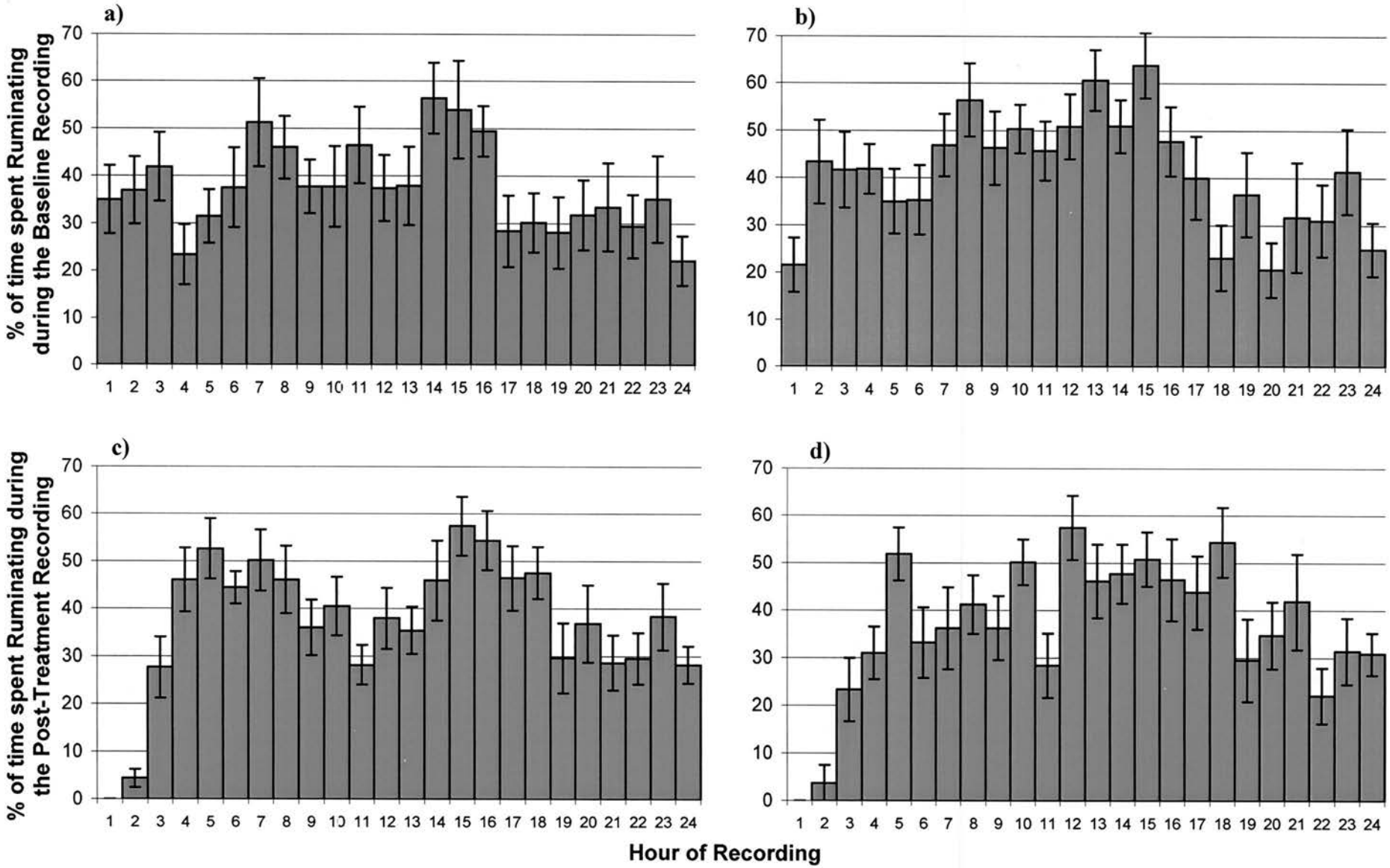


Figure 5.6 The mean (error bars indicate standard error) percentage of time spent Ruminating during each hour of 24h recordings. a) minimum space allowance baseline, b) maximum space allowance baseline. c) minimum space allowance post-treatment, d) maximum space allowance post-treatment. N = 11 sheep in each condition.

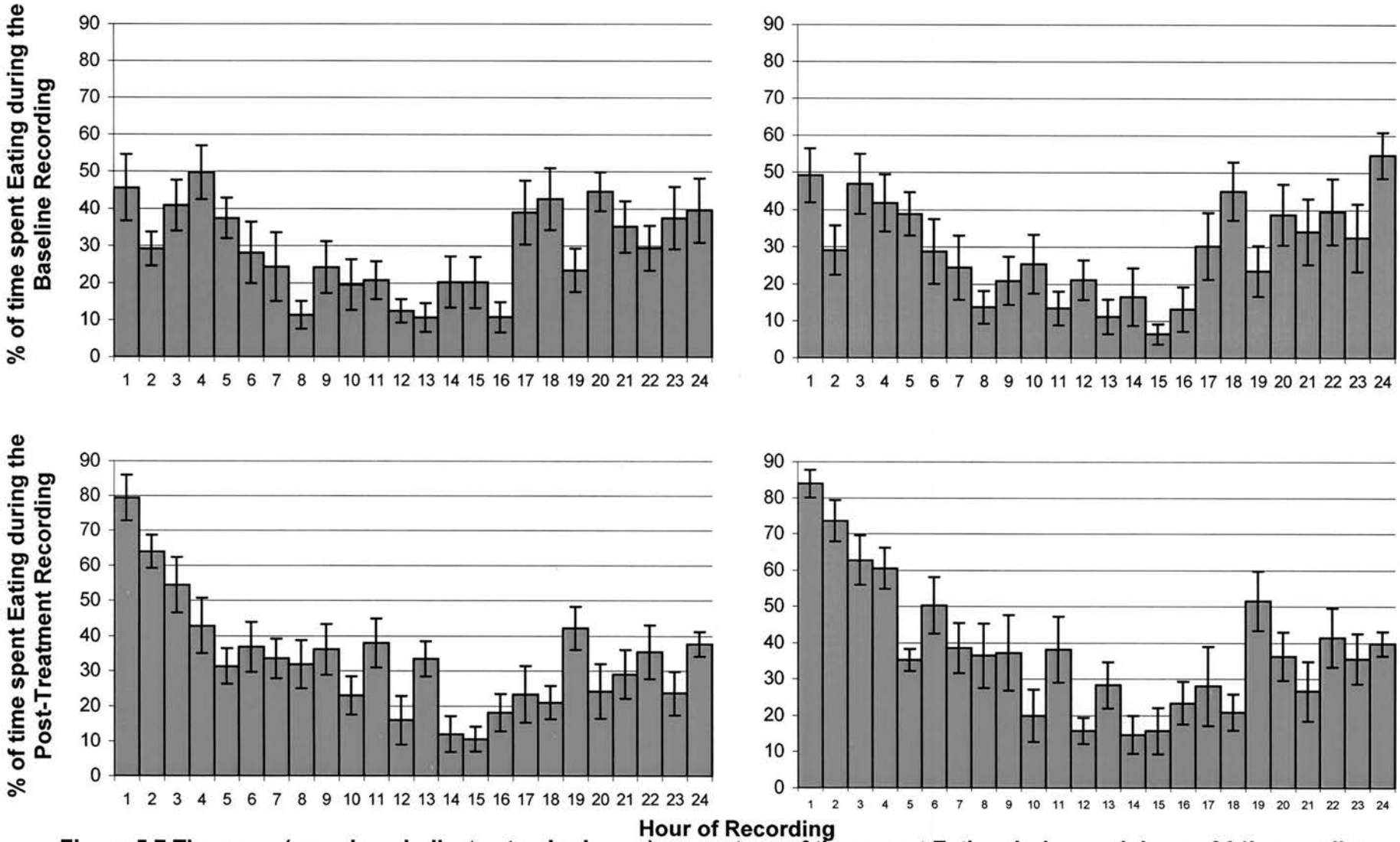


Figure 5.7 The mean (error bars indicate standard error) percentage of time spent Eating during each hour of 24h recordings. a) minimum space allowance baseline, b) maximum space allowance baseline. c) minimum space allowance post-treatment, d) maximum space allowance post-treatment. N = 11 sheep in each condition.

