Studies of Chronic Inflammatory Pain in Lambs after Rubber Ring Castration and Tail-docking: self-administration of analgesic and neurohistochemistry to validate behavioural assessment.

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A thesis submitted towards the degree of

Doctor of Philosophy

at the University of Edinburgh

2004

Abstract

Lambs castrated and tail-docked (c+td) by tight rubber ring experience severe acute pain lasting up to 3 hours. The use of behaviour to assess the severity of this pain has been validated. Within 5-7 days of application of rubber rings to the neck of the scrotum and the tail, chronic inflammatory lesions form at the sites of the rings. These lesions take 6-7 weeks to heal and can become severely infected. The presence of these chronic inflammatory lesions has been associated with the infrequent expression of the behaviours used to assess acute pain from c+td. It has been proposed that the occurrence of these behaviours is indicative of chronic inflammatory pain, but the use of behaviour has not been validated for the assessment of this pain. The aim of this thesis was to test the following hypotheses:

1. Lambs undergoing castration and tail-docking by tight rubber ring experience chronic inflammatory pain for up to six weeks, in association with chronically inflamed lesions.
2. The chronic inflammatory pain experienced by lambs is sufficient to induce changes in their behaviour.
3. Quantification of these behavioural changes constitutes a valid measure of the chronic inflammatory pain experienced by rubber ring castrated and tail-docked lambs.

Two methods were used to determine the presence of chronic inflammatory pain. The analgesic self-administration paradigm has been used as a means of investigating the experience of chronic inflammatory pain in rats suffering from adjuvant induced arthritis (AA). Similarly, in the studies reported in this thesis, the ability of RR c+td lambs to self-administer a NSAID was used to determine the presence of chronic inflammatory pain. Up-regulation of the synthesis of AVP and reduction in the synthesis of CRH in the parvocellular portion of the hypothalamic paraventricular nucleus (pPVN) has also been detected in rats with AA. Using *in situ* hybridisation histochemistry, evidence of such changes in the control of the HPA axis were sought in the present studies, to confirm the presence of chronic inflammatory pain in RR c+td lambs. Throughout the self-administration and neurohistochemical studies, the severity of chronic inflammatory lesions and the expression of potential 'pain'

behaviours were recorded to determine the significance of these measures with respect to chronic inflammatory pain. Evidence of a relationship between lesion severity and the magnitude of the change in behaviour was also sought.

The studies reported in this thesis further support the relationship between assessments of lesion severity and quantified changes in behaviour that have been associated with acute pain. The validity of the use of subtle changes in posture and changes in feeding motivation to quantify chronic inflammatory pain was supported as the consumption of analgesic creep feed resulted in elimination of the abnormal expression of these behaviours. Some evidence that c+td lambs select more analgesic feed was also present in these studies, although the responses were not reliable within and between individuals. No evidence was found of changes in the expression of hormones in the HPA axis that are characteristic of chronic inflammatory pain in rats.

Definitive evidence of the experience of chronic inflammatory pain was not provided by either the self-administration paradigm or the neurohistochemical studies. The use of changes in behaviour to assess chronic inflammatory pain must still be validated, but it is proposed that the incidence of abnormal lying and changes in feeding motivation are reliably associated with the severity of chronic inflammatory pain.

It is possible that the unchanged expression of AVP and CRH mRNA in the pPVN is a reflection of differences in the mechanisms of control of the chronically activated HPA axis in the rat and the sheep. It is also considered likely that lambs were unable to learn about the pain relieving properties of the analgesic creep feed, because the study did not provide the appropriate opportunity to learn the association. It is suggested that lambs may be able to control their experience of pain by adopting changes in posture and through activation of local endogenous analgesic mechanisms, thus avoiding the need for activation of the pPVN or other central mechanisms and precluding the need for the lamb to select an analgesic treatment.

The evidence supports the assertion that chronic inflammatory lesions from RR c+td are painful, a conclusion that can be used to further oppose of the use of RRs commercially. The potential use of RR c+td lesions as a model of chronic inflammatory pain was demonstrated.

I hereby declare that this thesis is of my own composition and that all assistance has been duly acknowledged. The results presented herein have not been previously submitted for any other degree or qualification.

Anita E. Rennie.

Acknowledgments.

I would like to acknowledge the Medical Research Council for awarding me my research studentship. I am also extremely grateful to Schering Plough Animal Health for providing me with a supply of Finadyne © (flunixin meglumine) for use in my studies of self-administration of analgesic.

I would like to thank my thesis committee; Vince Molony, Barbara Sumner, Francoise Wemelsfelder and Jeremy Bradshaw, for their support throughout my studies. I would particularly like to thank Vince for his constructive criticism, patience and encouragement and for always making time for me when much needed. I am especially grateful to Barbara for introducing me to neurohistochemical studies and for her continued support during my times in the laboratory. I learned a huge amount from her and have the utmost admiration for her as a scientist and friend.

I would also like to thank Joyce Kent for all her practical help. Joyce's help was critical to the accomplishment of much of the on-farm work, I learned a lot from her and she was always good company. I would also like to thank other members of the University of Edinburgh Animal Welfare Research Group, who generated a fun and stimulating environment in which to work. Willie van Wijde provided administrative support for us all in the group, but I also valued her unending moral support and thank her for her friendship and all the laughs.

I would like to acknowledge the cheerful statistical assistance of Dr Iain MacKendrick of BioSS, who advised on analysis and constructively criticised the design of experiments.

My practical work was carried out on farms belonging to the Moredun Institute and I am very grateful to the Moredun and their staff for making the work possible. Jim Williams facilitated my work on the farms, ensuring that lambs were provided when required and that space was available for my studies. I would like to thank Arthur Watt, Jim Smith, Terry, Tony and Jim for providing me with all I needed on the farms – from pen gates to lamb creep feed, the work would have been so much harder without their help. Manus Graham and Tony also provided the necessary skills for stomach tubing lambs during the pharmacology trials.

I was assisted on the farm by several other people. Maxine Palagi and Lynn Meikle assisted during behavioural observations. Fritha Langford, Nia Ball and Kenny Rutherford also helped me on the farm during particularly busy times.

The protocol and standards used in my HPLC analysis were provided by the Department of Veterinary Pharmacology at the University of Glasgow and I would like to thank Professor Andrea Nolan and Ian Gibson for their assistance. I am very appreciative of assistance provided by Dr Celia Goodhew who demonstrated the use of the HPLC equipment and helped me trouble-shoot when problems arose. I am also grateful to Professor Graham Pettigrew for assistance with the HPLC analysis.

I would like to thank Robert Lee of the Department of Medical Statistics for discussion and advice on the most appropriate methods of pharmacokinetic analysis.

I am indebted to several individuals at the Roslin Institute. I would like to thank Peter Sharp for facilitating my use of equipment at the institute. I would also particularly like to thank Mike Gentle for the use of his photographic equipment and Sandra Wilson and Laura Dick for the use of image analysis software and the space to carry out my image analysis.

I would like to thank all the staff in the Division of Preclinical Veterinary Sciences who provided support during my studies, especially Colin Warwick for assistance with photography and Derek Penman and Phyllis Lyon for administrative assistance.

I am immensly grateful for the support of all my friends during my PhD studies, especially Nia, Helena, Annemarie, Mairi, Kenny, Martin, Fritha and Emma.

I also want to thank my parents and family for their never-ending support, and especially my Dad for reading drafts of chapters – I always knew he would be absolutely honest with me! Finally I want to thank my husband, Bruce for always being there – no matter what!

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Preface

List of Abbreviations

AA	Adjuvant induced arthritis
ACTH	Adrenocorticotrophic hormone
ADX	Adrenalectomy
AUC	Area under the concentration time curve
AUMC	Area under the concentration x time against time curve
AVP	Arginine vasopressin
BNST	Bed nucleus of the stria terminalis
c+td	Castration and tail-docking
CD	Castrated and tail-docked lambs with access to analgesic creep feed (castrated-drugged)
Cl	Clearance
Cmax	Maximum plasma concentration
CN	Castrated and tail-docked lambs with no access to analgesic creep feed (castrated-non-drugged)
CNS	Central nervous system
COX	Cyclooxygenase
CRH	Corticotrophin releasing hormone
DLWG	Daily live-weight gain
ELISA	Enzyme linked immunosorbent assay
F	bioavailability
FAWC	Farm Animal Welfare Council
FM	Flunixin meglumine
GDX	Gonadectomised
GLM	General linear model
HD	Handled control lambs with access to analgesic creep feed (handled-drugged)
HN	Handled control lambs with no access to analgesic creep feed (handled-non-drugged)
HPA	Hypothalamo-pituitary-adrenal axis
HPB	Hypophysial portal blood
HPLC	High performance liquid chromatography

icv	Intra-cerebroventricular
IIH	Insulin-induced hypoglycaemia
im	Intramuscular
ir	Immunoreactive
iv	Intravenous
IV1	Lambs receiving flunixin meglumine intravenously at a dose of 1mg/kg body weight
IV2	Lambs receiving flunixin meglumine intravenously at a dose of 2mg/kg body weight
LA	Local anaesthetic
LiCl	Lithium chloride
LWG	Live-weight gain
MAT	Mean absorbance time
mPVN	Magnocellular paraventricular nucleus
MRT	Mean residence time
NSAID	Non-steroidal anti-inflammatory drug
NST	Nucleus of the solitary tract
O1	Lambs receiving flunixin meglumine by oral gavage at a dose of 1mg/kg body weight
O2	Lambs receiving flunixin meglumine by oral gavage at a dose of 2mg/kg body weight
OT	Oxytocin
PBN	Parabrachial nucleus
PLC	Phospholipase C
POMC	proopiomelanocortin
pPVN	Parvocellular paraventricular nucleus
PVG	Piebald-viral-glaxo (rat strain)
PVN	Paraventricular nucleus
RR	Rubber ring
SAC	Scottish Agricultural College
SCN	Supra-chiasmatic nucleus
SON	Supra-optic nucleus

t1/2	Plasma half-life
Tmax	Time at which maximum plasma concentration was reached
VAS	Visual analogue scale
Vd	Volume of distribution

Chapter 1

Introduction

Chapter 1. Rubber Ring Castration and Tail-docking: painful routine procedures.

1.1. *Introduction*

Castration and tail-docking (c+td) are procedures carried out routinely on the majority of male lambs in the U.K. and in other lamb producing countries. Numerous reasons for carrying out these procedures have traditionally been given, including improvement of carcass quality, prevention of indiscriminate breeding and prevention of fly strike of the tail and breech. However, over the last 50 years, the practice has been questioned, from the perspective of both production and the welfare of farmed animals. Research has provided considerable evidence of severe acute pain following c+td by conventional methods. This acute pain has been successfully quantified using behaviour (Mellor and Murray, 1989a; Molony *et al*, 1993; Molony *et al*, 2002). Evidence suggests that lambs may also experience chronic pain lasting up to six weeks after treatment (Kent *et al*, 1999; Kent *et al*, 2000). However, the use of behaviour to quantify this pain has not been validated. The main aim of this thesis is to address the problem of recognition and assessment of chronic pain in lambs.

1.2. *The law controlling the practice of castration and tail-docking.*

In the U.K. several pieces of legislation regulate the routine practice of c+td. Firstly the Protection of Animals Act 1911-1988, as amended, and the Protection of Animals Act (Scotland) 1912-1993 as amended, are intended to prevent cruelty to animals. Under this legislation an act of cruelty is one that causes unnecessary and substantial suffering and it is an offence to carry out any operation 'without due care and humanity'. The performance of c+td is not considered to be cruel under the Protection of Animals Act, provided it is carried out in accordance with the requirements of The Protection of Animals (Anaesthetics) Act 1954-1982, as amended. According to this Act castration can be carried out, by constriction of the blood supply to the scrotum (for example by the application of a tight rubber ring (RR)), without the use of anaesthetic, provided that the device is applied within the first week of life. Castration by other methods (again without anaesthetic) may be

carried out in lambs up to 3 months of age. In lambs older than 3 months of age, castration must be carried out using anaesthetic. Under the Veterinary Surgeons Act 1966-1991, castration of lambs within the provisions of the Protection of Animals (Anaesthetics) Act 1954-1982 may be carried out by suitably trained and experienced lay person over the age of 18, but the castration of lambs over 3 months of age can only be carried out by a veterinary surgeon. Similarly, tail-docking by constriction of the flow of blood to the tail may be carried out by a trained and experienced lay person over the age of 18 in accordance with the Veterinary Surgeons Act 1966-1991, without anaesthetic, provided that the procedure is carried out within the first week of life in accordance with the Protection of Animals (Anaesthetics) Act 1954. Tail-docking can be carried out using any other conventional method at any age without an anaesthetic. The Agriculture (Miscellaneous Provisions) Act also influences the practice of c+td. Under section 2 of this Act, government ministers are given the power to introduce statutory instruments regulating specific procedures that may be carried out on agricultural land. One such piece of legislation is the Welfare of Livestock (Prohibited Operations) Regulations (1982) under which it is required that sufficient tail must be left after docking so that the vulva is covered in female sheep and the anus is covered in males. Under section 3 of the Agriculture (Miscellaneous Provisions) Act (1968) government ministers retain the right to introduce Codes of recommendations for the welfare of livestock (better known as the welfare codes) that are intended to promote good welfare by encouraging best practice. Under the Welfare of Livestock Regulations (1994) it is a legal requirement that anyone keeping livestock must be familiar with the welfare codes and, although these codes are not law, they may be used in court as evidence of poor practice if caretakers are charged with an offence related to their care of their livestock under the Agriculture (Miscellaneous Provisions) Act (1968). The codes of recommendations for the welfare of livestock (sheep) were revised in 2000 and include the recommendation that farmers should take time to consider whether c+td are necessary, for each flock in turn. It is also recommended that the pain and distress caused by the procedures and the potential stress caused by gathering and handling the animals should be taken into account in weighing up the necessity of the procedures. The welfare codes also recommend that castration should only be carried

out when a strong bond has developed between the ewe and her lambs and that surgical castration should only be carried out by shepherds in exceptional circumstances. They also state that tail-docking should only be considered necessary when there is substantial evidence to suggest that welfare problems will result if tail-docking is not carried out and that both c+td should be carried out together in order to avoid stress from double handling.

1.3. *Why Castrate and tail-dock?*

Legal acceptance of mutilations like c+td has come about mainly because of what Robertson (1965) describes as 'reputable historical background'. The procedures have traditionally been considered necessary within the farming community (Wohlt *et al*, 1982; Robertson, 1965) and are still considered necessary by many farmers in the U.K. (Hosie *et al*, 1996). However, the necessity and justification for carrying out both procedures have increasingly been the subject of debate. Substantial evidence of pain as a result of c+td now exists and with this knowledge comes the ethical requirement to ensure that the need for these procedures is both reasonable and substantial, rather than simply being the product of tradition. The rationale behind the practice of castration and that of tail-docking are somewhat different. A summary of the arguments for and against c+td are considered separately below.

1.3.1. *Arguments for and against castration.*

Castration of lambs is considered necessary for several interconnected reasons. All these reasons are related to husbandry and productivity and few benefits for the lambs can be recognised. Firstly it is argued that castration of lambs facilitates their management (FAWC, 1994; Hosie *et al*, 1996; Robertson, 1965). After castration of male lambs, all lambs may be housed or grazed together, irrespective of their sex, without the risk of indiscriminate breeding. Castration is thought to reduce the development of secondary sexual behaviours, including aggression and mounting (FAWC, 1994). This assumption has been quantitatively assessed in two- and three-week-old pigs in which aggression, mounting and attempts at mounting were reduced by both surgical and immuno-castration (Cronin *et al*, 2003). A reduction in aggression in both surgically and immuno-castrated bulls was also observed by Price

et al (2003). No quantitative study on the effect of castration on aggression and mounting behaviours in sheep were found, but it is likely that castration has a similar effect on behaviour in this species. A reduction in secondary sexual behaviour is desirable as the risk to stockpersons may be reduced (although the risk must be considered less from rams than from bulls or boars). The more frequent occurrence of mounting seen in entire males is considered likely to result in bruising. The presence of bruising has been considered to be an indicator of welfare problems before slaughter (Jarvis and Cockram, 1994) and is one of the most frequent causes of downgrading or rejection of the carcass after slaughter (Green *et al*, 1995) resulting in significant losses for the meat industry (Jarvis and Cockram, 1994; McNally and Warriss, 1996). However, evidence suggests that bruising does not necessarily occur as a result of high libido and mounting in ram lambs, but as a result of riding behaviour during transportation and handling at livestock markets and in the slaughter house (Jarvis and Cockram, 1994). This is supported by evidence that bruising in lamb carcasses is much more prevalent in lambs sold through livestock markets for slaughter than in those sold direct from the farm to the slaughterhouse (Jarvis and Cockram, 1995). As the majority of lambs are currently sent for slaughter through the market place, the higher incidence of bruising in these animals is itself a cause for concern both for production and welfare reasons (Jarvis and Cockram, 1995). Evidence in cattle suggests that, of the cattle sold for slaughter through livestock markets, young bulls showed the least bruising and their carcasses were rejected less frequently than steers and heifers (McNally and Warriss, 1996). This may be a result of more careful or permissive handling and smaller grouping of entire males, because they are considered to be more volatile and sensitive in character and are more likely to become aroused in stressful conditions (Field, 1971). If it could be shown that the greater expression of arousal in entire males is associated with a greater perception of stress by the individual, this argument might be considered the only benefit realised by the castrated individuals themselves. From a production point of view, it is known that stress before slaughter results in a reduction in meat quality, with an increase in dark cutting meat of lesser value (Voisinet *et al*, 1997), and thus it is argued that the potentially greater experience of stress in entire males will result in reduced meat quality after slaughter (Field, 1971).

Further arguments for castration are also related to productivity. It is commonly supposed that meat from entire males, including rams, produces an unpleasant odor and taste when it is cooked and is considered by many to render the meat unfit for human consumption (Field, 1971). Entire male cattle, pigs and sheep develop heavier musculature around the neck and shoulders than castrated animals. The value of cuts of meat from these areas is lower and it has been argued that castrates produce better cuts of meat and have a higher dressing percentage (the percentage of empty live weight that can be converted to saleable carcass) overall (Robertson, 1965). Wethers are known to fatten more quickly and to deposit more fat, which was in the past considered more favourable (Robertson, 1965). For these reasons, ram lambs, especially those with 'excessively male characteristics', were less likely to qualify for the 'Variable Premium', a government subsidy that was paid for each lamb that attained certain standards of conformation and fat quantity and distribution (FAWC, 1994; Hosie *et al*, 1996). The system of subsidies has now changed and premiums are no longer paid on the basis of lamb quality (the variable premium was phased out from 1989), but are instead paid on a headage basis for every ewe which has given birth or is at least one year old by the 15th of May of that year. The payment is supplemented if the stock are kept on poor grazing under the 'Less Favoured Areas' scheme (DEFRA, 2003; FAWC, 1994). Thus, qualification for variable premiums themselves can no longer be used to justify the use of castration on financial grounds. Furthermore, consumer demand now strongly favours lean meat. European policy has therefore also changed to favour the production of leaner meat (FAWC, 1994) and meat wholesalers are now willing to accept the leaner carcasses of ram lambs, even with excessive male characteristics (Anderson, 2001).

It has long been recognised that the effects of castration are less evident and that masculine characteristics are less objectionable in male lambs than in cattle and pigs (Robertson, 1965). Furthermore, evidence shows that, if fed on a medium to high plane of nutrition, ram lambs gain weight more quickly and show higher rates of feed conversion than wethers (Field, 1971; Mahgoub and Lodge, 1994; Notter *et al*, 1991; Robertson, 1965). It has also been shown that the dressing percentage obtained from rams fed on good quality feed is close to or equal to that for wethers, and may exceed it, if the weight of the testes (sold as 'lamb fries') is taken into account. It is likely

that this is a reflection of the fact that finished ram lambs can be sold before they reach puberty at 5 months of age, the age at which the characteristically male build begins to become apparent (Field, 1971). Robertson (1965) reviewed evidence from 8 studies using taste panel tests and found that meat from ram lambs was considered to be as flavoursome or more flavoursome than that from wethers in 6 out of 8 studies. It was also found that, although meat from ram lambs was considered slightly less juicy and tender, the differences were small and were not significant to result in downgrading of the meat (Robertson, 1965). In a more recent but similar taste panel study, Anderson (1996) found that meat from grass fed lambs was the most tasty and juicy and that there was no difference in taste between meat from rams lambs and wethers. In support of these conclusions the New Zealand Animal Welfare Advisory Committee in their 'Code of recommendations and minimum standards of welfare of sheep' state that no unpleasant smell and flavouring is present in meat from pre-pubescent ram lambs.

The more sensitive nature of ram lambs by comparison to wethers might certainly raise welfare and production concerns, as has been noted. However, as little difference has been found between the quality of meat from rams compared to wethers, the production concerns appear unfounded. Despite this, the tendency to handle entire male stock more carefully, minimising any effect of stress on meat quality, could improve the well-being of stock overall.

Undoubtedly the ability to run all lambs together ensures that the husbandry of wethers within flocks is easier than that of rams, but as has been noted, most farmers should be able to finish lambs and sell them before they reach puberty at 5 months of age (Anderson, 1996; Hosie *et al*, 1996). In a survey of castration methods, farmers considered it possible to separate and fatten male lambs that had not been successfully castrated before they reached puberty, irrespective of the farming system (Hosie *et al*, 1996), and it has been proposed that extending this practice for all male lambs may be possible on the majority of farms (Anderson, 1996). Anderson (1996) indicated that ram lambs could be run together with ewe lambs until reaching 5-6 months of age, but that after this time separation was advisable. According to Anderson (1996), leaving male lambs entire is now normal practice on hill farms in Scotland and Wales, where the lambs are often destined for sale in Europe. Although

some substantial changes in stock management are necessary if ram lambs are to be kept on as stock lambs, such changes are beginning to be accepted in the farming community. It has been shown that, if successfully managed, a non-castration policy can have considerable production advantages. In New Zealand in 1970, a non-castration policy (more than two thirds of male lambs) resulted in an increase in the production of meat by an estimated 1000 tonnes annually in 1970 (Field, 1971).

1.3.2. *Arguments for and against tail-docking.*

Tail-docking is carried out mainly to reduce the occurrence of 'fly-strike' (myiasis and pseudomyiasis by maggots of the blow fly family (Calliphoridae)) of the tail and breech area (FAWC, 1994; French *et al*, 1994a). Faecal and urinary soiling of the tail and breech area provides warm damp environment suitable for the development of larvae (Graham, 1997). It is argued that tail-docking reduces such soiling and therefore theoretically reduces susceptibility to strike (French *et al*, 1994a; Riches, 1941; 1942; Wohlt *et al*, 1982). Thus tail-docking is seen as a means of preventing a condition that can cause damage to the wool and dermis and in serious cases can result in loss of appetite and condition and potentially death (Henderson, 1990; Schmidt and Roberts, 1996). The reduction in soiling of the tail and breech area is also considered to be advantageous for other reasons (French *et al*, 1994a). It has been argued that tail-docking facilitates care during breeding and lambing (FAWC, 1994) and has been said to increase breeding efficiency in ewes (Wohlt *et al*, 1982). In many cases tail-docking may be carried out simply to improve the appearance of lambs, giving them a consistent and square shape favoured by buyers and meat producers (Wohlt *et al*, 1982; FAWC, 1994).

The relationship between fly-strike and tail-docking was first examined by Riches *et al* (1941; 1942) and later by French *et al* (1994b). French *et al* (1994b) studied the incidence of strike in seven farms in South West England. Lambs that had been tail-docked by RR in the first week of life were found to be less likely to be the victim of strike than those that had not been tail-docked. They also found that undocked lambs were more soiled with faeces and urine than docked lambs and a relationship between the extent of soiling and the occurrence of strike was found. It was pointed out that, as the docked and undocked lambs in this study were kept together in the

same flocks, no conclusion could be drawn about the likelihood of strike occurring in the flock as a whole, but only that, within the flock, strike was most likely occur in the undocked and therefore most soiled lambs. No differences in the survival of lambs were found in this study. Riches *et al* (1941; 1942) also found that susceptibility to fly strike was associated with tail-docking, but the methodology and conclusions of these studies were more complex. Riches *et al* (1941; 1942) examined the differences in strike susceptibility after the tail had been docked to a range of lengths. The results showed that tail-docking resulted in a reduced occurrence of fly strike only when the remaining tail was approximately 10 centimetres long, as is common practice in the U.K. today. Very short docking increased susceptibility to both tail and breech strike in comparison to docking tails to 10cm. Whilst long-tailed and undocked lambs were more susceptible to tail strike, they were no more susceptible to breech strike than lambs with tails docked to 10cm. Thus, it must be concluded that both the length of the remaining tail and the degree of soiling are critical factors in fly strike susceptibility. French *et al* (1992) found that there are regional variations in the incidence of fly-strike. As might be expected, this survey showed that the incidence of strike is more likely in warmer areas in the South West and South East of England and less in the North. Thus the need for tail-docking to reduce fly strike might be considered more necessary where climatic conditions are favourable for the flies and their larvae. However, as French *et al* (1994c) point out, the necessity of tail-docking merely demonstrates that the methods used to control scouring and the blowflies themselves are inadequate. Alternative methods of control of fly-strike include dipping, dagging and spraying and their use as alternatives to tail-docking have been promoted.

It has been argued that tail-docking increases breeding efficiency in ewes (Wohlt *et al* 1982). Evidence in support of this tenet was sought by Riches (1942), but no evidence of such an effect was found in a sample of 197 ewes, comprising undocked and medium- and short-docked ewes. Dagging can be carried out to reduced soiling of the tail and breech areas. Critical differences in the ease of management of docked lambs and ewes over undocked animals must be found before ease of management can to be used to justify the practice of tail docking. The docking of lambs' tails simply to improve the appearance of the lamb, although traditional, is considered

ethically unacceptable. The possibility of breeding sheep for short tails and for reduced wool on the legs, abdomen and breech has recently been raised in Australia (Scobie *et al*, 1999). Whilst the welfare implications of selection for such traits must be carefully evaluated, this proposition has the potential to reduce the extent of soiling, incidence of fly-strike and facilitate management without the need for tail-docking.

It must be recognised that, for farmers, production benefits are likely to be the main incentive for the voluntary practice of a non-c+td policy. Where the production benefits are not sufficient to induce a change in farming practice, despite evidence of adverse welfare effects, the law should take into account scientific evidence and ensure that the degree to which production considerations compromise welfare is minimised.

1.4. *Methods of castration and tail-docking.*

There are three principal methods by which lambs are castrated in the U.K.; application of a rubber ring (RR) to the neck of the scrotum, crushing the spermatic cords with a bloodless castrator and open or surgical castration involving the excision of the testes. Tail-docking is carried out using one of four methods; RR, surgical docking, a combination of surgery and bloodless castrator and the use of a hot docking-iron. Descriptions of these procedures and a summary of the risks known to be associated with their use is given in Table 1.1. C+td in the U.K. is most commonly achieved by the application of RRs because they are cheap, effective, quick and easy to use (Barrowman *et al*, 1954; Hosie *et al*, 1996; Kent *et al*, 2001).

1.5. *Can lambs experience pain in response to these routine mutilations?*

RR c+td was introduced to the U.K. from New Zealand in the early 1950s (Barrowman *et al*, 1953). The effects of this new procedure on lambs were investigated by Barrowman *et al* (1953; 1954). It was concluded in these early studies, that older lambs may experience significant pain as a result of RR c+td (Barrowman *et al*, 1953; 1954). Since then studies have been carried out in an effort

Procedure	Method	Description	Risks
Castration	A rubber-ring is placed around the neck of the scrotum using a commercially available elastrator to stretch the ring over the scrotum containing the testes.	Occlusion of the blood supply to the testes and scrotum resulting in ischaemia and necrosis of the tissue distal to the ring. The scrotum and it contents dry out and fall off after approx. six weeks. Nociceptors in the scrotum and testes are not immediately disabled. Successful castration is achieved provided both testes are secured distal to the ring and can therefore be determined immediately.	Inflammatory lesions form at the neck of the scrotum proximal to the ring, these may become infected and be associated with chronic inflammatory pain and an increased risk of fly strike (Fisher *et al* 1996).
	Bloodless castrator (Burdizzo) a large pincer-like instrument with smooth rounded opposed jaws and a double levered action. This instrument applies high crushing forces.	Each spermatic cord is crushed between the metal jaws of the instrument without breaking the skin. The innervation and blood supply to the testes are thus obstructed. The success of the procedure is determined several weeks later when/if atrophy of the testes becomes apparent. This method can be used alone, crushing each cord in turn and leaving enough blood supply between the crush lesions for the scrotum to survive. It can also be used in combination with a rubber-ring, when a single crush is applied across the width of both cords..	Swelling of the scrotum may occur as a result of internal haemorrhage. Anatomically accurate positioning of the clamp is necessary to ensure that the spermatic cord is trapped and the urethra is not trapped in the clamp. Positioning of the clamp is made much easier and less critical by the prior application of a rubber-ring in the 'combined' method.
	Open or surgical	The scrotum is incised with a sharp knife and the testes exposed. Testes removed by tearing, cutting or twisting. Cautery, and clamping to reduce haemorrhage may be used in some cases.	There is an increased risk of haemorrhage and prolapse of intestines may occur particularly if the inguinal canal is open and abdominal pressure is high. The risk of peritoneal infection is also higher.
Tail-docking	Rubber-ring applied at approximately the level of the fourth caudal vertebra	Occlusion of the blood flow to the tail resulting in ischaemia and necrosis of the tissue distal to the ring and severing both pairs of caudal nerves. The distal portion of the tail dries out and falls off after 4-6 weeks.	An inflammatory lesion forms at the site of the ring, this may become infected and be associated with an increased risk of fly strike (Fisher *et al* 1996).
	Open or surgical	Tail is cut off with a sharp knife at approx. the level of the 4th caudal vertebra.	Haemorrhage occurs and can occasionally result in death. There is also a risk of infection.
	Crushing with a Bloodless castrator before surgical removal of the tail	A bloodless castrator is applied at approx. the level of the fourth caudal vertebra and while clamped the tail [distal] is cut off. This combination of treatments reduces haemorrhage.	Haemorrhage may still occur. The risk of infection remains.
	Hot Docking-iron	The iron is applied at the level of the fourth caudal vertebra, severing the tail and cauterising the stump.	Risk of haemorrhage and infection is less than that for surgical docking without cautery, but the stump may take longer to heal and is more likely to become infected due to tissue damage.

Table 1.1. Principal methods by which lambs are c+td in the U.K.

to quantify pain resulting from RR c+td in lambs of different ages and to identify practical means by which the resulting pain can be reduced. The identification of the least painful means of castrating and tail-docking lambs might at first seem simple, but in practice the determination and quantification of pain in animals is difficult because the experience of pain is essentially a subjective perception (Kitchell and Johnson, 1985) and pain is a word used primarily to describe a human experience. Whilst the animals' responses to the experience of animal pain can be measured, the magnitude of the actual experience itself cannot be measured. Because, unlike most humans, animals are incapable of reporting their experience of pain, their ability to experience pain at all has been questioned. However, whilst there are some neuroanatomical differences in the peripheral and central mechanisms involved in the sensation and perception of pain, the mechanisms underlying pain in mammals as well as other vertebrates are considered to be very similar and it is this that permits the use of animals as models for the investigation of pain in humans. Animal and human studies have shown that the application of high intensity thermal, mechanical or chemical stimuli to somatic or visceral structures results in activity in the peripheral nociceptors (with Aδ- and C- afferent fibres). This activity is relayed via parts of the dorsal horn grey matter to the brain through the spinothalamic tract and other ascending pathways carrying nociceptive information. On reaching the thalamus, this information is relayed to the somatosensory cortex (perception of pain), parietal insular cortex and the anterior cingulate and limbic cortices (motivational-affective dimensions of pain in humans) (Kitchell and Johnson, 1985, Zimmerman, 1986; Guilbaud *et al*, 1994; Meyer *et al*, 1994; Woolf, 1994; Short, 1998). The neurophysiological, physiological and behavioural responses of animals to such stimuli, reported to be painful in humans, are generally similar to the recorded in humans. These responses appear to serve the same functions as those in humans, which are; withdrawal from the stimulus, minimisation pain, facilitation of healing, elicitation of help and modification of behaviour (learning) to prevent future exposure to the stimulus (Bateson, 1991; Molony *et al*, 1997). In order to move beyond the debate over the ability of animals to experience pain, Molony (1986; 1992) advocated that the study of animal pain continue under the assumption that animal pain serves the same function as human pain and that the experience is

similar, but that human and animal pain are not necessarily the same. Thus, Molony and Kent (1997) proposed the following definition of animal pain:

> Animal pain is an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues; it changes the animal's physiology and behaviour to reduce or avoid damage, to reduce the likelihood of recurrence and to promote recovery; unnecessary pain occurs when the intensity or duration of the experience is inappropriate for the damage sustained or when the physiological and behavioural responses to it are unsuccessful at alleviating it.
>
> Molony and Kent (1997).

The strategies that have been employed to determine the presence and severity of animal pain, in response to experimentally induced and naturally occurring stimuli and as a result of husbandry practices, are many. The validity, sensitivity and reliability of these measures is continually questioned in the literature, for example methods of assessment of post-operative pain resulting from beak-trimming in poultry, c+td in lambs and ovaro-hysterectomy in dogs were recently the subject of a critical review by Rutherford (2002). Methods used to recognise and assess the acute pain from RR c+td include electrophysiological studies, quantification of behavioural changes and quantification of HPA activation by measurement of changes in the plasma cortisol concentration.

1.6. *Recognition and assessment of acute pain from rubber ring castration and tail-docking.*

1.6.1. *Electrophysiological studies*

Cottrell and Molony (1995) made electrophysiological recordings of visceral afferent activity in the superior spermatic nerve in lambs in response to the application of RRs to the neck of the scrotum. The presence of a ring resulted in 'vigorous discharge' in nociceptors of the testes and pampiniform plexus (a network of veins associated with the testis and epididymus). Application of an extremely tight ligature (pressure unquantified) to the neck of the scrotum resulted in cessation of all nervous activity within two minutes and recovery of activity within 5 minutes of ligature removal. The RR resulted in the application of less pressure than the ligature and resulted in successive blockade of nerves of different conduction velocities (larger,

faster, myelinated fibres first). The pressure applied by a single standard RR was insufficient to block all afferent activity within an hour after application and afferent activity was recorded for up to 90 minutes after ring application. Whilst Cottrell and Molony (1995) measured visceral afferent activity in the superior spermatic nerve, it is likely that the RR castration has similar effects in the middle and inferior spermatic nerves (Molony and Wood, 1992). Cottrell and Molony (1995) found that nociceptors with afferent fibres in the superior spermatic nerve are not activated by short scrotum castration (where RR is applied at the neck of the scrotum with the testes proximal to the ring), but they did not measure activity in any of the somatic afferent nerves from the scrotum including the ilioinguinal nerve, caudal scrotal nerve or the scrotal branch of the superficial perineal nerve. As short scrotum castration by RR produces a behavioural response in lambs (Molony *et al*, 2002), it is likely that stimulation of somatic nociceptors is also important to the overall experience of pain in RR castrated lambs.

Graham (1997) carried out similar studies in lambs to measure activity in nociceptors in deep and superficial nerve trunks innervating the tail in response to the application of a RR at the level of the fourth caudal inter-vertebral space during tail-docking. In Graham's studies, afferent activity was again gradually reduced after application of the RR and complete conduction block was achieved in the preparations within 11-44 minutes. From these results no conclusion can be drawn as to the time taken for all afferent activity from the tail to cease after application of the ring. It was suggested that the rate at which conduction block was achieved would vary depending on whether the ring was placed at an intervertebral space or over bone.

As well as stimulating nociceptors in the neck of the scrotum and in the tail mechanically, the application of the RR results in total occlusion of the blood and lymphatic vessels to the scrotum within 1 minute, resulting in ischaemia (Cottrell and Molony, 1995) and is likely to have a similar effect on the tail. As mechanoreceptors in the portion of the scrotum and tail distal to the ring continue to respond to stimulation for some time after application of the RR, it has been proposed that ischaemia contributes to the eventual blockade of conduction (Cottrell and Molony, 1995). This view was supported by Graham (1997) who found that removal of the RR resulted in the recovery of afferent activity within a few minutes. Thus, it was

concluded that conduction block by the application of RRs is likely to be achieved as a result of a combination of factors including disruption of conduction by ischaemia and/or compression and failure of receptors as a result of the effects of ischaemia (Graham, 1997). Occlusion of the blood supply itself can result in an increase in visceral afferent activity (Grubb *et al*, 1990) and is likely to contribute to the overall sensation of pain because the pressure applied by the RR is insufficient to result in immediate cessation of afferent activity (Cottrell and Molony, 1995; Graham, 1997). However, neither one of these studies attempted to identify afferents that responded to chemical changes that occur as a result of ischaemia, for example those responding to hypoxia, hypoglycaemia, hypercapnia and acidosis. Chemoreceptors will also respond to inflammatory mediators released from damaged tissue under the ring, again contributing to the overall perception of pain. As veins are more superficial than arteries, it is likely that, after application of the RR, the pressure in the testes and tail increases before occlusion of the blood supply is complete. This is particularly true in larger lambs where the pressure of the ring is distributed over a larger area. This may result in increased sensitivity of the afferent nerves of the scrotum, testis and associated structures (Cottrell and Molony, 1995). The combination of ischaemia and the destruction of innervation ultimately results in necrosis of the scrotum and its contents, which eventually falls off.

1.6.2. *Performance as a measure of acute pain.*

Whilst studies of electrophysiology provide definitive evidence of nociceptive stimulation by the application of RRs, this work does not provide a means by which the severity of pain can be easily quantified in the field. Traditionally, the degree of pain suffered by animals as a result of procedures such as c+td has been estimated by identification and quantification of a decrease in daily live weight gain (DLWG) (Robertson, 1965). Assessment of DLWG is used in studies of pain in lambs and other ruminants after c+td. Wohlt *et al* (1982) found that lambs tail-docked surgically at 14, 28 and 42 days old showed a reduced DLWG during the two weeks after tail-docking compared to the two weeks before tail-docking. However, the authors found no significant difference in weight gain between lambs tail-docked surgically or by RR. Similarly, Ware *et al* (2000) found that tail-docked male lambs were lighter than

undocked lambs 8-12 weeks after tail-docking, although this effect was not observed in ewe lambs and was only seen on one of three study farms. Rhodes *et al* (1994) found no difference in DLWG following tail-docking of 16 day old lambs using a bloodless castrator in the first 14 days after treatment and Kent *et al* (2000) found no differences in DLWG in the 28 days after c+td of newborn lambs by RR. Castration itself has been shown to reduce weight gain in lambs in the long-term (Field, 1971), but as the effects of castration on growth (inhibiting development of secondary sexual characteristics) do not become apparent until puberty at approximately 5 months of age, growth checks at around the time of castration in young lambs are unlikely to be associated with emasculation. However, DLWG is subject to considerable variation between studies and cannot be considered sufficiently reliable to confirm the presence or absence of acute pain from c+td without further evidence.

1.6.3. *Behavioural assessment of acute pain from rubber ring castration and tail-docking and evidence supporting its use.*

Barrowman *et al* (1953) and (1954) used behaviour as a means of determining the presence and degree of acute pain experienced by lambs after RR c+td. The behaviour was not recorded in a quantified way, but was simply recorded descriptively. However, these were the first behavioural studies in which it was suggested that lambs experienced significant pain and distress as a result of c+td using RRs or the Burdizzo. Barrowman *et al* (1953) described the response of 16-39 day old lambs to RR castration and reported abnormal walking (sideways and backwards), rolling, falling, turning to the scrotum and 'great uneasiness' which occurred almost immediately after application of the rings until 20 minutes after treatment. Later Barrowman *et al* (1954) compared the responses of very young lambs (less than 1 week old) to either RR castration or tail-docking, with that of 21-30 day-old lambs. In this study, Barrowman *et al* reported that there were few or no signs of distress in lambs castrated at less than a week of age, instead the lambs appeared 'indifferent' to the procedure. Older lambs, castrated at 21-30 days of age, showed almost immediate behavioural signs of distress as described by Barrowman *et al* (1953). The expression of these behaviours reached a maximum 15 minutes after treatment but all lambs showed resting between 30 minutes to one hours after

treatment. It was noted that the response was extremely variable with some individuals seeming 'quite oblivious of interference'. After tail-docking by RR, young lambs showed some signs of mild distress, including restlessness, which lasted up to 15 minutes. In contrast, they recorded severe signs of distress in 21-30 day-old lambs in response to tail-docking, again including falling, rolling and kicking. These behaviours were apparent immediately after treatment and lasted for up to 30 minutes. The lambs subsequently showed quiet lying and resumed grazing, suckling and sleeping within 2 hours. Again Barrowman *et al* (1954) noted considerable variation between individuals. Barrowman *et al* (1954) concluded that the evidence of distress in lambs c+td under a week of age was limited, whilst older lambs showed significant behavioural signs of distress in response to both c+td. It was thus inferred that younger lambs are less able to perceive pain from c+td than older lambs and are considered likely to have been influential in the introduction of U.K. legislation restricting the use of what was at the time a new method of c+td, without an anaesthetic, to the first week of life.

In the first quantified behavioural study of 7 day-old lambs subjected to RR c+td or to tail-docking alone, Mellor and Murray (1989a) concluded that behaviour was a sensitive means of determining the severity and duration of distress from the procedures. In this study, in groups of over 50 lambs, a range of behaviours was recorded at specific, but irregular, intervals over the first 4 hours after treatment. Some of these behaviours had been previously described by Barrowman *et al* (1953; 1954). In direct conflict with the conclusions drawn by Barrowman *et al* (1954), Mellor and Murray (1989a) found that lambs undergoing c+td by RR at 7 days of age showed behaviours which they considered to be consistent with the experience of significant distress. The results of this study were later confirmed using the same behavioural observation methods (Mellor *et al*, 1991; Wood *et al*, 1991; Lester *et al*, 1996) and these behavioural methods were found to be sufficiently sensitive to detect differences between much smaller groups (between 6 and 18 lambs) of c+td lambs and controls.

Similar changes in behaviour were observed in response to RR c+td in studies using more detailed examination of active and postural behaviours (Molony *et al*, 1993; Kent *et al*, 1995; Molony and Kent, 1997; Kent *et al*, 1998). In lambs castrated by

RR at three ages (5, 21 and 42 days old), Molony *et al* (1993) recorded posture in a scan sample every 2 minutes for the first 96 minutes and every 6 minutes until 180 minutes after treatment. The postures recorded included; normal standing (S1), normal ventral lying with legs tucked in with the head either down (V1) or up (V2), abnormal standing postures including, just detectable abnormal gait with swaying and abnormal stance with trembling (S2) or kicking and stamping, walking backwards, resting or walking on the knees and falling over (S3), standing still with no movement or statue standing which can include trembling (SS) and abnormal lying postures including lateral lying with legs extended (as described by Mellor and Murray (1989a)) with the head up (L1) or down (L2) or with kicking and rolling (L3) and abnormal ventral lying with the hind legs either partially (V3) or fully (V4) extended. Restlessness was recorded on an all occurrences basis for the first 96 minutes after treatment. In later studies active behaviours, including restlessness, foot-stamping, rolling, lip-curling, tail-wagging, easing-quarters, head-turning and vocalisation, were recorded on a continuous, all occurrences basis (Kent *et al*, 1995; Molony and Kent, 1997; Kent *et al*, 1998). Differences in behaviour were found between c+td lambs and handled controls and in all of these studies, temporal differences in the expression of behaviours were found, as was described by Mellor and Murray (1989a). For example, during the first hour after RR c+td, Molony *et al* (1993) found that lambs of all ages showed restlessness, which gradually declined over the first hour after treatment. In contrast the duration of time spent in abnormal postures persisted for up to 3 hours after treatment. These results were confirmed in further studies (Kent *et al*, 1995; Molony and Kent, 1997; Kent *et al*, 1998) and it was noted that during the first hour after treatment the intensity of expression of abnormal behaviours may be such that almost no normal lamb behaviour is observed (Molony and Kent, 1997).

The welfare implications of these studies are clear. As RR c+td can only legally be carried out in the first week of life, the process of gathering and the application of the rings must be carried out at a time when the bond between the ewe and her lambs may not be strongly established. Lambs may be unable to show normal following and suckling after treatment and the chances of mis-mothering are high. Also, without suckling for a period potentially lasting several hours, young lambs may

grow weak and if these procedures are carried out within the first few hours after birth lambs may not suckle sufficient colostrum to gain the benefit of passive immunity. These problems can and do result in increased loss of life (FAWC, 1994).

In response to severe pain, animals may show vigorous escape or avoidance behaviours. In contrast, severe pain may also be avoided or controlled if the animal adopts certain postures that reduce stimulation of the affected region, for example by staying very still or stretching to avoid contact with the region (Sanford *et al*, 1989; Kent and Molony, 2003). On this basis, Molony *et al* (1993) hypothesised that the expression of a high frequency of active behaviour during the first hour after RR c+td was a response to initial severe inescapable pain resulting from stimulation of somatic and visceral nociceptors by the RR. This view is upheld by the electrophysiological analysis carried out by Cottrell and Molony (1995) who found that conduction block in visceral afferents was not complete within 90 minutes after application of the rings. Molony *et al* (1993) also proposed that some of the postures adopted were indicative of escapable pain, the severity of which could be reduced when lambs adopted the abnormal lying and standing postures observed over the 3-4 hours after treatment. The implication of this proposal is that pain that results in postural changes, but not in a significantly higher expression of active behaviour, is less severe than that which results in the frequent expression of active behaviour. Molony *et al* (1993) found few differences in the behaviour of lambs c+td at 5, 21 and 42 days old and concluded that there was no evidence to suggest that younger lambs experienced less acute pain than older animals, supporting the view of Mellor and Murray (1989a). Instead the evidence suggests that lambs in all three age groups experiences severe acute pain lasting up to 3 hours after application of the RRs. This was not consistent with the assertion by Barrowman *et al* (1953; 1954), that younger lambs show no signs or limited signs of distress as a result of c+td. Molony *et al* (1993) did find that restlessness was more evident in older lambs c+td at 42-days-old. The authors suggested that this difference was a function of the greater quantity of tissue contained within the ring, which reduced the pressure applied to underlying nerves by the ring and therefore increased the time taken to block the conduction of afferent activity, an assertion that was supported by the finding of Cottrell and Molony (1995) and Graham (1997).

1.6.4. *Measurement of HPA acitivity.*

Pain, as a stressor, results in activation of the hypothalamo-pituitary-adrenal (HPA) axis. Activation of the HPA axis results in an increase in the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland, which in turn stimulates the release of cortisol from the adrenal cortex. Measurement of cortisol can be used to quantify the degree of HPA activation and thus, indirectly, as a measure of stress resulting from routine husbandry procedures. Quantification of the concentration of cortisol in plasma or saliva has been used to assess the stress produced by routine procedures in sheep, including restraint, separation (Moberg *et al*, 1980) and mulesing, a routine mutilation that results in severe pain in lambs (Fell and Shutt, 1989). Whilst pain is undoubtedly a stressor resulting in activation of the HPA axis, many other components of the RR c+td procedure are likely to induce stress. For this reason, it is often difficult to determine the extent to which pain itself is responsible for the overall magnitude of the HPA response. The contribution of many extraneous variables can be taken into account by the inclusion of suitable controls in the experimental design. For example the systemic concentration of cortisol in sheep is affected by breed (Mellor and Murray, 1989b) age (Moberg *et al*, 1980; Mellor and Murray, 1989b) and, in lambs over 15 days of age, circadian and diurnal rhythms are also a factor (Parraguez *et al*, 1989). Additional stress from catch-up, restraint and even the effects of expectation as a result of previous experience can be controlled for. Some factors cannot be controlled for however, for example, RR c+td produces a vigorous active behavioural response. As exercise itself is a stressor, this behaviour may augment the overall cortisol response observed. The measurement of cortisol has been used in the study of acute pain associated with RR c+td, and has generally been found to support the use of behaviour as a means of assessing distress resulting from the procedures. However, interpretation of diverging behavioural and plasma cortisol data has proven difficult and in many cases the conflicts have not been resolved.

Mellor and Murray (1989a) compared the plasma concentrations of cortisol in c+td lambs and lambs that were tail-docked alone with that of handled control lambs. A further group of lambs were injected with a high dose of ACTH to provide a positive

control for activation of the HPA axis. A peak in the plasma concentration of cortisol was recorded 30 minutes after RR treatment in both c+td and tail-docked only lambs, but the magnitude of the response was significantly higher in lambs undergoing both c+td. The concentration of cortisol also returned to pre-treatment levels sooner in tail-docking only lambs than in c+td individuals (60 minutes and 90minutes respectively). The initial rate of increase in the concentration of cortisol was the same in c+td lambs as in the lambs treated with ACTH and it was concluded that the cortisol response to RR c+td was maximal. The maximum concentration of cortisol after ACTH administration was however much higher than that reached in castrated and tail docked lambs and occurred later. Mellor and Murray concluded that the overall cortisol response to RR c+td was not limited by the capacity of the adrenal gland to respond to stress. The magnitude of the maximum plasma concentration of cortisol in response to RR castration is variable with values of 40-239 nmol/l recorded (Mellor and Murray, 1989a; Lester *et al*, 1991; Wood *et al*, 1991; Kent *et al*, 1993; Kent *et al*, 1995; Dinniss *et al*, 1997; Graham *et al*, 1997; Kent *et al*, 1998; Sutherland *et al*, 1999; Sutherland *et al*, 2000). These differences are likely to reflect the different ages and breeds of lambs (Moberg *et al*, 1980; Mellor and Murray, 1989b; Kent *et al*, 1993) and conditions of treatment used in different studies. In contrast the peak plasma concentration of cortisol consistently occur at between 30-60 minutes after treatment and return to pre-treatment levels is consistently reached by 90-120 minutes after (Mellor and Murray, 1989a; Lester *et al*, 1991; Wood *et al*, 1991; Kent *et al*, 1993; Kent *et al*, 1995; Dinniss *et al*, 1997; Graham *et al*, 1997; Kent *et al*, 1998; Sutherland *et al*, 1999; Sutherland *et al*, 2000).

Mellor and Murray (1989a) compared changes in the expression of behaviour, over the four hours after treatment, with the changes in the plasma concentration of cortisol. A consistent association between the changing plasma concentration of cortisol and the occurrence of restlessness and immobility was found. The authors therefore concluded that restlessness and immobility provided useful indicators of marked distress. In contrast, lateral lying with neck extension, which was recognised as an abnormal posture in lambs, was not considered to be a useful indicator of distress because lambs continued to show these postures when the plasma concentration of cortisol declined. Thus, it might be concluded that these behaviours

were representative of exhaustion and/or hyperthermia occurring as a result of the vigorous expression of active behaviours soon after application of the rings.

Dantzer and Mormede (1983) emphasised the importance of considering psychological aspects of stressful experiences when interpreting the behavioural and physiological responses induced. In their discussion they maintained that if the behavioural and physiological responses to stress represent attempts to minimise the adverse effects of a stressor, their ability to cope must be judged on the basis of whether or not these attempts were successful. Molony *et al* (1993) suggested that the expression of a high frequency of active behaviour, in response to c+td, is representative of the experience of acute unavoidable pain and is associated with the barrage of afferent activity resulting from mechanical and ischaemic stimulation of nociceptors in the scrotum and testes (Cottrell and Molony, 1995). The association of these behaviours with maximal release of cortisol might be therefore considered representative of the inability or failure of the lambs to cope both physically and psychologically with exposure to the stressor and that the pain dominates the lambs' experience. In contrast, abnormal postures, as described by Mellor and Murray (1989a), may be used by the lamb to reduce the experience of pain (Chapman *et al*, 1985) and therefore represent the experience of avoidable pain (Molony *et al*, 1993), since these postures continue to be expressed during the time when the majority of activity in visceral and somatic afferents has ceased (Cottrell and Molony, 1995; Graham, 1997). The expression of such postural changes could therefore be taken as evidence of successful coping, resulting in a decline in the plasma concentration of cortisol, despite continued expression of abnormal lying postures. Thus, whilst the abnormal lying may not always decline at the same time as cortisol, such changes in posture may still be reliably associated with the experience of a degree of acute pain and may represent a useful indicator of controllable pain.

In contrast to the studies of Mellor and Murray (1989a), the behaviour of lambs c+td by RR at 4, 21 and 42 days of age, recorded by Molony *et al* (1993), and parallel analysis of the plasma concentration of cortisol, reported separately by Kent *et al* (1993), supports the use of changes in posture to assess acute pain. Initially lambs of all ages spent almost all their time in abnormal lying and standing postures, but their expression declined over the 180 minutes of observation, showing a strong

relationship with the decline in plasma cortisol. The peak and subsequent decline in time spent in abnormal lying postures recorded by Lester *et al* (1996), after c+td by RR, occurred at approximately the same time as the peak and decline in the plasma concentration of cortisol. However, Kent *et al* (1993) noted that observations of lateral lying were correlated with the expression of high frequencies of active behaviours and may be a consequence of such behaviour rather than necessarily associated with the experience of pain *per se*.

1.6.5. *Reduction in pain by local anaesthesia and analgesia.*

The behavioural and physiological responses of lambs during the first 4 hours after RR c+td can be eliminated or reduced by the prior administration of multiple injections of the local anaesthetics (LA) lignocaine or bupivacaine, into the tail and scrotum and its contents, to block nervous transmission (Wood *et al*, 1991; Dinniss *et al*, 1997; Graham *et al*, 1997; Kent *et al*, 1997). These results provide evidence that the behavioural and physiological responses to RR c+td in lambs are directly associated with the effects of afferent nervous activity caused by application of the RRs. Cottrell and Molony (1995) provided electrophysiological evidence to support this assertion, finding that vigorous activity in visceral nociceptors caused by the application of the RR was eliminated by intratesticular administration of 0.1ml of 2% lignocaine. Wood *et al* (1991) noted that the effects of lignocaine lasted much longer than expected in RR c+td lambs and it was suggested that its effects were prolonged by the restriction of blood flow from the scrotum, which prevented the distribution of the anaesthetic away from the site of injection. In comparison with other methods of pain relief, LA has been found to be a more effective means of reducing pain from c+td; including epidural administration of local anaesthetic (bupivacaine), analgesic spray (salicylate and cooling from alcohol evaporation) during tail-docking, and intramuscularly administered non-steroidal anti-inflammatory (diclofenac) used during tail-docking (Graham *et al*, 1997) and the opioid analgesics morphine and etorphine, the α_2 adrenoceptor agonists, xylazine and the non-steroidal anti-inflammatory, carprofen administered prior to c+td (Wood, 1991; Price and Nolan, 2001). The administration of LA is a legal requirement prior to c+td in lambs over three months of age. In order to investigate the use of LA more routinely in younger

lambs, the use of a high-pressure needleless injector to administer LA safely and efficiently has recently been tested on farms (Kent *et al*, 2004) and was favoured by farmers.

Evidence of the involvement of endogenous analgesia in reducing acute pain from RR c+td was found by Wood *et al* (1991). The opioid antagonist, naloxone was administered intravenously to RR c+td lambs to block the effects of endogenous opioids. Naloxone pre-treatment resulted in a significant increase in the amount of time spent lying laterally in comparison with c+td lambs that did not receive naloxone. A non-significant increase in the plasma concentration of cortisol was also recorded in these lambs. This hyperalgesia suggests that castration and tail-docking is sufficiently painful to induce some opioid dependent endogenous analgesia appeared to be small. Sheep are thought to gain relatively little benefit from the administration of exogenous opioids to relieve pain from c+td (Wood, 1991) and thus the stimulation of endogenous analgesia sufficient to result in a change in behaviour is, in itself, highly significant. This does not rule out the possible contributions of other endogenous analgesic mechanisms but the extreme behavioural responses described suggest that lambs cannot cope, with the pain produced by rubber ring castration and tail docking, by using endogenous analgesia.

1.6.6. *Tissue damage as a measure of acute pain.*

As mentioned in section 1.5, the assessment of pain is complicated by the fact that only the effects of pain can be measured rather than the pain itself. As an alternative, the degree of damage caused by a procedure, and the predicted severity of pain, can be used as a 'yardstick' against which the effects of pain can be compared and validated as indices for its assessment. This strategy relies on the assumption that the degree of pain experience is directly related to the quantity of tissue damage. In order to confirm the validity and reliability of the use of changes in behaviour to assess acute pain resulting from RR c+td, Molony *et al* (2002) carried out just such a process of validation. Molony *et al* (2002) subjected lambs to 6 RR treatments of varying severity. These treatments were, in descending order of severity; bilateral c+td, bilateral castration, unilateral castration, short scrotum castration, short scrotum castration with local anaesthetic, handled controls. Acute pain was assessed using the

behavioural parameters, described by Kent *et al* (1995). The plasma concentration of cortisol was also measured. These variables were used in a principle components analysis to identify which of them accounted for the majority of the variation in the data. These variables were then used in a discriminant analysis to identify those variables that could be used to assign the lambs to the correct treatments most accurately. Using data recorded during the first 60 minutes after treatment, 79% of lambs were accurately allocated to their correct treatment group. After simplification of the data, by using the sum of active behaviour including vocalisation, the sum of abnormal standing postures and the sum of abnormal lying postures, lambs were allocated to their correct treatments groups as accurately using 60 minutes of data and more accurately using data from longer observations. The addition of the plasma concentration of cortisol did not improve the efficiency with which lambs could be categorised into their correct treatment group and was therefore considered to provide a supplementary measure of RR castration pain under these conditions. This view is supported by Kent *et al* (1993), who found that the area under the plasma cortisol concentration against time curve did not appear to reflect the dramatically higher expression of abnormal behaviour in RR treated lambs. Indeed, in the majority of studies the concentration of cortisol returned to normal levels within 90-120 minutes (Mellor and Murray, 1989a; Lester *et al*, 1991; Wood *et al*, 1991; Kent *et al*, 1993; Kent *et al*, 1995; Dinniss *et al*, 1997; Graham *et al*, 1997; Kent *et al*, 1998; Sutherland *et al*, 1999; Sutherland *et al*, 2000), whilst abnormal behaviour was recorded for up to three hours after application of the rings (Wood, 1991, Molony *et al*, 1993; Kent *et al*, 1995; Molony and Kent, 1997; Kent *et al*, 1998). Kent *et al* (1993) suggested that, as the capacity of the adrenal gland to store and release cortisol is not limitless and responses to severe pain may be subject to a 'ceiling' cortisol response. By administration of a high dose of ACTH into otherwise untreated lambs Mellor and Murray (1989a), showed that the cortisol response to RR c+td does not represent the maximum possible release of cortisol from the adrenal gland. However, the mechanisms of cortisol release in response to c+td have not been elucidated and the factors controlling cortisol release in response to painful stimulation are different to those that result from systemic administration of exogenous ACTH. The endogenous HPA response is stressor specific and the

magnitude of the cortisol response is dependent upon subtle changes in the synthesis and release of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus, the sensitivity of target tissues to the release of these hormones and the magnitude of the ensuing pituitary ACTH response (see Chapter 8). Thus, it remains possible that the cortisol response to RR c+td may have reached a maximum (Kent *et al*, 1993). If the plasma concentration of cortisol is used as the method of assessment, less pain may be recognised than is actually experienced by the lambs after RR c+td.

Further analysis in the study by Molony *et al* (2002) showed that simplification of the data by grouping the 7 original treatments into 3 categories of pain; severe, moderate and mild or no pain and using an index of behaviour that included only abnormal lying and the sum of active behaviour during the first 60 minutes after treatment, the lambs were allocated to the correct pain category in 91% of cases. In practical, field conditions, when accurate and rapid assessment of pain in individual animals must be considered the ideal, categorisation of pain into three such brackets is likely to be sufficient to allow appropriate treatment to be initiated. More sensitive grading of pain could then be used under experimental conditions to determine the efficacy of treatment and to allow adjustments to the treatment. This study provides clear evidence that the degree of change in behaviour associated with RR c+td is directly related to the quantity of tissue damaged by the ring and that a simplified index of quantified behaviour can be used to assess acute pain associated with RR c+td.

Quantified behavioural measures of acute pain from RR c+td have been used to validate the use of visual analogue scales (VAS) for the qualitative assessment of acute pain of RR c+td. Such VAS's may be used by experienced or trained personnel for rapid, on-farm assessments (Kent *et al*, 2001). These 'on-farm' methods have also been used to find practical means by which pain from rubber ring castration and tail docking can be reduced.

1.6.7. *Assessment of acute pain caused by surgical and bloodless castration and tail-docking.*

C+td by methods other than RR results in different types of tissue damage and therefore different types of pain. The use of the same behavioural measures to

quantify and compare acute pain resulting from different methods of c+td has been attempted, with the aim of determining the relative severity of acute pain caused by these procedures. Whilst this work has generated useful information about the comparative effects of different methods of c+td, the limitations of the use of behavioural assessment, developed for the quantification of acute pain from RR c+td, to determine the relative severity of acute pain caused by other methods of c+td have become apparent. There is ample discussion in the literature to demonstrate the difficulties associated with using cortisol to compare the effects of different methods of c+td. A particular case in point is that of the study of surgical and RR c+td by Shutt *et al* (1988) who attempted to use behavioural and physiological changes to determine which method resulted in the experience of least pain. Shutt *et al* (1988) described the active behaviours that occur after RR c+td, which were broadly in accordance with those reported in other studies (Mellor and Murray, 1989a; Molony *et al*, 1993). They also described the responses of surgically treated lambs. Surgically treated lambs flinched and vocalised in response to the cutting of the scrotum and tail and the removal of the testes. When released they adopted a hunched posture with splayed legs and remained subdued in the corner of the pen. Shutt *et al* (1988) also found that the cortisol and β-endorphin response in surgically treated lambs was much greater than that in RR treated lambs. On the basis of this evidence, the authors concluded that surgical c+td was less painful than RR treatment. It was supposed that the significant release of β-endorphin was sufficient to reduce the perception of any pain that was present and it was considered that there was little behavioural evidence of pain. Several studies have since reported that the behavioural response to surgical c+td is very different from that observed in RR treated lambs (Molony *et al*, 1993; Lester *et al*, 1996). Molony *et al* (1993) found that the behaviour of surgically c+td lambs was characterised by abnormal standing postures, particularly in older lambs (21 and 42 days old). In comparison with RR treated lambs, surgically castrated and tailed docked lambs showed little restlessness. Similarly Lester *et al* (1996) reported behaviour predominated by abnormal standing with limited restlessness in surgically c+td lambs. The nature of abnormal standing in surgically treated lambs in these studies was qualitatively different from that seen in RR treated lambs. Rather than showing rolling kicking and restless behaviour like that observed in RR treated

lambs, surgically treated lambs showed abnormal standing, standing very still immediately after treatment, moving no part of their bodies, for several minutes at a time. This behaviour continued for at least 150 minutes after treatment (Molony *et al*, 1993; Lester *et al*, 1996). The only movement recorded during this 'statue standing' was trembling. Minimisation of movement is likely to restrict aggravation of damaged and sensitised tissue, thus reducing pain (Sanford *et al*, 1989; Kent and Molony, 2003), but no conclusion can be drawn about the comparative degree of pain experienced by surgically treated lambs, with respect to other methods of c+td, until the significance of statue standing can be elucidated. This can only be achieved by comparing responses to incremental stages of the surgical c+td procedure. Validation of the use of suppressed food and water consumption and body weight as a means of determining the severity of post-operative pain in rats has been investigated in this way (Liles and Flecknell, 1993). Activation of the HPA axis as a result of surgical c+td peaked at a similar amplitude to that recorded in RR treated lambs, but the peak occurred earlier and the concentration remained higher for longer in 5, 21 and 42 day old lambs (Kent *et al*, 1993). Lester *et al* (1996), also found that the cortisol response peaked earlier and remained elevated for longer in surgically treated lambs, but also recorded a higher peak plasma concentration of cortisol than was found in RR treated lambs. Thus, in both these studies the area under the concentration/time curve for plasma cortisol was significantly higher after surgical treatment. The earlier peak in the concentration of cortisol is likely to be the result of immediate tissue damage caused by the knife (Kent *et al*, 1993). Lester *et al* (1991) found that the plasma concentration of cortisol did not return to pre-treatment values until eight hours after treatment. As discussed in section 1.6.4, the conclusions that can be drawn from measures of activation of the HPA axis are also limited when comparing results between methods of c+td because the HPA axis can be activated by a huge number of uncontrollable variables, particularly when comparisons are made between treatments that result in different types and degrees of tissue damage. Surgical castration requires an incision to be made in the scrotum and the testes to be removed by cutting and traction (FAWC, 1994). Such tissue damage results in the immediate release of inflammatory mediators and they in turn stimulate the release of CRH further activating the HPA axis (Kent *et al*, 1995). The release of

inflammatory mediators results in the stimulation of peripheral nociceptors and in central and peripheral sensitisation. Inflammatory stimulation of the HPA axis may be accompanied by an increase in the severity of the pain perceived. Haemorrhage is also known to result in up-regulation of the HPA axis (Darlington *et al* 1992) and is often a consequence of surgical c+td (FAWC, 1994). Sheep show aversion to personnel encountered during previous aversive experiences (Fell and Shutt, 1989). Psychological aversion to the handler taking blood samples following surgical c+td is a likely consequence of the procedure. As the effects of RR c+td do not generally become apparent until 2-3 minutes after application of the rings (Shutt *et al*, 1988), and lambs show no apparent response to the action of ringing (Barrowman *et al*, 1954; Shutt *et al*, 1988), RR treated lambs are less likely to associate the experience of pain with the handler and thus will habituate to restraint for blood sampling more quickly than surgically treated lambs.

The behaviours exhibited by Burdizzo c+td lambs are also rather different from those expressed by RR treated lambs. After Burdizzo c+td, lambs characteristically show abnormal standing (particularly statue standing) and ventral lying postures with leg extension (Kent *et al*, 1995). Dog sitting, a ventral lying posture with all four legs tucked under the body as is seen in dogs, and trembling have also been described in these lambs (Kent *et al*, 1995). In Burdizzo treated lambs, the plasma concentration of cortisol reached a peak earlier and took longer to decline than that observed in lambs castrated with standard RRs, but was of smaller amplitude (Kent *et al*, 1995). The application of the Burdizzo for c+td crushes tissues in the spermatic cords, bone and muscles of the tail and connective tissues, as well as crushing the nerves and blood vessels. This injury is different again from that resulting from surgery and RR application. Like surgical c+td, tissue injury occurs at the time of treatment resulting in elevation of cortisol to reach a peak at a similar time to that reported in response to surgical treatment (Kent *et al*, 1993; Kent *et al*, 1995). HPA activation is also sustained for longer than that recorded in response to RR treatment (Kent *et al*, 1995), but again this could be mediated by inflammation, haemorrhage and psychological factors rather than being reliably indicative of the sustained experience of pain. In contrast to surgical treatment, the peak in plasma cortisol is less than that obtained after either surgery or RR treatment suggesting that the initial injury is not

as painful as that experienced during RR or surgery. This view is not consistent with that of Thornton and Waterman-Pearson (1997) who found more sustained hypoalgesia after use of the combined methods of c+td than was recorded in response to RR treatment, whilst hypoalgesia was most pronounced after surgical treatment. Thus when the degree of stimulation of endogenous antinociceptive systems was considered to be directly related to the severity of pain experienced, these authors proposed that rubber-ring castration and tail-docking could be considered the least painful method of castration and tail-docking.

1.7. *Chronic effects of rubber ring castration and tail-docking.*

Much of the quantitative research into the severity of pain resulting from RR c+td has been directed towards the determination of the severity of acute pain experienced by lambs during the first few hours after application of the RRs. However, concern regarding the chronic effects of the use of RRs has also been expressed. Within one week after application of the RRs, the ischaemic skin immediately proximal to and under the RR begins to break down and an open lesion forms (Barrowman *et al*, 1953; 1954; Fenton *et al*, 1958; Molony *et al*, 1995; Kent *et al*, 1997; Kent *et al*, 1999; Kent *et al*, 2000; Sutherland *et al*, 2000). These lesions show varying degrees of swelling, inflammation and sepsis and take 6-7 weeks to resolve (Barrowman *et al*, 1953; 1954; Fenton *et al*, 1958; Molony *et al*, 1995; Kent *et al*, 1997; Kent *et al*, 1999; Kent *et al*, 2000; Sutherland *et al*, 2000). In combination with measurement of the width of the castration lesions, an eleven point scale of lesion severity was developed by Molony *et al* (1995), taking into account swelling, erythema and infection. This scale has been used to follow the changes in lesion severity for six to seven weeks after application of the rings, until complete healing occurs. A gradual increase in lesion severity rises to a peak at around 3-4 weeks after treatment and subsequently gradual healing is observed (Molony *et al*, 1995; Kent *et al*, 1997; Kent *et al*, 1999; Kent *et al*, 2000). This scale of severity has also been used to compare lesions in calves and lambs following the use of different methods of c+td, including those intended to reduce acute pain, (Molony *et al*, 1995; Kent *et al*, 2000) and in lambs of different ages (Kent *et al*, 1999). In lambs, the severity of lesions increases with increasing age (Barrowman *et al*, 1954; Kent *et al*, 1999). As the age and

weight of the lambs increases the quantity of tissue contained within the ring increases, so that the force applied by the ring is spread over a greater area. As the integrity of the skin breaks down, the ring effectively cuts through the dying tissue. Thus, the more tissue displaced by the ring the greater the potential size of the lesion where the skin and underlying tissues were previously attached. Molony *et al* (1995) proposed that, because of the wider distribution of forces from the ring in older, larger lambs, an incomplete seal between the healthy tissue proximal to the ring and the necrotic tissue distal to the ring could slow the rate at which the scrotum and its contents dries out providing conditions suitable for the multiplication of bacteria and allow micro-organisms or their products to gain access of to living tissue. The use of the Burdizzo, in combination with the RRs, results in more rapid breakdown of tissues at the ring and healing of the lesion, but does not reduce the overall severity of the procedure (Kent *et al*, 2000). The use of local anaesthetic had no effect on the rate of lesion healing (Kent *et al*, 2000).

The presence of the lesion at the site of the RRs is in itself a good indicator that chronic inflammatory pain is present in these animals. Chemical nociceptors in the vicinity of the lesion are activated by inflammatory mediators released from damaged cells, including bradykinin, serotonin and hydrogen ions (see Millan, 1999 for a review). The sensitivity of these nociceptors is also increased by inflammatory mediators, including bradykinin, the prostaglandins, leukotrienes, histamine, serotonin, interleukins and substance-P which is released from stimulated sensory afferent. Thus, the threshold of nociceptor activation is reduced, the occurrence of spontaneous nociceptor activity is increased and activation in response to non-noxious stimulation occurs (Levine and Taiwo, 1994). These electrophysiological changes mean that in the presence of inflammation, more pain is perceived in response to noxious stimulation (hyperalgesia), pain is perceived following non-noxious stimulation (allodynia) and pain may occur in the region in the absence of stimulation. By analogy pressure sores in humans occur as a result of ischaemia, typically in patients who are bedridden and unable to move themselves frequently to restore circulation. As the necrotic tissue comes away, patients are left with inflammatory lesions of varying severity in the dermal layers. These lesions are reported to be extremely painful (Young, 1990; Hanks, 1991; Jepson, 1992).

The amputation of the scrotum and its contents and of the tail results in the destruction of the distal portion of the nerves of the scrotal and tail regions. In limb amputations, the remaining portion of the transected nerve cells produces axonal processes in order to re-innervate the amputated tissue. When the growth of these processes towards the target peripheral structure is obstructed, the axonal processes intertwine forming balls termed neuromata. These neuromata can spontaneously produce nervous impulses, particularly at the site of injury, and can result in peripheral and central sensitisation and therefore chronic, intractable, neuropathic pain (Devor, 1994). Neuromata have been observed in chickens following beak amputation (Bernard and Gentle, 1985) and in dogs after tail-docking (Gross and Carr, 1990) and have been associated with self mutilation in this species. Neuromata have also been found in lambs, in the end of the remaining tail (French and Morgan, 1992) and may also be present in scar tissue at the site of scrotum amputation. The presence of neuromata suggests that chronic neuropathic pain may occur after RR c+td.

Measurement of DLWG has been used to determine the presence of any long-term effects of rubber ring castration and tail-docking on productivity. Reduced appetite and consequentially, weight loss or reduced weight gain are associated with chronic pain in both humans and animals (Chapman, 1985; Kitchell and Johnson, 1985; Zimmerman, 1986; Short 1998), thus long-term changes in LWG in lambs following RR c+td could be associated with chronic pain from the lesion. Whilst slight differences in DLWG are apparent in cattle after RR castration, evidence of an effect on this production parameter are not convincing, providing little evidence of significant chronic pain. In cattle, Fenton *et al* (1958) found that the LWG in RR castrated calves was lower than that seen in control calves by at least 1kg per week throughout the first five weeks after application of the rings. The differences were greatest during the third and fourth weeks after treatment (2.05 and 2.07kg/week for weeks 3 and 4 respectively), when inflamed lesions were apparent in most or all of the calves. However, the difference was only significant at $P<0.05$ on week four. Similarly, Molony *et al* (1995) found a difference in DLWG of approximately 0.1kg between control calves and those castrated with RRs, but in this case the difference

was not significant. Kent *et al* (2000) found no differences in DLWG between RR c+td lambs and controls.

Studies in both calves and lambs have shown that some of the abnormal behaviours used to measure the severity of acute pain from RR c+td are observed throughout the 6 weeks after treatment, although with much lower frequency. A gradual increase in the frequency of expression of these behaviours has been observed, peaking at 3-4 weeks after treatment and coinciding with the peak in lesion severity (Kent *et al*, 1999; Kent *et al*, 2000). The expression of abnormal behaviours has proven to be a valid means of quantifying acute pain associated with RR c+td, but as the responses of the animal are different depending on the degree of chronicity of the pain, there is no recognised method by which chronic pain can yet be quantified in animals (Sanford *et al*, 1986). A relationship between the severity of chronic inflammatory lesions from c+td and the expression of some abnormal 'acute pain' behaviours has been recognised and it is possible that their quantification may provide a means of assessing putative chronic inflammatory pain from these procedures in lambs. However, the presence of chronic pain (as apposed to discomfort) in these lambs is yet to be determined and the incidence of abnormal behaviours is low and highly variable (Kent *et al*, 1997; 1999; 2000).

1.8. *The need for recognition and assessment of chronic inflammatory pain in lambs after rubber ring castration and tail-docking.*

In 1994 the Farm Animal Welfare Council submitted a report on the welfare of sheep making recommendations to agriculture ministers that proposed changes to the practice of RR c+td (FAWC, 1994). FAWC accepted that there was substantial evidence of acute pain following RR c+td and of little difference in the degree of acute pain suffered in lambs treated between 1 day and 6 weeks of age. It was also noted mis-mothering and loss of lambs were common problems of castrating lambs in the first week of life. FAWC therefore recommended that the use of RRs should be permitted, without an anaesthetic, in lambs up to six weeks of age, although this position was to be reviewed when alternative methods of c+td were identified.

1.9. *Aims of this thesis.*

Thus, FAWC's recommendations emphasised the importance of determining the presence and significance of chronic inflammatory pain in association with c+td lesions and the need for validation of possible methods of quantification of this pain. The recognition of chronic inflammatory pain from RR c+td and the validation of methods of assessing this pain were the main aims of the studies reported in this thesis. For the purposes of this thesis the phrase 'chronic inflammatory pain' refers to pain lasting 6-7 weeks, in association with chronic inflammatory lesions produced by rubber ring castration and tail-docking. This includes ongoing pain resulting from continuous activation of sensitised nociceptors at the site of the lesion and episodes of acute pain from incidental stimulation of sensitised nociceptors. The contribution of central sensitisation to this pain is unclear. Amputation of the scrotum, its contents and the tail could result in the formation of neuromas and chronic neuropathic pain could be experienced both during and beyond resolution of the chronic inflammatory lesion. Thus neuropathic pain is not considered in these studies.

The studies were carried out to test the following hypotheses;

1. Lambs undergoing castration and tail-docking by tight rubber ring experience chronic inflammatory pain for 6-7 weeks, in association with chronically inflamed lesions.
2. The chronic inflammatory pain experienced by lambs is sufficient to induce changes in their behaviour.
3. Quantification of these behavioural changes constitutes a valid measure of the chronic inflammatory pain experienced by rubber ring castrated and tail-docked lambs.

Two strategies were used to test these hypotheses. First, in studies using a self-administration of analgesic paradigm, the ability of lambs to make a motivational, discriminative choice was tested, based on their experience of pain and prior knowledge of the consequences of their actions. It was hypothesised that lambs experiencing chronic inflammatory pain would learn about the benefits of consuming a feed containing an analgesic and subsequently select this feed in preference to a similar alternative that did not contain analgesic. Second, neurophysiological

evidence of changes in the regulation of the HPA axis that have been associated with the presence of chronic inflammatory pain from adjuvant induced arthritis, were sought in the parvocellular region of the paraventricular nucleus of the hypothalamus. In both sets of studies, the results were examined with respect to the expression of the abnormal behaviours validated for the quantification of acute pain from RR c+td and in relation to the severity of the chronic inflammatory lesions.

Chapter 2

General Methodology

Chapter 2. General Methodology

2.1. *Animals*

All animals were made available from the stock of Moredun Research Institute (Clinical Division) and were returned to the flock after experiments, following veterinary inspection, unless otherwise stated.

2.2. *Breeds*

Three breeds of lambs were used for this research: Dorset x Finnish Landrace, Greyface x Suffolk and Scottish Blackface. The Dorset ewes were lambed in December and January allowing experiments to be carried out during January and February. The Greyface and Blackface ewes were lambed from March to May and provided lambs for experiments from April to August. It was necessary to change breeds from Greyface x Suffolk lambs in the year 2000 to Scottish Blackface lambs in 2001 as Moredun Institute changed stock during the course of this project. This change was part of the Moredun Institute's program to eliminate problems with disease on their farms. It is considered that this change affected results.

2.3. *Management*

The lambs were housed, with their dams, in pens bedded with straw. All animals were provided with fresh water and hay *ad libitum*. Ewes were fed 500g of ESCA Ewe Nuts (SAC, Seafield Mill, protein 18%, fibre 8%) daily, which was divided between a morning and evening feed. The lambs were given constant access to lamb creep feed (Nustart Lamb Creep Pellets, Pye-Frankland Balanced Feeds, protein 18%, fibre 8%) and had access to their dam to suck milk. The supply of creep feed was topped up twice daily unless otherwise stated. The animals were brought into the experimental environment one week prior to any treatment. At least 3 days before the experiment began, and at 25-days of age, the lambs were treated with a coccidiostat (Vexocan, Janssen Laboratories Ltd., 1mg diclazuril/kg body weight, 0.25% w/v solution). A second dose was given before the lambs were returned to the flock. Before being returned to the flock all lambs were vaccinated (Heptivac) and wormed

(3% Levacide Drench, Norbrook Laboratories Ltd., Newry, 7.5mg levamisole hydrochloride per kilogram of body weight.)

2.4. *Weighing*

All lambs were weighed at the beginning of each experiment, at least 3 days after being brought into the experimental environment. Whilst the lambs were small enough they were weighed using a spring balance. The balance was first calibrated using a 10kg standard weight. A specially designed sling, which supported the animal by the chest and abdomen, was then used to suspend each lamb under the balance. Where it was necessary to obtain lamb weights throughout an experiment, lambs of 7 weeks of age and above were weighed using a flat bed scale with a crate designed for lambs and a digital display unit. These scales allowed lambs to walk on and off with little handling. The flat bed scales were also calibrated using a 10kg standard weight.

2.5. *Blood sampling*

At least 3 days after being brought into the experimental environment, wool was shaved from a wide area of the ventral neck of each lamb to expose both jugular veins. Blood samples (volume depending on experiment) were taken from alternate jugular veins to minimise vascular damage. 21 gauge 1-inch needles were used and samples were taken into heparinised tubes (Sarstedt monovettes) that mixed the blood and heparin as blood is drawn. The samples were immediately cooled to <4°C in ice and, within 15 minutes, were centrifuged at 3000 rpm for 10 minutes. The plasma was divided between two plastic tubes and stored at -20°C unless otherwise stated. The two tubes of plasma could then be used for two separate analyses (HPLC flunixin and colorimetric analyses for plasma protein or ELISA for prostaglandins). All blood analyses were completed within 9 months of the sample being taken. All tubes were labelled with the experiment number, lamb identification number and sample number. Jugular blood samples could usually be taken within approximately 30 seconds and any effects on the lamb appeared to be minimal.

2.6. *High performance liquid chromatography for extraction and quantification of flunixin meglumine in plasma*

The concentration of flunixin meglumine in the plasma of animals that had been administered the drug orally or intravenously, was measured using isocratic, reverse-phase high performance liquid chromatography (HPLC). The specifications of this technique were recommended and developed in the Department of Veterinary Pharmacology, University of Glasgow.

2.6.1. *Chemicals and solutions*

The following solutions were prepared for use in the extraction procedure:

Citrate/Phosphate Buffer

0.5818g of di-Sodium hydrogen orthophosphate (N_2HPO_4) and 1.67g of citric acid powder were weighed out and transferred to 100ml volumetric flask. Distilled water (glass) was added to make 100ml of solution. The pH was adjusted to 3.0 using 5M hydrochloric acid. This buffer was stored at 4°C and was used within 60 days (two months).

Mobile Phase

1 litre of mobile phase (eluant) was made up using a ratio of 70:30 acetonitrile/ glass distilled water. 5ml of acetic acid was added to each litre of mobile phase. Mobile phase was filtered before use using a 0.2μm nylon filter.

Standard Compound Solutions

A range of concentrations of standard solutions of flunixin meglumine was provided by Ian Gibson at the Department of Veterinary Pharmacology, University of Glasgow. The method used to prepare these solutions was as follows. 100mg of flunixin meglumine was weighed out using an analytical balance (Mettler AE106). This was transferred to a 100ml volumetric flask. Mobile phase was added to bring the total volume of solution to 100ml, producing a solution of concentration

1000μg/ml. Further standard solutions of flunixin meglumine were made by serial dilutions of the 1000μg/ml solution. Solutions were provided at the following concentrations; 0.10μg/ml, 0.25μg/ml, 0.50μg/ml, 1.00μg/ml, 2.50μg/ml, 5.00μg/ml, 10.00μg/ml.

2.6.2. *External standards*

Four standard solutions at a range of concentrations were used as external standards, in the HPLC extraction procedure, in order to determine how efficient the extraction process was. The peak area (mV) for each concentration was compared with that of internal standard of the same concentration to determine the percentage of recovery of flunixin meglumine from plasma samples after extraction. The peak areas (mV) for the range of external standards were also used to provide a measure of linearity of the standards, ensuring that standard preparation had been accurate. The concentrations of standards solutions used as external standards were 0.10μg/ml, 0.25μg/ml, 0.50μg/ml and 1.00μg/ml.