5.5 Discussion

5.5.1 Behaviour during the 29-h space restriction

There were differences in the amount of lying behaviour in sheep during the space restriction period between sheep in $0.3m^2$/sheep space and those in the $0.8m^2$/sheep space. Sheep in the $0.33m^2$/sheep group spent less time lying during the space restriction period than those in the $0.83m^2$/sheep group. Initially (and during a pilot study), there were difficulties in achieving a space difference between the conditions. This was because the experimental sheep were all large, Dorset cross ewes and the 'packing sheep' were smaller Scottish Blackface cross ewes, the larger Dorsets were able to dominate the packing sheep to the extent that even those in the minimum space allowance condition were able to lie down at will. This was rectified by removing the smaller (less than 42kg) packing sheep from the study and swapping them for Dorset sheep. The experimental sheep were no-longer able to dominate the others during the space restriction period and a difference between the two conditions was seen.

During the space restriction period, sheep in the maximum space allowance condition spent less time standing and were able to change their posture more frequently than sheep in the minimum space allowance condition. At the start of the space restriction period, sheep in the maximum space condition were seen exploring the pen and pen mates and eating bedding. In addition, sheep that were in the maximum space allowance group had space to move around and interact with other sheep in the confinement pen. Aggressive interactions (head butting-not quantified) were always observed within the first two to 4h of the space restriction period in the maximum space pen. In both conditions, the experimental sheep was 'an outsider' compared to those that had been housed in the group pen and were used as 'packing' sheep (even though many sheep were from the same overall flock). In the maximum space condition, the sheep had the space to carry out full aggressive interactions (facing off, rearing and head butts). Every maximum sheep was subjected to aggressive interactions from the packing sheep. In the minimum space allowance condition, aggressive interaction was observed on two occasions and consisted of side swipe head butts, which was all that the reduced space would allow.

It is possible that the sheep would find the mixing associated with the restricted space allowance period stressful, and this may have an effect on their subsequent sleep patterns (e.g. Cespuglio et al. 1995; Dewasmes et al. 2004). However, there were no major, demonstrative aggressive interactions seen in the second 14h period of restricted space, so it is possible that any social-stress sleep effects would have been reduced by the time the EEG was recorded.

In the maximum space allowance condition, all five sheep were easily able to lie down together. When all laying together it was noted that they seemed to attempt to avoid lying down facing one another. The sheep would lie along the sides of the pen, with one sheep lying in the middle, which is the characteristic lying pattern of sheep in pens (Huston, 1984). There is anecdotal evidence to suggest that when sheep have the space to do so, they adopt a position so they can lie down without facing another individual (Geist, 1971), and this is especially so for lower ranked individuals. In the minimum space allowance condition, it was more difficult for all five sheep to lie down together. Only on two occasions was this observed during the first 14h of the space restriction period. When sheep did lie down in the minimum condition, the remaining sheep often stood on her, pawed at her, or engaged in wool chewing of the sheep lying down. In observations from slaughterhouse lairages, Kim et al (1994) found sheep in low space allowances often walked over each other and tread on each other's limbs. Sheep in the maximum space allowance condition were able to lie down for longer bouts, as they were less likely to be disturbed by other sheep. It can be seen in figure 5.1 that sheep from the minimum space allowance showed more individual variation in lying behaviour than the sheep in the maximum space condition, possibly due to more disturbance from other sheep and less comfort. Overall, sheep in the minimum space allowance condition lay down less than those in the maximum space allowance condition. These results are consistent with other studies that found that sheep spent less time lying down during penning at low space allowance than at higher space allowances (e.g. Jarvis and Cockram, 1995).

However, during the second space restriction period, sheep in the minimum space allowance condition were seen to lie down together more frequently than they had in the first 14h space restriction. In fact, in both treatment conditions, sheep lay down more in the second 14h space restriction period than the first and lay down

quicker in the second 14h period than the first. There are three possible explanations: first that the sheep had become habituated to their conditions and their pen mates and therefore found it easier to lie all together, and second, that the sheep were becoming physically fatigued by the conditions and would lie down even if it was difficult to do so. The third is partly due to an experimental limitation, as the second 14h space restriction period coincides with the period of the day that sheep would 'naturally' spend inactive (i.e. from 2300h onwards), so sheep may be motivated to lie more at this time of day then at other times, even if it was difficult to do so. In a study by Koehl, et al (2002) rats were seen to alter their subsequent REM sleep pattern according to when in the circadian cycle a restraint stress was applied. To test if the circadian phase is the most important factor in whether the sheep are likely to lie down, further research is needed, carrying out space restriction commencing at varying times in the day. To investigate whether physical fatigue would encourage sheep to lie sooner, sheep could be made to be fatigued in a standardised fashion (e.g. on a treadmill) and then submit them to the space restriction period.

Although the minimum space allowance treatment was not entirely successful at preventing the sheep from lying down during the 'simulated transport' duration, they did lie down less than the sheep in the maximum space allowance treatment. Sheep from the maximum space allowance condition were able to lie down together with their heads resting on the bedding in a relaxed posture. It is probable that sheep in the maximum condition were able to sleep during the space restriction period. Again, sheep in the maximum space allowance condition could lie down with the head down more than sheep in the minimum condition. Sheep from the minimum condition would probably have been able to sleep at some points during the 29h, although it may have been more fragmented than in the baseline.

Although the sheep were habituated to wearing the electrophysiological equipment (that is, they had worn the harness previously and it could be attached without restraining the sheep), it is possible that the experimental sheep were more fatigued by carrying extra weight and may have laid down more frequently than they would have if not carrying the extra weight. This would have affected sheep in both treatment conditions and therefore may not have affected the overall post-treatment result between treatment groups.

Care should be taken in attempting to extrapolate these results to that of transport. In actual transport, there would be more stressors involved than just the restricted space, these could include: novelty, the vehicle movement, noise and vibration. In addition, transport itself may be more tiring to sheep than just restricted space in pens, as the animals must adjust their position to maintain balance in the moving vehicle and this may affect their lying behaviour during transport and post-transport. It is probable that sheep transported at the same space allowances as those presented here would lie down less often than the sheep in the space restricted pens.