2.6.2. *Preparation of internal standards*

500μl of blank plasma (ovine plasma containing no flunixin meglumine) was aliquoted into six labelled (S1-S6) ground-necked glass tubes. 50μl of mobile phase was aliquoted into tube S1. 50μl of 0.5μg/ml, 1.0μg/ml, 2.5μg/ml, 5.0μg/ml and 10.0μg/ml standard flunixin meglumine solutions were aliquoted into tubes S2, S3, S4, S5 and S6 respectively. The tubes were then vortexed for 10 seconds. This provided plasma samples containing flunixin meglumine (spiked samples) at concentrations of 0μg/ml, 0.05μg/ml, 0.1μg/ml, 0.25μg/ml, 0.50μg/ml and 1.00μg/ml (one in ten dilution of standards).

2.6.3. *Extraction of flunixin meglumine from samples*

500μl of each plasma sample was aliquoted into a 15ml ground-necked glass tube. The rest of the procedure was carried out in a fume cupboard; 200μl of phosphate/citrate buffer was added to each tube and the contents of the tube was vortexed for 10 seconds. 6ml of chloroform was added to each tube to extract

flunixin meglumine. The tubes were stoppered and mixed for 20 minutes on a slow rotary mixer. The samples were then centrifuged at 3500rpm for 20 minutes (Model J-6B, Beckman Coulter Bioresearch). Centrifugation separated the contents of the tube into two phases; an aqueous phase that was separated from a solvent phase by a layer of protein. The aqueous phase was discarded using a glass Pasteur pipette. The protein layer was pushed aside. 5ml of solvent phase was transferred to a clean glass tube. These extracts were evaporated to dryness under a stream of oxygen-free nitrogen using a heated dry block (Techne dri-block) at 50°C. The dried extracts were stored at 4°C overnight.

2.6.4. *Reconstitution of flunixin meglumine extracts*

The dried down extracts were removed from the fridge and left to warm to room temperature for 30 minutes. The extracts were then reconstituted using a minimum of 200µl of mobile phase. The volume of mobile phase used to reconstitute the extract was increased depending on the expected concentration of flunixin meglumine in the sample.

The extracts from internal standards were reconstituted using the following volumes of mobile phase, 200µl, 200µl, 500µl, 200µl, 500µl to extracted standards of concentrations 0µg/ml, 0.05µg/ml, 0.1µg/ml, 0.25µg/ml, 0.50µg/ml and 1.00µg/ml respectively. After adding the required volume of mobile phase, the tubes were vortexed for 30 seconds and then sonicated for 2 minutes.

2.6.5. *HPLC detection of flunixin meglumine*

An HPLC system, provided by the department of Pre-clinical Veterinary Medicine was used to detect and measure flunixin meglumine in the extracts. This system consisted of a solvent degasser (Phenomenex, DEGASSEX, DG 4400) to remove bubbles of gas from mobile phase (eluant) and gradient pump (Pharmacia, 2248) set to pump the eluant through the column at a flow rate of 1.0ml/minute and a maximum pressure of 3000psi, a sampling valve with a 100µl sample loop to load each sample, a guard column (C8, Octyl, MOS, 4.0mm x3.0mm) and a C8 (Prodigy 5, 250mm long, 4.6mm diameter, 5µ packing density, Intersil, Hichrom Ltd) column, a U.V. detector (Pharmacia, LKB VWM 2141), to detect peaks of the components of

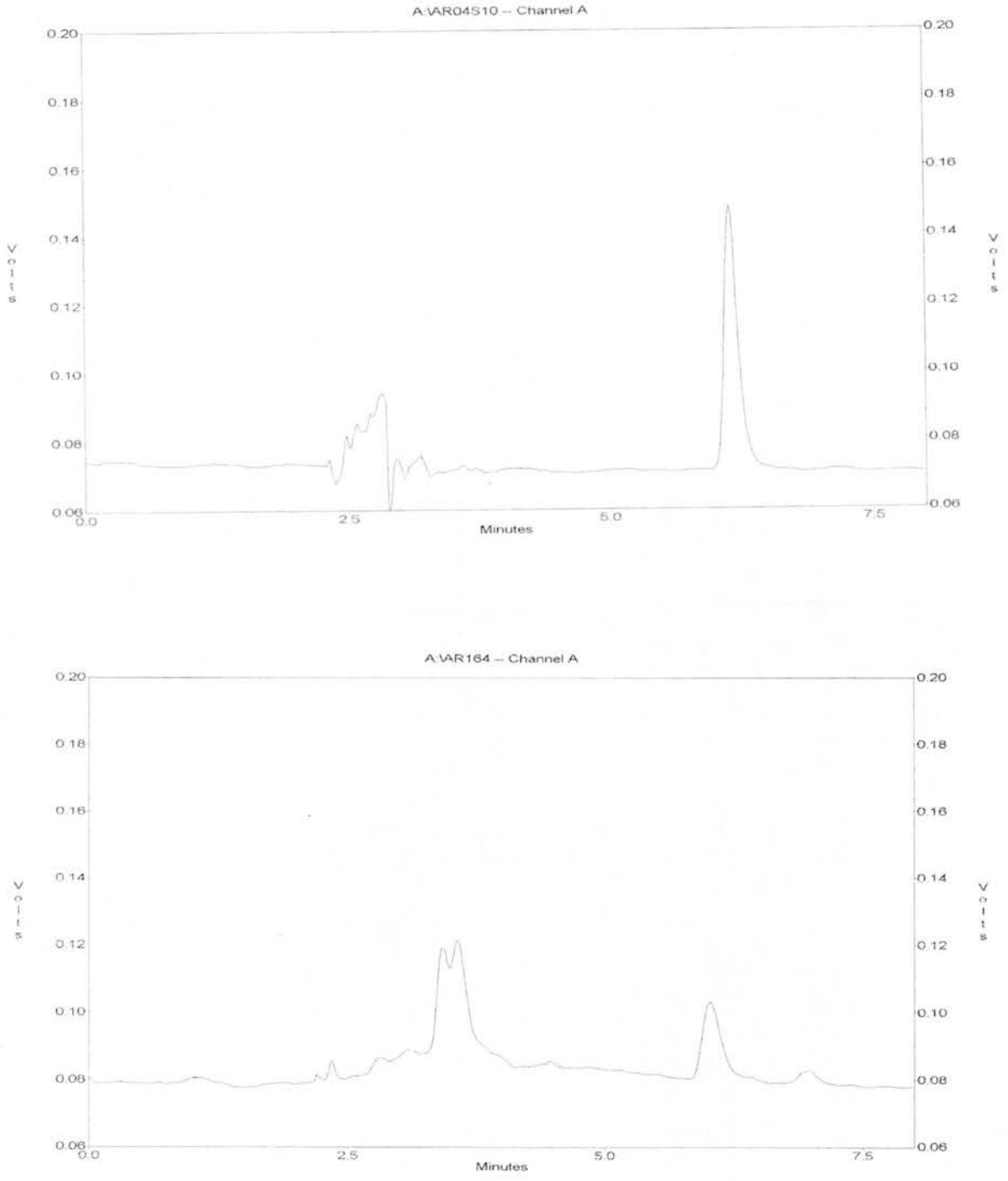
the sample as they come off the column, set for a wavelength of 287nm and a detection range of –0.1 – 1.0V and EZCROM (Scientific Software Inc., CA, USA) HPLC data integration software for PC.

Initial equilibrium of the system was obtained with mobile phase by purging all the lines and then allowing mobile phase to flow through the whole system for 10-15 minutes. Before analysis of samples, the four external standards (concentrations 0.10μg/ml, 0.25μg/ml, 0.50μg/ml and 1.00μg/ml) and the extracted internal standards 0μg/ml, 0.05μg/ml, 0.1μg/ml, 0.25μg/ml, 0.50μg/ml and 1.00μg/ml) were quantified on the HPLC.

100μl of each external or reconstituted internal standard was injected through the sampling valve, thus loading the sample loop. The valve was then rotated pushing the sample into the eluant stream. The peak representing flunixin meglumine came through the column after approximately 7 minutes. EZCHROM was used to calculate the area under the peak (mV). After every four samples an external standard was used to ensure no drift would interfere with the quantification process.

The extracted plasma samples were then analysed using the same technique.

Figure 2.2 Chromatogram output from HPLC analysis of samples containing flunixin meglumine. (a) the chromatogram following HPLC of an external standard at a concentration of 1.0μg/ml of FM (b) an internal (extracted) standard at the same concentration.



2.6.7. *Quantification of plasma concentration of flunixin meglumine*

For calculation of the plasma concentrations of flunixin the linearity (R^2) of external and internal standards must be > 0.97.

The percentage recovery (%) of flunixin meglumine for each internal standard was calculated in order to test the efficiency of the extraction technique. The following equation was used.

$$\% \text{ recovery} = (int_p/ext_p) \times ext_c \times (vol_{rec}/vol_{plas}) \times (100/83.33) \times (1/int_c) \times 100$$

where:

int_p = area of peak of internal standard

ext_p = area of peak of external standard

int_c = concentration of internal standard

ext_c = concentration of external standard

vol_{rec}= volume of mobile phase in which extracted internal standard was reconstituted

vol_{plas}= volume of original plasma sample

83.33= % of solvent taken to measure recovery (5ml/6ml)x100

A mean percentage of recovery of >90% was required for all internal standards for each batch of samples.

The plasma concentration of flunixin meglumine in each sample was then calculated according to the following equation:

$$\text{Concentration} = sam_p/ext_p \times ext_c \times vol_{rec}/vol_{plas} \times 100/83.33 \times 1/\text{eff} \times 100$$

where:

sam_p= area of peak of sample

eff = mean percentage recovery for run

83.33= % of solvent taken to measure recovery (ie (5ml/6ml)x100)

The limit of quantification for this technique was 0.05μg/ml.

2.7. *Treatments*

Lambs were allocated to treatment group on the basis of live-weight so that each group contained lambs of a range of weights and so that the mean live-weight for groups were approximately equal.

Three treatments were used:

Lambs were castrated using tight rubber rings. The rings used had an outside diameter of 15mm and an inside diameter of 5mm (Paragon Rubber Co Ltd.). In order to carry out castration the lambs were inverted. Both testes were then squeezed down into the scrotum. The ring was stretched using an elastrator and the scrotum and testes were pushed through the ring until it could be released around the neck of the scrotum.

Tight rubber rings were also used to dock the tail. The length of the tail was felt so that individual vertebrae could be distinguished. The ring was released so that the ring was placed between two vertebrae as far as possible. Tails were docked so that the remaining tail would just cover the anus. The whole castration and docking procedure normally took no longer than 1 minute. Castration and tail-docking (c+td) were usually carried out together as one treatment unless otherwise stated.

Control, handled lambs were up-turned in the same way as c+td lambs. The testes were squeezed down into the scrotum and the tail was held. No rubber rings were applied before the lamb was released. This procedure took less than 30 seconds.

In the self administration study described in chapter 6 and the first neurohistochemistry study described in chapter 9, lambs were castrated at 4 weeks of age. In the second self administration study (described in chapter 7) and in the second neurohistochemistry study the lambs were castrated at 6 weeks of age.

2.8. *Assessment of chronic inflammatory lesions*

The chronic inflammatory lesions resulting from c+td were examined and assessed twice weekly. Each lamb was caught and turned over for inspection. The presence or absence of the tail and scrotum was noted. The width of the lesion was measured to

the nearest millimetre, using vernier callipers. Subjective assessment of the severity of the lesion was made using the scale described by Kent *et al* (2000) (see Table 2.1).

Table 2.1. Eleven-point scale used for subjective assessment of the severity of lesions as described by Kent *et al* (2000).

Score	Definition
0.0 + 0.5	Intact skin with no swelling or reddening. Complete healing with no residual scab.
1.0 + 1.5	Swelling but skin intact or healing lesion with a scab.
2.0 + 2.5	Severe swelling but skin intact. Also narrow, reddened, ulcerated wound around the perimeter of the ring, little or no exudate, and slight swelling. Healing lesion showing scab with underlying scar tissue and exudate
3.0	Wide reddened ulcer surrounding ring, no exudate present. Large lesion, with exudate and swelling if tail or scrotum gone.
3.5	Reddened lesion with pus and localised swelling.
4.0 + 4.5	Reddened lesion with exudate, pus and extensive swelling.
5.0	Large reddened lesion with much exudate and pus, necrotic tissue and extensive swelling. Advice from N.V.S. required.

2.9. *Behavioural analysis*

Observations of behaviour were made by direct observation of the lambs using 3 experienced observers. Observers were first trained to recognise behaviours, either by direct observation of c+td lambs or by observation of c+td lambs on video tapes. Observers were spaced in strategic positions around the penned area and on raised platforms so that every animal could be observed from at least two angles at all times. The experimental animals were divided into manageable batches for observation. Each batch included an equal number of animals from each treatment group as far as possible. No more than 12 animals were observed at any time unless otherwise stated. Observations were continuous for 2 hours or 3 hours as stated for each experiment. Observations were recorded directly onto laptop computers using 'The Observer' behavioural analysis software (Noldus Information Technology). All

observations were made according to the ethogram described in Table 2.2 below. This ethogram is adapted slightly from that described by Molony *et al* (1995) for assessment of chronic pain in calves. The behaviours 'chew' and 'horn' were added to the original ethogram (Molony *et al*, 1995) as these behaviours occurred frequently in penned lambs in at least one experiment. Slight adaptations to the ethogram for each experiment are described where necessary, for example Greyface x Suffolk and Dorset Finn lambs do not have horns suitable for use in 'horn' behaviour.

Active behaviours (see table 2.2) were recorded continuously throughout the observation time. Every occurrence of behaviour was recorded providing frequency data. Using 'The Observer' software, this method of observation is described as 'focal animal sampling with multiple actors' and each occurrence of a behaviour is described as an 'event'.

Postures of lambs were recorded during scan samples every 6 minutes throughout the observation time. At each scan the lambs' postures and behaviours were recorded. This data provided an accurate estimate of the proportion of time that lambs spent in each posture and the proportion of time that lambs spent eating, idling, sleeping and ruminating. Using The Observer software, postures are described as 'behavioural states'. The posture for each animal is recorded first and each posture is then qualified with a 'modifier' behaviour in which the lamb is occupied. For example a lamb may be described as standing normally (S1, the behavioural state), eating (the modifier).

Two of the observers were the normal handlers of the animals. During the week prior to the start of the experiment these handlers spent as much time as possible in the barn with the animals in an effort to minimise the effect of the observers on the sheep. Observers were seated on the platforms for at least 5 minutes before observations started to allow the animals time to settle.

Table 2.2. Ethogram used during behavioural observations of lambs adapted from that described by Molony *et al* (1995)

Behaviour	Abbreviation	Definition
Active Behaviour		
Restlessness	rst	Combined frequency of getting up and lying down. Each recording accounts for one incidence of getting up and one incidence of lying down. Incidences of lambs rising half way and then lying down again were included.
Easequarters	eq	Movement of front or hind leg that is not sufficiently violent to be considered a kick. Movement of the whole body or hind quarters and tensing of the hind leg, during resting, in standing or lying positions after which the location of the animal does not move.
Head turn	ht	Turning of the head beyond the shoulder to reach the back, flank, navel, scrotum and inside and outside of hind leg. Grooming actions included.
Footstamp/ kick	fsk	Violent raising and lowering of a leg whilst standing, or use of the leg to kick out in standing or lying posture.
REQ score	REQ	Combined score of activity calculated by summing rst, fsk and eq.
Itch quarters	iq	Scratching or rubbing of hind quarters and side on an inanimate object.
Horn	hn	Scratching by tilting of head upwards and backwards and/or to one side so that one or both horn comes into contact with body.
Chew	cw	Mouthing or chewing bars of pen.
Teat seek	ts	Attempts to suckle whether successful or not.
Tail wag	tw	Rapid sideways movements of the tail. Wagging immediately before teat-seeking not included. Each separate bout of wagging recorded as one occurrence.
Play	pl	Butting others and objects, gambolling, pawing, jumping onto and down from obstacles. Each bout of play behaviour recorded as one incidence.
Posture		
Normal lying	V1, V2	Ventral lying with legs tucked under the body with head down (V1) or or up (V2).
Abnormal lying	V3,	Ventral lying with legs partially or fully extended and sitting on hind-quarters (dog sitting).
Lateral lying	LL	Lying on side with one shoulder flat on the ground. Legs extended with head up or down.
Normal standing	S1	Standing still, walking or playing with no apparent abnormalities.
Abnormal standing	SS, S2	Standing abnormally still for more than 10 seconds, especially with hind legs slightly apart and with tail tucked in and back arched (SS). Standing or walking unsteadily with notable abnormalities.
Eat	e	Eating hay, concentrate, straw-bedding and drinking. Can be performed lying or standing
Idle	i	Describes behaviour when the lamb is not performing other behaviours in ethogram. Can be lying or standing.
Ruminate	r	Regurgitation of food for further mastication, characteristic chew motion of jaw, preceded by regurgitation of bolus and followed by swallowing of bolus. A bout of rumination contains pauses in between boluses of 5 to 10 seconds when the lamb prepares to regurgitate the next bolus.
Sleep	s	Lying with eyes closed. Can be shown in any of the lying postures.

Chapter 3

Pharmacokinetics of Flunixin Meglumine

Chapter 3. Pharmacokinetics of Flunixin Meglumine in Lambs: oral and intravenous administration.

3.1.1. *Introduction*

There are no analgesics that are licensed for use in sheep. This complicates the selection of a suitable analgesic for use in this species, both by veterinarians in practice and for research purposes and could therefore compromise the welfare of the animals. In studies of self-administration of analgesics, the use of narcotic analgesics may be problematic because of the potential for the development of physical dependence and tolerance associated with the administration of these drugs over a long period of time. For this reason a non-narcotic alternative was sought.

3.1.2. *Non-steroidal anti-inflammatory drugs*

Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of non-narcotic, anti-inflammatory drugs that have proven to have potent, anti-pyretic and analgesic properties. The principle mechanism by which these drugs act, first proposed by Vane (1971), is by inhibition of the biochemical pathway leading to the production of prostaglandins. This mechanism is now classed as one of the defining characteristics of the NSAIDs (Higgins and Lees, 1984).

Following tissue damage, phospholipids are released from cell membranes. These phospholipids are broken down by phospholipase A_2, releasing arachidonic acid. Arachidonic acid is converted by the enzyme cyclo-oxygenase (COX) into unstable intermediate compounds and then converted to prostaglandins and thromboxanes. It is also broken down by lipoxygenase producing leukotrienes (Cunningham and Lees, 1994). The prostaglandins are constituents of the inflammatory soup of mediators that can sensitise peripheral nociceptors, thus lowering their threshold of activation (Lascelles, 1996). They are also involved in vasodilation and increase of blood vessel permeability (Cunningham and Lees, 1994).

Two isoforms of COX exist (Smith *et al*, 1994). COX-1 is a constitutive enzyme expressed in most tissues. The eicosanoids produced by this isoform are critical for the maintenance of normal homeostatic functions, including maintenance of the

gastrointestinal (GI) tract mucosa, platelets and blood flow. The second isoform, COX-2, is synthesised by macrophages and inflammatory cells, only after stimulation by cytokines and is central to the production of inflammatory prostaglandins (Papich, 1997).

3.1.3. *Cyclo-oxygenase inhibition*

Most NSAIDs inhibit both COX-1 and COX-2 and have been found to cause vomiting, ulceration, bleeding and perforation of the GI tract in dogs (Vonderhaar and Salisbury, 1993). These toxic effects are mainly caused by the inhibition of COX-1 (Papich, 1997), but may also be caused by direct irritation of the gut mucosa by the tablet itself (Haslock, 1998). These effects are more severe in young animals as their hepatic and renal clearance mechanisms are not mature and the drug takes longer to be cleared from the system (Papich, 1997).

Some NSAIDs are more specific to the inhibition of one isoform of COX than to the other, illustrated by the COX-2: COX-1 ratio, (i.e. the ratio of concentrations required to inhibit COX-2 and COX-1). For example aspirin has a very high COX-2: COX-1 ratio (i.e. it requires a higher concentration of drug to inhibit COX-2 than COX-1), whilst those of ibuprofen and carprofen are amongst the lowest (Papich, 1997). Thus, in order to reduce the adverse effects of NSAIDs, the search for new NSAIDs has centred on the development of COX-2 specific drugs.

3.1.4. *Central action of NSAIDs?*

According to the theory that NSAIDs act by inhibition of COX and therefore prostaglandin production, the action of NSAIDs should be dependant on the presence of inflammation. Without inflammation, the drugs should, theoretically, have no effect on the threshold of response to noxious stimulation. However, there is evidence that NSAIDs (a particular example is carprofen) can induce analgesia in the absence of inflammation. NSAIDs have been reported to act on the central nervous system, preventing central sensitisation after peripheral injury by inhibiting spinal glutamate and substance P receptors (Malmberg and Yaksh, 1992). A thalamic site of action has also been proposed (Jurna and Burne, 1990).

3.1.5. *Flunixin meglumine; a NSAID*

The NSAID flunixin is the tri-fluoromethyl derivative of clonixin, and is administered as the N-methyl-d-glucamine salt, flunixin meglumine (FM) (Ciofalo *et al*, 1977). FM is classified as a carboxylic acid (Cheng *et al*, 1998b) and is a weak acid with a pH of 5.82 (Johansson and Anler, 1988). It is licensed for use in dogs, horses and cattle (Welsh *et al*, 1993) and has been shown to have potent analgesic effects in humans (Zederfeldt *et al*, 1977) mice, rats and monkey (Ciofalo *et al*, 1977), in the donkey (Cheng *et al*, 1996) and goat (Anderson *et al*, 1991). The pharmacokinetics and efficacy of the drug in sheep have recently been investigated, but these investigations are incomplete.

3.1.6. *Flunixin meglumine in the sheep*

In a study of the pharmacokinetics of FM in sheep, Welsh *et al* (1993) found that there was no significant difference between the kinetic characteristics of the drug after intravenous administration at 1.0 and 2.0mg/kg body weight, indicating that the concentration of FM in plasma is directly proportional to the dose in this species. The drug was rapidly distributed in plasma (2.3 and 2.7 minutes respectively), a rate similar to that found in cattle (9.6 minutes; Anderson *et al*, 1990). The elimination half-lives of FM at 1.0 and 2.0mg/kg i.v. (229.8 and 205.9 minutes) were also similar to those found in cattle (188.4 minutes; Anderson *et al*, 1990) and in dogs (220.2 minutes; Hardie *et al*, 1985). However, the mean body clearance rate was slower than that found in cattle (Hardee *et al*, 1985; Anderson *et al*, 1990), horses, donkeys (Coakley *et al*, 1999) and dogs (Hardie *et al*, 1985).

3.1.7. *Intra-muscular administration of flunixin meglumine in sheep*

After intra-muscular (i.m.) administration to sheep at 1.1 and 2.2mg/kg body weight, Welsh *et al* (1993) found that the bioavailability of FM was at least 70%, which is again, similar to the bioavailability found in cattle after i.m. administration (Anderson *et al*, 1990). FM was found to be absorbed rapidly from the site of i.m. injection, reaching a maximum concentration of 5.9±0.47μg/ml within 45 minutes after a 1.1mg/kg i.m. dose was administered. Welsh *et al* (1993) concluded that the

i.m. route of administration may therefore be suitable in the treatment of acute pain in the sheep.

3.1.8. *Efficacy of flunixin meglumine in sheep*

Further studies have shown that FM is a potent analgesic in sheep. When FM was administered intravenously it significantly attenuated the development of mechanical hyperalgesia after the application of a tourniquet to the forelimb of sheep (Welsh and Nolan, 1994). It was also found to attenuate hyperalgesia resulting from abdominal surgery (Welsh and Nolan, 1995) and hyperalgesia resulting from repeated subcutaneous injections of carrageenan (Welsh and Nolan, 1994).

Studies of COX inhibition have shown that, in sheep, FM inhibits COX-2 more strongly than COX-1 (Cheng *et al*, 1998b). Thus FM has some COX-2 selectivity in this species.

The anti-inflammatory effects of NSAIDs are often found to last longer than would be expected from the plasma concentration of the drug (Cheng *et al*, 1998a). Studies in other species have shown that FM preferentially penetrates inflamed tissue, from which it is eliminated more slowly than from normal tissue (Cheng *et al*, 1998a). However, in a tissue cage study in sheep, FM was found in high concentration in both inflamed and non-inflamed tissue and was eliminated more slowly from these tissues than from plasma (Cheng *et al*, 1998a). As it was expected that the concentration of FM would be higher in inflamed tissue, Cheng *et al* (1998a) proposed that in sheep, less of the drug may be bound to protein than in other species. This would enable penetration of non-inflamed tissue, in which the blood supply and permeability of blood vessels was normal.

3.1.9. *Flunixin meglumine has central actions in sheep*

Chambers *et al* (1995) examined the mechanisms by which FM induces analgesia. They tested the effects of i.v. administration on the threshold of noxious mechanical stimulation in both healthy and lame sheep (foot rot). Serum thromboxane levels were measured to assess COX inhibition. FM treatment produced a small rise in noxious threshold of both healthy and lame sheep, indicating analgesia. Two neurotransmitter systems, considered to be of major importance in the processing of

nociceptive information in the sheep were examined: the opioidergic system and the α_2-adrenergic system. When these systems were blocked by pre-treatment with antagonists (naloxone and atipamezole respectively), the rise in pain threshold to noxious mechanical stimulation caused by FM was prevented. This evidence suggests that FM produces analgesia in the absence of inflammation and that central mechanisms, mediated via opioidergic and α_2-adrenergic pathways, are involved (Chambers *et al*, 1995).

3.1.10. *Oral administration of flunixin meglumine*

In studies in cattle, by Odensvik (1995) and Odensvik and Magnusson (1996), oral administration of FM, by consumption of feed coated with drug granules, was found to be as effective in inhibition of prostaglandins as i.v. administration. Cmax was reached by 3.5 hours. Inhibition of prostaglandins was found within one hour and continued until 30 hours when the last sample was taken. It was proposed in these studies that oral administration could provide an alternative to i.v. administration of FM in cattle. FM is also administered orally in horses and dogs. In a pilot trial by this research group, FM was incorporated into feed and administered to lambs at a dose of 1.1mg/kg, the drug reached a Cmax of 1.38 μg/ml within 30 minutes of administration. However, no information on the pharmacokinetics of FM after oral administration in sheep has been found in the literature.

3.1.11. *Aims of the present study*

While the pharmacokinetic and pharmacological knowledge on the use of FM in sheep is growing, certain information was required for the successful use of FM in studies of self-administration. I.v. doses of both 1.0 and 2.0mg/kg have been recommended for sheep in the literature (Welsh *et al*, 1993; White and Taylor, 2000) and no study has been found that recommends a dose rate for oral administration in this species.

Pharmacokinetic data from oral administration was required to allow direct comparisons with data from i.v. and i.m. administration studies and with oral data from cattle and other species. This information was also required for better design of the protocol for self-administration studies. For example, the rate at which the

plasma concentration of the drug rises in lambs dictates the extent of the delay between consumption of drugged feed and the onset of analgesic effects.

Papich (1997) recommended that in general, animals over six weeks of age can be treated as adults with respect to drug administration. However, as the animals used in studies of self-administration span this age group (i.e. 4-12 weeks of age), it was considered important to determine the pharmacokinetics of the drug in lambs of this age, for comparison with data from older animals.

The efficacy of FM after oral administration in sheep is also unknown. As discussed in section 3.1.8, the inhibition of prostaglandins can provide an estimate of efficacy. The measurement of 15-keto-13,14-dihydro-prostaglandin $F_2\alpha$ has been recommended in sheep as this 1st stage metabolite of $PGF_2\alpha$ has a longer half-life in plasma than $PGF_2\alpha$. It is therefore more accurately measurable and less susceptible to breakdown during sample collection and analyses than the parent compound (Odensvik, 1995; Zarco *et al*, 1988a; 1988b).

No account of the effects of FM on the general behaviour of sheep or any other species, have been found. Any subtle changes in behaviour resulting directly from the administration of FM could be misinterpreted during self-administration studies, when behaviour was to be examined for changes in response to chronic inflammatory lesions. Thus, examination of the behaviour of healthy lambs, which were administered with FM, was considered necessary so that this information could be taken into account in future studies of lamb behaviour involving FM administration.

The aims of this study were therefore to determine the pharmacokinetics of FM in 6-week-old lambs after oral and i.v. administration at 1.0 and 2.0mg/kg body weight, to estimate the efficacy of FM by measurement of 15-keto 13,14-dihydro-prostaglandin $F_2\alpha$ inhibition and to identify changes in behaviour associated with FM administration.

3.2 *Methodology*

3.2.1 *Animals and treatments*

Twenty eight, six-week-old, healthy, twin, male lambs (Finnish Landrace x Dorset) were housed in groups with two sets of twin lambs (4 lambs) and two ewes in each 4x4m, straw-bedded pen. The animals were subject to the normal management procedures outlined in chapter 2 part 2.3. Briefly, animals had continuous access to fresh water and hay. The ewes were fed 500g of ESCA ewe nuts daily. The lambs were given constant access to lamb creep feed (Pye-Frankland Balanced Feeds, Lamb Creep Pellets), in a creep area enclosed within the pen, and had constant access to their dams to suck milk. Four days prior to treatment the animals were brought into the experimental environment and the lambs were weighed. The lambs were divided between the following groups with weight balanced as far as possible across the groups.

IV1 = Intravenous administration of flunixin meglumine (FM) at a dose of 1mg/kg body weight.
IV2 = Intravenous administration of FM at a dose of 2mg/kg body weight.
O1 = Oral administration of FM at a dose of 1 mg/kg body weight.
O2 = Oral administration of FM at a dose of 2 mg/kg body weight.

Each pair of twins was assigned to receive either oral or intravenous administration and one lamb from each pair received flunixin at either 1 or 2 mg/kg body weight.

3.2.2. *Administration routes*

Finadyne ® Solution (Shering-Plough Animal Health, flunixin meglumine) was administered intravenously at doses of either 1 or 2mg/kg body weight into one jugular vein.

For oral administration the dose of Finadyne ® Granules (Shering-Plough Animal Health, flunixin meglumine) was mixed with 10ml distilled water. The lamb was caught and a stomach tube was introduced into the reticulo-rumen by either an experienced shepherd or the named veterinary surgeon. The suspension of Finadyne

® was introduced using a syringe and was flushed through with 50ml of distilled water.

3.2.3. *Blood sampling*

Blood samples were taken at different time intervals depending on the route of administration of FM. Lambs that received an intravenous dose of FM were sampled at 2, 5, 10, 20 and 30 minutes and 1, 2, 4, 8, 12, and 24 hours after administration. Lambs that received an oral dose of FM were sampled at 30 and 45 minutes and 1, 2, 4, 6, 8, 12, and 24 hours after administration. Blood samples were taken as described in Section 2.5. Briefly, 7ml samples were taken alternately from the left and right jugular veins starting at the side contralateral to the site of injection of the drug to avoid contamination. The samples were taken into heparinised tubes, immediately cooled in ice and, within 15 minutes, were centrifuged at 3000 rpm for 10 minutes. Plasma was stored at -20°C.

3.2.4. *Behavioural observation*

Behavioural observations were recorded directly onto 'The Observer' behavioural analysis software while watching the lambs. Two pens (8 lambs) were observed, simultaneously, by two experienced observers. Two observation periods of 30 minutes were made 3 and 5 hours after administration of the drug, using the ethogram described in Section 2.8. The behaviour 'chew' was not recorded on this occasion.

3.2.5. *High performance liquid chromatography for extraction and quantification of flunixin meglumine in plasma.*

The concentration of FM in plasma, of animals which were administered the drug orally or intravenously, was measured using isocratic, reverse-phase high performance liquid chromatography (HPLC) as described in section 2.6.

3.2.6. *Enzyme-linked immunosorbent assay for 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$.*

In order to provide some estimate of the efficacy of Finadyne ® at the doses provided during this trial, enzyme-immuno assay for 15-keto-13,14-dihydro-prostaglandin $F_{2\alpha}$ was attempted. As there is no commercially available kit for quantification of this prostaglandin, the method had to be developed from its basic components. Within the time available the method was not sufficiently successful to produce reliable results, however a reliable standard curve was produced and slight modifications to the method could prove sufficient to produce reliable results. The protocol as developed so far can be found in appendix A.

3.2.7. *Pharmacokinetic analyses*

The plasma concentration of FM for each lamb was plotted against time and was subjected to non-compartmental (regression) analysis using the computer program WINNONLIN (version 4.0 Lexington, KY, USA). Two models were used for the analysis. For orally administered doses of FM, the extra-vascular administration model from the WINNONLIN library of non-compartmental analyses was used. The data for intravenously administered doses were analysed using the bolus IV administration model. The area under the plasma concentration versus time curve (AUC or zero moment curve) and area under the product of the concentration x time versus time (AUMC or first moment curve) for both i.v. and oral data were calculated using the linear trapezoidal rule, until Tmax was reached. The log trapezoidal rule was used to calculate AUC and AUMC after extrapolation of the data from the last sample to infinity. The last four points on the concentration time curve were used to fit the regression and therefore estimate the slope, lambda z (λz), of the terminal elimination phase. The maximum concentration (Cmax), the time at which the maximum concentration (Tmax) occurred and the elimination half-life ($t_{½}$) were also calculated in WINNONLIN. From these values, further pharmacokinetic parameters were calculated according to the following equations:

Mean Residence Time (MRT) – the arithmetic average time that each drug molecule remains in the system.

$$MRT = AUMC/AUC$$

Apparent Volume of Distribution (V_d) – Volume of fluid required to contain the total amount of drug in the body at the concentration observed after i.v. administration.

$$V_d = (Dose_{iv} \times MRT) / AUC_{iv}$$

Mean Absorbance Time for Oral Administration (MAT) – the arithmetic average time that each drug molecule takes to be absorbed into the plasma compartment.

$$MAT = MRT_o - MRT_{iv}$$

Bioavailability (F) – The proportion of the dose that reaches the blood (therefore biologically active) after oral administration, taking into account absorption and metabolic degradation.

$$F = (AUC_o / AUC_{iv}) \times 100\%$$

Total Body Clearance (Cl) – Rate of elimination of drug by metabolism or excretion.

$$Cl = Dose / AUC$$

3.2.8. *Statistical analyses*

Statistical analyses were carried out using Minitab 9 and Genstat 5.2 statistical analysis packages. All data were tested for normality using the Anderson Darling test for normality in Minitab. Parametric and non-parametric tests were subsequently used where appropriate. None of the data were successfully normalised by transformation.

General linear models (GLM), a version of analysis of variance (ANOVA), were used to evaluate differences in the concentration of FM and pharmacokinetic parameters after administration at doses of 1 or 2 mg/kg by oral and i.v. routes. Variation between groups as a result of the route of administration, the dose and interactions between the route and dose was sought. Where necessary, post hoc Student's T-tests were performed to determine where the differences lay. Students T-tests or Mann-Whitney U tests were used to examine differences between parameters that applied only to two of the groups.

Generalised linear models (regression analysis) were used to compare behaviour between groups. Where differences occurred, post hoc T-tests were used to determine where the differences lay. Paired T-tests or Wilcoxon Sign rank tests, depending on the distribution of the data, were used to determine differences between behaviour of lambs before and after the administration of FM, irrespective of the route of administration and dose.

3.3. *Results*

3.3.1. *Plasma concentration of flunixin meglumine*

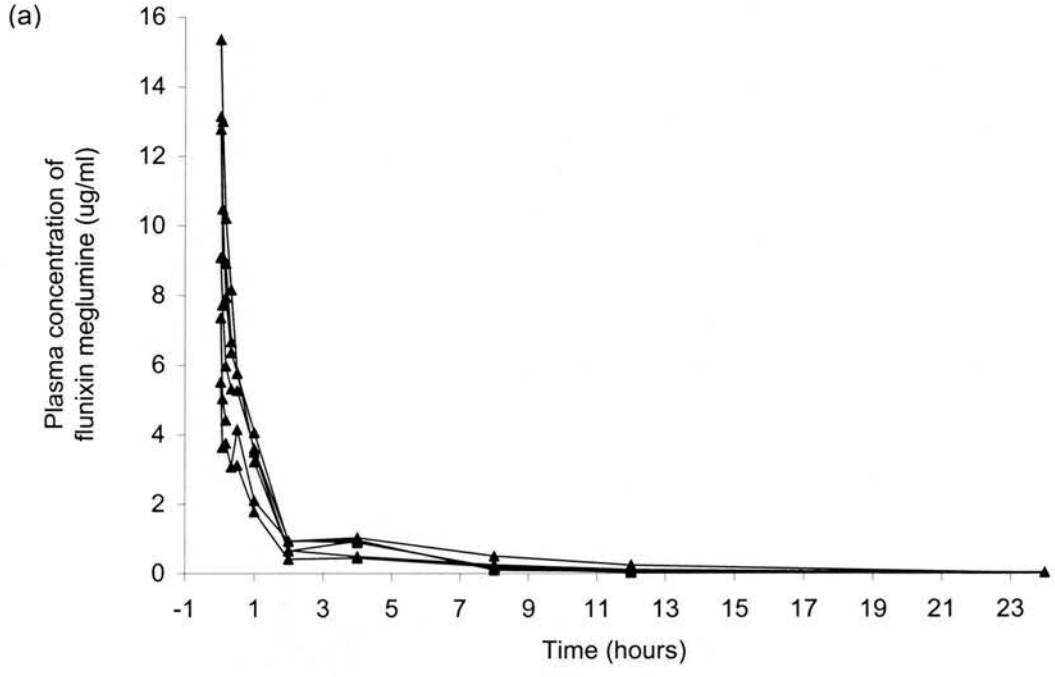
The changes in the plasma concentration of FM, after i.v. and oral administration at 1mg/kg and 2mg/kg, for all animals are presented in figure 3.1a-d. The mean plasma concentration for each treatment group at each time point is shown is also shown in table 3.1. with the statistical significance of the differences between groups shown in table 3.2.

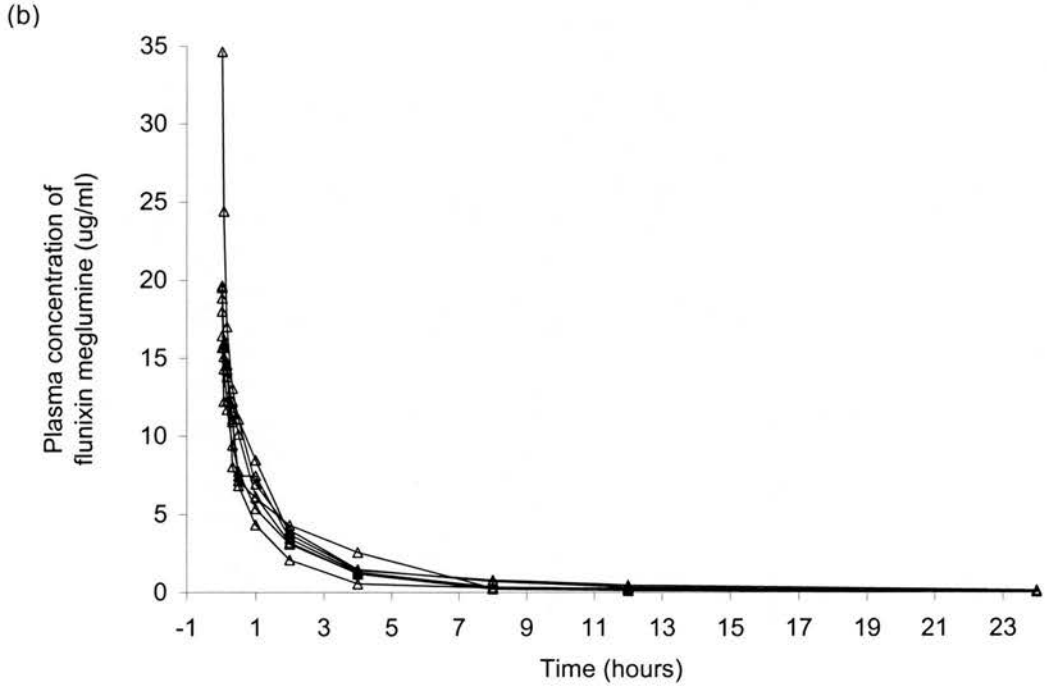
During the first two hours after administration, the plasma concentrations of FM were different depending on both the route of administration and on the dose. These differences were statistically significant at $P<0.01$. The difference in plasma concentration of FM was most noticeable after 30 minutes when the concentration was shown by GLM to be highly statistically different between groups both in respect of the dose and the group ($P>0.0001$ see table 3.2). As expected, the maximum concentration of the drug in plasma (see table 3.1) was highest in IV2 animals (20.39μg/ml ±7.71) and lowest in O1 lambs (0.91μg/ml ±0.34).

After oral administration the plasma concentration of FM had reached a maximum by the time the first sample was taken for 1 individual receiving 1.0mg/kg and 3 individuals receiving 2.0mg/kg. It is likely that the true maximum plasma concentration of FM in these animals occurred earlier than the first time point. The maximum plasma concentration of FM occurred later for one individual receiving 2.0mg/kg FM (at 2 hours after administration).

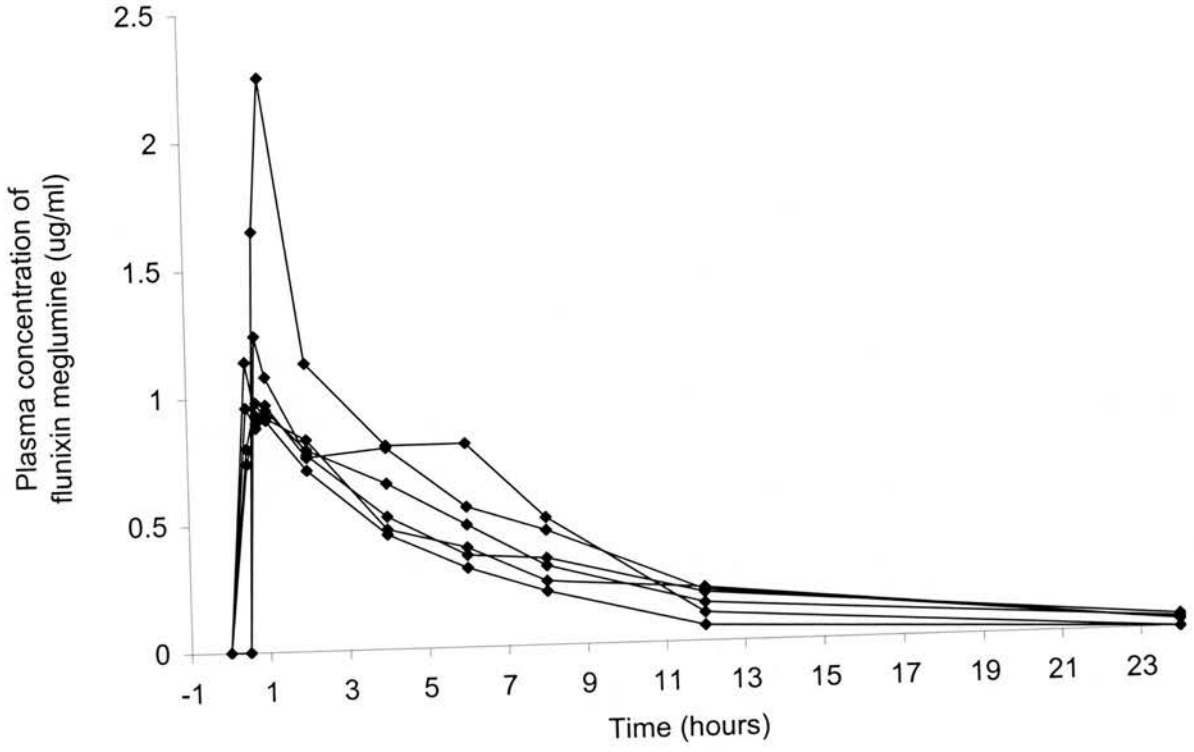
After 4 hours no difference in the plasma concentration of FM was found between lambs receiving an oral or i.v. dose (GLM, $F_{3,25}=0.79$, $P=0.384$). However, there was still a significant difference after 4 hours, between the plasma concentrations of FM in lambs receiving FM orally at the 1.0 and 2.0 mg/kg (Students T-test, $T_{1,13}=-6.89$, $P=0.0002$). This difference in plasma concentration of FM between lambs dosed orally at the two doses remained significant 12 hours after administration (0.15±0.06μg/ml and 0.32±0.12μg/ml for O1 and O2 lambs respectively), but there was no difference between them 24 hours after drug administration.

Figure 3.1. Change in concentration of FM in plasma of 6-week old lambs during the first 24 hours after administration of the drug. (a) i.v. administration at 1.0mg/kg. (b) i.v. administration at 2.0mg/kg. (c) oral administration at 1.0mg/kg. (d) oral administration at 2.0mg/kg.





(c)


Plasma concentration of flunixin meglumine (ug/ml)
2.5
2
1.5
1
0.5
0
-1
1
3
5
7
9
11
13
15
17
19
21
23
Time (hours)

(d)

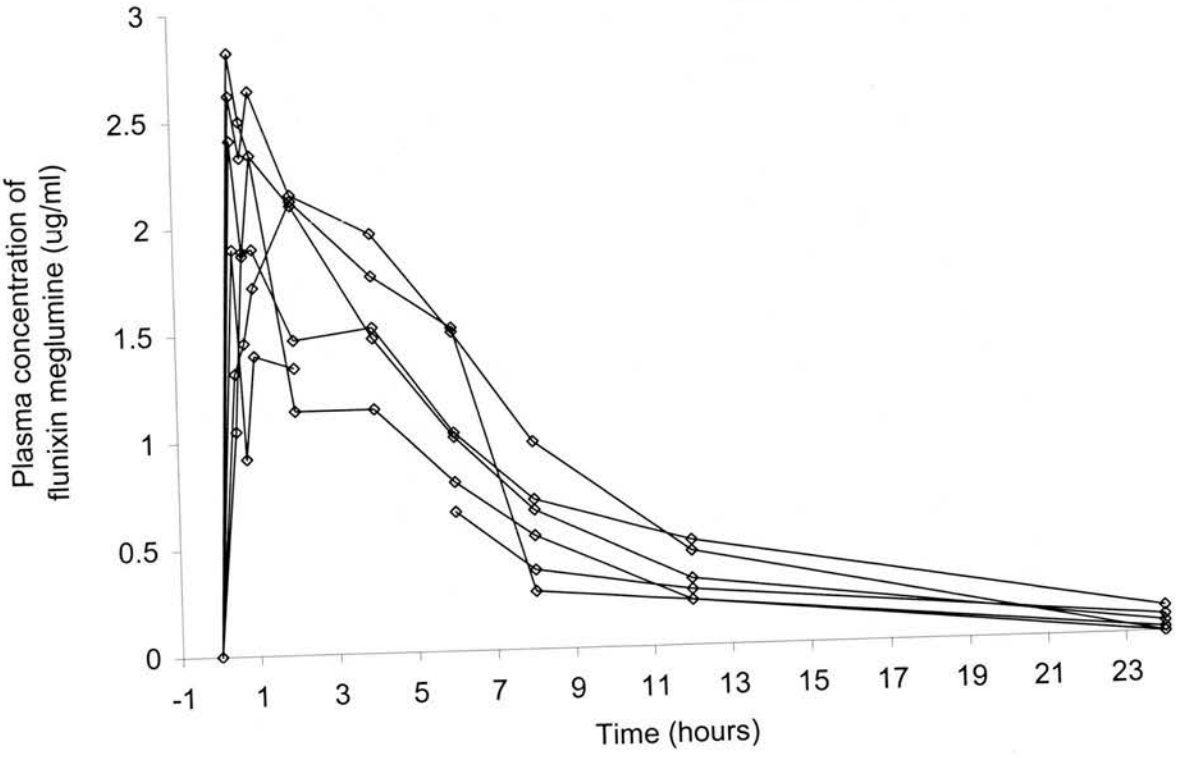

Plasma concentration of flunixin meglumine (ug/ml)
3
2.5
2
1.5
1
0.5
0
-1
1
3
5
7
9
11
13
15
17
19
21
23
Time (hours)

Table 3.1 The mean (±SEM) concentration of FM in plasma during the first 24 hours after administration at 1 and 2 mg/kg either orally or intravenously to 6-week old lambs. with the statistical significance of the differences between groups shown in table 3.2.

	Mean Plasma Concentration of Flunixin meglumine (ug/ml)								
Time (h)	I.V. (1mg/kg)	(+/-SEM)	I.V. (2mg/kg)	(+/-SEM)	Oral (1mg/kg)	(+/-SEM)	Oral (2mg/kg)	(+/-SEM)	Significance
0	0	0	0	0	0	0	0	0	
0.03	11.1	4.19	20.39	7.71	-	-	-	-	**
0.08	8.593	3.25	16.22	6.13	-	-	-	-	**
0.17	7.374	2.79	13.98	5.28	-	-	-	-	***
0.33	6.03	2.28	10.9	4.13	-	-	-	-	***
0.5	5.13	1.94	8.77	3.31	0.91	0.34	2.26	0.89	***
0.75	-		-		1.10	0.42	2.20	0.83	*
1	3.82	1.44	6.38	2.41	1.18	0.45	2.43	0.92	***
2	0.83	0.31	3.39	1.28	0.83	0.31	1.88	0.71	***
4	1.09	0.41	1.37	0.52	0.61	0.23	1.64	0.62	*
6	-		-		0.48	0.18	1.09	0.41	**
8	0.28	0.10	0.41	0.15	0.34	0.13	0.60	0.23	*
12	0.11	0.04	0.23	0.09	0.15	0.06	0.32	0.12	*
24	0.01	0.00	0.04	0.02	0.03	0.01	0.05	0.02	

Table 3.2. Results of GLM and *post-hoc* analyses of the differences in concentration of FM in plasma of 6-week old lambs after administration orally or intravenously at 1 or 2 mg/kg.

Time (h)	GLM		I.V. (1mg/kg) vs I.V. (2mg/kg)	Oral (1mg/kg) vs Oral (2mg/kg)	I.V. (1mg/kg) vs Oral (1mg/kg)	I.V. (2mg/kg) vs Oral (2mg/kg)
	Route	Dose				
0	-	-	-	-	-	-
0.03	-	-	T=-3.29, P=0.0095	-	-	-
0.08	-	-	T=-3.96, P=0.0023	-	-	-
0.17	-	-	T=-5.46, P=0.0003	-	-	-
0.33	-	-	T=-4.69, P=0.0009	-	-	-
0.5	F=97.13, P=0.000	F=22.61, P=0.000	T=4.09, P=0.022	T=-3.49, P=0.013	T=8.05, P=0.0005	T=7.65, P>0.0001
0.75	-	-	-	T=-2.65, P=0.038	-	-
1	F=34.17, P=0.000	F=11.37, P=0.003	T=-2.59, P=0.029	T=-2.72, P=0.023	T=-2.05, P=0.0023	T=9.27, P>0.0001
2	F=15.14, P=0.001	F=86.02, P=0.000	T=-8.68, P<0.0001	T=-4.54, P=0.0039	T=0.00, P=1.000	T=4.29, P=0.0013
4	F=0.79, P=0.384	F=4.89, P=0.037	T=-0.72, P=0.49	T=-6.89, P=0.0002	T=1.49, P=0.19	T=-1.03, P=0.33
6	-	-	-	T=-4.25, P=0.0022	-	-
8	F=1.51, P=0.232	F=0.41, P=0.530	T=-1.21, P=0.25	T=-2.58, P=0.032	T=-0.81, P=0.44	T=-1.57, P=0.15
12	F=2.21, P=0.150	F=16.28, P=0.000	T=-2.12, P=0.057	T=-3.46, P=0.0071	T=-0.94, P=0.37	T=-1.38, P=0.19
24	F=0.91, P=0.349	F=4.80, P=0.038	T=-1.75, P=0.12	T=-1.30, P=0.23	T=-1.35, P=0.21	T=-0.43, P=0.68

3.3.2. *Pharmacokinetic parameters*

A summary of the pharmacokinetic parameters calculated in this study is presented in table 3.3 as the mean (±SEM). The results of statistical tests of the significance of the differences in these parameters between groups are summarised in table 3.4.

The Cmax for all four groups were significantly different, confirming results reported above. The Cmax for the 2.0mg/kg dose was approximately twice that for the 1.0mg/kg dose after both oral and i.v. administration. The time at which Cmax (Tmax) occurred did not differ between groups O1 and O2 ($W=52$, $P=1.00$), however. One individual, in group O1, showed a Cmax that was twice that of the other individuals in the group (2.25μg/ml). A second individual, in group IV2, also showed a much higher Cmax than the other individuals in the group (34.6μg/ml). The data for plasma FM concentration from a third individual showed very abnormal, fluctuating results and was eliminated from further analysis.

The MAT for groups O1 and O2 were the same (3.14± 0.55 and 3.14± 0.56 respectively, $T_{1,13}=0.01$, $P=0.99$). This shows that the drug was absorbed from the gut after oral administration at the same rate irrespective of the dose used.

There was a significant effect of route of administration on the elimination half-life (t½). t½ of FM appeared to be slightly higher after oral administration (GLM, route $F_{3,25}=4.31$, $P=0.049$). Further analysis showed that the difference in t½ was only significant between O1 and IV1 ($T_{1,13}=2.26$, $P=0.045$), the groups receiving the lower dose and was the result of a particularly low t½ in the IV1 group.

The MRT was significantly higher in lambs that were administered the drug orally than in those receiving an intravenous dose (GLM, route $F_{3,25}=33.12$, $P>0.0001$), reflecting the time taken for the drug to reach the blood after administration. The dose administered did not however, significantly affect MRT.

The initial examination of the differences in AUC, using GLM, indicated that the effect of the dose of drug administered had a more significant effect on the AUC than the route of administration ($F_{3,25}=20.61$, $P>0.0001$ and $F_{3,25}=14.17$, $P=0.001$ respectively). Post-hoc t-tests showed that the AUC for all groups differed significantly at $P>0.05$.

Table 3.3. Means (±SEM) of the pharmacokinetic parameters for FM administered to 6-week old lambs either orally or intravenously at 1 or 2 mg/kg.

Parameter	Units	Mean I.V. (+/-SEM) 1mg/kg n=7	Mean I.V. (+/-SEM) 2mg/kg n=7	Mean Oral (+/-SEM) 1mg/kg n=7	Mean Oral (+/-SEM) 2mg/kg n=6
CMAX	ug/ml	13.21 (+/-1.56)	23.87 (+/-3.43)	1.16 (+/-0.19)	2.64 (+/-0.36)
tMAX	h	-	-	0.75 (0.75-1)	1.00 (0-1)
MAT	h	-	-	3.14 (+/-0.55)	3.14 (+/-0.56)
t1/2	h	3.04 (+/-0.46)	4.25 (+/-0.49)	4.65 (+/-0.54)	4.67 (+/-0.46)
MRT	h	3.16 (+/-0.46)	3.54 (+/-0.38)	6.30 (+/-0.58)	6.67 (+/-0.69)
AUC	ug.h/ml	13.22 (+/-2.13)	23.34 (+/-2.78)	6.88 (+/-0.93)	14.76 (+/-1.61)
AUMC	ug.h2/ml	42.60 (+/-10.87)	81.90 (+/-14.09)	42.56 (+/-5.69)	96.54 (+/-11.92)
F	%	-	-	61.63 (+/-16.45)	69.45 (+/-12.50)
Cl	ml/h/kg	20.79 (+/-3.25)	19.58 (+/-1.94)	-	-
Vd(ss)	ml/kg	67.05 (+/-17.36)	63.53 (+/-8.34)	-	-

Table 3.4. The results of GLM and *post-hoc* analyses to determine differences in pharmacokinetic parameters between groups after the administration of FM either orally or intravenously at doses of either 1 or 2 mg/kg. *Post-hoc* tests were either Student's T-tests or Wilcoxon sign rank tests depending on the distribution of the data.

Parameter	GLM Route	Dose	*Post-hoc* comparison I.V. (1mg/kg) vs I.V. (2mg/kg)	Oral (1mg/kg) vs Oral (2mg/kg)	I.V. (1mg/kg) vs Oral (1mg/kg)	I.V. (2mg/kg) vs Oral (2mg/kg)
CMAX	F=11.10, P=0.000	F=10.25, P=0.004	T=-2.83, P=0.022	T=-3.58, P=0.006	T=-7.65, P=0.0003	T=-6.16, P=0.0008
TMAX	-	-	-	W=52.0, P=1.00	-	-
MAT	-	-	-	T=0.01, P=0.99	-	-
T1/2	F=4.31, P=0.049	F=1.57, P=0.222	T=-1.78, P=0.10	T=-0.03, P=0.98	T=2.26, P=0.045	T=-0.63, P=0.54
MRT	F=33.12, P=0.000	F=0.47, P=0.500	T=-0.62, P=0.55	T=-0.41, P=0.69	T=4.18, P=0.0015	T=-3.97, P=0.0033
AUC	F=14.17, P=0.001	F=20.61, P=0.000	T=-2.87, P=0.015	T=-4.24, P=0.0022	T=-2.73, P=0.026	T=-2.67, P=0.026
AUMC	F=0.43, P=0.516	F=17.72, P=0.00	T=-2.21, P=0.049	T=-4.09, P=0.0035	T=0.00, P=1.00	T=0.79, P=0.440
F	-	-	-	T=-1.29, P=0.22	-	-
Cl	-	-	T=0.32, P=0.76	-	-	-
Vd	-	-	T=-0.18, P=0.86	-	-	-

This result is shown more clearly by the comparison of AUMC between groups. The GLM of AUMC showed that the effect of the route of administration of the drug did not significantly affect (at $P>0.05$) the magnitude of the AUMC ($F_{3,25}=0.43$, $P=0.516$), but that the dose at which the drug was administered was still extremely important ($F_{3,25}=17.72$, $P>0.0001$). In fact the AUMC after administration of FM at 2.0mg/kg was approximately twice that from administration of FM at 1.0mg/kg irrespective of the route of administration.

The bioavailability of FM after oral administration was 61.63% (±16.45) and 69.45% (±12.50) for O1 and O2 lambs respectively. These values were not statistically significantly different at $p>0.05$.

Similarly, the elimination rate (Cl) and the volume of distribution ($V_{d(ss)}$) did not change between groups IV1 and IV2 ($T_{1,13}=0.32$, $P=0.76$ and $T_{1,13}=0.18$, $P=0.86$ respectively) showing that these parameters were also independent of dosage.

3.3.3. *Behavioural analyses*

The results of behavioural observations are shown in tables 3.5 and 3.6. The route of administration and the dose administered has little effect on the behaviours expressed by lambs in any group. When the behaviour of all the lambs after the drug had been administered was compared with the behaviour of the same lambs on the previous day, again there was little difference in the behaviour expressed. However, during control observations the day before drug administration, lambs spent more time eating than they did after the drug was administered ($T_{1,7}=2.54$, $P=0.038$). There were no differences in any other behaviour expressed.

Table 3.5. Summary of behavioural observations in 6-week old lambs after administration of FM orally or intravenously at doses of 1 or 2 mg/kg. Data are expressed as median (Q1-Q3) or mean (±sd) depending on the distribution of the data. The data recorded during the two 30 minute observation periods was first summed to provide a measure of behaviour over the full hour of observation time. (a) summary of posture and behavioural states data. (b) summary of the frequency of active behaviour.

(a)

Behaviour	I.V. 1mg/kg	I.V. 2mg/kg	Oral 1mg/kg	Oral 2mg/kg
S1	8 (6-22)	20 (16-32)	12 (8-26)	16 (8-26)
V1	0 (0-2)	0 (0-2)	4 (0-4)	0 (0-4)
V2	28 (14-28)	16 (8-24)	24 (12-26)	20 (10-24)
V3	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
eat	4 (2-16)	8 (2-8)	12 (4-26)	8 (6-24)
idle	18.29 (+/-11.04)	25.71 (+/-11.04)	8.57 (+/-9.07)	9.14 (+/-9.72)
ruminate	12 (4-18)	4 (2-8)	12 (2-20)	0 (0-14)
sleep	0 (0-0)	4 (0-4)	4 (0-6)	0 (0-4)

(b)

Behaviour	I.V. 1mg/kg	I.V. 2mg/kg	Oral 1mg/kg	Oral 2mg/kg
restlessness	2.86 (+/-1.70)	2.57 (+/-1.57)	2.29 (+/-2.19)	3.86 (+/-1.51)
easequarters	1.14 (+/-3.42)	0.86 (+/-2.57)	2.00 (+/-1.46)	1.57 (+/-1.07)
teatseek	1 (1-2)	2 (1-2)	1 (0.5-2)	1 (0.5-1)
tailwag	0 (0-0)	0 (0-0.5)	0 (0-0)	0 (0-0)
play	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
headturn	0 (0-0)	1 (0-2)	1 (0-3.5)	2 (0-2)
rub quarters	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)

3.4. *Discussion*

3.4.1. *Absorption parameters*

Maximum plasma concentration of flunixin meglumine

The maximum plasma concentrations of FM after i.v. administration were 11.1 ±4.19μg/ml and 20.39 ±7.71μg/ml for 1.0 and 2.0 mg/kg doses respectively. These Cmax values were much lower than those previously observed in adult sheep (approximately 22 and 34μg/ml for 1.0 and 2.0 mg/kg doses respectively) (Welsh *et al*, 1993). In cattle, Odensvik (1995) found a Cmax of 16.16 ±5.28μg/ml after i.v. administration at 2.2mg/kg. This is likely to be a reflection of the larger volume of distribution in this species (Odensvik, 1995).

After oral administration of FM at 1.0 and 2.0mg/kg the Cmax values achieved were 1.18 ±0.45μg/ml and 2.43 ±2.43μg/ml respectively. These concentrations are lower than those achieved after i.m. administration of FM at 1.1 and 2.2 mg/kg in sheep (5.9 ±0.47μg/ml and 11.0 ±1.97μg/ml respectively). This reflects differences in the ease with which FM can reach the blood after administration by different routes. After oral administration, incomplete systemic availability of the drug generally results, as the drug may be metabolised in the intestinal mucosa or liver (Baggot, 1992). The pH of the rumen is 5.5-6.5 (Odensvik, 1995). The trapping of ions in the rumen can inhibit absorption significantly, if the drug administered is alkaline. As FM is a weak acid with a pH of 5.82, ion trapping should not represent a significant threat to absorption from the rumen. However, the lambs in this study were not weaned and the suckling of alkaline milk may have neutralised the rumen environment sufficiently, such that ion trapping was a significant factor reducing absorption. If absorption was inhibited in this way, lower Cmax values might be expected after oral administration.

The Cmax achieved after administration at a dose of 2.0mg/kg in the present study was twice that achieved after oral administration of the drug, at a dose of 2.2mg/kg, in adult cattle (0.9 ±0.05μg/ml) (Odensvik, 1995). This may be partially because the drug was administered with a feed, but may also be due to the time taken for absorption into the plasma compartment and the large volume of distribution in this species (Odensvik, 1995). As discussed above, it was the Cmax values after i.v. administration that were lower than those expected in sheep. This evidence refutes

the proposal that the suckling of milk inhibited the absorption of FM from the rumen in this study.

The Cmax achieved after i.v. administration at 1.0mg/kg was approximately half that achieved after administration at 2.0mg/kg i.v. A similar result was found for the Cmax achieved after oral administration at the two doses in this study and is consistent with data from intramuscular (i.m.) administration in a previous pharmacokinetic study in sheep (1.1 and 2.2mg/kg doses) (Welsh *et al*, 1993). This indicates that in sheep, between doses of 1.0-2.2 mg/kg, the concentration of FM in plasma is directly proportional to the dose, irrespective of the route of administration.

Time taken to reach maximum plasma concentration of flunixin meglumine

The times taken to reach Cmax (Tmax) were 0.75 (0.75-1) hours and 1.0 (0-1)hours for 1.0 and 2.0mg/kg respectively, after oral administration. These times were not significantly different at $p<0.05$. After i.m. administration of FM in adult sheep, at 1.1 and 2.2mg/kg, Tmax occurred at or before 0.75 and at or before 1 hour after administration respectively. This shows that, in sheep, FM is as quickly absorbed into plasma after oral administration as it is after i.m. administration.

A study by Pyorala *et al* (1999) showed that FM caused the worst tissue damage (of the drugs tested, including phenylbutazone) in cattle after i.m. administration at 2.2mg/kg, destroying around 80g of tissue around the site of injection. Severe tissue lesions of this sort may persist for months (George *et al*, 1995) and have serious welfare and economic implications, particularly if several injections were required over a period of time. Oral administration of FM could therefore prove to be a valuable alternative to i.m. administration.

After oral administration, at 2.2 mg/kg, in cattle, FM took much longer to reach Cmax (3.5 ±1.0 hours). This time included the time taken for cattle to completely consume feed containing the granules of drug. However, the concentration of FM in plasma was sufficient to inhibit prostaglandins within the first hour (Odensvik, 1995). It was concluded that FM reaches effective plasma concentrations within a few minutes after administration either orally or by i.m. administration in cattle. It is likely that the same is true in sheep.

Mean absorption time

The mean absorption times (MAT), which is the arithmetic average time that each drug molecule takes to be absorbed into the plasma compartment, were 3.14 ±0.55 hours and 3.14 ±0.56 hours for 1.0 and 2.0 mg/kg doses respectively. The MAT and Tmax data show that over this range, the dose administered makes no difference to the rate of absorption of FM after oral administration in 6-week-old lambs. MAT was much longer in cattle (6.3 ±1.8 hours) (Odensvik *et al*, 1995). It would appear therefore that the rumen in 6-week-old lambs represents less of a barrier to FM absorption than in adult cattle. Differences in absorption time between these studies are also likely to be related to the larger volume of distribution in cattle.

Volume of distribution

The volumes of distribution ($V_{d(ss)}$) previously found in sheep, after 1.0 and 2.0 mg/kg i.v. doses (166.2 ±37.2 ml/kg and 151.8 ±26.3 ml/kg) (Welsh *et al* 1993) were more than twice those found in the lambs in this study (67.5 ±17.36 ml/kg and 63.53 ±8.34 ml/kg). The $V_{d(ss)}$ found in this study are also low in comparison with that found in dogs after a 1.1mg/kg i.v. dose (181 ±79.0 ml/kg) (Hardie *et al*, 1985). As mentioned above, the $V_{d(ss)}$ for FM in cattle (782 ±237 ml/kg) (Odensvik, 1995) after an i.v. dose at 2.2mg/kg, is extremely high by comparison with that in sheep and dogs. There was no effect of dose on the $V_{d(ss)}$ in the present study, which is consistent with previous studies in sheep (Welsh *et al*, 1993).

3.4.2. *Elimination parameters*

Clearance

The dose rate did not affect the rate of clearance (Cl) of FM from plasma. Cl was 20.79 ±3.25 ml/kg/hr and 19.58 ±1.94 ml/kg/hr for 1.0 and 2.0 mg/kg i.v. doses respectively. Cl in these lambs was very low in comparison with adult sheep; 36 ±1.8 ml/kg/hr and 42 ±1.8 ml/kg/hr (Welsh *et al* 1993). Cl in sheep has been found to be much lower than in dogs; 63.6 ±13.9 ml/kg/hr (Hardie *et al*, 1985) and cattle; 115 ±11 ml/kg/hr (Odensvik, 1995). Although differences in the modelling of the data in different studies was considered to be a possible cause of these interspecies

differences, it was concluded by Welsh *et al* (1993), that sheep simply clear the drug more slowly than other species. In the present study, non-compartmental analyses were carried out using the same programme and models as those used by Odensvik (1995). Modelling discrepancies therefore cannot account for differences in the Cl observed between these trials. It is likely that the difference between Cl in lambs and adult sheep is caused by the relative immaturity of hepatic and renal clearance in these younger animals (Papich, 1997).

Elimination half-life

In the present study the elimination half-lives (t½) after i.v. administration at 1.0 and 2.0 mg/kg were 3.04 ±0.46 hours and 4.25 ±0.49 hours respectively. Again, the dose having no effect on t½ at $p< 0.05$. This is comparable with data found in adult sheep at the same dose rates; 3.83 and 3.43 hours respectively (Welsh *et al*, 1993), and with that found in dogs after i.v. administration at 1.1mg/kg; 3.67hours (Hardie *et al*, 1985). Although the Cl of FM from plasma was very low in lambs in this study, the $V_{d(ss)}$ was also very low. Therefore, there was no overall difference in t½ between this study and the study by Welsh *et al* (1993) in sheep. However, the t½ found in cattle after i.v. administration at 2.2mg/kg was much longer; 5.2 hours (Odensvik, 1995), despite the relatively rapid Cl, reflecting the much larger $V_{d(ss)}$ found in cattle. After oral administration the t½s were 4.65 ±0.54 hours and 4.67 ±0.46 hours, with dose having no effect at $p<0.05$. There was no difference between t½ between oral and i.v. administration routes at the higher dose rate in this study. After i.v. and oral administration at 1.0mg/kg however there was a small but significant difference in the t½. This may reflect the fact that the $V_{d(ss)}$ was slightly higher (although not significantly) at the lower dose, whilst the Cl at 1.0mg/kg was almost exactly the same as that for the 2.0mg/kg dose. However, in cattle, the t½ was found to be slightly higher after oral administration than i.v. administration at 2.2mg/kg although this difference was also not significant. This evidence suggests that a trend for elimination to take slightly, but not significantly, longer, after oral administration, such that small differences in the $V_{d(ss)}$ between oral and i.v. routes, as seen in the present study at the lower dose, can push this trend into significance. It should be

noted that the means for the t½ presented in this study are arithmetic means and not harmonic means as quoted in some other studies.

3.4.3. *Parameters incorporating absorption and elimination*

Mean residence time

The mean residence time (MRT) is the arithmetic average time that each drug molecule remains in the system. In the present study, the MRT after oral administration was twice that observed after i.v. administration, but was not affected by the dose at $p<0.05$. This is also comparable with data from cattle in which the MRT after oral administration was also twice that observed after i.v. administration; 12.7 ±1.0hours and 6.4 ±1.6 hours for oral and i.v. administration respectively (Odensvik, 1995). This reflects the time taken for absorption after oral administration

Area under the zero moment curve

The area under the concentration-time curve (AUC) (or area under the zero moment curve) is directly proportional to the total amount of drug introduced to the plasma compartment, irrespective of the rate at which it enters. In the present study the AUC after the 2.0mg/kg dose was approximately twice that observed after administration at 1.0mg/kg, irrespective of the route of administration. This is further evidence that the plasma concentration of FM is directly proportional to the dose rate over this range. This relationship was also observed after oral and i.v. administration in cattle (Odensvik, 1995) and after i.m. administration in sheep (Welsh *et al*, 1993).

Area under the first moment curve

The area under the concentration x time- time curve (AUMC) (or area under the first moment curve) further illustrates the direct relationship between the plasma concentration of FM and the dose administered, by increasing the importance of the elimination and absorption phases of the curve. As absorption and elimination after oral administration take slightly longer, these phases are larger on the first moment curve after oral administration. Thus, the difference between the AUMC for groups O1 and IV1 and between O2 and IV2 is reduced to insignificance, but the AUMC for administration at 1.0mg/kg remains half that observed at 2.0mg/kg.

Bioavailability after oral administration

Incomplete systemic availability results after oral administration of FM. The drug may be destroyed by 'first pass' metabolism in the liver or intestinal mucosa. The absorption of the drug also depends on its ion content and that of the gut content. If significant ion-trapping occurs in the gut, less of the drug will be absorbed. These factors are particularly important in ruminant species. The large capacity of the rumen, accommodating continuous fermentation, significantly dilutes the drug, the histological structure of the epithelium slows absorption and higher efficiency of metabolism by the liver all act to reduce the bioavailability of the drug, in comparison with carnivorous and monogastric species (Baggot, 1992). Comparison of AUC for i.v. and oral administration at the same dose provides an estimate of how much of the oral dose reaches the plasma and is therefore biologically active. This is termed bioavailability (F). After i.m. administration in sheep the bioavailability of FM was estimated to be 70%. However, this is likely to be an over estimation as the doses used were not the same for the two routes of administration (1.1 and 2.2mg/kg i.m. and 1.0 and 2.0mg.kg i.v.). The bioavailability in the present study, after dosing at 1.0 and 2.0mg/kg both orally and i.v., was 61.6 ±16.5% and 68.5 ±12.5% respectively. The difference was not significant at $p<0.05$. This is comparable with the bioavailability of FM after i.m. administration in sheep (Welsh *et al*, 1993) and with that from oral administration in cattle at 2.2mg/kg; 60.1 ±5.0% (Odensvik, 1995).

3.4.4. *Prostaglandin inhibition*

Attempts to measure the inhibitory effects of FM on prostaglandin were unsuccessful in this study. However, in comparison with data from studies of prostaglandin inhibition evidence suggests that the plasma concentrations of FM achieved in this study after oral administration at both 1.0 and 2.0mg/kg should be sufficient to significantly inhibit prostaglandins. After oral administration of FM at 1.0 and 2.0mg/kg the Cmax values achieved were 1.18 ±0.45μg/ml and 2.43 ±2.43μg/ml respectively, reached 0.75 and 1 hours after administration.

The Cmax achieved after oral administration of FM at a dose of 2.2mg/kg, in adult cattle was 0.9 ±0.05μg/ml (Odensvik, 1995). This maximum was achieved 3.5 ±1.0 hours after administration (but included the time taken to consume the granules). However, the plasma concentration of FM after oral administration was sufficient to inhibit prostaglandins by as much as i.v. administration at the same dose within an hour of administration. The concentrations of FM achieved after oral administration, at 1.0mg/kg, in the present study exceed those in cattle after oral administration at 2.2mg/kg (Odensvik, 1995) by 0.5 hours after administration. This indicates that, in the present study, the plasma concentration of FM may well have been sufficient to induce inhibition of prostaglandins within the first 30 minutes after administration.
Also, Cheng *et al* (1998a) studied of the selectivity of FM for inflamed tissue and the resulting inhibition of inflammatory prostaglandins. They found that the Cmax of FM in exudate was 1.82 ±0.21μg/ml, which was sufficient to inhibit inflammatory prostaglandins by 100%. They also found that a plasma concentration of 0.00012 ±0.00006μg/ml of FM was sufficient to inhibit inflammatory prostaglandins by 50%, 144 hours after administration.

3.4.5. *Behaviour*

Observation of the behaviour of lambs in this study revealed no gross behavioural abnormalities that might be associated with NSAID toxicity. There was a reduction in the amount of time spent eating after administration of FM irrespective on the treatment group in comparison with pre-treatment observations. Chambers *et al* (1995) identified the opioidergic system and the α_2-adrenergic system as possible means by which FM could act centrally to induce analgesia, in the absence of inflammation. One of the unwanted effects of opioids is appetite suppression. The decrease in eating after FM administration could thus be a reflection of actions of FM through opioidergic pathways. This might be tested by the co-administration of an opioid antagonist such as naloxone. Given this hypothesis, it is paradoxical that no differences in the time spent eating were found between groups during the post-treatment phase. This refutes the former proposal as the concentration of the drug and consequently the route of administration and dosage should be important factors in the development of appetite suppression. The similarity in the occurrence of eating

however does indicate that the oral gavage methodology itself had no confounding effect in comparison with i.v. dosing.

3.4.6. *Conclusion*

The results of this study indicate that oral administration of FM is a suitable, or possibly better, alternative to i.v. and i.m. administration in sheep, even for the treatment of severe acute pain. FM reached plasma quickly after oral administration at both doses and at concentrations sufficient to significantly inhibit inflammatory prostaglandins. This indicates that the delay between consumption of drugged feed, during self-administration studies, and the onset of analgesic effects is short, and is probably less than 30 minutes. Published evidence supports the conclusion that oral administration is as effective in terms of absorption time and level of prostaglandin inhibition as i.m. and i.v. administration respectively. As FM is known to penetrate and remain in inflamed tissue for longer than in plasma (Cheng *et al*, 1998a), it is likely that the inhibition of prostaglandins persists for longer than the time taken for elimination from the plasma compartment. The bioavailability of FM after oral administration in lambs was also as high as that achieved after i.m. administration in sheep (Welsh *et al*, 1993). The results indicate that an oral dose of 1.0mg/kg should be sufficient for use in self-administration trials

FM administration induced a decrease in the amount of time spent eating, irrespective of the route of administration and the dose. This result must be considered during interpretation of behavioural analyses during later trials using FM.

Chapter 4

Preference Studies and Self-administration to determine Chronic Inflammatory Pain

Chapter 4. Preference studies and the use of self-administration to determine the presence and significance of chronic inflammatory pain.

4.1. *Introduction*

The behavioural and physiological signs of chronic pain are highly variable within and between subjects (Short, 1998). They are also dependent on the specific condition and the degree of chronicity encountered (Kitchell and Johnson, 1985). Self-administration of analgesic was first used to determine the presence of chronic inflammatory pain by Colpaert *et al* (1980; 1982), in studies of adjuvant-induced arthritis (AA). The self-administration protocol was used in these studies to compare the consumption of an analgesic fluid in arthritic rats with that of controls, in a preference test paradigm.

In the studies reported in this thesis, self-administration of analgesic was used to determine the presence of chronic inflammatory pain from castration and tail-docking in lambs. These studies were based on those by Colpaert *et al* (1980; 1982) in rats. In this chapter, the relevance of preference testing to assess the subjective experience of pain in animals will be evaluated. The use of such tests to investigate mechanisms by which conditioned feed associations develop in sheep will then be reviewed. Finally, the use of preference testing theory to determine the presence of chronic inflammatory pain from analgesic self-administration studies in rats will be discussed.

4.2. *Assessment of welfare: the importance of subjective feelings/experience*

It is generally agreed that how an animal feels and the strength of those feelings determines, to a greater or lesser extent, that animal's welfare (Dawkins, 1990). In order to properly assess animal welfare, we must have a means of determining the type and strength of animals' subjective feelings. The inability of animals to communicate their feelings verbally remains a challenging barrier (Duncan, 1992), limiting the refinement of our assessments. Whilst the experience of pain in animals remains a controversial subject, it is commonly accepted in the field of animal welfare research, that whilst animal pain may not be exactly the same experience as

that in humans, it serves the same function and is similarly aversive (Molony, 1986; Molony, 1992; Molony and Kent, 1997; Dawkins, 1990). The presence of pain can therefore seriously affect welfare.

4.3. *Use of preference testing to assess welfare*

Using a strategy adopted from early psychological research (Kilgour *et al*, 1991), scientists have attempted to overcome the barrier of communication between humans and animals by providing the animal with an opportunity to express their feelings in preference tests. In such preference tests, animals are effectively asked how they feel by allowing them to 'vote with their feet' (Duncan, 1992). In order to make a choice in a preference test, the animal must have some understanding of the available choices. To facilitate this, most preference paradigms incorporate a simple pre-test conditioning period in which animals are given an opportunity to learn the association between their own actions, the characteristics of the stimulus provided and the positive or negative consequences of the experience of that stimulus (Lawrence and Illius, 1997). The positive or negative consequences of the animal's behaviour in response to the stimulus are reinforcers for that behaviour (Hogan and Roper, 1978), because they increase or reduce respectively the likelihood of that action occurring again. The animal thus learns to make an operant response in order to gain or avoid the stimulus. A simple association may be learned between the act of locomotion and proximity to the stimulus, but more complex abstract associations may also be learned (Lawrence and Illius, 1997). The animal is then given the opportunity to express an informed choice between the options experienced during the conditioning period. Adaptations of this methodology have been used widely in animal welfare research in order to draw conclusions about the animal's well being.

In studies in sheep, Rushen (1986b) used latencies of approach as a simple measure of the relative aversive nature of electro-immobilisation and mechanical restraint after sheep had experienced both handling procedures during conditioning. Alternatively, Y- and T-mazes provide a simple and controlled environment in which animals must choose between two stimuli. The animals are again given a conditioning period in which they learn the position and nature of the two stimuli in the maze separately. They are subsequently given access to a maze containing both

stimuli and allowed to make a choice between them. This approach was also used to measure the relative aversion to different restraint methods in sheep (Rushen, 1986a). In other preference studies, animals' use of space may be used to determine preferred aspects of their environment (Gonyou, 1991). The use of space by animals given access to more than one controlled environment may be used to infer preferences for certain aspects of that environment (Nicol, 1997). This technique was used by Dawkins (1981) to assess cage size preferences in laying hens. Similarly, but in a less controlled paradigm, use of space was used to demonstrate preferences of sheep and goats for sheltered areas of their environment just prior to parturition (Gonyou and Stookey, 1983; O'Brien, 1983). This methodology has also been used in pharmacology to determine the positive and negative consequences of drug administration (Carr *et al*, 1989). In such studies animals are conditioned using drug administration associated with a distinctive location, and a second location that is not associated with the drugged state. The preference for both locations is subsequently tested giving a measure of conditioned place preference. The technique has been used to assess positive reinforcement from intrinsically rewarding drugs (Mucha and Iverson, 1984) and as a model to evaluate the pain-relieving properties of analgesics (Sufka, 1994).

Measurement of the strength of preferences or aversions gives an indication of the extent to which welfare may be compromised by the inability to access or avoid a stimulus (Dawkins, 1983; Duncan, 1992). In an expansion of operant conditioning and associative learning studies, Dawkins (1983) hypothesised that the economic theory of consumer demand (already used in the field of behavioural ecology) could be used to demonstrate the strength of preferences in animals. According to this theory, consumers alter their purchasing patterns to maximise utility (satisfaction), depending on the availability of economic resources or the cost of the desired items. If income remains the same but the cost of desired items increases, the purchase of essential items should be maintained whilst the purchase of non-essential or luxury items should decline. Similarly if the cost of goods remains stable but income is reduced, the purchase of such luxury items should again decline.

In more complex operant conditioning tests, animals must learn to perform multiple actions in order to gain a reward or avoid an undesirable stimulus. For example, in

studies of feeding motivation, sheep were required to learn to push through a weighted door in order to reach a ration of feed. When sheep had learned the initial task, they were then required to do more work in order to reach the feed by adding weight to the door. Thus, the motivation to reach the feed was tested (Jackson *et al*, 1999). Alternatively the availability of 'currency' may be reduced by limiting the time available for performance of a selection of behaviours. The relative necessity of litter over food was thus tested in laying hens and it was found, unsurprisingly, that the need for litter was less than that for feed after feed deprivation for 18 hours (Dawkins, 1983). It is hypothesised that if an animal is highly motivated to carry out a behaviour (as demonstrated in such operant tests) then non-performance of that behaviour may constitute significant suffering (Dawkins, 1990).

4.4. *Criticisms of preference testing*

Whilst preference testing is elegant in theory, it is also simplistic and has received justified criticism on several levels. In any choice paradigm, the preference shown can only ever be relative to the options given, whether in a simple or complex environment (Gonyou, 1991; Duncan, 1992). Thus the selected stimulus may either be the most preferred or the least aversive of the available options, a difference with major implications for the welfare of the animals concerned (Duncan, 1992). In free choice tests, the decision to spend a specified duration of the available time in one environment over another may represent an active choice rather than an expression of aversion for one particular environment or a preference for another (Duncan, 1992). Also, as Lawrence and Illius (1997) point out, use of consumer demand theory may result in over restriction of time 'currency' or increases in cost, to the extent that the animal only has the time or the motivation to express the most critical behaviours, thus only fundamental physiological needs are detected (Lawrence, 1986).