The effect of the minimum space condition was that the sheep could not all lie down together with their heads resting, in the characteristic sleep posture. Two experimental sheep in the minimum condition were never observed to be lying with their head down throughout the 29h space restriction period. When the sheep were split into analysis groups according to the amount of time spent lying down with head down, six of the eight sheep in the less than 3 % lying group were from the minimum space allowance treatment. There were no sheep from the minimum space allowance treatment in the more than 8 % lying with head down analysis group. From these results, it seems unlikely that most sheep would get any sleep during transport when transported at space allowances of $0.3m^2$/sheep or less. Sheep may be able to get some light Non-REM sleep in a less than fully relaxed position (in a similar fashion to cattle when prevented from lying –Ruckebusch, 1974, further research is needed to ascertain if sheep show similar results). The total loss of muscle tone during REM sleep requires sufficient space to lie down with the head resting, so from these results it appears unlikely that sheep in low space allowances would be able to undergo REM sleep available to those in higher space allowances. Therefore, we can assume that the sheep from the minimum space allowance condition were (at least partially) REM sleep deprived for 29h.

5.5.2 Behaviour and sleep post-treatment

There were treatment effects of reduced space on the quantity and spectral quality of sleep in the 24-h post-treatment. An aim at the start of this study was to find if the treatments would reduce or increase rest and sleep post-treatment. Sheep that had been subjected to the maximum space allowance for 29-h lay down less

post-treatment. Sheep that had been subjected to the minimum space allowance for 29-h had more REM sleep post-treatment.

The latencies to both Non-REM and REM sleep were higher for both treatment groups post-treatment as compared to baselines. The first hour post-treatment was spent in the company of the researcher during EEG attachment and this could have influenced the behaviour. It would have been preferable to not have to reattach during the post-treatment period, but refitting during the treatment period with the packing sheep present and in restricted space proved too difficult. Initially, the full electrophysiological equipment was going to be put onto the sheep to record from the sheep during the space allowance period. However, the packing sheep were able to pull on and break the equipment and therefore post-treatment recording only was decided upon. During the first hour post-treatment, the animals had their electrophysiological equipment refitted and most spent that time standing and eating. On the majority of sheep, the equipment was refitted without restraining the sheep in any way. In addition, most sheep spent the majority of the second hour post-treatment standing and eating.

Sheep from both treatment groups took longer to lie down with the head up and to lie down with the head down post-treatment as compared to the baselines. Sheep from the maximum space allowance condition took longer to lay down with the head down than the minimum condition sheep in the 24-h post treatment. The increased latency to sleep may be indicative of a priority to eat before resting. These results suggest that sheep spent the first couple of hours post-treatment feeding to reduce the motivation to feed that had built up over the previous 14h. Maximum space allowance sheep seemed to have a greater priority for eating compared to sheep from the minimum space allowance. Maximum space allowance sheep spent, on average, over 60 % of each hour eating for the first 4h, minimum space allowance sheep spent less than 60 % of each hour eating after the first 2h post treatment. These results are comparable with those found by Cockram et al. (1996) who observed that sheep transported at $0.22m^2$/sheep lay down more in the first 12h post-transport than sheep transported at $0.41m^2$/sheep. Moreover, Cockram et al (1996) found that the sheep transported at $0.41m^2$/sheep spent more time eating in the first 12h post-transport than those transported at $0.22m^2$/sheep. It is possible that sheep

that have experienced near total lying deprivation in low space allowances during transport may have a lower priority for eating in lairage. It is important to note that it is likely that both groups of sheep would have been equally motivated to feed, as both groups experienced the same food withdrawal period. Therefore, the differences in resting and eating behaviour between the treatment conditions are due to the extra lying restriction at the low space allowance and probably the additional stress of this procedure.

Sheep from both conditions took longer to start to ruminate post-treatment compared to baseline and sheep from the maximum space allowance ruminated less post treatment than baselines. Sheep would have started the baseline condition after a long period of ad-lib food. Sheep in the baseline condition would have therefore had substrate with which to ruminate within the first hour of the baseline if they were motivated to do so. Any food eaten during the 1h 'rest-period' in the middle of the treatment would have probably been ruminated during the second 14h of space restriction, although rumination was not recorded, as it was not always observable due to the lack of clarity of the time-lapse video recordings.

There were no differences in total time spent, mean bout duration or the number of bouts of post-treatment Non-REM sleep between the treatment conditions. This perhaps suggests that sheep were not Non-REM sleep deprived during the 29h space restriction period. However, sheep from both conditions, showed an increase in slow frequency (delta 0.1-4Hz) waves within the EEG traces during Non-REM sleep taken after the space restriction period. The sheep from the minimum space condition had a higher average percentage of delta waves in Non-REM sleep than those from the maximum space allowance condition. An increase in the 'intensity' of Non-REM sleep as measured by an increase in the % of delta waves has been shown in rats (e.g. Tobler and Borbély, 1986; Meerlo et al. 1997) and humans after prolonged wakefulness. In addition, the intensity of Non-REM sleep may be governed by the waking experience, an increase in delta waves during subsequent Non-REM sleep is found in rats (e.g. Meerlo et al. 2001) and mice (e.g. Lancel et al. 2003) after a stressful social defeat. Sheep may have been Non-REM sleep restricted (if not deprived), getting less Non-REM sleep and more fragmented sleep during the space restriction period than they would have had with more space.

The increase in delta waves seen during the Non-REM sleep may be indicative of sleep deprivation, but there may also be a relationship between the increased intensity of post-treatment sleep and the stress of the treatment procedure. (The stressors might include frustration from being unable to lie down, the mixing with unfamiliar sheep and hunger and thirst.) However, Cockram et al (1996) found when plasma cortisol concentrations were measured from sheep being transported at different space allowances that the response reduced over the transport time suggesting that sheep found the novelty stressful, but not the cumulative effect of lying deprivation during transport. Sheep from the minimum space allowance had, on average, a higher percentage of delta waves in Non-REM sleep than those from the maximum space allowance group, they were certainly more restricted in laying down –perhaps more sleep restricted –but also possibly more stressed by their situation. One way to fully understand this relationship would be to record the EEG from sheep during the space restriction period (and stop the packing sheep from destroying the electrodes) and/or measure other physiological indicators of stress such as cortisol.

Sheep from the minimum space allowance spent more time in REM sleep in the post-treatment 24-h than in baselines and as compared to sheep from the maximum space allowance condition. The bout duration was not different, but there were significantly more bouts of REM sleep post-treatment than in the baseline. These increases in the amount of REM were seen in the first 12h of the post-treatment 24h. This suggests that the sheep required the 12h time in which extra resting could occur for recovery from the 29h of space restriction at the $0.3m^2$/sheep level. Whether the extra REM sleep was needed as the sheep had undergone REM sleep deprivation, or whether it was a stress recovery method for the sheep that had undergone 29h space restriction at low space allowances is not clear. In an experiment where rats were given injections of doses of corticosterone, Non-REM was affected (a decrease in overall slow wave sleep) but REM sleep was not affected (Vázquez-Palacios et al. 2001). Whereas, Cespuglio et al (1995) showed that a short intense stress in rats resulted in an increase in REM sleep during the subsequent sleep period.

The increase seen in REM sleep in sheep from the minimum space allowance as a result of lying deprivation was also illustrated when the groups were split into groups according to how much time they had spent lying down with their head down during the space restriction period. Here, those that had lain down head down for less than 3% of the 29h period were found to show more REM sleep in the subsequent 24h than those who spent 8% or more of the 29h period lying down head down.

A further interesting change in REM sleep post treatment was seen in the pattern of REM and Non-REM sleep. In the baseline, sheep from both groups had less then 5% of their sleep periods going straight from awake to REM sleep, without any time spent in Non-REM sleep. Sheep from both conditions showed an increase in the percentage of sleep periods going directly to REM sleep from wakefulness during the first 6h post-treatment. This increase was greater in the sheep from the minimum space allowance condition than those in the maximum space allowance condition. After the first 6h the percentages of sleep periods going directly to REM sleep were not significantly different from the baseline. In normal adult sheep, sleep direct to REM is the least usual form of sleep cycle. More commonly, sleep cycles start with Non-REM and proceed to REM (Ruckebusch, 1972). The next most common cycle involves only Non-REM. Foetal sheep have a high percentage of their sleep proceeding straight to REM (Szeto and Hinman, 1985).

Sheep from both treatment conditions had a tendency to show an increase in the density of eye movements during 10s epochs of REM sleep post-treatment as compared to baselines. This gives further evidence of a change in the quality of sleep post-treatment, not just the quantity. There is evidence to suggest that eye movement density in REM sleep is affected by chronic stress in humans (Douglass et al. 1992). It is unclear what this could imply for sheep after 29h of space restriction.

5.6 Conclusion

Stocking at a space allowance of $0.3m^2$/sheep for 29h decreases the amount of resting behaviour and almost certainly reduces sleep during the period of space restriction. This experiment has shown that sheep penned at low space allowances for the maximum journey time experience changes in the quantity of REM sleep and

the quality of REM and Non-REM sleep, post space restriction. Even for sheep penned at a higher space allowance of $0.8m^2$/sheep, there are post-space restriction changes in the quality of sleep, however the effects are more marked in sheep penned at the low space allowances. It is likely that sheep transported at the space allowances reported here would be able to rest less often during transport than when penned in this experiment. As discussed (chapter 4) there are many factors which could affect the welfare of sheep during transport, over and above the space allowance factor (e.g. driving events, see Cockram et al. 2004).

The lairage period during a maximum permitted journey, at least 1h with food and water, may be an area of concern regarding the welfare of sheep. In this experiment, only two sheep lay down during the 1h 'rest-period', whereas for the majority of the sheep the time was spent eating and drinking (eating first, followed by drinking). Allowing for the probability that a transported sheep may be more fatigued than a penned sheep, it is possible that the majority of transported sheep would not be able to rest in a 1h rest stop during a journey. The answer may be to increase the minimum rest stop time to allow sheep to rest after they have eaten.

In an experiment into the relationship between sleep restriction and responses to stress in rats, Meerlo et al (2002) found that after sleep restriction, the ACTH response to a restraint stress was reduced, but the plasma corticosterone concentration was not affected. It is possible then, that sheep that have been REM sleep deprived during transport may have an altered stress response to subsequent stressors, especially if unable to undergo recovery sleep after the deprivation. This may be exacerbated by repeated REM sleep deprivation such as in a repeated transport-market-transport-lairage situation. Here it would be important for sheep to be given appropriate, undisturbed conditions to recover and adequate space to allow all animals to lie down together. It is also important to bear in mind that sheep may behave differently in lairage, depending on what they have experienced before their arrival (direct from farms, via markets, etc) (e.g. Cockram et al. 2000; and in cattle: Cockram, 1990). If sheep were to be transported without adequate time to recover from previous transport, then there is a risk that the sheep would be fatigued. Under legislation, animals may only be considered fit to be transported if there is no presence of fatigue (MAFF, 1997). A method of solving this dilemma might involve

assessing current legislation on space allowance during transport, to ensure that sheep were transported at a density that allowed them all to lie down and rest simultaneously and therefore have less requirement for increased rest post-transport.

Chapter 6. General Discussion

6.1 The aims of the project

- To develop a non-invasive method of recognising sleep in sheep with simultaneous behavioural observation and electrophysiological recordings.
- To validate the reliability of the non-invasive electrophysiological techniques to record an EEG that could be used to differentiate between sleep and wakefulness in sheep and to characterise sleep in sheep
- To test the hypothesis that potentially aversive waking experiences could affect the subsequent sleep and rest behaviour of sheep.

6.2 The methodology

6.2.1 The electrode attachment procedure

The attachment procedure was undoubtedly stressful for the sheep even after gentling procedures. This was shown by the high heart rates during the first couple of minutes of starting a recording (see chapter 3) and by the observed (but not quantified) high respiration rates during equipment attachment in most sheep. The reaction of the sheep during the attachment more pronounced in some sheep than others (possibly accounting for some of the post-treatment variation between sheep in behaviour/sleep). Some of the sheep required light restraint (i.e. holding their head), especially when attaching the head electrodes (the sheep were never tied at any point). Other individuals, however, would lie down next to the experimenter and ruminate during head electrode attachment. Most sheep were in between these two extremes: not relaxed enough to lie down while the experimenter was in the pen, but not requiring any restraint. Fresh hay was always provided during attachment and most sheep spent the procedure feeding. Gentling had an effect in reducing the behavioural indicators of stress (e.g. escape behaviours reduced with each progressive gentling exercise). The least gentling was carried out during the novel environment experiment, because it was impossible to carry out intensive handling of the sheep at pasture. Here, there were the highest percentages of sheep that showed escape behaviours during electrode attachment. All of the other procedures took place after the sheep had been in individual pens for two weeks, with at least

one hour per day spent gentling each individual sheep. It would have been interesting to have quantified the reactions of sheep during the attachment procedure to see if there were any effects of sheep response to attachment post-treatment.

6.2.2 Electrode reliability

A period before the start of the preliminary project had been used to assess: the effectiveness of various electrode designs, adhesive products and methods of electrode protection. If this initial time had not been spent assessing the feasibility of the products used in the study, it is possible that the reliability of the electrophysiological equipment to remain on the sheep for 24-h recordings would have been much reduced. In the preliminary study, many recordings were abandoned before the 24-h had been reached, as the electrodes would become detached from the skin of the sheep. Baldock et al (1987) found that skin surface electrode reliability was low (55% successful) when recording the ECG of free-ranging sheep. Initially, the reliability of the electrodes in the preliminary study was similar to that of Baldock et al, and to get three satisfactory recordings from each of the first pair of sheep took several recordings from each sheep. The breakthrough in electrode reliability in the preliminary study came with the arrival of the electrode adhesive strip developed by Aston University (Birmingham, UK). This substance was a gel that could be cut into strips to stick over the electrodes and stick on either side of the scalp. It seemed especially useful in keeping the electrodes in place as it absorbed liquid, but remained sticky, so if the sheep were to sweat (which they often did as their heads were wrapped in crepe bandage and topped with a fibre-glass helmet), the electrode adhesive strip would stay in place. The electrodes also seemed to remain stuck to the skin surface when covered in the strip, retaining a reasonable impedance (the strip absorbing sweat helping to maintain ideal impedance). The subsequent recordings were more successful and there were less electrode failures, most 24-h recordings could be used for analysis. In addition, practice and well-gentled animals were invaluable in maintaining a high electrode reliability.

Electrode reliability became a concern again during the three main experiments and new methods had to be developed in order not to lose too many recordings. The novel indoor environment experiment posed problems as the

baseline and control recordings had to be successful from every EMG site (as well as the EEG), because they were made from sheep at pasture and there were no video recordings to rely on. Extra waterproofing of equipment (see chapter 3) was used to ensure electrodes did not become detached. Even with extra precautions, there was an approximately 25% failure rate for recordings during this experiment. Recordings that failed during the baseline were repeated on both sheep from the pair, but any that failed on the movement or post-movement recording day were discarded from the data.