Variations in preferences within and between animals may also occur as a result of prior experience of the stimulus. If an animal has little relative experience of one stimulus, for example wood-shaving litter for a battery reared hen, it is likely to approach it with caution initially. Later however, with more experience of the substrate, these birds show a strong preference for litter over wire flooring (Dawkins,

1981). The degree of arousal and the environmental conditions (e.g. temperature) may also influence choices made. For these reasons, animals may express different preferences in their natural environments and the validity of highly controlled testing paradigms has been questioned (Lawrence and Illius, 1997)

According to traditional operant conditioning theory (theory of equipotentiality), all animals should be able to learn to perform any behaviour in order to reach any desirable target (Roper, 1983). However a great deal of evidence against this hypothesis has since been found. It is now considered that the ability of animals to learn associations between their actions and the target stimulus are subject to species specific and task specific constraints. Associations between certain pairings of action and reinforcer may simply be incompatible (Sevenster, 1973). In these circumstances an animal may appear not to be very highly motivated to perform a behaviour (Dawkins and Beardsley, 1986). This was demonstrated in a study by Young *et al* (1994), in which pigs were required to interact with different types of manipulanda to obtain a food reward. The pigs in this study were better able to learn to manipulate a wooden 'arm' with their mouths than to push a paddle with their snouts to receive a food reward. It has been suggested that reduced ability to learn an association in such cases demonstrates that the animal is being asked to make associations between behaviours that belong to different motivational systems (Hogan and Roper, 1978; Dawkins and Beardsley, 1986). Thus an animal is better able to learn behaviours, for example in association with food, that are species typical feeding behaviours (Young *et al*, 1994). For these reasons choices offered to animals must be sensible, natural choices to which animals are likely to be able to express a sensible preference.

In the use of preference testing theory to study the subjective feelings of animals, it is assumed that animals will make decisions that are good for their welfare (Duncan, 1992). However, an animal will show a preference on the basis of how it feels at that moment in time and such preferences may not be of benefit to long-term welfare. Temporal variations in motivation to perform behaviours occur and it has been supposed that most animals do not have the ability to plan for the whole of a day (Dawkins, 1990). Dawkins (1983) used the terms proximate and ultimate needs to describe differences in motivation to perform behaviour at different times. Behaviours that are required in order for the animal or its offspring to survive, like

eating, drinking and predator vigilance, might be considered ultimate needs, having significant evolutionary function. Whereas proximate needs are those motivated behaviours that are not essential for the animals' ultimate survival. Dawkins notes that in a captive environment proximate and ultimate needs become disassociated such that, whilst dust bathing in chickens is a proximate need that relates directly to an ultimate need in the wild, there is less need to maintain feathers for warmth and predator evasion in captivity (Dawkins, 1983). For this reason preference studies have moved away from short-term instantaneous samples of preference, to studies determining preference over longer periods of time (Lawrence and Illius, 1997).

4.5. *Conditioning in response to feeding in sheep.*

Domestic sheep are selective close grazers (Angus, 2000) that consume a wide variety of plant species (Zahorik *et al*, 1990). The reasons for the extent of the variation in their diet are not fully understood (Early and Provenza, 1998). However, sheep select food on the basis of conditioned associations between the characteristics of the food and unique, systemic and hedonic consequences of consuming that food (Arsenos and Kyriazakis, 1999). In other words, they show preference and aversion for feed types on the basis of previous experience.

Garcia and Koelling (1966) demonstrated the classical (or Pavlovian) conditioning of an association between a food's characteristics and the unique consequences of its ingestion, in rats. They showed that rats given a novel tasting liquid to drink later showed aversion to that liquid when sickness was induced experimentally, using toxins or x-radiation, soon after ingestion. Thus the rats learned to associate the outcome (sickness) with the taste of the novel liquid. It is thought that all animals use such conditioned food associations, in conjunction with internal feedback of physiological needs to consider the choices available in the immediate environment and to optimally select their diet (Provenza, 1996).

4.5.1. *Central and peripheral mechanisms controlling conditioned food associations*

Many mechanisms for the control of feed intake have been proposed (see Novin, 1983 for a review) and no attempt to provide a complete review of the topic will be made here. Instead pathways that are generally accepted as important to the control

of feed intake and the formation of conditioned food associations will be summarised. Sensory characteristics, unique to each food are dictated by its chemical and physical properties. These characteristics are sensed by chemo-, osmo- and mechanoreceptors at all levels of the gastrointestinal tract (Glenn and Erickson, 1976; Anil *et al*, 1993; Mbanya *et al*, 1993; Smith, 1996). It has been shown that feed intake cannot be controlled by the selective stimulation of one modality specific group of gastrointestinal receptors, but that the relative importance of stimulation of each group of receptors is dependent on the degree to which other groups are being stimulated. Thus, the degree of gut distension (mechanoreceptor stimulation) required to significantly reduce food intake is dependent upon the chemical and osmotic characteristics of the feed (Anil *et al*, 1993; Mbanya *et al*, 1993). Anatomical and electrophysiological studies have shown that sensory information from these receptors is transmitted along visceral and gustatory nerves to synapse in the nucleus of the solitary tract (NST) in the medulla. Axons project from the NST to the parabrachial nucleus (PBN). Visceral and gustatory neurones are also known to project to the hypothalamus and bed nucleus of the stria terminalis (BNST) and to the prefrontal and orbitofrontal cortex (the limbic forebrain) (Glenn and Erickson, 1976; Norgren, 1983; Novin, 1983). The importance of these pathways in the integration of visceral and gustatory information has been shown in studies in which lesions of these pathways reduced the ability of rats to develop conditioned food associations (Anil and Forbes, 1980; Crawley *et al*, 1984; Spector *et al*, 1992). Gustatory projections to the thalamus and cortex are accepted, but the degree to which these areas are influenced by visceral afferent information is unknown (Norgren, 1983). In studies in which the activity of the cortex was experimentally reduced using anaesthesia or functional decortication by the application of potassium chloride onto the surface of the brain, it has been shown that conditioned associations between foods and the systemic and hedonic consequences of consuming it can occur without the need for cognitive function (Buresova and Bures, 1973; Burmudez-Rattoni *et al*, 1988; Provenza *et al*, 1994). Thus, it is thought that conditioned associations develop through (sub-cortical) automatic processing of food characteristics and sensory feedback (Provenza *et al*, 1994). Evidence indicates that the limbic system and the emetic system (Grant, 1987; Mitchelson, 1992), serotonin

(Leibowitz and Alexander, 1998), noradrenalin (Miner, 1992; Wang *et al*, 1999), dopamine (Berridge, 1996), cytokines (Weingarten, 1996), arginine vasopressin (Brownson *et al*, 2002) are involved in the formation of conditioned food preferences and aversions. It is likely that the full complexity of the nervous and hormonal interactions involved in the formation and expression of feed preferences is yet to be realised.

4.5.2. *Conditioning of nutritional value of foods in sheep.*

Classically conditioned associations between the characteristics of a given food and its nutritive value have been demonstrated in sheep. For example, Villalba and Provenza (2000a) conditioned lambs by delivering an intra-ruminal infusion of starch during grazing on straw, a forage of low nutritive value. In control animals, water was delivered into the rumen instead. After a period of food restriction, lambs that were given straw paired with starch infusion during conditioning, ate more straw than lambs that were given straw paired with an infusion of water. Further studies have shown sheep do not simply select the most nutritious food, but that their diet selection is dependant on the animal's nutritional status in relation to the nutrients provided by available foods. In a study by Kyriazakis *et al* (1996), lambs were experimentally infected with a sub-clinical burden of the intestinal parasitic helminth *Trichostrongylus columbriformis*. Typically, helminth parasitism reduces growth rate and disrupts absorption of protein from the small intestine (Coop and Jackson, 2000). In this study, infected lambs were found to select foods containing a higher concentration of protein than uninfected control animals (Kyriazakis *et al*, 1996). Cosgrove and Neilzen (2000) demonstrated that helminth infected lambs are also able to select a diet with a higher protein concentration (clover-rich) in the more complex and natural field environment. These studies both found that the diet selection made by the lambs reduced the nutritional deficit induced by sub-clinical parasitic infection, resulting in recovery of growth rate. Furness (1988a) reported remarkable evidence that wild and feral populations of ruminants are also able to dramatically alter their diet in order to minimise the effects of nutrient deficiencies. During observations of ground nesting tern and Arctic skua populations on the islands of Foula and Rhum, Furness and his colleagues observed characteristic

mutilations to fledglings that included head, leg and wing amputations, but no damage to the body. In many cases mutilated birds had survived the attack and the amputation had healed. Whilst it was not understood why the body of the birds had not been consumed, these attacks were at first put down to predation by small carnivores. However, during nocturnal observations of semi-feral Shetland sheep on Foula, one individual was observed to use its nose to flip over a tern chick and bite off its legs (Furness, 1988b). Two more sightings of this behaviour were observed in the flock of sheep of Foula and mutilated birds accounted for 5% of the 4000 chicks hatched that year. Similar behaviour was observed in red deer on Rhum (Furness, 1988a). Close examination of the mutilated birds showed that in many cases the skin of the leg or wing remained intact, but that the bones had been selectively removed. It was proposed that the sheep and deer selected the bones in order to attenuate calcium and phosphorus deficiencies resulting from poor quality grazing in their island habitat (Furness, 1988a), although this hypothesis was not tested.

Mechanisms also exist by which sheep prevent excessive intake of any nutrient. Excessive consumption of nutrients may have as serious adverse effects as deficiency of nutrients (Forbes and Kyriazakis, 1995). Preference for food of a given nutritive value will vary depending on the nutritive status of the animal (Provenza *et al*, 1996) and is inversely proportional to the number of recent feedback events (Hogan and Roper, 1978). This theory was demonstrated by Wang and Provenza (1996) who showed that lambs fed flavoured feeds that were paired with intra-ruminal infusions of energy or protein, subsequently preferred flavours that had previously been associated with protein or energy respectively.

Sheep are also able make dietary selections that attenuate the adverse effects of over-ingestion of nutrients. For example, Cooper *et al* (1996) fed sheep with a barley-based ration of high grain content, a feed that can cause ruminal acidosis. These sheep preferred a ration of feed mixed with sodium bicarbonate over a second ration that did not contain sodium bicarbonate. In a similar study, Phy and Provenza (1998a) found that grain-fed lambs preferred fluids containing sodium bicarbonate or lasacosis. Both sodium bicarbonate and lasacosis reduce grain induced ruminal acidosis (Ha *et al*, 1983; Chow *et al*, 1994).

Nutrient specific preferences or aversions represent points along a continuum. For example ruminants tend to prefer foods that are highly digestible resulting in greater nutritive feedback (Provenza, 1996). However, if the rate of nutrient release is too high, the animal may become ill (Cooper *et al*, 1996) and aversion to the food will follow (Provenza *et al*, 1996). This was demonstrated by Arsenos and Kyriazakis (1999) who used intra-ruminal infusions of high and low concentrations of casein (a source of nitrogen) as nutritive stimuli. After conditioning in which a novel food was paired with a low dose of casein, lambs showed a preference for that food. However lambs receiving a high dose of casein later showed aversion to the novel feed.

Evidence shows that sheep are able to select foods to rectify nutrient deficits, avoid over-ingestion of specific nutrients and attenuate the consequences of over-ingestion of nutrients. Thus, there is strong evidence that as a result of conditioning, sheep are able to balance nutritive utility against an internal feedback system that provides information on the animals' long-term nutritional status. This enables them to show preference or avoidance towards available feeds along a continuum, selecting those that will optimally satisfy their nutritive requirements at that time (Forbes and Kyriazakis, 1995).

4.5.3. *Conditioning of toxicological status of foods in sheep.*

Under natural and captive grazing conditions sheep encounter plant species that contain varying quantities of phytotoxins that have toxic effects on consumption (Angus, 2000). In good conditions, when sufficient alternative grasses and herbs are available, sheep are able to minimise their consumption of toxic plants (Angus, 2000). However, in less favourable conditions it may become necessary for herbivores to consume more phytotoxin-containing plants in order to satisfy nutritional requirements. It is generally accepted that sheep select a diet that avoids or minimises consumption of phytotoxins whilst maintaining optimal nutritional status (Westoby, 1974; Belovsky and Schmidt, 1994). In the majority of studies of conditioned aversions to toxins in sheep (Provenza, 1996; Kyriazakis *et al*, 1998), lithium chloride (LiCl) was the toxin used to study the development of conditioned associations. For example, in ewes and lambs, 2% of LiCl incorporated into a palatable feed of rolled barley or rabbit pellets was sufficient to induce conditioned

aversion to that feed (Thornhallsdottir *et al*, 1987). In their study conditioned aversion was maintained, without repeated exposure, for up to 60 days. In a study by Launchbaugh and Provenza (1994), lambs were exposed to a range of novel feeds into which LiCl was incorporated at a range of concentrations. The strength of aversion was found to be proportional to the concentration of LiCl. In a further study Hills *et al* (1999) used sulphur to induce feed aversion, and again higher doses of the toxin were associated with stronger aversion to the feed in which it was incorporated. Kyriazakis *et al* (1998) used oxalic acid, a component of many plant species commonly eaten by ruminants, to study conditioned food aversion. Over consumption of oxalic acid results in the formation of calcium oxalate crystals in cells causing cell damage and reduced calcium availability. The effects of oxalic acid ingestion are more general than those of LiCl, which specifically stimulates the emetic system, and therefore better resemble naturally occurring toxicity. The results of the investigation support the conclusions of the studies using sulphur and LiCl outlined above, with lambs showing greater aversion to higher concentration of the toxin under these more natural conditions. It should be noted that conditioned aversions are typically observed in animals that have not shown overt symptoms of toxicity (Provenza, 1996) suggesting that sheep have evolved strategies to reduce the risk of toxicosis from ingestion of poisonous plants.

On encountering a novel food, sheep show a tendency to sample only small amounts initially (Launchbaugh and Provenza, 1994). This food neophobia is considered to be a behavioural adaptation that helps to prevent toxicosis when sampling potentially toxic novel foods (Chapple and Lynch, 1986). If illness does not occur, consumption of novel feeds is gradually increased (Chapple and Lynch, 1986; Chapple *et al*, 1987a; 1987b). It is frequently observed that, although conditioned food aversions may be developed following a single experience of the food (Burritt and Provenza, 1991), aversions are not absolute. Sheep show a tendency to continue sampling small amounts of aversive feeds after an initial aversion has been established (Thornhallsdottir *et al*, 1987; Launchbaugh and Provenza, 1994; Hills *et al*, 1999) and will increase their intake when the feed is no longer associated with toxin-induced illness (Thornhallsdottir *et al*, 1987; Provenza, 1996). This behaviour is consistent with the theory that sheep use conditioned associations to optimise

nutrient intake but minimise toxin intake. A further protective adaptation observed by Launchbaugh and Provenza (1994) is that the aversion learned in response to illness is generalised to foods with similar characteristics. In their study, generalisation of aversion became more persistent as the dose of toxin, and thus degree of malaise, was increased. The risk of toxicosis from related plant species may thus be reduced in natural conditions.

4.5.4. *Constraints on conditioning of associations in sheep.*

As discussed in section 4.4 learning of conditioned associations is subject to species specific and test specific constraints (Garcia and Koelling, 1966; Sevenster, 1973; Roper, 1983; Hogan and Roper, 1983; Dawkins and Beardsley, 1986). Conditioned responses to foods are no exception to this rule.

4.5.4.1. *Discrimination between feeds.*

Firstly, in order to develop and maintain a conditioned association, the animal requires cues by which foods may be distinguished. No association can be learnt if two foods of differing nutritional and toxicological status do not differ in any other respect (Forbes and Kyriazakis, 1995). Studies have shown that the relative value of perceptible cues for discrimination between foods varies between species (Wilcoxon *et al*, 1971), but that those characteristics that can be most reliably related to the specific food are generally the most useful (Forbes and Kyriazakis, 1995).

Flavour and smell are determined by the biochemistry of the food and are therefore intrinsically linked to the unique consequences of consuming the food (Provenza, 1996). For this reason, flavour and smell represent reliable cues by which foods of a particular nutritive and toxicological status may be identified and accordingly, are be the cues that are most relied upon during feed selection. In experimental manipulations of diet selection in ruminants, cattle, goats and sheep, have been found to be able use flavour as a cue to discriminate between the nutritive and toxicological value of feeds (Zahorik, 1990; Kronberg *et al*, 1993; Arsenos and Kyriazakis, 1999). In nature, flavour and taste cues are very reliably linked to specific consequences and as a result associations between certain types of flavour and consequence are easier for the animal to learn. For example sweet flavours are naturally preferred to sour or

salt (Sclafani, 1995) and associations between sweet flavour and carbohydrate infusion are easier to learn than carbohydrate infusions paired with salt of sour flavours (Ramirez, 1996). Further, sheep show a natural aversion to strong flavours and smells (Launchbaugh and Provenza, 1994; Baumont *et al*, 2000), probably because more intense flavours and smells are naturally associated with phytotoxin-containing plants (Launchbaugh and Provenza, 1994). However, these constraints are not absolute and sheep also appear able to learn associations between unusual pairings of flavour and smell, if exposure is maintained. For example, in a study to examine the effect of flavour strength on the maintenance of conditioned flavour aversions, lambs initially consumed less of the food containing the strongest concentration of oregano flavouring and showed aversion to this flavour when it was paired with a dose of LiCl. Subsequently however, lambs were given the flavoured feed that was not paired with LiCl for one day and on the next day they increased their intake of strongly flavoured feed. Thus when the strongly flavoured, but nutritious food was no longer associated with malaise, the lambs overcame their natural aversion to the strong flavour and the previously conditioned flavour aversion (Launchbaugh and Provenza, 1994).

Texture is also highly specific to particular feeds and it is considered likely that touch is used by sheep to distinguish feeds (Baumont *et al*, 2000). Evidence suggests that sheep tend to select feeds with physical characteristics that make them easier to manipulate and therefore increase rate of intake (Kenney and Black, 1984; Inoue *et al*, 1994). Again, this constraint is not absolute and sheep have been found to be able to learn associations between foods that are difficult to manipulate and therefore have a slow rate of ingestion, such as straw, and the highly nutritious stimulus of protein infusion (Villalba and Provenza, 2000b).

According to the argument that intrinsic characteristics of a feed are the most reliable cues to consequence, the location of the feed should not be as valuable an indicator as flavour, smell and texture as there is often no direct association between location and the nutritional value or toxicological status of the food (Forbes and Kyriazakis, 1995). However, evidence in chickens suggests that the location of the food can be used as a cue if sensory information about the food is limited (Steinruck *et al*, 1990 as cited by Forbes and Kyriazakis, 1995). In Steinruck's study, changing the position

of the test feeds resulted in a decline in selection of the preferred option. The preference was reinstated after a short time in which the location of the feeds was relearned. During foraging, in natural conditions with diverse feeding options, sheep must find a balance between many factors that have an impact on optimal diet selection, including the spatial distribution of plants within a feeding site (Baumont *et al*, 2000). Thus location of feeds may be relevant to diet selection in sheep, as the position of a desired feed may be learned over time. However, it is generally considered that food location is less valuable as a cue for the sub-conscious development of conditioned food associations and that the location-food association becomes the principle cue only when no other cue is available to distinguish feeds (Forbes and Kyriazakis,1995).

4.5.4.2. *Hedonic characteristics of feeds.*

The sensory characteristics of feeds may stimulate hedonically motivated feeding behaviours and change the strictly physiological basis of the control of diet selection (Baumont *et al*, 2000). Flavour, smell and texture are sensory stimuli for which animals may develop hedonic preferences independent of nutritive or toxicological considerations. This may explain why sheep often consume clover to beyond the point of satiety, which can cause illness as a result of over-ingestion of cyanogenic compounds (Parsons *et al*, 1994). However it appears that, in common with nutritionally influenced diet selection, sheep base hedonic selection on the degree of variety. For example, Wang and Provenza (1996) found that lambs ate more of their high-energy diet if it contained a variety of feeds, rather than one feed.

4.5.4.3. *Effect of experience on diet selection.*

As mentioned in section 4.5.3, food neophobia is often expressed when novel foods are offered. Sheep show a tendency to sample small amounts of a novel food until it is determined whether that food is associated with negative post-ingestion consequences (Launchbaugh and Provenza, 1994). It has been found that if familiar flavours and odours are mixed with novel foods, neophobia is reduced (Van Tien *et al*, 1999). Conditioning of an association between a novel feed and negative consequences occurs more easily than an association between a familiar and

previously harmless feed and negative consequences. For example, in a study by Burritt and Provenza (1991), lambs were given a selection of novel and familiar feeds paired with a dose of LiCl. When the learning of conditioned taste aversions were later tested, conditioned aversions were found with novel but not familiar feeds. If all the foods with which they were tested were familiar, the lambs avoided the food that they had eaten the most of during the conditioning meal (Phy and Provenza, 1996). There is some evidence that even flavours experienced in their mothers milk or *in utero* result in conditioned associations in lambs (Nolte and Provenza, 1992). Social experiences also influence preferences as lambs benefit from their mothers experience by learning what to eat from them. Thornhallsdottir *et al* (1987) showed that lambs housed individually ate more of a novel feed than singly housed ewes and than lambs housed singly with their ewe, even after the feed had been paired with LiCl infusion to make them ill. It has also been suggested that social influences can greatly reduce individual variability in food intake (Chapple *et al*, 1987a; 1987b; Forbes and Kyriazakis, 1995)

4.5.4.4. *Effect of delay on the development of conditioned associations.*

It is often considered that the consequences of consuming foods are only realised after some temporal delay because of the need for digestion and absorption of nutrients and toxins (Arsenos *et al*, 2000). This is in conflict with the traditional view of the rules of association and conditioning, that in order to learn an association, the consequence must be realised within a few seconds or minutes after the action (Roper, 1983). In order to investigate the ability of animals to learn despite delays in consequence, time delays have been incorporated into experimental paradigms. Zahorik and Houpt (1981) concluded that lambs do not possess the neurophysiological mechanisms for long-delay learning. However, more recent evidence suggests that sheep do indeed possess the capacity to learn negative and positive conditioned associations despite significant delays (30mins – 8 hours) in consequence (Burritt and Provenza, 1991; Arsenos *et al*, 2000).

4.5.5. *Selection of feeds to attenuate illness.*

As has been discussed in section 4.5.2, studies have shown that sheep are able to select food to minimise the effects of nutrient deficiencies (Kyriazakis *et al*, 1996) and to reduce the effects of excessive intake of nutrients (Phy and Provenza, 1998b). By extension, it is considered possible that animals can select foods for medicinal purposes. Observational evidence of food selection for medicinal purposes is widely reported in the primate literature. For example in three chimpanzee populations along the Western shores of Lake Tanganika, thirteen plant species have been identified as being selected for medicinal purposes. Individual chimpanzees showing symptoms of discomfort and disease have been observed to consume these plants and evidence, for example reduced faecal parasite egg counts, suggests that they do indeed have some medicinal properties. Many of these plants were also used by native human populations to reduce parasite burdens, relieve menstrual pain and headaches and attenuate malaise. At least half have been shown to have pharmacological properties consistent with claimed ethnomedicinal effects (Huffman and Wrangham, 1994).

4.6. *Chronic inflammatory pain and self-selection of analgesic.*

In the 1950s and 60s adjuvant-induced arthritis (AA) was developed in the rat in order to study the pathophysiology of arthritis and to determine the therapeutic effects of anti-inflammatory compounds on the condition (Colpaert *et al*, 1980). AA develops 11 to 16 days after the sub-cutaneous injection of heat-killed *Mycobacterium butyricum*, resulting in oedema and inflammation around paws (especially hind) and joints and radial swellings in the tail associated with each intervertebral disc (Colpaert, 1987). The development of the condition varies between individuals, but may persist for several months with the appearance of deforming joint lesions in some individuals (Pearson, 1963). Rheumatic disease causes chronic pain in human patients (Burckhardt, 1984) and AA in the rat is considered to be a satisfactory model of the condition. It was suggested that AA might also be useful as an experimental model of chronic inflammatory pain (Colpaert *et al*, 1980). However, Colpaert *et al* (1980) considered the evidence insufficient to move forward with studies in which the relevance of AA as a model of chronic pain was only assumed. In order to show unequivocally that AA was

associated with chronic pain, Colpaert *et al* (1980) exploited the ability of rats to learn conditioned associations between consumptive behaviour and post-ingestion consequences. It was argued that, in arthritic rats, consumption of a solution containing analgesic would result in pain relief, inducing a conditioned association between the characteristics of the fluid and analgesia. In a pre-test training period, during the first 7 days after adjuvant innoculation, control rats (n=24) and AA rats (n=24) were first familiarised with a palatable saccharin and glucose solution containing no analgesic provided for one hour each day. At the start of the next week the rats were deprived of water for two days. On the third day they were offered a second solution that contained suprofen (0.5mg/ml) and small amounts of sodium hydroxide during a one-hour conditioning period. On days 4 to 7 of the protocol the rats were given the opportunity to choose between these two fluids during an hour-long test period. No other fluid was available to the rats. Measures of body temperature, weight and paw diameter were made to assess the progress of the inflammatory process in rats with AA. The volume of each fluid consumed during the test was measured. The deprivation, conditioning and then choice protocol was continued until 77 days (11 weeks) after inoculation of AA rats. Following inoculation, the body temperature of adjuvant inoculated rats initially increased but then returned to near normal, before increasing again over the last 3 weeks of the study. The sum of the diameter of the hind paws and tibiotarsal joints was used as a measure of inflammation. Inflammation increased after the first week, rising to a peak between 3 and 4 weeks after inoculation when the mean diameter was nearly 25% greater than that in control animals. Inflammation then declined, before levelling out to a plateau at 6 weeks after inoculation until the end of the study. Inflammation in AA rats did not decline to reach the diameter measured in control rats. The body weight of AA rats declined dramatically during the first 4 weeks after innoculation, losing more than 20% of their body weight in this time. The weight of control rats showed no decline and increased gradually throughout the study. The lowest body weight in AA rats coincided with the peak in inflammation at 21-28 days after inoculation. AA rats were found to consume more suprofen solution than control animals, but this difference was only found to be significant during weeks 4-7 (28-49) after inoculation, slightly after the peak in inflammation. The difference in

suprofen consumption was greatest 5 weeks after inoculation when AA rats consumed 10% more than controls. Suprofen consumption then declined to near control levels by 8 weeks after inoculation. At the peak of consumption the volume of suprofen solution consumed provided a dose of 5.9mg/kg, a dose nearly 5 times that required to significantly reduce inflammation (Awouters *et al*, 1975 as cited in Colpaert *et al*, 1980). It was concluded that rats suffering from AA self-administered more suprofen solution than control rats and that the data were consistent with the hypothesis that AA is associated with significant chronic pain.

In discussion of these results, Colpaert *et al* (1980) raised several significant criticisms of their methodology which were later discussed again in analyses of a later study by the same author (Colpaert, 1987). Firstly, the relevance of the study as a means of demonstrating chronic pain was questioned as it is possible that the rats with AA may have selected suprofen in order to reduce inflammation rather than to reduce the experience of pain. This criticism was put forward because the dose of suprofen self-administered by rats with AA was considered to be sufficient to significantly attenuate inflammation (Awouters *et al*, 1975 as cited in Colpaert *et al*, 1980). The presence of a time-delay between the peak in paw diameter and the peak of suprofen intake is inconsistent with this idea. However it is has been argued that although paw diameter can give an indication of the progress of inflammation in AA, there are components of the inflammatory process that are not well represented by this measure of inflammation (Rainsford, 1982). It is therefore possible that suprofen intake was reinforced by alleviation of inflammatory processes and pain not indicated by paw diameter (Colpaert, 1987).

In 1980, Colpaert *et al* suggested that the time-delay between the peak in inflammation and the peak in suprofen intake in AA rats could be explained by the slow onset of effects of the NSAID, resulting in a delay in acquisition and extinction of the conditioned association. This is in accordance with previous studies in rats which indicated that the development of conditioned associations can occur despite delays in consequence, but that acquisition of the association can take longer under these conditions (Garcia and Koelling, 1966). It is also conceivable that a delay in consequences would result in a delay in extinction of the conditioned association as rats continued to seek reward. Unconditioned aversion to the taste of the suprofen

solution is likely to further delay the acquisition of a conditioned association between the aversive taste and positive post-ingestion consequences.

Colpaert (1987) suggested that because rats were given only sweet solution to drink in the week following inoculation, those with AA may have developed a conditioned aversion to its taste because of its association with the onset of disease. Thus whilst this conditioned aversion may not have been strong enough to overcome the unconditioned aversion to the suprofen solution, it may have resulted in slightly elevated suprofen intake in arthritic rats in comparison to controls.

Whilst it is conceivable that rats with AA consumed more suprofen than control rats in order to reduce pain or inflammation or because of conditioned aversion to sweet solution, an alternative explanation could be proposed, an idea supported by Shaman *et al* (1993). The development in the arthritic condition may result in changes in the relative hedonic or nutritive status of the suprofen and sweet solutions. In lambs, physiological imbalances caused by nutrient deficiency (Furness, 1988a; 1988b) and excess (Wang and Provenza, 1996) and by parasitism (Kyriazakis *et al*, 1996) induced changes in diet selection that attenuated adverse effects. Thus it seems reasonable to suppose that AA in the rats resulted in changes in the relative preference for the flavour of the two test solutions. Thus either preference for sweet solution might be reduced, indirectly increasing the intake of suprofen, or the preference for suprofen solution might increase. The pattern of change in relative preference would still be associated with that of the development and resolution of inflammation and could show a time lag with respect progression of inflammation, because of a delay between changes in the inflammatory process and realisation of adverse effects.

It should be noted that the differences in intake of suprofen solution between groups was less than 10% for the majority of the study and that the variability of intake within groups was high. Colpaert *et al* (1980) artificially reduced this variation within groups, by combining the data for the four tests days of each week and it was on this data that statistical analyses were based. Thus, the difference between groups was exaggerated. Because the variability in suprofen intake within groups was high, it is likely that the variation in the active concentration of the suprofen in the blood was also high. However, as the plasma concentration of suprofen was not measured,

the significance of differences in plasma suprofen between groups could not be determined. In a study of self-administration of carprofen in lame broiler chickens, the effect of variation in feed intake on the resulting plasma concentration of analgesic was demonstrated (Danbury *et al*, 2000). Danbury *et al* (2000) used a similar protocol to that described by Colpaert *et al*, 1980; 1982), using lame and sound broiler chickens with access to drugged and undrugged, differently coloured feeds. After a conditioning period, the birds were given a choice of the two feeds. A formula to accommodate the continuing overall increase in lameness was incorporated into the analysis and the results indicated that lame birds with access to analgesic ate significantly more analgesic feed than sound (control) birds ($P<0.001$) and showed a slight preference for analgesic feed over the undrugged alternative. However there was no significant difference in the plasma concentration of carprofen, suggesting that the differences in intake observed were insufficient to result in measurable differences in the active concentration of the drug in blood. Thus the evidence of self-administration was weakened.

In order to address some of their own criticisms of the original study, Colpaert *et al* (1982) demonstrated self-administration using the opiate analgesic fentanyl. Fentanyl has little anti-inflammatory action and a rapid onset of analgesic effects after oral administration (Niemegeers *et al*, 1976 as cited by Colpaert *et al*, 1982; Colpaert *et al*, 1982). Thus, the use of fentanyl eliminated the possibility that AA rats selected analgesic for anti-inflammatory properties and reduced the likelihood of a delay in acquisition of the association. The protocol was adapted from that in the previous study. Suprofen solution was replaced with fentanyl dissolved in tap water at a rate of 0.008mg/ml and two further control groups (one untreated (n=29) and one inoculated with adjuvant (n=28)) were included in a second experiment. In this second experiment, rats were not exposed to the analgesic solution but had a choice of only sweet solution or tap water, to control for differences in the hedonic and nutritive value of the solutions. The frequency of vocalisation was recorded as a measure of 'irritability', a behavioural sign characteristic of chronic pain in humans (Procacci *et al*, 1979). The intake of fentanyl solution in AA rats was initially higher than that in control animals and declined more slowly. Fentanyl intake in AA rats significantly exceeded that in control animals during weeks 3, 4 and 5 of the study. It

was also noted that the peak of fentanyl intake in AA rats occurred during the third week after inoculation and coincided with the peak of inflammation and thus there was no time-delay between decline in intake of analgesic and decline in inflammation. In the analgesic control groups, water consumption was very stable throughout the study and accounted for a mean of 7% of the total fluid intake in both control and AA rats. This was higher than the consumption of fentanyl dissolved in water by the control rats indicating that the taste of fentanyl itself was aversive. The intake of fentanyl solution in AA rats exceeded 7% of total fluid intake during the first four weeks after inoculation, indicating that AA rats selected more fentanyl than control rats despite the aversive flavour of the analgesic. The volume of analgesic fluid consumed by AA rats corresponds to a dose of 0.44-0.24mg/kg, a dose known to increase tail-flick latencies in acute pain studies (Niemegeers *et al*, 1976 as cited by Colpaert *et al*, 1982). However it was noted that this dose did not appear sufficient for control rats to overcome the aversive nature of the fentanyl solution and find the narcotic properties of the opioid solution reinforcing for hedonic reasons. A peak in the rate of vocalisation was observed that coincided with the onset and peak of inflammation and fentanyl intake and was also considered to be consistent with the presence of chronic pain. Similarly an initial sharp decline in body weight and subsequently lower growth rate, was observed in AA that did not receive analgesic. A reduced growth rate was also found in the previous study (Colpaert *et al*, 1980) and is significant as it is characteristic of a depression-like condition associated with chronic pain in humans (Zimmerman, 1986). A comparison of the weight of AA rats with and without access to analgesic would have provided a potential means of determining the significance of the effect of analgesic administration. However, the body weights of AA rats with access to analgesic were not measured and this comparison could not be made. Rats with AA vocalised more often than control animals in this study (Colpaert *et al*, 1982). Because fentanyl has no anti-inflammatory action, the increased intake of the analgesic solution in this study could not have been induced by conditioned association of an anti-inflammatory effect. Further, arthritic rats with a choice of sweet solution and water (but no analgesic) did not select more water than non-arthritic rats. This evidence refutes the suggestion that rats developed a conditioned aversion to the sweet solution during the post-

inoculation familiarisation period, inducing a measurable increase in consumption of analgesic solution. The evidence given in this study does not answer the criticism that the relative hedonic status of sweet versus analgesic solution might be altered by the arthritic condition. This effect could also be closely associated with the temporal progression of the disease.

Also, as in the previous study, the variation within groups was such that a significant effect of AA on fentanyl intake could only be found when the data for the four days of choice in each week were combined, thus artificially reducing variation. Again the plasma concentration of analgesic was not measured. The dose of fentanyl reached a maximum of 0.44mg/kg, a dose sufficient to increase thresholds in tail-flick latency tests. However fentanyl intake only differed between groups by a maximum of 11% throughout the study. Again concentrations of fentanyl in blood were not measured and it is therefore not known whether the small difference in intake of fentanyl, accompanied with wide variation, resulted in a significant difference in the plasma concentration of fentanyl between groups.

In a further study reported in 2001, Colpaert *et al* incorporated many more controls into the self-administration protocol in order to show with more certainty that self-administration of fentanyl occurred as a result of analgesic reinforcement in a chronic pain condition and not as a result of hedonic sensations associated with the opioid analgesic. Using the self-administration protocol reported in two previous studies Colpaert *et al* 1980, 1982), rats were given the opportunity to experience the effects of the consumption of fentanyl solution in a conditioning period. They were then given the choice between the analgesic solution and a familiar and highly palatable sweet solution containing glucose and saccharin in a preference test. Within this experimental paradigm, further treatment groups (n=9-17) were included in order to control for opioid dependence in control and arthritic rats. In one group, rats were fitted with a sub-cutaneous osmotic mini-pump that delivered the steroid dexamethazone at a constant rate. Similarly pumps delivering naloxone hydrochloride and fentanyl citrate were implanted sub-cutaneously in other groups. The pumps were implanted on day 14 and removed again on day 28 after inoculation and the effects of these treatments on the occurrence of self-administration of fentanyl were recorded. It was concluded that self-administration had occurred,

reducing the sensation of pain from AA. Firstly AA rats consumed more fentanyl solution than control animals, in keeping with previous studies. Continuous infusion of dexamethazone, dramatically reduced inflammation and resulted in a dose dependant decline in intake of fentanyl solution in AA rats. At the highest dose of dexamethazone, fentanyl intake was reduced to the same level observed in control rats and is consistent with a reduction in pain caused by the steroid treatment. Infusion of naloxone also reduced fentanyl self-administration, although by less than dexamethazone (55% and 65% reductions respectively). However, it is known that intermittent reinforcement of a behaviour once linked with positive reinforcement, results in a more stable maintenance of that behaviour than a reliable reinforcement schedule (Roper, 1983). This may explain why naloxone treatment did not result in total loss of the self-administration phenomenon. However, according to this theory an initial increase in fentanyl consumption would have been expected.

Infusion with fentanyl also resulted in a decline in fentanyl self-administration. This result indicates that self-administration occurred as a result of analgesic reinforcement of fentanyl consumption and not because of hedonic reinforcing properties of the opioid solution. Both dexamethazone and fentanyl treatment resulted in an increase in the overall consumption of fluid during the hour-long test period whilst naloxone treatment did not. This result refutes the suggestion (Shaham *et al*, 1993) that the perception of the taste of the analgesic solution is altered somehow in the arthritic condition resulting in hedonic reinforcement of the solution in arthritic rats.

A criticism, identified by Colpaert (1987), that applies to all these studies and remains unanswered by changes in protocol is that, whilst self-administration of analgesic may indeed represent the presence of chronic intractable pain from AA, it could also be a reflection of the presence of acute secondary pain as a result of hyperalgesia and allodynia. The presence of chronic pain cannot therefore be determined absolutely from these studies.

4.7. *Conclusion*

In conclusion, whilst preference testing and the ability of animals to learn conditioned associations may be used to aid determination of subjective experiences,

these studies are subject to many species and individual specific constraints that limit conclusions. Despite this, these techniques have been used with success to study factors involved in the selection of foods in sheep and many of the behavioural and physiological mechanisms that result in specific selections have been deduced. It has been shown that sheep are able to learn to select feeds on the basis of learned positive and negative associations between characteristics of the feed and the nutritive and toxic effects of their consumption. In contrast with traditional views of associative learning, it has also been shown that sheep are capable of learning these associations when there is a considerable delay between cue and consequence. Previous experience, social influences and natural constraints on learning modify preferences and the aversions shown by individuals and these effects must be controlled for in preference studies. The ability of rats to learn conditioned associations between the characteristics of fluids and the effects of their consumption has been used in preference studies to determine the presence of chronic inflammatory pain from AA. These studies indicate that rats are able to learn the association between a novel-tasting liquid containing an analgesic and its pain-relieving properties, thus indicating the experience of pain in rats with AA.

Chapter 5

General Methodology for Self-administration studies

Chapter 5. Self-administration studies: General Methodology

5.1. *Animals and management*

Single, male lambs were housed in straw bedded pens (1.6 x 3.2m) with their dams. Pens were adjacent to one another and encompassed a creep area in which lambs could obtain feed without competition from the ewe. In keeping with general management procedures (see Section 2.2), the animals had *ad libitum* access to hay and fresh water at all times. The ewes were fed 500g of ESCA Ewe Nuts (SAC, Seafield Mill, protein 18%, fibre 8%) daily. Lambs had access to their dam to suck milk. Lambs also had access to creep feed (Nustart Lamb Creep Pellets, Pye-Frankland Balanced Feeds, protein 18%, fibre 8%) which was provided in accordance with the protocol required for the self-administration experiments.

5.2. *Experimental feeds*

Two experimental feeds were used in the self-administration protocol:

The un-drugged feed was commercially available creep feed (Nustart) with nothing added. To produce analgesic feed, powdered creep feed pellets (Nustart) were mixed with Finadyne® granules (flunixin meglumine, Shering-Plough Animal Health) and then repelleted (Roslin Institute Feed Mill). In these studies Flunixin meglumine (FM) was to be administered orally at a dose of 1.1mg/kg of body weight. The drug was incorporated so that 1.1mg of FM was contained within 2.8g of re-pelleted creep feed. A full dose for a 40 kg lamb was therefore contained in 112g of feed. The re-pelleted creep pellets were the same diameter as the original creep feed but were paler in colour. It was considered likely that the analgesic feed had a different taste to the un-drugged feed although no attempt was made to confirm or define this taste difference.

5.3. *Treatments*

The 28 lambs were weighed and assigned to four groups so that live-weight was balanced across groups, with seven lambs in each group (n=7). The location of the lambs within the barn was balanced as far as possible so that an equal number of lambs from each group were positioned towards the outside, inside and close to

aisles. This was to attempt to ensure that any variables resulting from the experimental environment had an equal effect across groups.

The animals were brought into the barn one week prior to any treatment to ensure that they were acclimatised to the environment and that they were eating creep feed. The daily consumption of creep feed was recorded, for each lamb, during the week prior to the beginning of the experiment, so that the amount of creep feed available could be adjusted to account for appetite.

Two groups of lambs were castrated and tail-docked (C) using tight rubber rings. Two groups of lambs were handled (H), as necessary for castration and tail-docking, but no rings were applied. (A full description of the methods for castration and tail-docking and handling of controls can be found in Section 2.7.)