The transport experiment was less affected by electrode detachment. The sheep remained undercover, so waterproofing was not a big problem and the sheep did not have the space to run around (which they were seen to do in the pasture on a few occasions). The failures that did occur were connected with the electrical equipment needed to power the on-vehicle video recorders and a breakdown of the vehicle itself.

The initial plan had been to record all of the usual electrophysiological measurements during the space restriction period of the space allowance experiment. However, electrode failure during this experiment was not due to electrode detachment, but to loss of equipment from the attention of the 'packing' sheep, especially in the maximum space allowance condition. Even after coating the electrode leads in chilli oil or mustard, the leads were often chewed and destroyed during the 29-h period (some sheep had exotic tastes!). Due to budget restraints, the decision was made to restrict the electrophysiological measures during the space restriction period to the leg EMG alone. It was interesting that electrode lead chewing was only seen on one occasion when the sheep were in a social group at pasture, but was seen during every space restriction period. It is impossible to ascertain the reasons for the chewing. However, it is likely that the lack of food, boredom and social mixing were involved. The equipment on the sheep that were in the maximum space allowance condition were more affected as the sheep had more room for movement and social interaction than in the minimum space allowance condition.

6.2.3 Effects of the equipment on the behaviour of the sheep

As discussed in chapter 2, the initial design of the equipment resulted in differences in behaviour when wearing the equipment as compared with the companion animal not wearing any equipment. The results gained in the preliminary experiment would have been particularly affected, as the new harness design was not used until the start of the validation experiments. The behaviour was affected in the opposite way from what was expected: the sheep spent more time lying down when wearing the equipment than without the equipment. The harness was pinpointed as the main problem by watching the behaviour of sheep wearing the harness alone; the helmet alone and the behaviour of the sheep after being made to stand for two hours (equating to the instrument attachment time). Therefore, the preliminary results may be called into question, as sheep did lie down for longer when wearing the equipment. They also spent more time in the lying head down position. Therefore, it is possible that sheep wearing the electrophysiological equipment spent more time sleeping than the sheep that were not wearing the equipment (although this was impossible to test using present methods). It is interesting that the sleep results gained in the preliminary study were very similar to those found by using more invasive electrophysiological methods, but that the present experiment showed that sheep lay down for longer than those from the previous studies. Perhaps the sleep was not affected by the electrophysiological equipment in the same way as the behaviour (except for the response to the stress during the fitting procedure). The harness seemed to be the main cause of the alteration in behaviour, probably due to the leather straps pulling at the fleece of the sheep, and the tendency for the Embla to slide around the back of the sheep may have unbalanced them. The sheep may have attempted to remain still (by lying down more), to lessen the extent of wool pulls or to lessen the unbalancing caused by the Embla.

The new harness was developed from a design already used in physiological studies of sheep (Goddard et al. 1998), and was adapted specifically to reduce the slippage of the harness round the back of the sheep. In addition, the new harness was made of a softer material, with webbing straps that had quick release fasteners, easily fastened to lessen the extent of fleece pulling when tightened. If time had allowed, the preliminary experiment would have been repeated to ensure that the sleep results had not been affected by the harness in the same way as the behaviour.

However, as the sleep results were so similar to previous studies (e.g. Ruckebusch, 1972), the decision was made to continue with the validation part of these studies.

6.2.4 The use of the Somnologica software in sheep

The sleep scoring programme used throughout these studies was developed for use in human sleep research and medicine. Therefore, it was possible that it may not have worked in sheep. There were a number of manual additions that were carried out during each scoring session to reduce the error due to sheep related behaviour that the programme was not set up to recognise. These manual additions (e.g. always scoring the sheep as awake unless it was in the lying head down posture) may have themselves resulted in error. It is possible that sheep can get some light Non-REM sleep while standing (similar to lying deprived cattle, Ruckebusch, 1974) and quite likely that sheep were experiencing drowsy wakefulness/light Non-REM sleep during rumination (e.g. Ruckebusch, 1972), but the present method was unable to reliably detect sleep when muscle activity was present. However, deeper Non-REM sleep and REM sleep result in loss of muscle tone and require a relaxed sleep posture. Therefore, it is likely that the methodology used here was able to recognise the deeper sleep.

Healthy, adult humans tend to sleep in a monophasic manner (sleeping in one long bout with microarousals) with a sleep cycle that alters from one stage of sleep to the next over a series of minutes (e.g. taking 90 minutes from sleep onset to the first stage of REM sleep). The sleep staging system devised by Rechtschaffen and Kales (1968) analyses 30s epochs for frequency and amplitude in order to determine the sleep stage for that epoch. Each sleep stage can last up to 30 minutes or so, and the staging epochs are short enough that one epoch containing artefact is not going to alter the decision made on the stage of sleep during the bout. If the total sleep bout of sheep lasts about 15 minutes, then 30s epochs used to score the sleep may have been much too long to effectively pick up on the nuances of sleep in sheep. Sheep have such short bouts of sleep they may change from one stage of sleep (especially different stages of Non-REM) to the next in a shorter period of time than humans. Even though there were differences in frequency during the Non-REM sleep, the scoring software consistently scored the Non-REM sleep as Stage I or Stage II,

rarely Stage III and never Stage IV. It is possible that altering the settings in Somnologica to allow it to score using shorter epochs may have produced different results when scoring Non-REM sleep. However, the complex probability relationships used by the scoring system to effectively score the Non-REM sleep needed to be changed in conjunction with the epoch length. As the programme had not been used to score sheep sleep before, it would have been difficult to know how to alter the scoring-probabilities efficiently. Therefore, the programme was left unaltered for these series of studies.

6.3 The results

The results from the preliminary experiment and the three investigations are summarised in Table 6.1.

Table 6.1 A summary of the results of the series of investigations relating to sleep in sheep

Experiment	Main Results Relating to Sleep in Sheep
Preliminary Study	Total Sleep % of 24h =17.3% Non-REM Sleep = 14.5 % REM Sleep = 2.8 %
Novel Environment	**At Pasture**: • Less sleep than in preliminary study • Sleep mainly at night with short bouts in the middle of the day. **During 24-h post-movement as compared with pasture:** • 30% increase in the time spent in REM Sleep • Tendency for an increase in REM bouts • Latency to sleep reduced • No difference in amount of Non-REM Sleep • Increase in the % of delta waves in Non-REM Sleep • Flattened profile of sleep in 24-h (i.e. less sleep at night and more in daylight) **During 7d post-movement compared with pasture:** • No difference in Non-REM Sleep • No difference in the amount of REM Sleep • Increase in the density of eye-movements in REM Sleep • Flattened profile of sleep in 24-h (i.e. less sleep at night and more in daylight)
8-h Road Transport	**During 8-h Transport:** • No Sleep • Reduced lying **During 16h post-transport as compared with baseline:** • Latency to REM increased • Fewer bouts of REM in 1st 8-h post-transport • Fewer, shorter bouts of Non-REM in 1st 8-h post-transport • Spent less time in Non-REM Sleep • Increase in the % of delta waves in Non-REM Sleep in the 1st 8-h post-transport
29-h Space Restriction	**During Space Restriction:** • Less lying in 0.3m^2/sheep space allowance than in 0.83m^2/sheep **During post-treatment 24-h as compared with baseline** • Latency to sleep (both REM and Non-REM) reduced • No difference in amount of Non-REM Sleep • Increase in the % of delta waves in Non-REM Sleep for both treatment groups (greater in 0.3m^2/sheep) • Increase in the time spent in REM Sleep in 0.3m^2/sheep • Increase in the number of REM Sleep bouts in 0.3m^2/sheep • All differences in sleep occurred within the first 12-h of the post-treatment 24-h.

The changes seen in the amount of sleep post-treatment are subtle. However, they are consistent, as in each case at least eight out of the ten treatment sheep showed the same direction and magnitude of response. This suggests that the effect that is being shown in the statistics is a 'real' one, and not affected by carrying out many statistical tests, allowing one to be significant 'by chance'. Other researchers have found that changes in sleep after potentially stressful waking events are subtle, and easily missed if only the total 24-h sleep is measured (e.g. Webb and Friedman, 1971). Moreover, in the present investigations, the effects were seen even when the individual responses of the sheep were variable. The number of bouts of REM sleep and the bout length of REM sleep were the two least variable factors (with small standard errors), whereas latency to sleep and latency to REM sleep were extremely variable between sheep.

Individual differences in sleep and responses to stress in sheep, could relate to age differences, breed, physiological state (e.g. reproductive state, health, etc). The sheep varied in age from young adult (post-lambing gimmer) to older ewes. Human sleep amount and quality differs considerably with age (e.g. Hume et al. 1998). There is evidence to suggest that adult sheep spend less time sleeping than neonatal lambs (e.g. Grant et al. 1995; Das, 2001), but it is unclear whether the age differences used in this project would have affected the results. Although our sheep were nominally of the same breed (Dorset cross) the breed with which they were crossed was variable among the sheep across the whole series of studies. The most common second breed was Finn, and others used were Suffolk and Texel. These breeds may have differed in their temperament and also in the morphology of the head, potentially altering the quality of the EEG recordings. All the sheep used on the experiments were physically healthy, although some animals did become lame; these were always treated and declared healthy before continued use on the project. Reproductive status can affect the sleep of humans (Mauri et al. 1988). The sheep used in this project were not in-lamb, but some animals may have come into oestrus and this could have affected their sleep.

6.3.1 The effects of potentially aversive treatments on subsequent REM sleep

In the novel environment and the restricted space allowance experiments, an increase in the times spent in REM sleep was seen post-treatment (in the treatment sheep of the novel environment experiment and the sheep in the minimum space allowance condition). Both groups of sheep showed an increase in the number of REM sleep bouts that accounted for its increased percentage of the 24-h post-treatment. In addition, in both groups there was no significant increase in the mean REM sleep bout length post-treatment (as seen also in rodents: Bonnet et al. 1997; Dewasmes et al. 2004; Tang et al. 2004; Pawlyk et al. 2005). This seems to show that the bout length of REM sleep in adult sheep is relatively inelastic, but the overall amount of REM sleep in a 24-h period can be altered by experience during prior wakefulness (i.e. the bout length remained the same post-treatment, but the number of bouts increased). Humans with clinical depression have shorter latencies to REM sleep (therefore altering the bout length of Non-REM sleep) and more bouts of REM sleep, but the mean bout length of REM sleep is usually unaffected (e.g. Boivin, 2000, Rotenberg et al. 2002).

None of the conditions resulted in a reduction of REM sleep post-treatment. Other studies have shown situations where treatment conditions can reduce subsequent REM sleep. These include painful conditions in humans and rats (e.g. Drewes et al. 1998; Anderson and Tufik, 2000 for humans and rats respectively). In humans, anticipatory anxiety about an event the following day (Åkerstedt et al. 2004) and the 'first night effect' of movement to a novel environment also affect sleep (e.g. Agnew et al. 1966, see chapter 3). It was found that in rodents, an extreme experience during wakefulness, likely to cause fear (e.g. foot shocks Sanford et al. 2003) reduced subsequent REM sleep. It seems that for both humans and rats, a reduction in REM sleep can be associated with an extreme change in psychological state in the previous period of wakefulness. Sheep may also show similar reductions in REM sleep after particularly extreme fearful experiences during wakefulness. However, this direction of response was not seen in the treatment sheep during the three 'aversive husbandry procedure' experiments; perhaps the situations did not represent fear or extreme anxiety for the sheep. (It is possible that the one or

two individuals in each experiment that reacted with decreased REM sleep did so because they had feelings of extreme fear/anxiety during the treatment.)

The fact that the majority of sheep reacted with an increase in REM sleep rather than a decrease was somewhat unexpected. It had been hypothesised that the novel inside environment would have induced anxiety during the first 24-h and that the 8-h road transport would also have had an extreme anxiety effect. It is possible that sheep react differently to extreme anxiety to that of other animals. However, in my opinion, this is unlikely as the changes in sleep were consistent with a stressful event but not one associated with extreme fear or anxiety. All sheep were extensively handled and gentled in a habituation process in an attempt to reduce the stress associated with electrode attachment. This meant that most sheep were extremely tame and easy to handle. Most sheep no-longer behaved in the same way as a non-tamed sheep (e.g. sheep could be loaded onto the transport vehicle by following the experimenter!). There is the possibility that the gentling regime reduced the responses of the sheep to the experimental treatment procedures. However, Hargreaves and Hutson (1990) showed that gentled sheep had a reduced stress response (heart rate and flight distance) to events that occurred when the 'gentler' was present, but no reduction when other people were involved. During the present investigations, the 'gentler' was present to attach the electrophysiological equipment to the sheep, but otherwise spent little time with the sheep during or post-treatment.

Other factors could have reduced the extremity of the responses to the treatment conditions. The inside penning used as the novel environment was quiet, with very little human activity during the post-treatment period, which helped reduce post-moving activity. In addition, the companion sheep was familiar with the environment and may have had an influence on the behaviour of the treatment sheep (Cook et al. 1996). The transport journey was carried out in optimum conditions: the additional stressors usually associated with transport, such as reduced space and social mixing and wet bedding were not present, perhaps reducing any extreme reactions in the treatment sheep. In the restricted space experiment, there were social interactions between treatment sheep and the packing animals that may have induced extreme anxiety after a social defeat (similar to rats e.g. Meerlo et al. 1997, see

below). However, the aggressive encounters were observed during the first two to three hours of the treatment and therefore any subsequent affect could dissipate by the post-treatment period (after 29-h of treatment).

REM sleep is proposed to have a function, or at least a relationship, with learning and memory consolidation in humans (e.g. Maquet, 2001, Bódizs et al. 2001). Laureys et al (2001) found that the cortex experienced more activity during the subsequent REM sleep after learning a task. Humans have more bouts of REM sleep and a greater density of rapid eye movements during REM sleep after learning a task as compared with controls given a reading task (Smith and Lapp, 1991). In non-human animals, REM sleep is also related to learning and memory. If rats are REM sleep deprived after a period of training, they are subsequently less able to carry out the task than non-deprived controls (Pearlman, 1979).

All of the three main experiments carried out within these investigations probably included elements of learning and the need of some memory consolidation. All of the experiments would have required habituation (a form of learning) from the sheep as they became used to their new situation. The restricted space experiment involved social interaction, where it was required to learn or remember the novel or familiar members of the original flock. It is possible that the changes seen in REM sleep post-treatments were related to learning (habituation) and/or memory consolidation, rather than stress.