One castrated and tail-docked (C) group and one handled (H) group of lambs were then assigned to 'drugged' (D) treatment. These animals had access to one hopper containing analgesic feed and a second hopper containing un-drugged feed during the self-administration experiment. The second castrated and tail-docked (C) group and the second handled (H) group of lambs were assigned to 'non-drugged' (N) treatment. These animals had access to two hoppers, both containing un-drugged feed during the self administration experiment. Both hoppers were placed at the back of the creep area for all lambs. These treatments are outlined in Table 5.1.

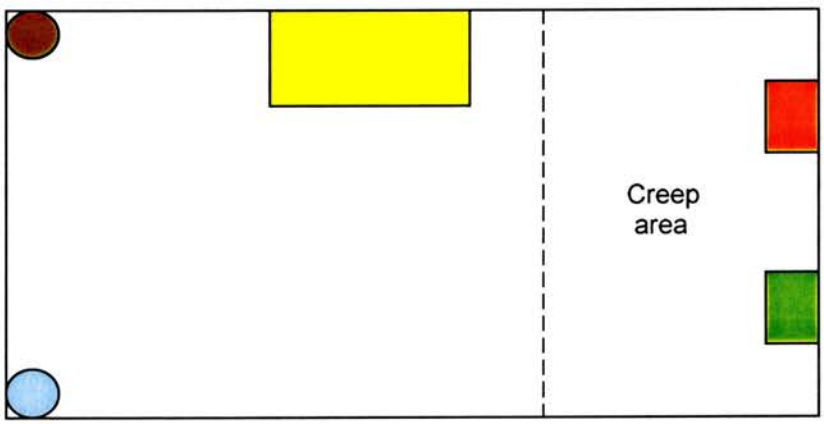
Table 5.1. Treatments used for each of the four groups of lambs during self-administration of analgesic experiments.

		CASTRATION TREATMENT	
		Castrated and Tail-docked (C)	Handled Control (H)
DRUG TREATMENT	DRUGGED- (D) Choice of analgesic feed and un-drugged feed in preference test.	N= 7 lambs Castrated and tail-docked Drugged **(CD)**	N= 7 lambs Handled Control Drugged **(HD)**
	NON-DRUGGED- (N) Two hoppers containing un-drugged feed in preference test.	N= 7 lambs Castrated and tail-docked Non-drugged **(CN)**	N= 7 lambs Handled Control Non-drugged **(HN)**

5.4. *Pen layout for experimental procedure*

Figure 5.1 (a) A diagrammatic representation of the pen layout during the self-administration experiments. The lamb and ewe had access to water (blue) and hay (yellow). The ewe was fed her daily ration in a feed bucket (brown) to which the lamb had limited access. A creep gate divided the pen into two areas so that the lamb had access to the whole pen but the ewe could not enter a test area where the test feeds were available. The hoppers containing the analgesic (red) and un-drugged (green) test feeds were placed at the back of the pen, but the side at which the different feeds were placed was balanced across groups. CN and HN lambs were given two hoppers containing un-drugged feed. (b) Photograph showing pen layout during self-administration experiments.

(a)



(b)

5.5. *Colorimetric determination of total plasma protein and albumin*

The total concentration of protein and the concentration of albumin in the plasma of the lambs was determined by quantitative colorimetric analysis. The long-term administration of flunixin meglumine, a non-steroidal anti-inflammatory drug, may cause adverse effects. Increased plasma protein may indicate dehydration potentially caused by reduced absorption of liquid, because of reduced integrity of the gut wall. Total plasma protein and albumin were therefore measured to provide some indication of any adverse effects of the NSAID.

5.5.1. *Assay for total protein*

The total protein analysis kit (Sigma Diagnostics) is based on the biuret reaction, where the copper ions in the blue alkaline reagent, react with the peptide bonds of the proteins producing a purple solution with a maximum absorbance at 540nm. The intensity of the colour is proportional to the concentration of protein in solution.

5.5.2. *Total protein assay protocol*

Plasma samples were removed from storage at -20°C and thawed at room temperature. They were inverted several times once thawed.

10 tubes were labelled blank (B) (distilled water), two controls (C1 and C2) (one of normal and one of abnormal protein concentration (Accutrol™ Sigma Diagnostics), and protein standards (S1-S5) (concentrations of 2, 4, 6, 8, and 10 g/L) (Sigma Diagnostics). Sufficient tubes were also labelled for each animal sample (1,2…).

1.0ml of total protein reagent (Sigma Diagnostics) was aliquoted into each tube. 20µl of distilled water, was aliquoted into tube B. 20µl of normal protein concentration control was aliquoted into tube C1 and 20µl of abnormal protein concentration control was aliquoted into tube C2. 20µl of each standard was aliquoted into tubes S1-S5. Finally 20µl of each sample was aliquoted into the sample tubes. The tubes were then inverted gently several times to ensure thorough mixing. The tubes were incubated for 10 minutes at room temperature.

200µl from each tube was aliquoted, in duplicate, into a 96 well plate for measurement of absorbance (Dynex MRX TCII Microplate Reader and Revelation software, © Dynex Technologies U.K. Ltd). The absorbance was measured at

540nm. The total protein concentration was then calculated according to the following equation.

Total Protein (g/L) = $(A_{sam}/A_{standard}) \times C_{standard}$

where A_{sam} = absorbance of sample

$A_{standard}$= absorbance of standard

$C_{standard}$= concentration of standard

5.5.3. *Assay for albumin*

The albumin reagent (Sigma Diagnostics) used contains bromcresol green (BCG). Albumin binds to BCG producing a blue-green colour with a maximum absorbance at 628nm.

5.5.4. *Albumin assay protocol*

Plasma samples were removed from storage at -20°C and thawed at room temperature. They were inverted several times once thawed.

10 tubes were labelled blank (B) (distilled water), two controls (C1 and C2) (one of normal and one of abnormal albumin concentration (Accutrol™ Sigma Diagnostics), and albumin standards (S1-S5) (concentrations of 2, 4, 6, 8, and 10 g/L Sigma Diagnostics). Sufficient tubes were also labelled for each animal sample (1,2...).

1.0ml of BCG (Sigma Diagnostics) was aliquoted into each tube. 10µl of distilled water, was aliquoted into tube B. 10µl of normal albumin concentration control was aliquoted into tube C1 and 10µl of abnormal albumin concentration control was aliquoted into tube C2. 10µl of each standard was aliquoted into tubes S1-S5. Finally 10µl of each sample was aliquoted into the sample tubes. The tubes were then inverted gently several times to ensure thorough mixing. 200µl from each tube was aliquoted, in duplicate, into a 96 well plate for measurement of absorbance (Dynex MRX TCII Microplate Reader and Revelation software, © Dynex Technologies U.K. Ltd). The absorbance was measured at 628nm within 1 minute. Albumin concentration was then calculated according to the following equation.

$$\text{Albumin (g/L)} = [(A_{sam} - A_{blank})/(A_{standard} - A_{blank})] \times C_{standard}$$

where A_{sam} = absorbance of sample

$A_{standard}$= absorbance of standard

A_{blank} = absorbance of blank

$C_{standard}$= concentration of standard

Chapter 6

Self-administration of Analgesic to determine Chronic Inflammatory Pain 1

Chapter 6. Self-administration of analgesic to determine the presence of chronic inflammatory pain from castration and tail-docking of lambs.

6.1. *Introduction*

Feed aversion, the avoidance of foods that have been associated with a negative consequence after consumption, is a well-known phenomenon that has been induced experimentally in many species including sheep (Garcia and Koelling, 1966; Kronberg *et al*, 1993; Thornhallsdottir *et al*, 1987; Olsen *et al*, 1989; Zahorik *et al*, 1990; Nolte *et al*, 1998). More controversially, animals (including sheep) have been reported to selectively consume food in order to obtain specific nutrients or medicinal properties in circumstances of naturally occurring or experimentally induced ill-health (Furness, 1988a; 1988b; Huffman and Wrangham, 1994; Kyriazakis *et al*, 1996; Ramirez, 1996; Arsenos and Kyriazakis, 1999). Thus, these animals are considered to be capable of learning an association between the act of consuming a feed and either a negative or positive consequence of that action. It has been shown that sheep are capable of making this association even when the consequence of consuming the food is temporally dissociated from the act of food consumption by between 30 minutes and 8 hours (Burritt and Provenza, 1991; Arsenos *et al*, 2000).

In the early 1980s Colpaert *et al* (1980; 1982) used the ability of rats to make such associations to validate the use of experimentally-induced arthritis as a model of inflammatory pain. Colpaert *et al* (1980) postulated that, if rats with adjuvant-induced arthritis experienced chronic inflammatory pain, they would learn the association between the positive pain-relieving consequences of consuming a liquid containing analgesic. Although variation between individuals in these studies was high and no measure of the concentration of the analgesic in the blood was taken, rats that had been inoculated with *Mycobacterium butyricum* consumed slightly more of the analgesic fluid than did vehicle-inoculated control animals. Colpaert *et al* (1980; 1982) concluded that these rats had indeed learned the association between analgesia and consumption of a novel-tasting liquid. The results of these early investigations were recently confirmed in a more conclusive study (Colpaert *et al*,

2001). Using the same experimental paradigm as before, Colpaert *et al* (2001) again found that rats with experimentally induced arthritis consumed more of the analgesic liquid than control animals. However, when inflammatory pain was reduced by the infusion of fentanyl citrate, naloxone and dexamethasone from subcutaneously implanted pumps, the selection of analgesic fluid over a highly palatable sucrose solution diminished, indicating that the initial selection of the analgesic fluid was likely to have been associated with the consequent induction of analgesia.

During the first 6 weeks after c+td by RR, lambs express behaviours that have been validated for the assessment of acute pain (Kent *et al*, 2000). These behaviours are expressed infrequently (by comparison with the acute behavioural response), however the presence of ths behaviour is associated with chronic inflammatory lesions resulting from c+td and suggests that lambs may experience chronic inflammatory pain. The expression of such behaviours in sheep may be considered particularly significant, as the behavioural indication of disease in this prey species should be considered maladaptive (Bateson, 1991). However, it is also possible that the intermittent expression of 'pain' behaviours is indicative of only mild discomfort or transient irritation. Consequently the use of these behaviours to assess chronic pain must be validated.

The purpose of this study is to determine whether the chronic inflammatory lesions resulting from RR c+td are associated with chronic pain, which the presence of 'pain' behaviours would suggest, and to validate the use of these behaviours to assess this pain. As lambs have been shown to be capable of learning the association between the consumption of a feed and subsequent beneficial effects, it is hypothesised that lambs showing pain behaviours in association with chronic inflammatory lesions experience pain and that they will demonstrate their experience of pain by learning the positive post-ingestion consequences of consuming a novel feed containing an analgesic. A pilot study was carried out by Rutherford (1999), investigating the ability of c+td lambs to self-administer the non-steroidal anti-inflammatory drug (NSAID), flunixin meglumine (Finadyne © Shering Plough Animal Health). In Rutherford's study c+td and handled control lambs were given a training period starting 7 days after c+td. On the first day of training lambs were

offered a feed containing flunixin meglumine in a hopper at one side of the back of the pen. The following day an undrugged feed was offered in a different hopper placed at the other side of the back of the pen. Training continued alternating feeds each day for eight days. The lambs were then offered a choice of both feeds over a three-week period until lesions resulting from RR c+td had healed. Both the feed containing flunixin meglumine and the undrugged feed were also mixed with grass meal in an effort to avoid neophobia of the drugged feed (Van Tien *et al*, 1999). A quantity of each of these feeds sufficient to give lambs a 1.1mg/kg dose of NSAID was sprinkled over ordinary creep feed in each of the two hoppers. The behaviour of the lambs was observed weekly throughout the study using methods described by (Molony *et al*, 1995). The severity of lesions from c+td was also recorded using methods described by (Kent *et al*, 2000).

The presence of a self-administration phenomenon was not proven in Rutherford's study. Several factors in the methodology are considered to have reduced the power of the study. Initially, when lambs were 15-18 days old, the volume of feed they consumed was small and potentially insufficient for lambs to have consumed an efficacious dose of analgesic creep feed during training. Also the variation in the weight of feed consumed was very high throughout the study (e.g. 113.2 ±132.2g in one of the pens). This may have been related to high variation in the severity of the lesions. However, full results of lesion severity were not presented and thus this interpretation of the data could not be tested. As the lambs grew older they ate more feed and the relative proportion of feed taken from the analgesic hopper decreased. The feed hoppers were weighed 24 hours after the daily allowance of feed was given. It is considered likely that the critical selection of feed occurred during the first 3-4 hours within the 24-hour period and that any preference may have been disguised by prolonged access to the feeds. Furthermore, lambs appeared to consume feed containing grass meal selectively (Rutherford, personal communication) and therefore any motivation to consume feed containing NSAID may have been disguised by a confounding motivation to eat feed containing grass meal that was available in both hoppers.

Despite these conflicting issues, small differences in the weight of analgesic feed consumed were detected. During the first week of choice testing (15-20 days after

c+td) c+td lambs ate a significantly greater proportion of their feed from the hopper containing NSAID creep feed. Handled control lambs showed no significant preference for food from either hopper. However, after the first week of choice testing and until the lesions had healed (22-37 days after c+td) neither c+td lambs nor handled controls showed a preference for either feed. There were some differences in the behaviour of c+td lambs in comparison with handled controls, particularly in the incidence of easing quarters. It was suggested that these differences were associated with chronic inflammatory pain.

The methodology of the present study is based on that described by Rutherford (1999). However, several important changes were incorporated in order to reduce the degree of variation and thus increase the power of the experiment. Firstly, lambs were c+td at 4 weeks of age, and were trained at 5-6 weeks of age, in an effort to ensure that they would be consuming sufficient creep feed during training and to provide the best possible opportunity for the lambs to learn the post-ingestion consequences of consuming analgesic creep feed. The increase in age and therefore size was also expected to result in more severe c+td lesions (Kent *et al*, 1999), thus ensuring that all lambs developed lesions of sufficient severity to be more consistently associated with 'pain' behaviours (Kent *et al*, 1999) and the experience of putative chronic pain. No flavouring was used to disguise the taste or smell of the NSAID, thus removing the potential for a confounding preference of this flavour and at the same time providing a cue, in addition to that of location, by which lambs could identify the feeds. Evidence suggests that, whilst lambs may be able to use location as a cue to distinguish between foods (Forbes and Kyriazakis, 1995; Baumont *et al*, 2000), the cues of taste and smell are more reliably associated with the consequences of feed consumption and therefore the association between smell and taste and post-ingestion consequences should be easier for the lambs to learn (Launchbaugh and Provenza, 1994).

6.2. Methodology

6.2.1. *Animals and management*

28 four-week-old, single, male lambs (Greyface x Suffolk) were housed, with their dams, in adjacent, straw-bedded pens (1.6 x 3.2m) with a separate creep area (in accordance with management procedures outlined in Section 5.1). In keeping with general management procedures (see Section 2.2), the animals had *ad libitum* access to hay and fresh water. Ewes were fed 500g of concentrated feed daily and lambs had access to their dam to suck milk.

6.2.2. *Experimental feeds*

Two experimental feeds were used for the self-administration protocol.

The un-drugged feed was simply commercially available creep feed (Nustart Lamb Creep Pellets, Pye-Frankland Balanced Feeds, protein 18%, fibre 8%)

The analgesic feed was made by combining this creep feed with Finadyne® granules and repelleting the mixture as described in Section 5.2. The re-pelleted creep feed was slightly paler than the original creep feed and was more brittle. It is also considered likely that this feed had a distinctive taste and smell.

6.2.3. *Treatments*

The 28 lambs were weighed and assigned to four groups, each with 7 lambs, so that live-weight was balanced across the groups. The location of the lambs within the barn was balanced to ensure that any variables resulting from the experimental environment had an equal effect across groups.

The daily consumption of creep feed was recorded for each lamb during the week prior to the beginning of the experiment so that the volume of creep feed available could be adjusted to account for appetite.

The treatment groups were as described in Section 5.3. and Table 5.1

6.2.4. *Self-administration protocol*

The self-administration protocol was started 6 days after castration. During the first 8 days of the protocol, the lambs were subject to a training regime where they were

given the opportunity to learn the associations between the benefits of consuming either analgesic or un-drugged feeds and the location and taste of these feeds.
Subsequently lambs were offered a choice of both analgesic and un-drugged feeds for five days in each week. The comparative volumes of analgesic and un-drugged feed consumed were measured on choice days, providing an estimate of preference.
On the last two days of each week, all lambs were given access to only un-drugged feed, to minimise any unwanted effects of the long-term administration of NSAID and in accordance with the manufacturer's recommendations for its use in horses.
The lambs were weighed once each week to ensure the correct dose of analgesic could be administered to each lamb.
The self-administration protocol was continued for 6 weeks while the chronic inflammatory lesions developed and then healed.

6.2.4.1. *Pen layout for experimental procedure*
The layout of the pen was as described in section 5.4

6.2.4.2. *Training Regime*
Two hoppers were introduced to each pen at the left and right sides of the back gate of the creep area, approximately 0.50m apart, as illustrated in Figure 5.4.
On the first two days of training, lambs with access to analgesic feed (CD and HD) were given a quantity of analgesic feed (sufficient to provide a dose of 1.1mg/kg body weight) placed in one hopper on top of un-drugged feed. The total amount in the hopper was equal to the daily allowance of feed for each lamb. The second hopper was left empty.
On the third and fourth training days, the hopper that had contained analgesic feed was left empty. The second hopper was filled with a volume of un-drugged feed equal to the daily allowance for each lamb. The hopper in which analgesic feed was placed did not change nor did the position of the hoppers within the pen.
The lambs in groups with no access to analgesic feed (CN and HN) were also given two hoppers at the back of the creep area. On the first two days, one hopper was filled with a volume of un-drugged feed, equal to the daily allowance for each lamb, and again the second hopper remained empty. On the third and fourth days of

training the first hopper was left empty and the second hopper was filled with a volume of un-drugged feed equal to the daily allowance for each lamb. Again, the position of the hoppers within the pen was not changed. The hopper containing feed was thus alternated every two days for the eight training days.

In order to show preference, the lambs had to learn to make the association between the benefits of consuming analgesic or un-drugged experimental feeds and the sensory characteristics of the feeds (taste, smell, texture and location (left or right)). To facilitate learning, it was essential that the lambs consumed the entire dose of analgesic feed during training. The quantity of feed available during training was thus restricted to half the appetite of each lamb. The hoppers were removed from the pen and weighed after 3 and 23 hours.

6.2.4.3. *Preference testing*

Following training, lambs were offered a choice of both experimental feeds in the designated hoppers on the first five days of each week. Each hopper contained a volume of feed equivalent to the full daily allowance so that lambs could obtain all their food from one hopper. The hoppers were removed from the pen and weighed after 3 and 23 hours.

During preference testing no restrictions were made on the volume of feed consumed, up to a maximum daily allowance of 500g. The lambs therefore, with access to both hoppers, could consume up to 1000g of creep feed each day.

The side (left or right) of the pen at which analgesic feed could be found for each lamb was selected at random using random number generation (Microsoft Excel). For the purposes of analysis an 'analgesic' hopper was nominated at random for lambs in the un-drugged groups (CN and HN). The proportion of the total feed consumed that was taken from the analgesic hopper (CD+HD) or nominated analgesic hopper (CN+HN) was calculated and used in statistical comparisons.

6.2.5. *Behaviour at hoppers*

In order to determine, not only the choice of feed that lambs selected, but also the way in which the selection was made, the behaviour of lambs at the hoppers was

filmed, using CCTV cameras, for subsequent analysis. The latency, frequency and duration of visits to each hopper was recorded.

6.2.6. *Lesion assessment*

The chronic inflammatory lesions resulting from c+td were examined and assessed twice each week. Each lamb was caught and inverted. The presence or absence of the tail and scrotum were noted. The width of the lesion was measured, using callipers, to the nearest millimetre. Subjective assessment of the severity of the lesion was made using the scale described by Kent *et al* (2000) as described in Table 2.1. Using this system, the severity of the lesion was scored on a scale of 0 - 5 in increments of 0.5.

6.2.7. *Behavioural analysis*

Observations of behaviour were made, once each week, on the second day of preference testing. Two observation periods, for 2 hours each, were made between 14.00 and 19.00. The methods used are explained fully in Section 2.8. Three experienced observers were spaced around the penned area on raised platforms to ensure that every animal could be observed from at least two angles at all times. The animals were divided into 3 manageable batches for observation (one group of 12 lambs and two groups of 8). Each batch included an equal number of animals from each treatment group. Observations were recorded directly onto computers (Dell Latitude Laptops) using The Observer behavioural analysis software (Noldus Information Technology). All observations were made according to the ethogram shown in Table 2.2. The behaviours 'chew' and 'horn' were omitted from the ethogram on this occasion because these behaviours were not observed in this breed of lamb. The frequencies of active behaviours were recorded, continuously, throughout the observation time. Postures of lambs were recorded during scan samples every 6 minutes. At each scan, the lamb's posture and behaviour was recorded.

Two of the observers were the normal handlers of the animals. During the week before the start of the experiment these handlers spent as much time as possible in the barn with the animals in an effort to minimise the effect of the observers on the

sheep during behavioural observations. Observers were seated on the platforms, above the sheep, for at least 5 minutes before observations started, allowing the animals time to settle.

In between the two observation periods, the hoppers were weighed (3 hour sample), the lambs were blood sampled and assessments of chronic inflammatory lesions were made.

6.2.8. *Blood samples*

4ml blood samples were taken on the second preference test day (according to the methods described in Section 2.5), between 3 and 4 hours after the test feeds were first placed in the pen, between the two behavioural observation periods and after the hoppers had been weighed. The samples were immediately cooled to <4°C on ice and were centrifuged at 3000rpm for ten minutes, within ten minutes of drawing. Plasma was transferred to labelled plastic tubes and stored at -20°C until required for analysis.

6.2.9. *Colorimetric determination of total protein in plasma*

The concentration of total plasma protein was determined using quantitative colorimetric assay as described in Section 5.5.

6.2.10. *High performance liquid chromatography for extraction and quantification of flunixin meglumine in plasma.*

The plasma concentration of flunixin meglumine for all lambs receiving drugged feed, was measured using isocratic, reverse-phase high performance liquid chromatography (HPLC) as described in section 2.6.

6.2.11. *Statistical analysis*

General linear models (GLM) were used to determine differences between groups in the weight of lambs, the consumption of feed during training, the weight of creep feed eaten during choice, the proportion of feed eaten from the analgesic hopper (first transformed using log-tan transformation), the frequency of visits and proportion of total duration spent at the analgesic hopper (transformed using log-tan

transformation) during choice and the concentrations of flunixin meglumine (after square root transformation) and total protein in plasma. Further GLMs were used to examine the effects of analgesic feed consumption during training (as a covariate in the analysis) on the proportion of feed taken from the analgesic hopper (transformed using log tan transformation). In this analysis, a regression is used initially to determine the effects of the covariate before ANOVA is used to examine the differences between groups resulting from treatments. GLMs test the significance of the effects of c+td and analgesic treatments and the interaction between the two treatments on the variable in question. GLMs were carried out for each sample point during the study. Fisher's approach was used *post-hoc* to determine where the differences lay.

Student's t-tests were used to compare the width of castration lesions and Mann-Whitney U tests to compare the severity scores for c+td lesions. Regression analysis was used to identify any relationship between the size of the lesion and the proportion of feed consumed from the analgesic hopper (no transformation).

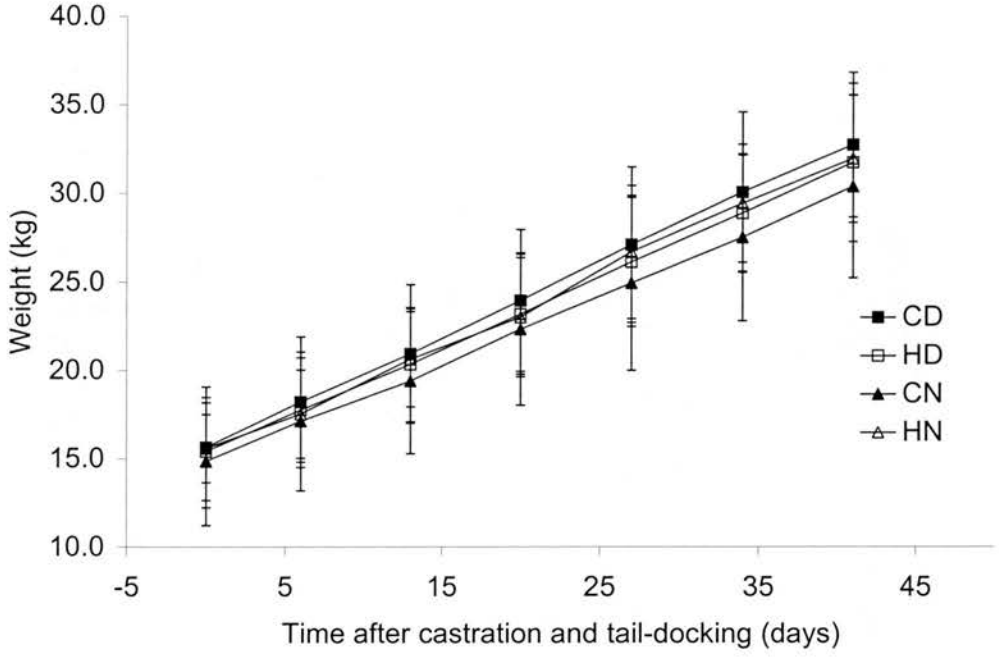
Genstat (version 5) was used to carry out generalised linear models on behavioural data. Differences identified using the generalised linear model were examined more closely *post-hoc* using Fisher's approach to determine where differences lay. Data on active behaviours were analysed using Poisson distribution and the logarithmic link function, whilst data on posture were analysed using the Binomial proportions model and Logit transformation provided in the Genstat package.

6.3. Results

6.3.1. *Weight of lambs*

At the start of the study the lambs were divided into groups with their weights balanced across treatments. Thus, there were no initial statistically significant differences in the weight of lambs between treatments at $P<0.05$. The average weight of lambs in each group more than doubled from approximately 15kg to 31kg over the six weeks of the trial. There was some evidence that lambs in group CN put on weight more slowly, thus their weight was increasingly lower than that of the other lambs (see figure 6.1). However, this effect did not reach statistical significance at any time in the trial.

Figure 6.1. Change in live-weight (kg) of lambs over the duration of the trial, expressed as mean (±sd), n=7.



6.3.2. *Training*

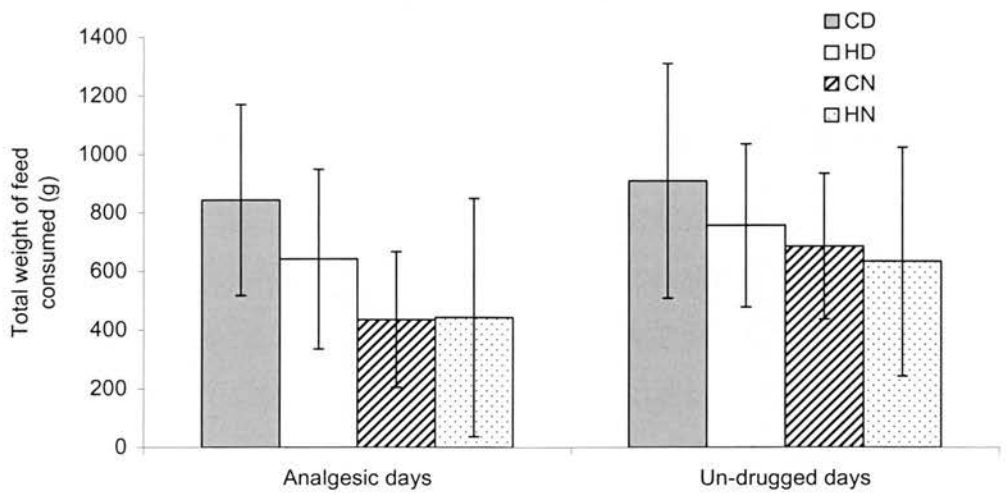
The consumption of feed on the four days when lambs had access to the analgesic hopper was summed to give a total for feed consumption for each lamb on analgesic training days. The same was done for feed consumption on un-drugged days. These results are shown in figure 6.2. On analgesic training days, when the lambs in groups CD and HD had access to analgesic creep feed, the total feed consumption was

significantly affected by drug treatment ($F_{3,25}$= 6.17, P=0.020), although c+td did not have a significant effect at P>0.05. Further analysis using Fisher's approach revealed that CD lambs ate significantly more in total than those groups without access to analgesic ($t_{3,25}$=6.13, P>0.0001 and $t_{3,25}$=6.25, P<0.0001 for HN and CN lambs respectively). HD lambs also ate significantly more in total than both groups of lambs without access to analgesic ($t_{3,25}$=3.04, P=0.0054 and $t_{3,25}$=-3.16, P=0.0041 for HN and CN lambs respectively). Although no difference in the total weight of feed consumed on 'analgesic' training days was found between CN and HN lambs ($t_{3,25}$=-0.11, P=0.9107), it was noted that CD lambs ate significantly more than HD lambs ($t_{3,25}$=3.09, P=0.0049).

Thus, on training days where drugged food was available to CD and HD lambs, these lambs ate more than those that had access to only un-drugged feed (groups CN and HN). In contrast, on days when only un-drugged feed was available, there was no significant effect of c+td, drug or the interaction between them on the quantity of feed consumed. The trends in food consumption remained the same however.

Although all lambs consumed feed on days when access to only the 'analgesic' hopper was offered, the variation within groups in the weight of feed consumed during training was high. Some lambs ate very little of either feed during training.

Figure 6.2. Mean (±sd) total feed consumption (g) for analgesic and un-drugged training days (n=7).

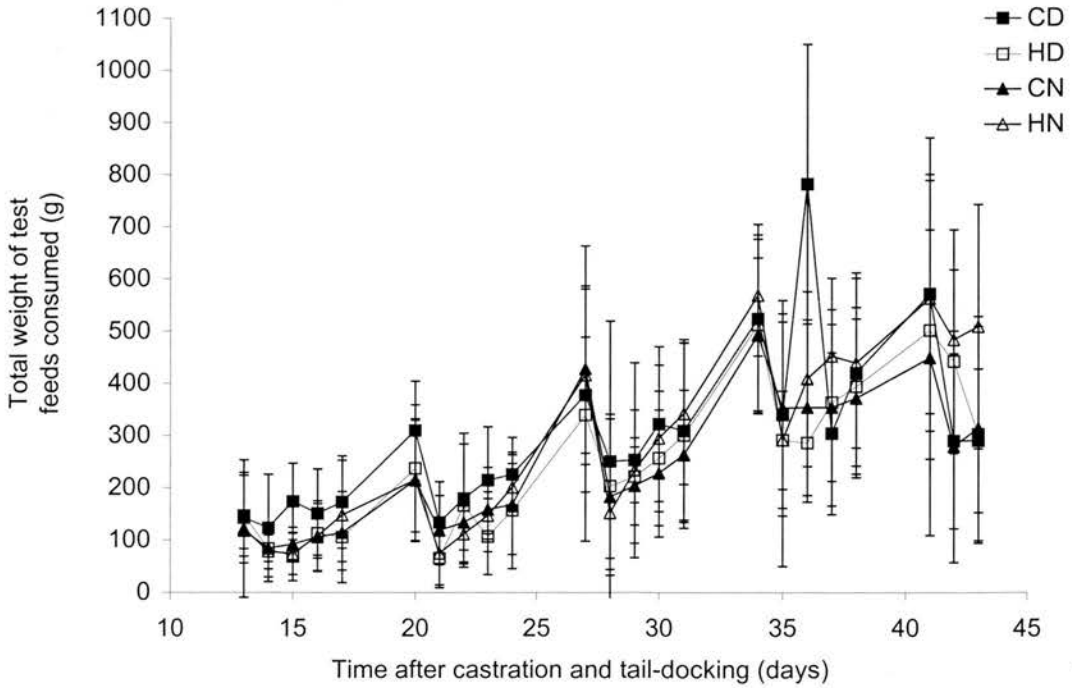


6.3.3. *Choice test*

Total weight of feed consumed

The total weight of feed consumed throughout choice testing is shown in figure 6.3. There was little difference in the total weight of feed consumed by lambs in each group. However, on day 21 c+td lambs consumed significantly more feed than handled controls ($F_{3,25}$=5.07, P=0.034), a result confirmed using Fisher's tests. On day 36, analysis using GLM indicated that there was a significant effect of c+td, analgesic treatment and of the interaction between c+td and analgesic treatment on the total feed consumed. Further investigation using Fisher's approach revealed that CD lambs ate significantly more than the other groups of lambs ($t_{3,25}$=9.84, $t_{3,25}$=13.08, and $t_{3,25}$=11.29 at P<0.0001 for HN, CN and HD lambs respectively). The analysis also revealed that HN lambs ate significantly more than HD lambs ($t_{3,25}$=-3.23, P=0.0035). On day 42 a significant effect of c+td treatment was found again (with no associated effect of analgesic), however the pattern in the data was different on this occasion with CN and HN lambs consuming significantly more than CD and HD lambs. Thus, despite the occurrence of occasional differences in feed consumption, no consistent differences were observed. The only temporally consistent pattern within the data was that lambs ate more on the first day of choice in each series of five days. On the second day of choice in each cycle, lambs ate much less, but subsequently increased their intake again over the remaining three days of testing in each cycle. This pattern was consistent across groups.

Figure 6.3. Mean (±sd) total weight (g) of test feed consumed during choice testing throughout the trial.



Self administration analysis

The proportion of feed that was taken from the analgesic hopper by lambs in all groups over the course of the study is shown in figure 6.4. Analyses to determine differences in the proportion of test feed taken from the 'analgesic' hopper showed that on days 30, 34, 37, 38, and 43, there were significant differences between groups as a result of analgesic treatment but that c+td had no effect on feed selection (see table 6.1a). Post-hoc analysis using Fisher's approach confirmed that lambs with access to analgesic feed (CD and HD) ate significantly less from the 'analgesic' hopper than those in groups CN and HN (i.e. lambs without access to analgesic feed). In order to determine whether training had been effective, the weight of feed consumed on 'analgesic' days during training was used as a covariate in a GLM used to assess differences in the proportion of total feed that was taken from the 'analgesic' hopper. The inclusion of the consumption of 'analgesic' feed during training as a covariate accounted for variance in the analysis on days 13,20,22,23,28,36,37,41 and 42 of preference testing. However, as can be seen from table 6.3b, the significance of the effect was not consistent across the study. Also, the regression coefficients for the covariate were negative throughout the study, indicating an inverse relationship between the weight of 'analgesic' feed eaten during training and the proportion of feed taken from the analgesic hopper during the choice test, i.e. the more analgesic feed eaten during training, the lower the proportion taken from the analgesic hopper during subsequent choice.

Figure 6.4. Mean (±sd) proportion of test feed consumed from the analgesic hopper during choice testing throughout the trial, n=7.

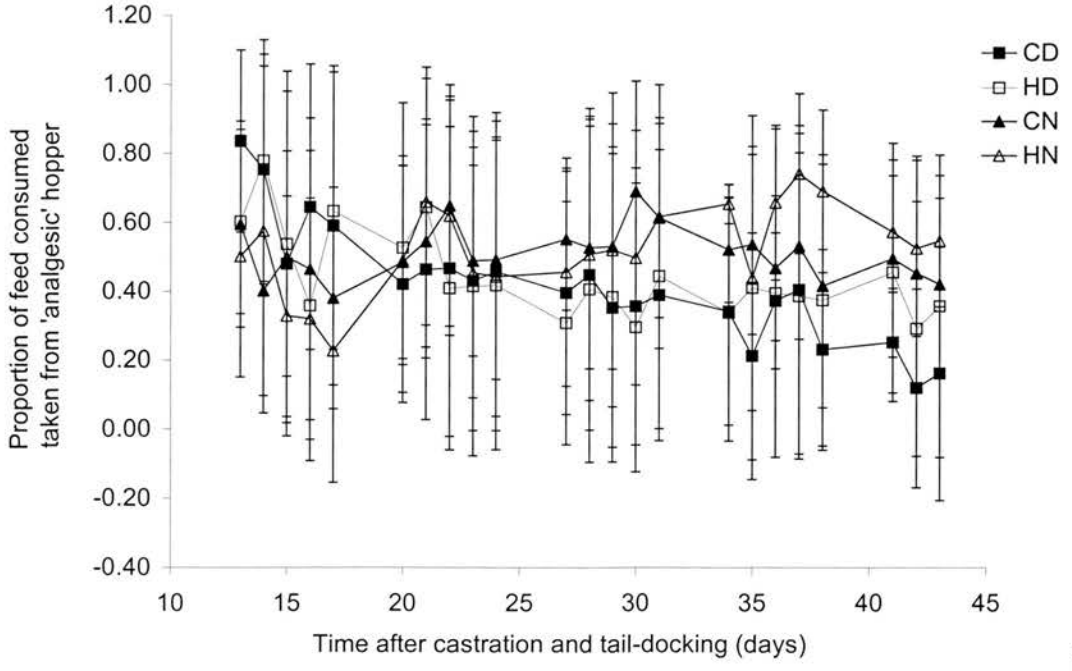


Table 6.1. (a) Results of GLM analysis of the proportion of feed taken from the 'analgesic' hopper for all groups. The significance of the effect of c+td and analgesic treatments and the effects of the interaction between these two treatments are shown. (b) Results of GLM analysis of the proportion of feed taken from the 'analgesic' hopper for all groups this time incorporating the effects of treatments and their interaction and also the effects of the covariate of training 'analgesic' feed consumption and its interaction with treatments is taken into account.

(a)

Variable		Time after castration and tail-docking (days)																		
		13	14	15	16	17	20	21	22	23	24	27	28	29	30	31	34	35	36	37
castrated	F(3,25)	1.380	1.480	0.700	1.590	0.140	0.150	0.070	0.510	0.000	0.660	0.720	0.640	0.010	0.620	0.010	0.030	0.020	0.320	0.100
	P	0.253	0.236	0.411	0.220	0.712	0.700	0.788	0.483	0.965	0.426	0.405	0.433	0.938	0.440	0.937	0.874	0.887	0.578	0.753
drugged	F(3,25)	1.490	0.800	0.060	0.000	3.940	0.120	0.130	0.770	1.030	0.190	0.590	0.890	0.610	**4.980**	4.170	**6.500**	0.520	2.700	**4.600**
	P	0.235	0.380	0.816	0.964	0.059	0.737	0.721	0.388	0.320	0.665	0.448	0.354	0.444	**0.035**	0.052	**0.018**	0.480	0.113	**0.042**
cast x drug	F(3,25)	3.660	0.920	0.060	0.600	0.750	0.280	0.870	0.320	0.000	0.070	0.020	0.080	0.000	0.200	0.540	0.880	1.700	0.310	0.770
	P	0.068	0.347	0.807	0.447	0.396	0.601	0.361	0.574	0.960	0.794	0.895	0.784	0.965	0.660	0.468	0.358	0.204	0.585	0.388

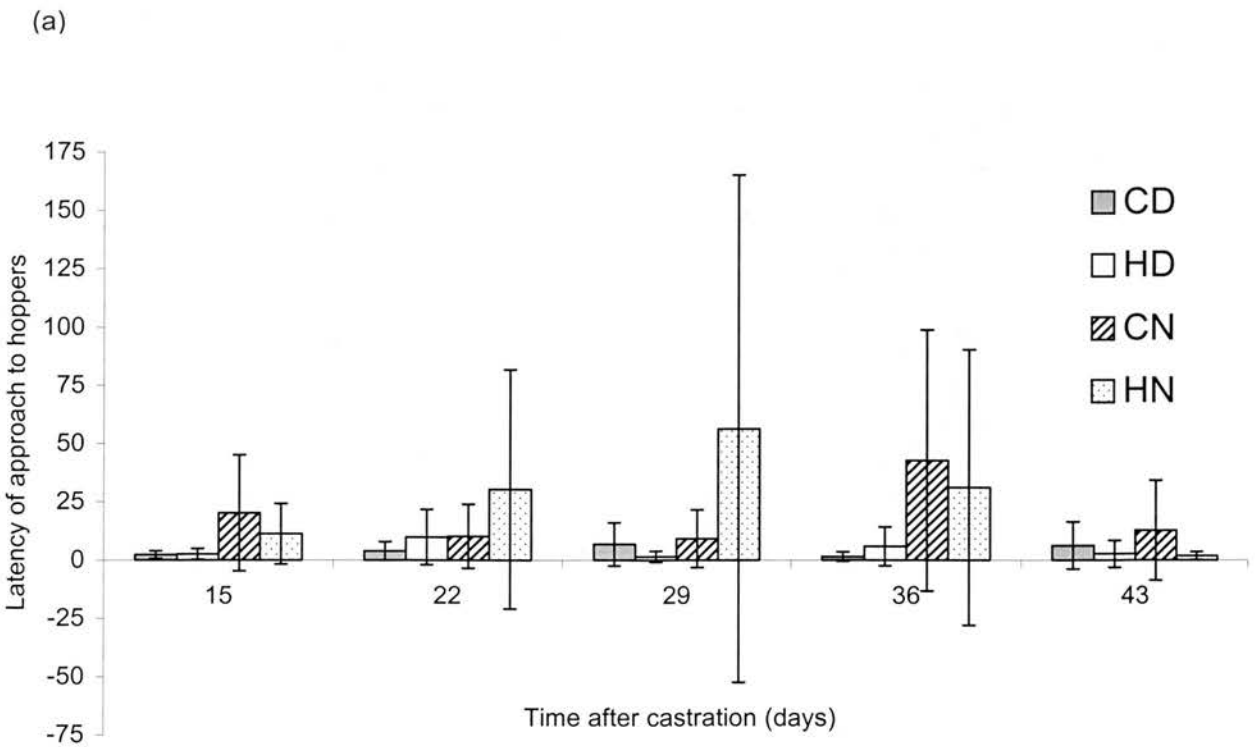
(b)

Variable		Time after castration and tail-docking (days)																		
		13	14	15	16	17	20	21	22	23	24	27	28	29	30	31	34	35	36	37
castrated	F(6,22)	3.070	1.200	1.610	0.400	0.410	**5.040**	1.610	1.950	3.810	0.200	0.160	3.010	2.260	0.400	0.660	1.660	0.540	2.010	**16.460**
	P	0.095	0.286	0.220	0.533	0.530	**0.036**	0.218	0.178	0.065	0.658	0.690	0.097	0.148	0.532	0.424	0.211	0.469	0.170	**0.001**
drugged	F(6,22)	3.760	1.100	0.590	1.160	1.120	1.890	0.310	1.450	1.560	0.260	0.010	0.030	0.450	0.890	0.620	0.140	0.970	0.590	2.010
	P	0.067	0.308	0.453	0.294	0.303	0.183	0.583	0.241	0.225	0.618	0.910	0.857	0.511	0.356	0.440	0.709	0.336	0.450	0.170
cast x drug	F(6,22)	**8.120**	0.260	1.020	0.010	1.080	0.320	1.070	0.000	0.360	0.000	0.130	0.340	0.380	0.590	0.690	0.180	0.940	0.260	1.670
	P	**0.010**	0.614	0.323	0.924	0.310	0.577	0.312	0.945	0.555	0.967	0.724	0.567	0.543	0.450	0.417	0.674	0.344	0.618	0.210
train dr	F(6,22)	**5.200**	0.360	0.000	1.470	0.010	1.570	6.470	0.710	1.510	2.630	1.800	2.070	2.930	1.550	0.750	0.720	5.600	**4.900**	0.710
	P	**0.034**	0.556	0.986	0.239	0.915	0.224	0.019	0.408	0.233	0.120	0.194	0.165	0.102	0.227	0.396	0.406	0.028	**0.038**	0.408
cast x train dr	F(6,22)	1.270	0.560	3.370	1.840	0.820	**7.850**	1.840	**4.530**	**6.090**	0.940	0.910	**5.680**	3.190	1.350	0.970	2.940	1.210	2.720	**20.320**
	P	0.274	0.463	0.081	0.190	0.376	**0.011**	0.189	**0.045**	**0.022**	0.344	0.350	**0.027**	0.088	0.258	0.335	0.101	0.283	0.114	**0.000**
drug x train dr	F(6,22)	0.790	0.940	1.170	2.230	0.130	2.540	0.000	3.210	2.850	0.160	0.000	0.010	0.350	0.020	0.000	2.790	1.300	1.740	0.180
	P	0.385	0.343	0.292	0.151	0.726	0.126	0.988	0.088	0.106	0.694	0.998	0.917	0.561	0.876	0.952	0.109	0.267	0.201	0.674

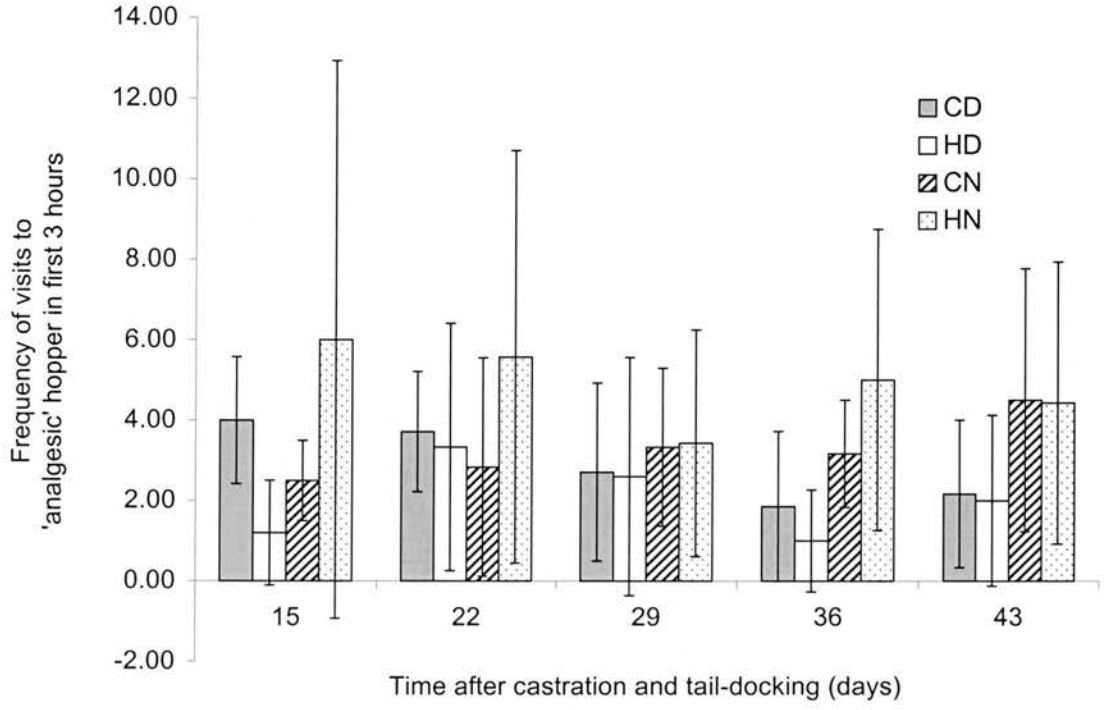
6.3.4. *Behaviour at the hoppers*

There were few real differences in the latency of approach to the hoppers and the frequency and duration of visits to the 'analgesic' hopper. The variation in the latency to approach hoppers was high (see figure 6.5a), but was less in the groups with access to the analgesic feed. The latency of approach seemed to be consistently lower in CD and HD lambs throughout, although this difference was only significant on day 15 after c+td ($F_{3,23}$=7.16, P=0.018). Towards the end of the trial, lambs in groups CD and HD visited the hopper less frequently than the lambs in groups CN and HN. This effect of analgesic treatment was significant on day 36 ($F_{3,23}$=8.23, P= 0.009) (confirmed by post-hoc testing) and neared significance on day 43 ($F_{3,23}$=4.14, P=0.055). These results are shown in figure 6.5b. As can be seen in figure 6.5c, lambs in group CD appeared to spend longer at the analgesic hopper during day 15, this difference however was found to be non-significant at P<0.05.

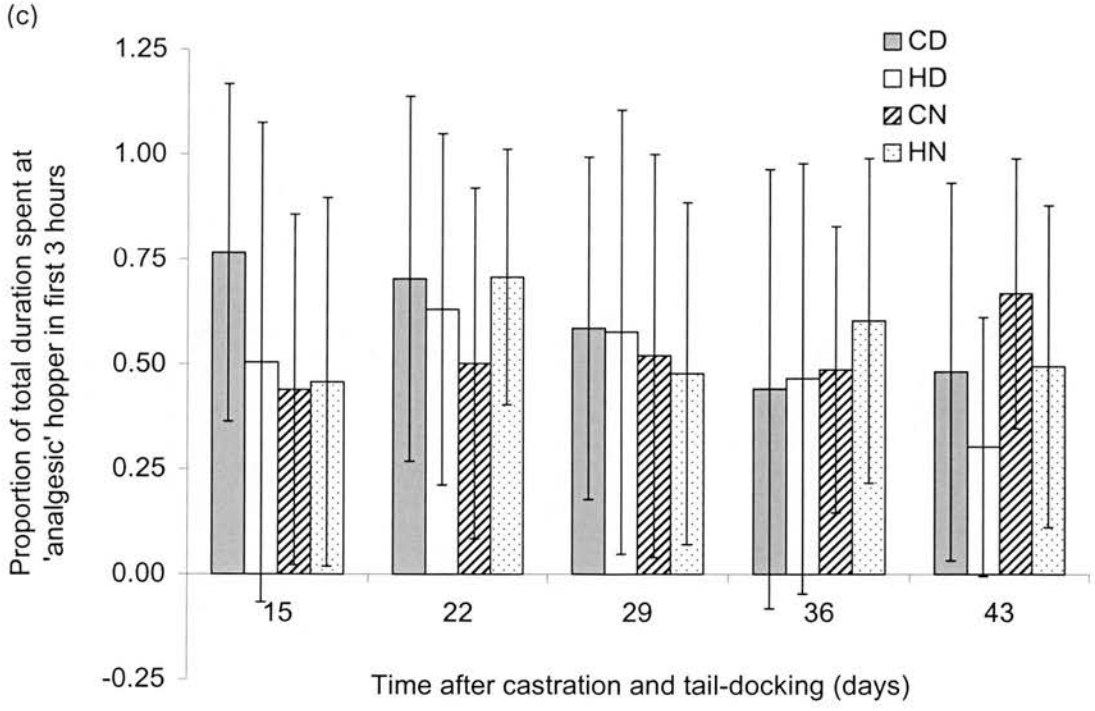
Figure 6.5. Behaviour of lambs at the test hoppers during the first three hours after the hoppers were placed in the pen, n=7. (a) Mean (±sd) latency of approach to hoppers. (b) Mean (±sd) frequency of visits to the 'analgesic' hopper. (c) Mean (±sd) proportion of the total duration of time spent feeding that was spent at the 'analgesic' hopper.



(b)


14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
-2.00
Frequency of visits to 'analgesic' hopper in first 3 hours
CD
HD
CN
HN
15
22
29
36
43
Time after castration and tail-docking (days)

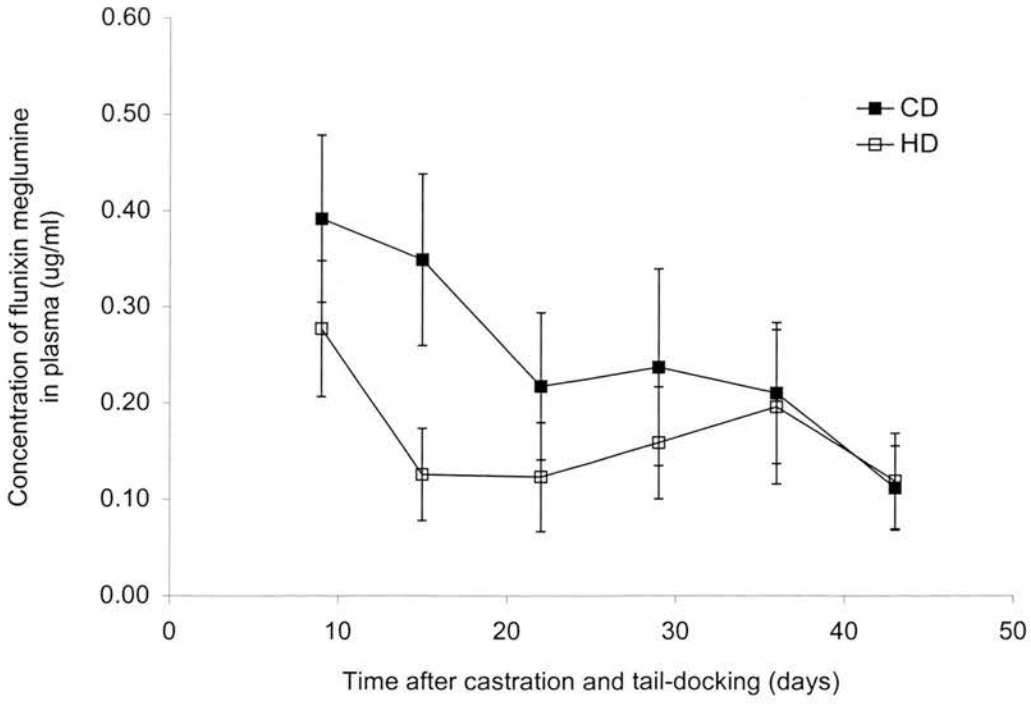
(c)


1.25
1.00
0.75
0.50
0.25
0.00
-0.25
Proportion of total duration spent at 'analgesic' hopper in first 3 hours
CD
HD
CN
HN
15
22
29
36
43
Time after castration and tail-docking (days)

6.3.5. Plasma concentration of flunixin meglumine

The mean (±SEM) plasma concentrations of flunixin meglumine 3-4 hours after test feeds were first given, in lambs in groups CD and HD are presented in figure 6.6. The plasma concentration of flunixin meglumine in HD lambs was highest during training (0.28 ±0.09μg/ml) but dropped to 0.13 (±0.06)μg/ml during the first week of choice. The plasma concentration remained at around this lower level for the remainder of the trial. In CD lambs, the mean plasma concentration of flunixin meglumine was also high during training (0.39± 0.10μg/ml) but remained high during the first week of choice (0.35± 0.11μg/ml). The difference between the plasma concentration of FM in CD and HD lambs was significant at this point (day 16) ($F_{1,13}$=4.86, P= 0.048). After the first week however, the plasma concentration of FM in CD lambs also declined and there were no further significant differences in concentration between the two groups for the remainder of the trial. A synopsis of this statistical analysis is shown in table 6.2. A GLM was carried out in which the proportion of the total feed consumed that was taken from the analgesic hopper was included as a covariate in order to determine whether proportion of feed taken from the analgesic hopper provided a good measure of drug intake. However the analysis showed that the proportion of feed taken from the analgesic hopper had no influence over the plasma concentration of FM.

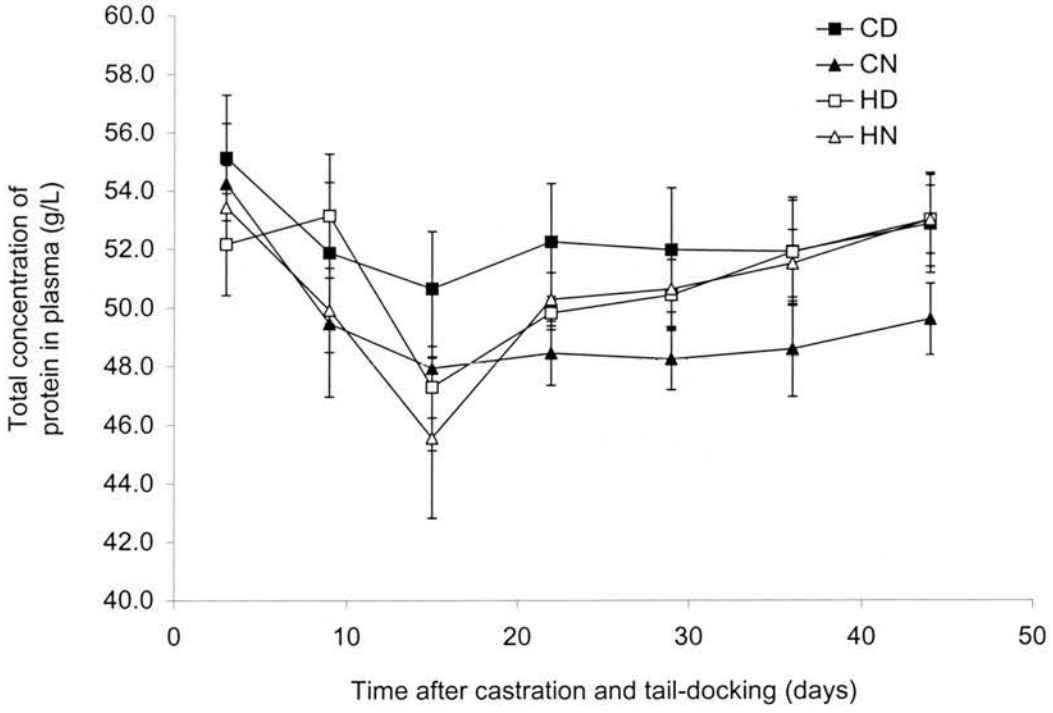
Figure 6.6. Mean (SEM) concentration (μg/ml) of FM in plasma 3-4hours after test feeds were given, in CD and HD lambs for the duration of the trial, n=7.



6.3.6. *Total plasma protein*

The mean (±SEM) concentrations of protein found in plasma are shown in figure 6.7. There was an initial decline in plasma protein from pre-trial values on day 3 after castration, which continued through training and into the first week of preference testing. However, the concentration of plasma protein rose again slightly throughout the remainder of the trial. These changes occurred in all groups and statistical analyses revealed no significant differences as a result of treatment. When the concentration of flunixin meglumine in plasma was added to the analysis as a covariate for groups CD and HD, it was not found to have influenced the plasma concentration of total protein within groups of lambs.

Figure 6.7. Mean (SEM) total concentration (g/L) of protein in plasma in CD and HD lambs over the duration of the trial, n=7.



6.3.7. *Severity of lesions*

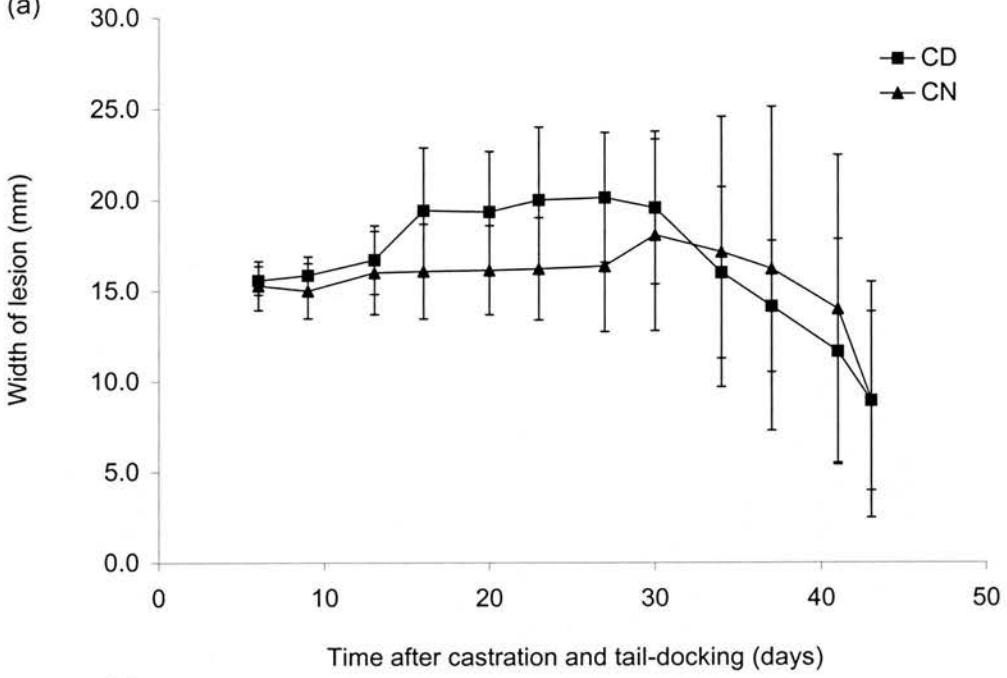
The width and score of scrotal lesions and the score of tail lesions for lambs in groups CD and CN are presented in figure 6.8. The severity of scrotal and tail lesions reached a peak 20 days after castration in CD lambs. This was slightly later than that observed in CN lambs (peak at day 13 and day 16 for scrotal and tail lesions respectively). There were some significant differences between the severity of lesions observed in lambs with and without access to analgesic. Scrotal lesions in CD lambs were consistently wider than those in CN lambs between days 16 and 27 after treatment. This difference neared but did not reach significance at $P<0.05$ throughout this period. On day 20 however, the severity score of scrotal lesions in CD lambs was significantly higher than in CN lambs (W=69.0, $P=0.032$).

The scrotums and tails of CD lambs appeared to drop off earlier than those of CN lambs (scrotum - median (Q1-Q3)= 34 (34-41) and 41(37-41) days and tail - mean (±sd) = 27.6 (± 10.4) and 27.5 (±7.2) days for CD and CN lambs respectively). However statistical analysis showed that that the differences were not significant (Mann-Whitney W=24.5, $P=0.443$; Student's t-test $T_{1,13}=0.01$, $P=0.999$ for scrotums and tails respectively). In a regression analysis, the weight of the lamb had no effect on the proportion of feed that was consumed from the 'analgesic' hopper. However, when regression analyses were carried out on the width of the lesion and the plasma concentration on FM in CD lambs, a positive relationship was found that reached significance on day 20 ($F_{1,6}=6.23$, $P=0.032$), just before the width of lesions reached a maximum in CD lambs.

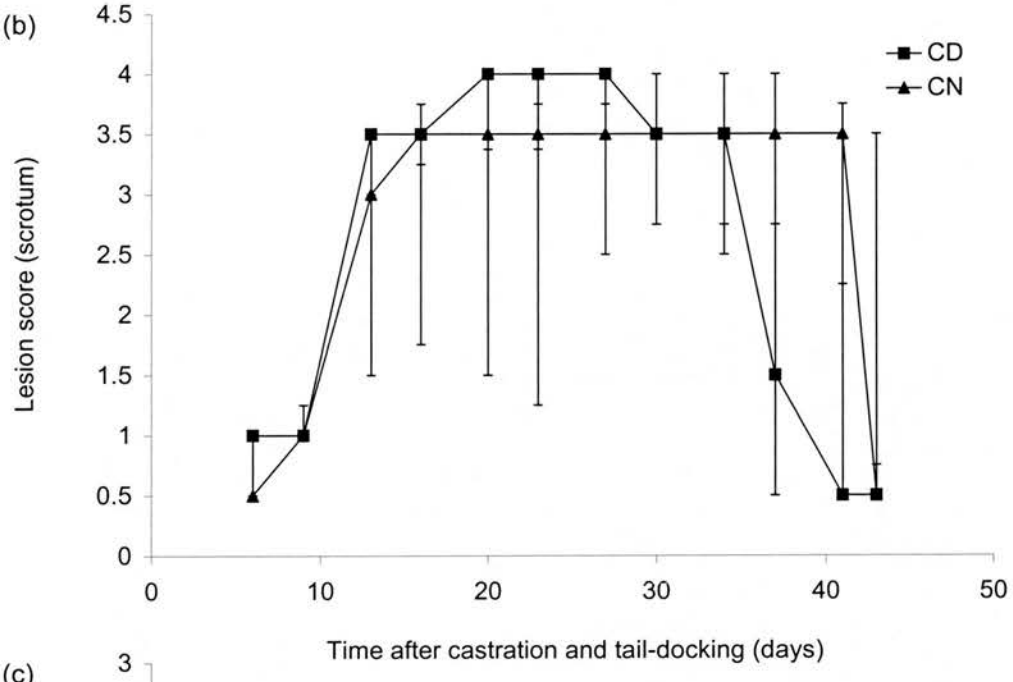
The weight of lambs was used as a covariate in a GLM to test for differences in lesion width between CD and CN lambs. On days 13 and 20 after treatment the weight of lambs had a significant effect on the width of the lesion ($F_{1,11}=11.92$, $P=0.006$ and $F_{1,11}=6.23$, $P=0.032$ for days 13 and 20 respectively), whilst analgesic treatment had no significant effect. Pictures of the changing severity of a chronic inflammatory lesion over the 6-weeks after ring application in a representative lamb are shown in Appendix D.

Figure 6.8. Severity of lesions from c+td of CD and CN lambs (n=7) (a) Mean (±sd) width of lesions (mm). (b) Median (Q1-Q3) score of scrotal lesions. (c) Median (Q1-Q3) score of tail lesions.

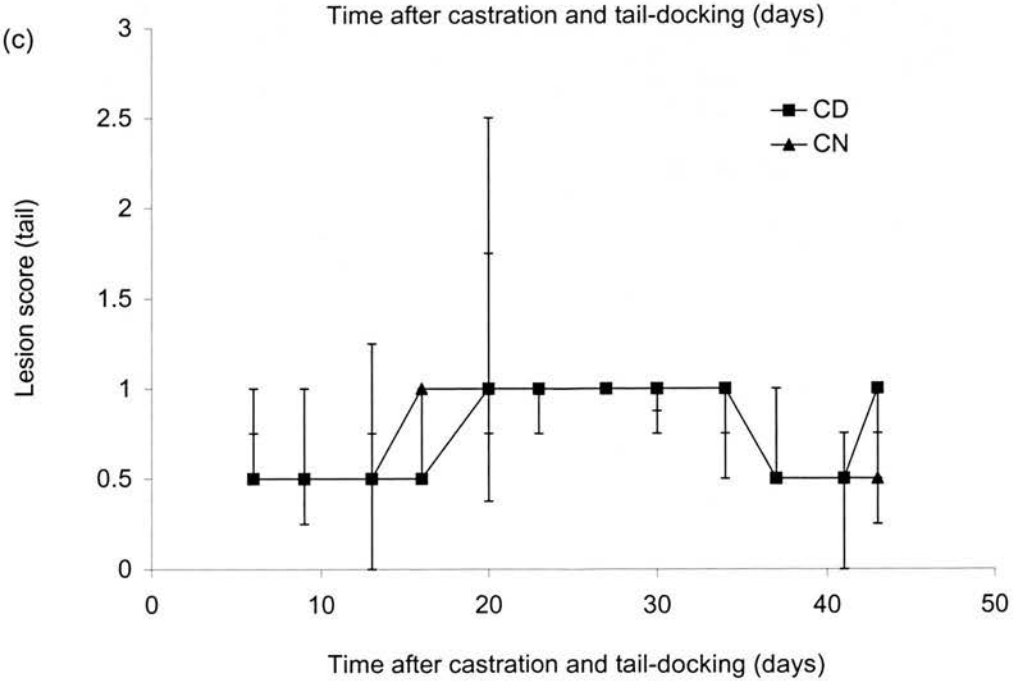
(a)


Width of lesion (mm)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
10
20
30
40
50
CD
CN
Time after castration and tail-docking (days)

(b)


Lesion score (scrotum)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
CD
CN
Time after castration and tail-docking (days)

(c)


Lesion score (tail)
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
CD
CN
Time after castration and tail-docking (days)

6.3.8. *Behavioural analysis: postures*

S1

There was no consistent significant pattern of expression of S1 during the trial in any group (see figure 6.9a and b). The total number of observations in which S1 posture was recorded was lower in CN lambs and the highest in CD lambs but this difference was not significant. A significant effect of both c+td and analgesic treatments (although not the interaction between them) on S1 posture was found during training (day 9). *Post-hoc* analyses using Fisher's tests showed that HN lambs were observed significantly less frequently in this posture than the other lambs ($t_{3,24}$=2.13, P=0.0428 (CN), $t_{3,24}$=2.92, P=0.0073 (HD), $t_{3,24}$=2.83, P=0.0090 (CD)). On day 15, a significant effect of c+td ($t_{3,24}$=-2.39, P=0.017) and of the interaction between the effects of c+td and analgesic treatment ($t_{3,24}$=2.51, P=0.012) on the time spent in S1 posture was found. *Post-hoc* Fisher's testing revealed that CN lambs were observed in S1 posture significantly less frequently than HN lambs ($t_{3,24}$=-2.39, P=0.024), but that no other differences existed between the groups.

V1

Lambs in group HN appeared to show consistently more V1 posture during the trial (see figure 6.9c and d). However statistical analysis showed that only c+td had a significant effect on day 9 ($t_{3,24}$=-2.22, P=0.036) and post-hoc analysis showed that HN lambs only showed significantly more V1 posture than CN lambs ($t_{3,24}$=-2.22, P=0.036). The effect of c+td neared significance again on day 22 ($t_{3,24}$=-1.80, P=0.071) and in the sum of observations for the whole trial period ($t_{3,24}$=-1.89, P=0.072). There were no other consistent differences in the expression of V1 posture.

V2

Slightly more consistent differences were found in the expression of V2 posture (see figure 6.9e). Significant effects of c+td were found on days 29 ($t_{3,24}$=2.28, P=0.023) and 36 ($t_{3,24}$=2.11, P=0.034) and an effect of the interaction between c+td and analgesic treatment was also found on day 36 ($t_{3,24}$=-1.98, P=0.048). Post-hoc analysis showed that this result indicated only that CN only showed significantly

more V2 posture than HN lambs and that no other statistically significant differences were present. Overall these differences were reflected in the total number of observations in which lambs were recorded in V2 posture (see figure 6.9f). Again c+td had a significant effect ($t_{3,24}$=2.12, P=0.044) and post-hoc analysis showed that CN lambs spent more time in V2 posture than HN lambs ($t_{3,24}$=2.13, P=0.043) .

Normal postures

Overall there was no significant difference in the expression of normal postures (a sum of postures expressed frequently by control animals) during the trial. The variation in the expression of these postures was wide. There were no consistent trends in the sum of normal postures (see figure 6.9g and h).

Abnormal postures: V3, SS and LL

Statue standing (SS) did not occur at any time during the trial. There was dramatic variation in the expression of V3 posture, with few individuals accounting for most of the behaviour. No significant differences in its expression were found between groups (see figure 6.9i). Overall, CN lambs appeared to show slightly more in total of this posture, whilst its occurrence in CD lambs was similar to that seen in the handled lambs (see figure 6.9j). HN lambs showed very high within group variability in the expression of this behaviour. There were also no significant differences in the expression of LL posture (see figure 6.9k). In total there were no significant differences in the expression of abnormal postures between groups (see figure 6.9l). However c+td lambs had a greater tendency to show abnormal lying than handled lambs, when the total expression of abnormal postures was summed (see figure 6.9m). Variability, particularly in HN lambs, was extremely high and no significant differences were found.

Behavioural states

Eating

The number of observations that CN lambs spent eating (see figure 6.9n) declined during the first 4 weeks after castration, reaching a low of 6.4(±2.1) observations on day 29. This low coincided with the peak in the width of lesions and occurs in a time period during which maximum scores of severity were recorded for scrotal and tail lesions. The number of observations in which CN were observed eating then increased. None of the other groups showed a temporal pattern to the number of observations spent eating and there were no statistically significant differences between the groups at any point during the trial or when the behaviour was combined for the whole trial (see figure 6.9p).

Idling

C+td animals had a tendency to show more idling than handled lambs (see figure 6.9q). Analysis using generalised linear models indicated that c+td had a significant effect on the occurrence of idling behaviour on day 36 ($t_{3,24}$=3.01, P=0.003). Post-hoc Fisher's approach testing showed that again only CN lambs showed significantly more of this behaviour than HN lambs ($t_{3,24}$=3.01, P=0.006) although the difference between CN and HD lambs neared significance ($t_{3,24}$=2.00, P=0.057). The effect of c+td on the occurrence of idling was also close to significance ($t_{3,24}$=1.92, P=0.066) when observations of this behaviour were summed for the whole trial (see figure 6.9r)

Rumination

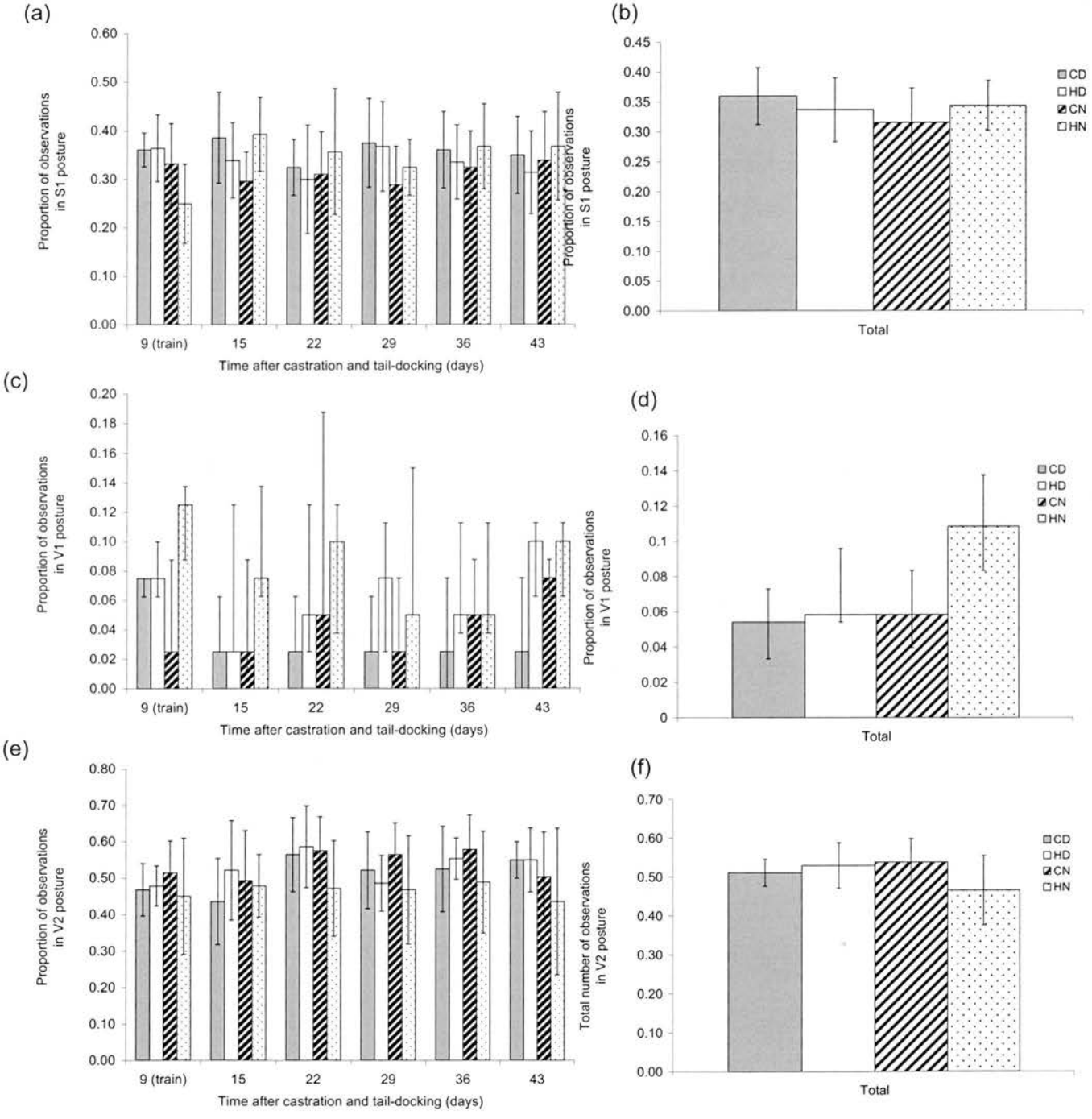
There was no obvious consistent pattern in the occurrence of rumination over the whole trial (see figure 6.9s and t). However, on day 22, an effect of analgesic treatment on the number of observations spent ruminating was observed ($t_{3,24}$=2.63, P=0.015). This effect on rumination was also seen when the occurrence of the behaviour was summed for the whole trial ($t_{3,24}$=2.74, P=0.012). Post-hoc testing showed that on day 22 HD lambs ruminated significantly more than HN lambs ($t_{3,24}$=2.63, P=0.014). This difference was also significant when the behaviour was

summed for the whole trial period ($t_{3,24}$=2.74, P=0.011). No statistically significant differences were found in the occurrence of the behaviour between any other groups.

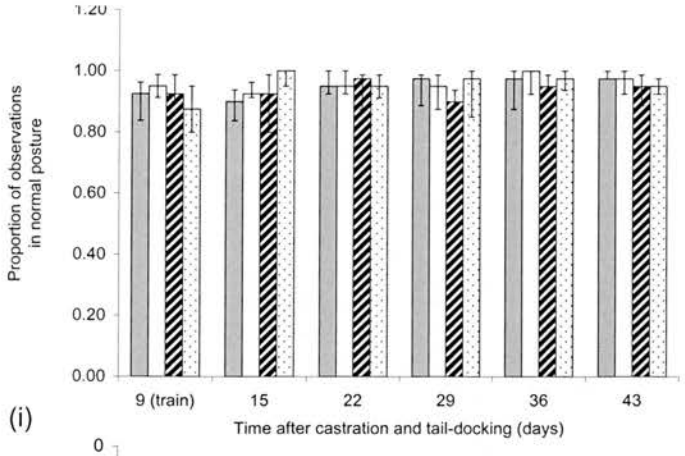
Sleeping

Finally, c+td had a significant effect on the number of observations in which sleeping was recorded on day 9 after treatment ($t_{3,24}$=2.41, P=0.024). Post-hoc testing revealed that HN lambs slept significantly more than CN lambs ($t_{3,24}$=2.26, P=0.033). C+td lambs showed a tendency to sleep less than handled animals for the remainder of the trial, although no significant differences were found during each weekly set of observations or when the behaviour was summed for the trial (see figure 6.9u and v).

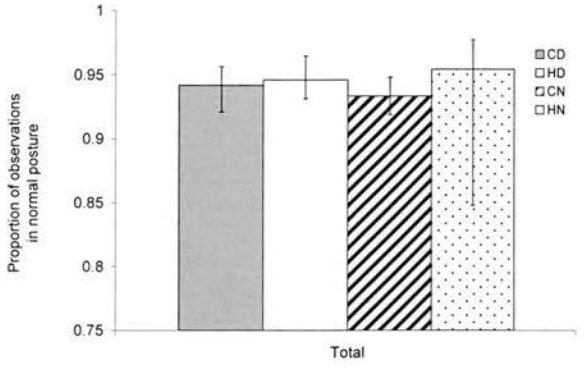
Figure 6.9. Proportions of observations that lambs were observed in postures and behavioural states during two 2 hours observation periods with scan samples every 6 minutes. **(a)** Mean (±sd) proportion of observations in **S1** posture for each weekly observation throughout the trial. **(b)** Total mean (±sd) proportion of observations observed in **S1** as a summary for the whole study period. **(c)** Median (Q1-Q3) **V1 (d)** Total mean (±sd) **V1 (e)** Mean (±sd) **V2 (f)** Total mean (±sd) **V2 (g)** Mean (±sd) **normal (h)** Total median (Q1-Q3) **normal (i)** Median (Q1-Q3) **V3 (j)** Total median (Q1-Q3) **V3 (k)** Total median (Q1-Q3) **LL (l)** Median (Q1-Q3) **abnormal (m)** Total median (Q1-Q3) **abnormal (n)** Mean (±sd) **eating (p)** Total mean (±sd) **eating (q)** Mean (±sd) **idling (r)** Total mean (±sd) **idling (s)** Mean (±sd) **ruminating (t)** Total mean (±sd) **ruminating (u)** Mean (±sd) **sleeping (v)** Total mean (±sd) **sleeping**.