6.3.2 The effects of potentially aversive treatments on subsequent Non-REM sleep

The amount of Non-REM sleep was less affected by treatment than REM sleep. However, post-8-h-transport sheep had fewer shorter bouts of Non-REM sleep. In addition, the sheep from the maximum space allowance condition had fewer bouts of Non-REM sleep (the amount of Non-REM sleep was unchanged in the other groups of sheep).

The evidence from the behaviour of both the post-transport and the maximum space allowance groups suggests that eating was a higher priority than sleeping. Although the transport group lay down more in the first 8-h post-transport they spent most of this time eating. The maximum space allowance group lay down

less in the first 6h post-treatment and spent more time eating than compared with the baseline period.

Perhaps Non-REM sleep was less important to these two groups of animals that had been food deprived during the treatment. Sheep from the minimum space allowance condition were also food deprived, but they were more affected by rest/sleep deprivation during the 29-h treatment than the sheep from the maximum space allowance condition. The sheep from the maximum space allowance condition were able to lie down easily during the treatment; those from the minimum space allowance condition were not (see chapter 5). Although feeding was a high priority for the sheep from the minimum space allowance condition, it did not supersede sleeping. The minimum space allowance group had a similar amount of Non-REM sleep and an increased amount of REM sleep post-treatment as compared with baselines.

Although the amount of Non-REM sleep seemed less affected by treatment than REM sleep, the quality of the waveform of Non-REM was affected by every treatment imposed in this study. The Somnologica sleep software enabled in-depth spectral analysis of areas of the EEG trace and sleep power band analysis. The sleep power band analysis gave information about the change in frequency as the percentage of the waveform that fell into each category (delta, theta, alpha, sigma and beta). Post-treatment, Non-REM sleep differed in its frequency to pre-treatment baselines. There was, in each case, an increase in the percentage of delta waves (0.1-4Hz) post-treatment. This indicated that, although the Non-REM sleep appeared unchanged in bout length and bout number, there was a significant alteration in the 'intensity' of Non-REM sleep.

Why should this change in the percentage of slow waves in Non-REM sleep occur post-treatment? There are two explanations. First, the 'intensity' of Non-REM sleep has been shown to increase after prolonged wakefulness: indeed in rats there seems to be a direct relationship between increasing frequency of Non-REM and increased time spent awake (Tobler et al. 1986; Endo et al. 1997). In human sleep deprivation studies, where stressful procedures are not necessary to keep the subjects from sleeping, an increase in Non-REM 'intensity' has been seen after increased time since last asleep (e.g. Brunner et al. 1990; Wichniak et al. 2003). Therefore, the

changes in Non-REM post-treatment in sheep could be due to increased periods of wakefulness.

However, a second explanation can be used, in which stressful events during wakefulness have been shown to affect the percentage of delta waves in subsequent sleep. Rats subjected to a stressful social defeat showed an increase in delta waves in subsequent Non-REM sleep that was greater than rats that had been kept awake for the same amount of time by gentle handling from a familiar person (Meerlo et al. 1997). Indeed, these differences -between rats that had been exposed to a stressor and those that had not- were still present if both treatment and control rats were kept awake for a further five hours after the social defeat. Therefore, the percentage of delta wave increase was still higher in rats that had been socially defeated even after prolonged wakefulness (Meerlo et al. 2001).

The increase in percentage of delta waves in Non-REM sleep of sheep post-treatment is the most compelling evidence that sheep found each of the husbandry procedures (used as treatments) stressful. In the novel indoor environment experiment, both controls and treatment sheep experienced an equal period of time in forced wakefulness and potential stressful situation (i.e. during the electrode attachment procedure). The only difference was that the treatment sheep was moved to the novel environment, and here we see the increased 'intensity' of Non-REM sleep. In addition, this alteration in the spectral properties of sleep was not due to the physical conditions of the novel environment, but solely the psychological effect of the change from one environment to the other, as it was not present seven days post-movement.

The transport experiment was more complex, inasmuch as the journey (approximately eight hours long) was spent awake by all the treatment sheep. Moreover, although the control sheep lay down less during the treatment period, nevertheless they slept during this time, and the time since last sleep was shorter than for the treatment sheep. Therefore, it is possible that the increase in the percentage of delta waves seen in sleep post-transport could have been due to either the stress of the transport, the increased length of wakefulness caused by the transport, or a combination of these factors. However, as previous studies have shown, road transport is a stressful experience for sheep (e.g. Cockram et al. 1996;

Cockram et al. 1997). The present study showed alterations in behaviour (fewer and shorter lying bouts and less rumination) that indicate that transport was not a comfortable period for the sheep. It is probable that the treatment sheep did undergo a stress response because of transport. As Meerlo et al (2001) have shown, the effect of a stressful experience can still be present in rats even after a period of 'stressless' sleep deprivation. Therefore it is probable that the increase in delta waves of Non-REM sleep post-transport was, at least in part, due to the stress of transport.

In the space allowance experiment, sheep from both the minimum and the maximum space allowance conditions showed an increase in the percentage of delta waves in the Non-REM post-treatment. Those sheep in the minimum space allowance condition had a greater increase in delta waves than the minimum space allowance group. The postural time budget results from the space allowance experiment suggest that the sheep in the minimum space allowance condition may have been partially sleep deprived (i.e. deprived of REM sleep), or at least sleep disturbed. It seems likely that sheep in the maximum space allowance condition would not have been as affected as those in the minimum condition in relation to sleep deprivation, as all sheep could lay down 'head down' at the same time. What conclusions can be drawn from the increase of delta waves in Non-REM sleep after experiencing either of these space allowance conditions? There are a number of reasons why sheep from both groups may have found the space restriction period stressful. First, there was social mixing, which did involve aggressive interaction, although this would be expected to affect the sheep most during the first few hours of the space restriction period. Second, there was the possible frustration and/or hunger and thirst, as sheep were without food for 14-h periods. Indeed, if there were stress responses in sheep due to food/water restriction, they may have occurred in the second 14-h period, and were therefore more likely than responses of a stressor that occurred in the first 14-h period to affect the subsequent 24-h sleep. Finally, sheep in the minimum space allowance condition may have shown a greater difference in delta waves in Non-REM sleep as they had been partially sleep deprived. Once again, the difference seen in post-treatment Non-REM sleep provides evidence that sheep found the treatment stressful.

6.4 Future Research

The results from these studies have shown that there are measurable effects on the sleep of sheep after they have experienced husbandry procedures during wakefulness. The changes that occurred in sleep post-treatment in the three 'aversive husbandry procedure' experiments were subtle and may not have been easy to measure with behaviour alone. The spectral analysis of the EEG during sleep after events during wakefulness showed that the frequency of the EEG changed post-treatment. This result would have been impossible to obtain using behaviour alone. However, the expense, the relative inefficient use of time (both in gentling the animals, the attachment procedure and the analysis of the data), the stress of the attachment period and the variable reliability of the procedure may be off putting for future research using this method. The disadvantages, as outlined above, should not be allowed to overshadow the benefits of exploring both circadian rhythms, and rest and sleep as a method of understanding an animal's response to an experience. Sleep is not sufficiently studied and under-represented in the body of animal welfare research.

However, there is some potential for measuring sleep and rest in more detail without having to resort to electrophysiological studies. It is very unusual to have the type of lying (i.e. the exact posture) recorded in time budget experiments of farm animals (although, see Krohn and Munksgaard, 1993). If detailed behavioural observation is carried out it is possible to ascertain when an animal is definitely asleep, and it is usually possible to observe the eye-movement part of REM sleep from behaviour. Recoding the exact posture, muscle relaxation, whether the eyes are open or shut and noting if the animal has a preferred sleeping site would give an approximate idea of sleep amount, especially if accompanied by detailed behaviour observation (including eye and ear movements during sleep). A detailed description of animal behaviour during the 'inactive' periods of the day would be able to give much more information on the effects of stressors on animals than just measuring lying alone.

One aspect of sleep that would be useful to understand is that of the motivation to sleep. It would be interesting for future research to investigate how important sleep is for an animal. All animals, including humans eventually have to

sleep after a period of prolonged wakefulness, and humans report that the desire to sleep after a period of sleep deprivation is very strong. If a method could be developed to test how much an animal would work to be able to sleep (in a similar way that animals are tested to perform other behaviours using consumer demand theory, see Dawkins, 1983), the motivation to sleep could be tested. This may be very difficult as the tiredness of the animal may confound the amount that they will work in order to have access to sleep, so that a non-fatiguing method would have to be developed. However, this has been developed for the demand function of lying requirements in dairy cattle and I see no reason why this should not be extended to include sleep (Jensen et al. 2004 and 2005).

The electrophysiological techniques have potential for many future uses in animal welfare research. The most reliable electrodes -and the easiest to analyse- were the EMG electrodes on the hind-leg and the jaw. Both these measurements could have a variety of uses in animal welfare studies. For example, the jaw EMG provided a method for assessing rumination, not only bout and inter-bout lengths, but chew rate, chew rate variability and possibly the work of the muscles during rumination (e.g. Kemsley et al. 2003). Similarly, the hind-leg EMG was easy to analyse in terms of postural changes and may provide further information on how the muscles were working (and therefore muscle fatigue) during a physical task (e.g. Biedermann, 1991). Giovagnoli et al (2002) used the EMG from the legs of horses to assess balance preservation by the horses during transport. This technique could be used to record the EMG in other animals during transport and in other situations of forced exercise.

6.5 Conclusion

The aims proposed at the start of the project were completed satisfactorily, giving a non-invasive method of recording sleep in sheep. The methodology is not without its inherent difficulties: it is time consuming to carry out and to analyse; ten to twenty percent of the recordings made have to be discarded due to the unreliability of the non-invasive electrodes; and the methodology can be somewhat stressful during electrode attachment (both to the experimental animals and the experimenter!). However, when successful, the electrophysiological recordings made

using this methodology were a useful tool for understanding the behaviour of sheep and giving more information as to the activity of the brain and the mental state (wakefulness and the two main states of sleep) of sheep after experiences during wakefulness.

In addition, the use of non-invasive electrophysiology in conjunction with behavioural observation has the potential to be used in other animal science and animal welfare studies. Electromyography, in particular, may prove to be a useful device in future studies, especially in the study of fatigue in relation to animal transport.

Furthermore, I hope that this study will provide an impetus for more research into the sleep, rest and circadian rhythms of animals, specifically in relation to animal welfare. There is a large body of research using non-human animals as a model for humans within the field of sleep medicine. This should be used as a basis for an increase in the understanding of the importance of sleep to non-human animals and its relationship with the subjective experiences of animals during wakefulness.

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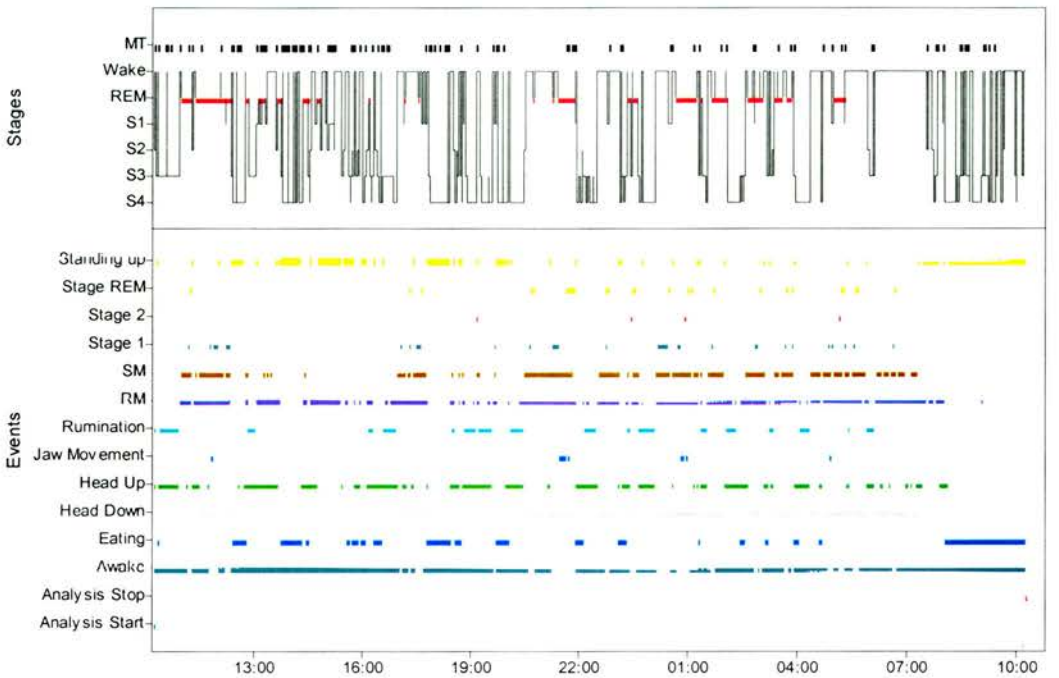
YOUR LOGO?

Somnologica
Printed: 6/30/2003, 15:32:47

Event Report

300403-p5shB, sheep B pair 5

Date of Recording : 4/30/2003 (24 hours and 30 minutes, starting at 10:13:52)



Type	Count	Total time [s]	Mean [s]	Index [1/h]	Shortest [s]	Longest [s]
MT	118	3540	30	-	30	30
Wake	99	31890	322,1	-	30	5040
REM	39	16650	426,9	-	150	1890
S1	49	2730	55,7	-	30	150
S2	69	3330	48,3	-	30	240
S3	96	10140	105,6	-	30	780
S4	70	18150	259,3	-	30	1500
Standing up	42	21500	511,9	1,7	8,9	7835
Stage REM	18	3954,2	219,7	0,7	52,7	630,6
Stage 2	4	579,6	144,9	0,2	23,6	241,9
Stage 1	22	6642,4	301,9	0,9	60,3	1048,8
SM	768	389,3	0,5	31,3	0,3	1,7
RM	3842	1479,3	0,4	156,8	0,2	1,3
Rumination	19	17247,8	907,0	0,8	136,7	1974,7
Jaw Movement	6	1543,6	257,3	0,2	28,8	661
Head Up	69	33900,3	491,3	2,8	7,2	2145,4
Head Down	33	30979,2	938,8	1,3	11,7	2599,7
Eating	23	22358,5	972,1	0,9	34,4	8194,2
Awake	26	73671,9	2833,5	1,1	60	16853,2
Analysis Stop	1	0	0	0	0	0
Analysis Start	1	0	0	0	0	0