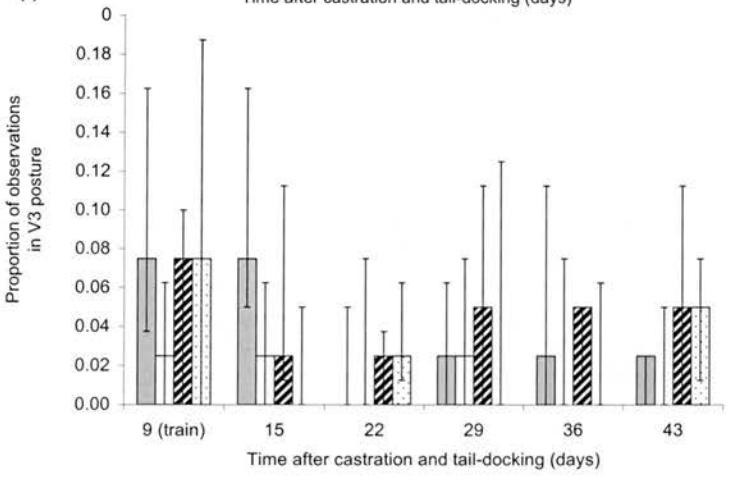
(g)


Proportion of observations in normal posture
1.20
1.00
0.80
0.60
0.40
0.20
0.00
9 (train)
15
22
29
36
43
Time after castration and tail-docking (days)

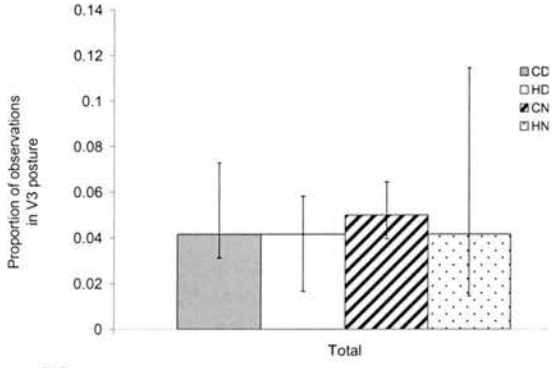
(h)


Proportion of observations in normal posture
1
0.95
0.9
0.85
0.8
0.75
CD
HD
CN
HN
Total

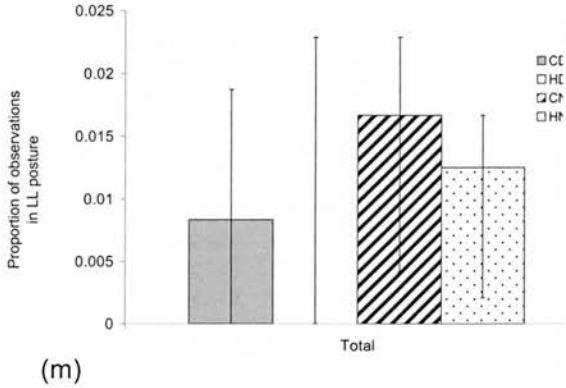
(i)


Proportion of observations in V3 posture
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
9 (train)
15
22
29
36
43
Time after castration and tail-docking (days)

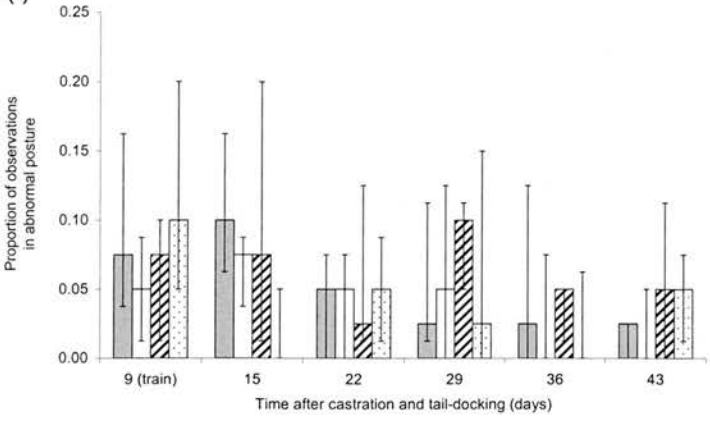
(j)


Proportion of observations in V3 posture
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
CD
HD
CN
HN
Total

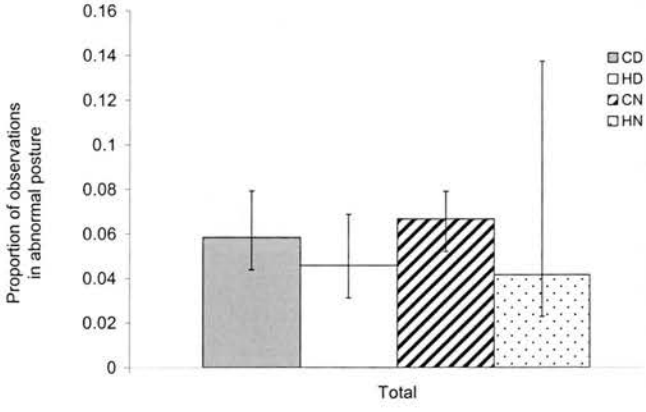
(k)

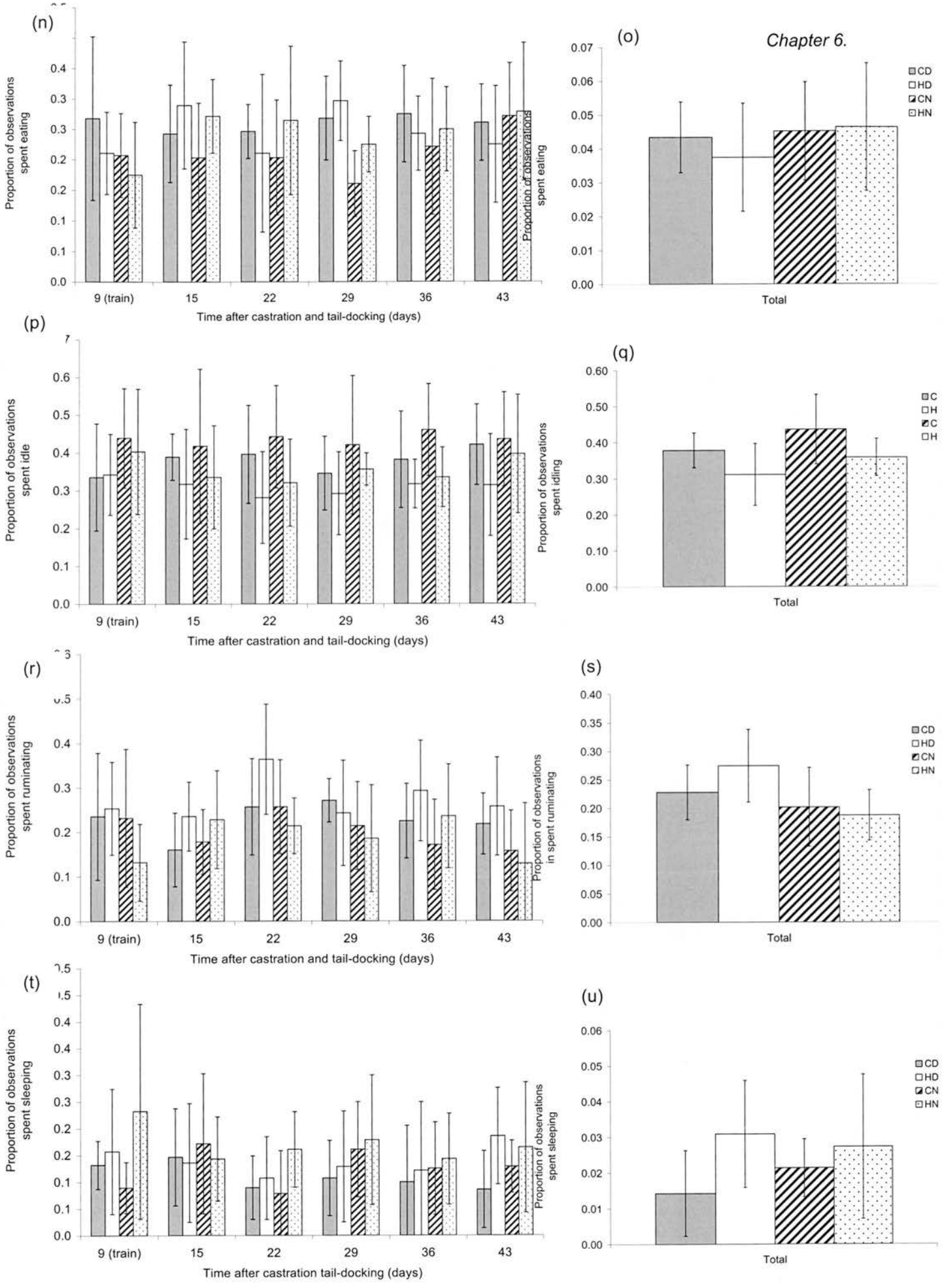

Proportion of observations in LL posture
0.025
0.02
0.015
0.01
0.005
0
Total

(l)


Proportion of observations in abnormal posture
0.25
0.20
0.15
0.10
0.05
0.00
9 (train)
15
22
29
36
43
Time after castration and tail-docking (days)

(m)


Proportion of observations in abnormal posture
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
CD
HD
CN
HN
Total


(n)
Proportion of observations spent eating
0.0
0.1
0.1
0.2
0.2
0.3
0.3
0.4
9 (train)
15
22
29
36
43
Time after castration and tail-docking (days)
Proportion of observations spent eating
(o)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
Total
CD
HD
CN
HN
(p)
Proportion of observations spent idle
0.0
0.1
0.2
0.3
0.4
0.5
0.6
9 (train)
15
22
29
36
43
Time after castration and tail-docking (days)
Proportion of observations spent idling
(q)
0.00
0.10
0.20
0.30
0.40
0.50
0.60
Total
C
H
C
H
(r)
Proportion of observations spent ruminating
0.0
0.1
0.2
0.3
0.4
9 (train)
15
22
29
36
43
Time after castration and tail-docking (days)
Proportion of observations in spent ruminating
(s)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
Total
CD
HD
CN
HN
(t)
Proportion of observations spent sleeping
0.0
0.1
0.1
0.2
0.2
0.3
0.3
0.4
9 (train)
15
22
29
36
43
Time after castration tail-docking (days)
Proportion of observations spent sleeping
(u)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
Total
CD
HD
CN
HN

6.3.9. *Behavioural analysis: frequency of active behaviour*

Restlessness

There was some indication that c+td lambs showed more restlessness than handled control animals as shown in figures 6.10a and b. On day 22 a significant effect of c+td on the expression of restless behaviour was found ($t_{3,24}$=2.27, P=0.023). Post-hoc analysis showed that both CN and CD lambs showed more restlessness than HN lambs ($t_{3,24}$=2.26, P=0.032, $t_{3,24}$=3.78, P=0.0009 respectively) and that the difference in the expression of the behaviour neared significance between CD and HD lambs ($t_{3,24}$=1.83, P=0.079). On day 43 a significant effect of c+td, analgesic treatment and their interaction was found and post-hoc Fisher's approach analysis indicated that HN lambs were significantly less restless than CN lambs ($t_{3,24}$=2.56, P=0.017). Han lambs also less restlessness than HD lambs although this difference did not reach significance ($t_{3,24}$=1.96, P=0.061). In total c+td lambs appeared to show more restlessness over the whole trial although this difference was not significant at P<0.05.

Easing quarters

CN lambs also appeared to show more easing quarters behaviour than the other lambs (see figures 6.10c and d). On day 43 a significant effect of c+td, analgesic treatment and their interaction was detected. Further analysis showed that CN lambs did indeed express more of this behaviour than HN lambs ($t_{3,24}$=3.44, P=0.002). Easing quarters also occurred more frequently in HD than HN lambs ($t_{3,24}$=2.08, P=0.048). In total, c+td was found to have an effect on the frequency of easing quarters behaviour, an effect that neared significance at P<0.05 ($t_{3,24}$=1.92, P=0.067). It appeared that c+td lambs showed more of this behaviour than handled controls.

Foot-stamping and kicking

There was no difference in the frequency of foot stamping and kicking between groups, although in total it appeared that c+td animals showed this behaviour more

frequently (see figure 6.10e). However, foot stamping occurred very infrequently in all groups, with one or two individuals accounting for most of the behaviour.

REQ

When the occurrence of the active behaviours described above (restlessness, easing quarters and foot-stamping) was summed for each observation time (see figure 6.10f) and totalled for the trial (see figure 6.10g) to give an REQ score. It was found that in general c+td animals showed these behaviours more frequently than handled controls, although this did not reach statistical significance at $p<0.05$. On day 43 a highly significant effect of c+td and the interaction factor was found as well as a significant effect of analgesic treatment. Fisher's approach showed that HN lambs showed significantly less active behaviour than both CN and HD lambs ($t_{3,24}=3.99$, $P=0.0005$ and $t_{3,24}=2.60$ $P=0.015$ respectively). CN lambs also showed more active behaviour. The effect of c+td on the frequency of active behaviour was also seen when the expression of behaviours was summed for the trial ($t_{3,24}=2.22$, $P=0.036$). Post-hoc Fisher's approach testing demonstrated that active behaviour was observed more frequently in CN than HN lambs ($t_{3,24}=2.05$, $P=0.048$) although the difference was not highly significant.

Tail-wagging

CN, CD and HD lambs showed tail wagging with increasing frequency until day 29 before their expression of the behaviour declined (see figure 6.10h), although in CD lambs this pattern was not as clearly defined as in CN and HD lambs. This pattern of behaviour was not observed in HN lambs however. On day 29 an effect of c+td on the frequency of tail-wagging was found ($t_{3,24}=2.46$, $P=0.021$). Post-hoc testing showed that HN lambs showed less tail-wagging than both HD and CD lambs ($t_{3,24}=2.49$, $P=0.020$ and $t_{3,24}=2.18$, $P=0.039$). When the behaviour was summed for the trial (see figure 6.10i), CD lambs showed the most tail wagging and the greatest variation in the expression of the behaviour, but the overall differences between groups were not significant at $P<0.05$.

Teat-seeking

There was an overall decline in the frequency of teat seeking in all groups over the duration of the trial, from approximately 9 down to 2 teat-seeking attempts in 4 hours (see figure 6.10j). There were no significant differences in the expression of this behaviour between groups at any time during the trial or when the total frequency of the behaviour was compared for the whole trial (see figure 6.10k).

Head-turning

There was wide variation in the expression of head turning with groups, a fact that may account for the lack of significant differences between groups in head turning to any target. Overall CD animals seemed to show more of the behaviour than the other lambs but again because of high variation this difference was not significant at any time or for the total frequency of this behaviour in the trial (see figures 6.10l and m).

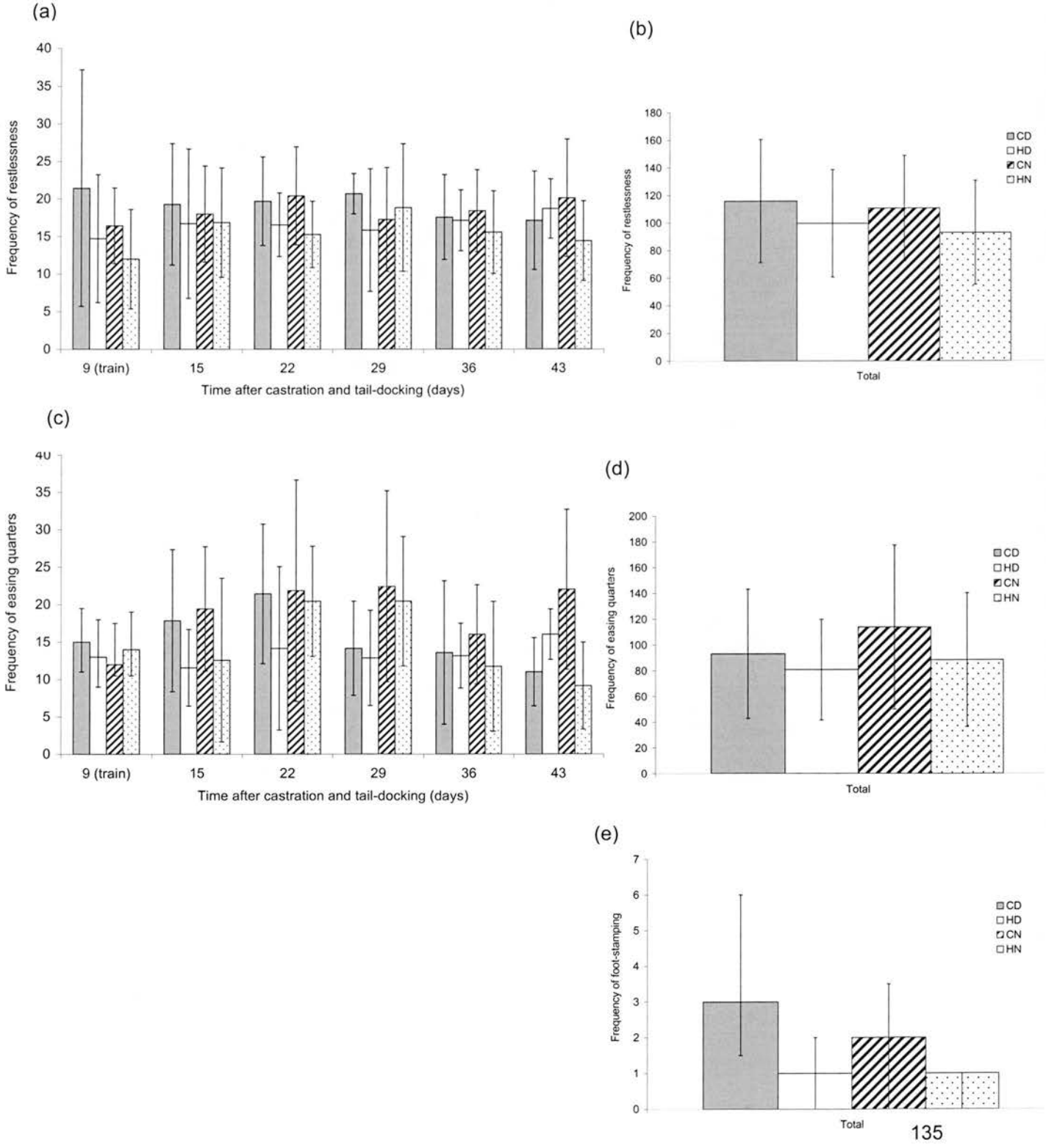
Play

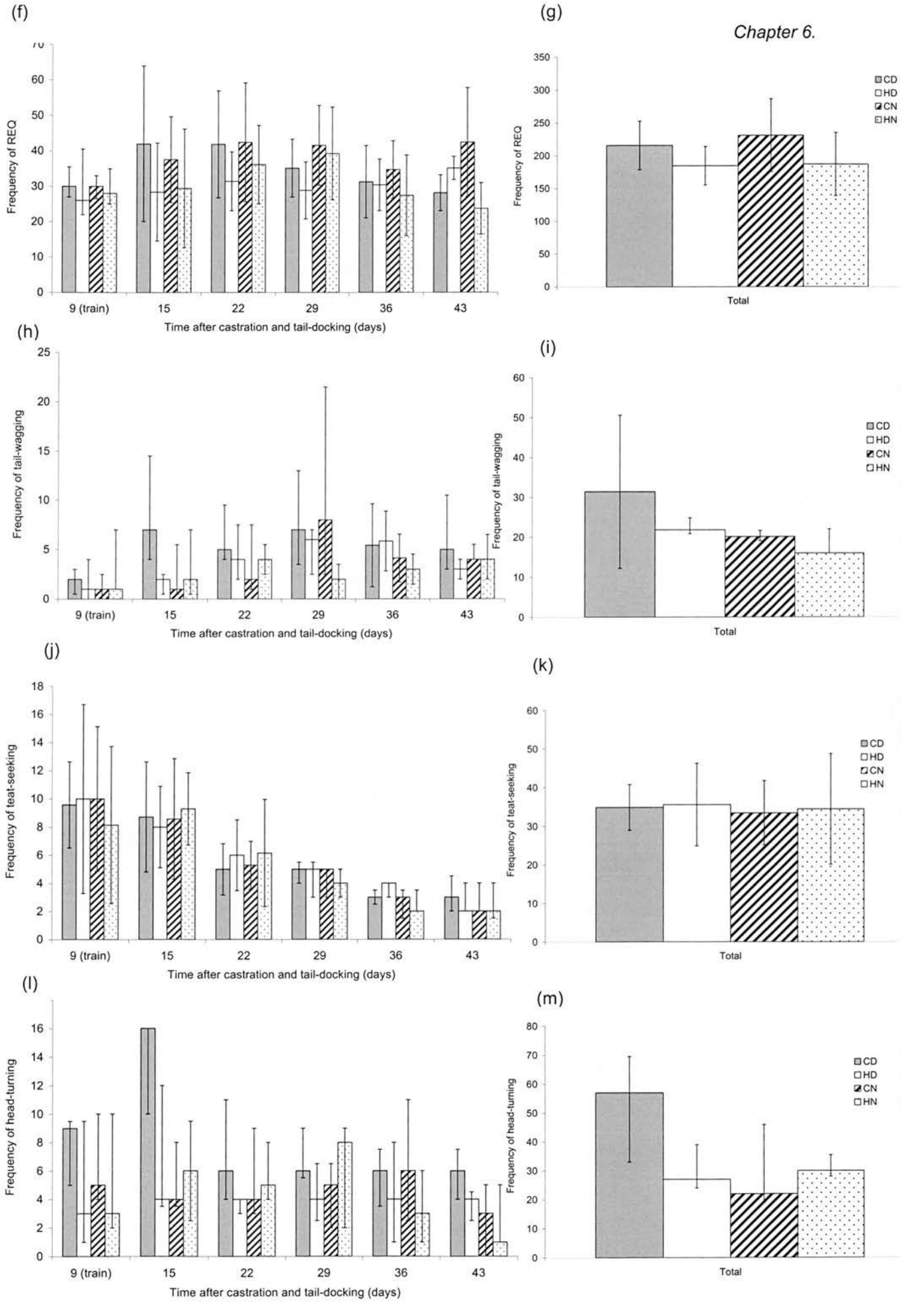
Play occurred but at very low frequency in all groups and again the variation within groups was high with some lambs expressing much more behaviour than others. There were no significant differences in the expression of play throughout the trial or when the behaviour was summed for the trial (see figure 6.10n and o).

Rubbing

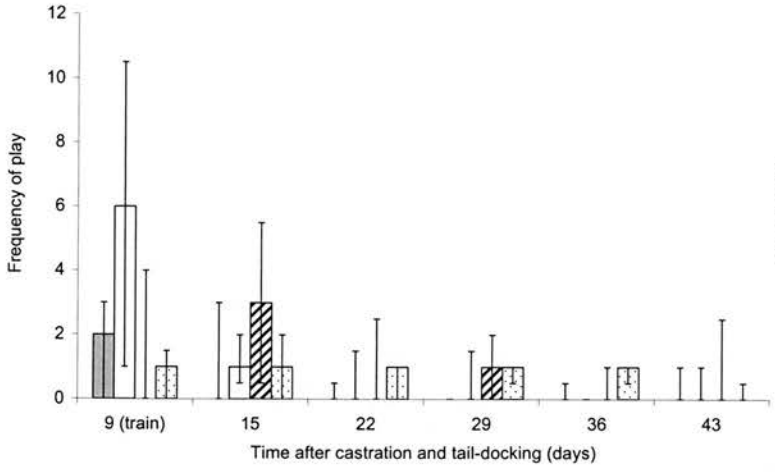
There was also wide variation in the expression of rubbing behaviour within groups (see figure 6.10p). On days 22 and 36 a significant effect of analgesic treatment and the interaction was found. Further analysis revealed that on day 22 there was a significant difference in the expression of rubbing between HN and HD lambs ($t_{3,24}$=3.11, P=0.005). Although this result appears inconsistent with graphical representation of the data, the extent of variation in opposite directions in these groups may account for this anomaly. On day 36 the difference between HN and HD lambs was found again with HD lambs expressing significantly more of the behaviour than HN lambs ($t_{3,24}$=2.19, P=0.037). Overall (see figure 6.10q) analgesic treatment had some effect on the occurrence of rubbing but this difference was close to but did not reach significance at $p<0.05$ ($t_{3,24}$=2.04, P=0.052).

Figure 6.10. Frequency of active behaviours observed during two, 2 hour long observation periods. **(a)** Mean (±sd) frequency of **restlessness** for each weekly observation throughout the trial. **(b)** Total mean (±sd) frequency of **restlessness** as a summary for the whole study period. **(c)** Mean (±sd) **easing quarters (d)** Total mean (±sd) **easing quarters (e)** Total median (Q1-Q3) **foot-stamping (f)** Mean (±sd) **REQ (g)** Total mean (±sd) **REQ (h)** Median (Q1-Q3) **tail-wagging (i)** Total median (Q1-Q3) **tail-wagging (j)** Mean (±sd) **teat-seeking (k)** Total mean (±sd) **teat-seeking (l)** Median (Q1-Q3) **head-turning (m)** Total median (Q1-Q3) **head-turning (n)** Median (Q1-Q3) **play (o)** Total median (Q1-Q3) **play (p)** Median (Q1-Q3) **rubbing (q)** Total median (Q1-Q3) **rubbing**.

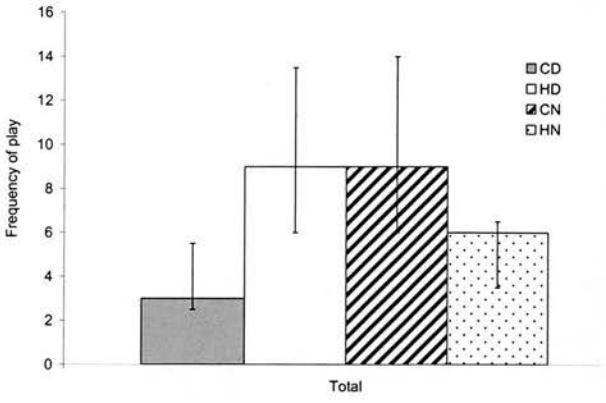



(f)
Frequency of REQ
Time after castration and tail-docking (days)
9 (train)
15
22
29
36
43
(g)
Frequency of REQ
Total
CD
HD
CN
HN
(h)
Frequency of tail-wagging
Time after castration and tail-docking (days)
(i)
Frequency of tail-wagging
Total
(j)
Frequency of teat-seeking
Time after castration and tail-docking (days)
(k)
Frequency of teat-seeking
Total
(l)
Frequency of head-turning
Time after castration and tail-docking (days)
(m)
Frequency of head-turning
Total

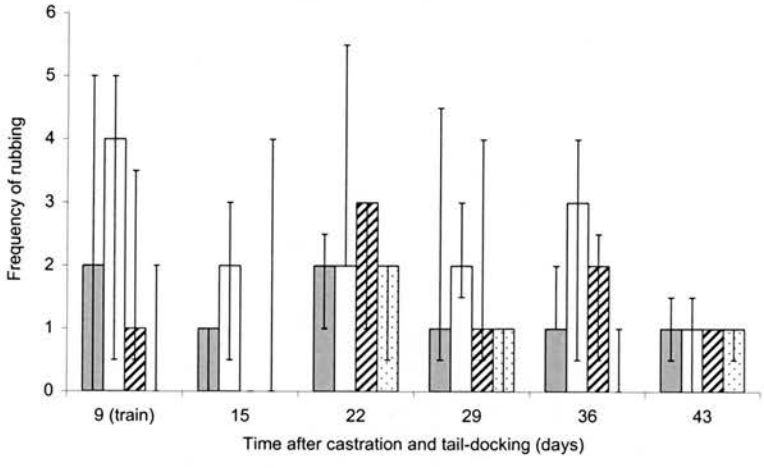
(n)


Frequency of play
12
10
8
6
4
2
0
9 (train)
15
22
29
36
43
Time after castration and tail-docking (days)

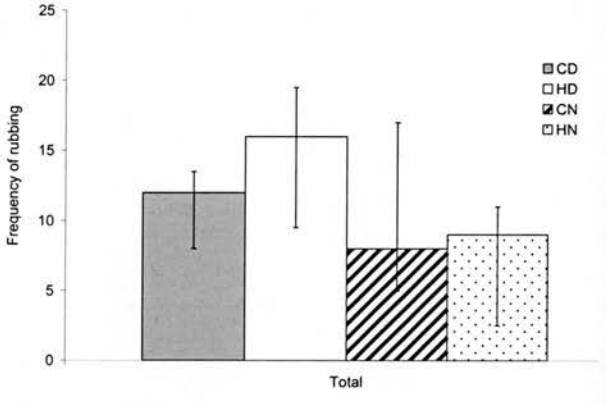
(o)


Frequency of play
16
14
12
10
8
6
4
2
0
CD
HD
CN
HN
Total

(p)


Frequency of rubbing
6
5
4
3
2
1
0
9 (train)
15
22
29
36
43
Time after castration and tail-docking (days)

(q)


Frequency of rubbing
25
20
15
10
5
0
CD
HD
CN
HN
Total

6.4. Discussion

6.4.1. *Physical evidence of chronic inflammatory pain?*

Weight and total food consumption.

Anorexia is a well accepted symptom of chronic intractable pain in both human and animal subjects, resulting in weight loss or reduced weight gain (Chapman, 1985; Kitchell and Johnson, 1985; Zimmermann, 1986; Short, 1998). Thus, lower daily live weight gain might be expected in CN lambs in this study as these lambs were subjected to a putatively chronically painful treatment without the benefit of analgesia. However, provided an efficacious dose of NSAID had been consumed, the effect of c+td on weight gain should be reduced or eliminated by the consumption of flunixin meglumine in CD lambs. In fact, c+td and analgesic treatments had no statistically significant effect on the weight of lambs. This result confirms those of previous studies in which c+td lambs showed the same daily live-weight gain as control animals (Kent *et al*, 2000). In the present study there were also no differences in the total weight of creep feed consumed between groups confirming that there was no effect of c+td on food intake.

Although differences between groups were not significant, the rate at which CN lambs gained weight appeared less in comparison with other lambs as the study progressed. Furthermore, the fact that CD lambs consistently showed a slightly (but not significantly) higher food intake than CN lambs, suggests that any effect of c+td on weight gain was eliminated by the consumption of analgesic and may have been induced by pain from c+td lesions. If the severity of the lesions had persisted rather than healed, a more significant effect on weight gain might have been observed. However, without statistically significant evidence, the presence of chronic pain cannot be inferred from weight data in this study.

Severity of lesions

The severity score of scrotal lesions observed in the present study was similar to that observed in a previous study on lambs castrated at four weeks of age (Kent *et* al, 1999). The peak in lesions severity occurred at a similar time after application of the RR (3-4 weeks) (Kent *et al*, 1999). There were some differences between the studies however. The mean maximum width of lesions in CN lambs was lower than

observed previously in lambs of this age and breed (18.1±5.28mm this study compared to 21mm observed by Kent *et al* (1999)) and in CD lambs in this study, the mean maximum severity score of scrotal lesions (score= 4.0 (3.5-4.0)) was slightly higher than previously recorded (score=3.6) (Kent *et al*, 1999). At their most severe, scrotal lesions in CD lambs were found to have a slightly higher score than those in CN lambs, a result that suggests that the NSAID may have had a negative influence on healing. However, further analysis revealed a significant influence of the weight of the lamb, but no effect of drug treatment, on the size of the lesion. Although no significant difference was found in the weight of lambs between groups, the weights of CN and CD lambs were the furthest apart (see figure 6.1). The slight difference in weight may have been sufficient to induce a significant difference in overall lesion severity, however, because of limitations of the lesion assessment system, this theory could not be tested on the lesion score data. Data presented by Kent *et al* (2000) showed that older, larger lambs develop more severe lesions than younger lambs. The evidence found in this study suggests that even very small differences in the size of the lamb may have a significant effect on the severity of the chronic inflammatory lesion.

6.4.2. *Behavioural evidence of chronic inflammatory pain?*

Zimmermann (1986) suggested several changes in normal species-specific behaviour that may occur in chronic pain syndromes. It should be noted that the changes in behaviour that do occur are likely to be specific to the particular type of chronic pain observed. The suggested list of changes includes guarding behaviour, expressed as changes in movement and posture, attention to a painful region, changes in sleep patterns, and changes in social behaviour. Such changes in behaviour have been identified in lambs in response to chronic inflammatory lesions from RR c+td using an ethogram developed to assess chronic pain from castration of calves (Molony *et al*, 1995) and adapted from methods used for the assessment of acute pain from c+td (Molony and Kent, 1993; Kent *et al*, 1995). Lambs c+td at 2 and 28 days of age have been found to show significantly more foot-stamping, tail-wagging and total activity and more abnormal postures, idling and suckling and less eating and playing than

their handled control siblings over the period that chronic lesion took to form and heal (Kent *et al*, 1999; Kent *et al*, 2000).

Some changes in the behaviour of lambs were observed in the present study. Handled control lambs spent more observations sleeping than CN lambs and, importantly, no significant difference in time spent sleeping was found between handled control lambs and c+td lambs with access to analgesic (CD lambs). This result is in accordance with the behavioural changes considered to be indicative of a chronic pain state in animals (Zimmermann, 1985) and indicates that the consumption of analgesic reduced the disturbance of sleep patterns in CD lambs. C+td lambs were also more restless than handled control lambs and a higher incidence of easing quarters was observed in CN lambs. These results are similar to those observed in previous studies (*Kent et al* 1999, 2000) in which active behaviour also increased in c+td lambs.

None of behavioural changes described above were consistently different between groups throughout the study and few showed any temporal pattern. However a temporal change was seen in the number of observations which CN lambs spent eating. This declined as the scrotal lesions became more severe and then increased again as the lesions healed. Although the occurrence of this behaviour was not significantly different from that seen in other lambs, no such pattern of behaviour was observed in CD lambs. This suggests that the pattern of eating observed in CN lambs was caused by pain from c+td lesions, but that this pain was reduced by consumption of NSAID in CD lambs. Despite there being no differences in the total weight of feed consumed, this result supports the suggestion that the small (and non-significant) differences in the weight of lambs may have been induced by the presence of the chronic inflammatory lesion.

Another behaviour that showed a temporal change was a steady decline in teat seeking observed in all lambs as ewes reduced access for suckling. A transient rise in tail wagging was also observed, but, as it occurred in both groups of c+td lambs and in handled controls, this change in behaviour cannot have been related to the formation of lesions and is likely to be indicative of an increase in the motivation to teat-seek as the ewes gradually restricted suckling bouts.

6.4.3. *Evidence of self-administration?*

In this study the hypothesis that lambs with lesions feel pain and that they will therefore learn to self-administer analgesic was assessed in two ways. Firstly the proportion of feed taken from the analgesic hopper was determined. Secondly the plasma concentration of FM was determined. Variation was expected in the proportion of drugged feed taken because of individual preferences. It was also expected that more variation would be generated when the NSAID was absorbed through the gut. Further, as the analgesic feed was placed on top of ordinary creep feed in the hopper it was possible for lambs to avoid the analgesic feed. Thus, it was necessary to determine whether differences in the proportion of feed taken from the analgesic hopper resulted in real differences in plasma FM.

Analysis of the proportion of feed taken from the 'analgesic' hopper provided no evidence that c+td lambs selected to consume a greater proportion of their feed from the 'analgesic' hopper than handled control lambs. Lambs with access to analgesic creep feed initially consumed the same proportion of their feed from the 'analgesic' hopper as CN and HN lambs, with similar variability between groups. Whilst there was a significant difference in the latency to approach the hoppers between drugged and un-drugged lambs on day 15 after treatment, there was no difference in the duration and frequency of visits to the hoppers and no relationship was found between the width of the lesion and the proportion of food taken from the analgesic hopper. In combination there is very little evidence that lambs in this study learned about the benefits of consuming analgesic creep feed and did not self-administer analgesic. However, when the plasma concentration of FM was analysed by GLM with the proportion of feed taken from the analgesic hopper as a covariate in the analysis, the results indicated that the proportion of feed taken from the analgesic hopper had no influence on the plasma concentration of FM. It may therefore be concluded that the proportion of feed taken from the analgesic hopper was a poor estimate of analgesic intake.

The plasma concentration of FM was considered to be a more appropriate measure of self-administration than the proportion of feed taken form the analgesic hopper, because it constituted a direct measure of drug intake. There was evidence in this data that CD lambs had in fact selected more analgesic than handled controls. The

concentration of FM was higher in CD lambs both during training and during the first three weeks of preference testing. This difference reached significance at $P<0.05$ during the first week of preference testing despite that fact that FM was measured 3-4 hours after first access to the feed and that the variation in concentration was high. It was on the first week of preference testing that a significantly shorter latency to approach the hoppers was detected in drugged lambs. This evidence is supported by the fact that there was a positive association between the size of castration lesions and the plasma concentration of FM for most of the study. This relationship reached significance at $P<0.05$ during the first and second week of choice testing (days 13 and 20 after c+td) when the severity of lesions was increasing.

By 20 days after castration (week 2 of preference testing), there was a decline in the plasma concentration of FM in plasma in both CD and HD lambs. On day 20 there was a highly statistically significant relationship between the width of the castration lesion and the plasma concentration of FM, indicating that those lambs with more severe lesions had taken in significantly more NSAID. Thus at this time, the plasma FM concentration in CD lambs was still related to the severity of the lesion. This suggests there was a decline in the requirement for analgesic despite the persisting physical severity of lesions. Subsequently however, the relationship between the severity of the lesion and FM concentration in CD disappeared. The decline in concentration occurred in both c+td lambs and handled controls and no significant differences in the plasma concentration of FM were found between the groups during this time. This evidence suggests that the width and subjective assessment of the severity of the lesions may not provide an accurate measure of the degree of pain suffered by the lambs in association with c+td lesions. Instead it suggests that the more severe pain is experienced prior to the development of the most severe manifestation of the lesion.

Another possible explanation for the decline in FM concentration, despite enduring lesion severity, is the possible development of an aversion to the analgesic feed. This aversion could be the result of a gradual onset of adverse effects of the NSAID in the GI tract and could simply have accelerated the rate at which the administration of analgesic reached extinction that would be expected when the lesions healed. Rutherford (1999) noted that lambs with access to analgesic appeared to rub

themselves on inanimate objects more frequently and concluded that this behaviour may be indicative a mild adverse effect of the NSAID. However, in the present study, this is considered unlikely as only HD lambs showed more of this behaviour than HN lambs, whist no differences were found between CD and CN lambs and the handled controls. Another behaviour that might be affected by gastric discomfort is rumination. Lambs with access to analgesic ruminated more than un-drugged lambs when the behaviour was summed for the whole trial period. This difference was not a reflection of a higher feed intake in these lambs (as far as can be assessed without quantification of hay and straw intake). Despite some behavioural suggestions of adverse effects of the analgesic, no evidence of changes in total plasma concentration of protein were found. In horses, the presence of hypoproteinaemia has been used as an indicator of detect gastric irritation, in the form of a protein-losing gastroenteropathy, after the oral administration of NSAIDs over a period of time (Lees *et al*, 1983; Higgins and Lees, 1984; Goodrich *et al*, 1998). The total concentration of protein in plasma was measured in this study as a means of detecting potential GI irritation. After an initial decline observed in all lambs, total plasma protein remained stable at approximately 50g/L for the remainder of the study and no differences were found between groups that had access to analgesic and control lambs indicating that no gastric toxicity was present. The measurement of total protein was the only measure made to assess toxicity from the NSAID and it is noted that NSAIDs may also cause hepatotoxicity and have some toxic effects on the kidneys. Some evidence of such toxic effects could be provided by the measurement of parameters including aspartate aminotransferase and the serum urea: creatine ratio for liver and renal toxicity respectively (Higgins and Lees, 1984) although this was not attempted in this study.

The consistent pattern of feed consumption shown by all the lambs suggests another potential cause for the development of aversion to the novel analgesic feed. The high intake of feed on day 1 of choice was followed by a slump in consumption on day 2. Lambs subsequently increased their daily consumption gradually over the three remaining days of choice testing each week. This pattern of feeding is typical of that seen when a mild aversion is learned after consumption of a certain feed to satiety (Early and Provenza, 1998). Such a response may have been caused by the reduction

on creep availability on the two 'off' days each week. Thus, novelty of test feeds, and potentially hunger, induced lambs to consume an excess of feed on the first day of choice testing each week. This excessive consumption resulting in satiety and possibly in mild indigestion and pain or malaise. CD and HD lambs had access to two feeds with distinct characteristics at specific locations. If these lambs consumed more of one or other of these feeds excessively on the first day of preference testing, they may have mistakenly learned to associate pain/malaise of indigestion and the taste of the feed and/or the position of that hopper. Thus the respective selections made by lambs in these groups would tend to converge despite any prior learning about the potential benefits of consuming each feed.

A final explanation for the decline in plasma concentration of FM in CD lambs, despite the severity of lesions remaining high is that the lambs' ability to retain the association they learned during training declines over time. It is well known that food preferences and even aversions are dynamic. The ability to continually test previously learned aversions in order to obtain sufficient nutrients is considered to be adaptive, provided animals do not consume too much of the previously aversive food. In the same way, if lambs did not eat sufficient drugged feed to obtain an analgesic effect on a sufficient number of occasions, it is possible that they unlearned the association between consuming the feed and its beneficial post-ingestion effects. Thus the use of one long training session at the start of the study at a time when putative pain from the lesions may not have been severe, might be considered insufficient. This conclusion would be inconsistent with evidence suggesting that sheep can remember positive associations between feeds and the consequences of consuming them for at least a year (Squibb *et al*, 1990).

6.4.4. *Conclusion*

Some behavioural and physical evidence of pain was present during this study. Also CD lambs appeared to have self-administered analgesic for a short period of time. However the difference in plasma concentration of FM between CD and HD lambs only just reached significance at $P<0.05$ and did not persist throughout the most severe stages of lesion development. Thus the evidence of a self-administration phenomenon in c+td lambs is inconclusive. The results also suggest that the method

of lesion assessment is not a reliable indicator of the degree of pain experienced. Several sources of extraneous variation were identified in the methodology. Elimination of these sources of variation would increase the power of future trials. Sources of variation include; the use of only one training period at the start of the study and the inclusion of ordinary creep feed in the analgesic hopper. These changes to the methodology and further redevelopments should be considered for future studies of self-administration of analgesic in lambs after RR c+td.

Chapter 7

Self-administration of Analgesic to determine Chronic Inflammatory Pain 2

Chapter 7. Self-administration of analgesic to determine chronic inflammatory pain from castration and tail-docking of lambs using a revised methodology.

7.1. *Introduction*

In the previous trial (described in Chapter 6) some evidence of self-administration of analgesic in c+td lambs was found. A higher plasma concentration of FM was found in CD lambs in comparison with handled controls indicating that CD lambs had selected to consume more analgesic creep feed than handled lambs (HD). However, the difference in plasma concentration of FM did not persist through the time when the lesions from c+td were at their most severe and reached statistical significance for only one observation during the trial. Furthermore variation was high between individuals. Thus, the evidence of a true self-administration phenomenon was considered to be inconclusive. Sources of extraneous variation were identified and modifications were made to reduce or eliminate this variation in the methodology used in the next study. It was undesirable to increase the numbers of lambs used both for ethical reasons and because the protocol would have been unmanageable with more animals.

In the previous study c+td and handled control lambs with no access to analgesic were included to control for side preferences not related to the presence of the analgesic. However, the proportion of feed consumed from the 'analgesic' hopper was found not to be a true measure of drug intake and therefore the weight of each feed consumed could not be used to provide evidence of self-administration. Instead, the concentration of FM in plasma was used for this analysis. As a result no comparison of side preferences could be made between lambs with and without access to analgesic, and this variable was not controlled for as planned.

Evidence from the previous study suggested that self-administration may have occurred initially, but a decline in plasma concentration of FM observed in CD lambs in the previous study occurred more rapidly than lesion healing and started before lesions reached a peak. This result suggests that there is no direct relationship between the measures of size and subjectively-assessed severity of the c+td lesions and the degree of pain experienced. This could not be fully statistically evaluated

because of limitations of the assessment method. It is also possible that something other than lesion healing initiated extinction of the self-administration phenomenon. The schedule used for training and the scheduled variations in the total quantity of feed given at different stages of the protocol were considered to be potential confounding factors that may have contributed to the premature extinction of self-administration.

Changes in behaviour thought to be indicative of pain were found in the previous study, however these changes were also inconsistent over time. The association between the occurrence of behaviours and the severity of lesions has been found to be more consistent in lambs that were castrated at 6 weeks of age than at 2 or four weeks of age (Kent *et al*, 1999). This was considered to be because the lesions found in older lambs were generally more severe than those found in younger lambs. In the study described in chapter 6, there was wide variation in the severity of lesions, which probably increased the overall variation in the motivation to select analgesic creep feed.

All the potentially confounding factors described above were taken into account when this present study was designed. Thus lambs were castrated at six weeks of age in an effort to obtain lesions that were consistently more severe and more likely to be associated with pain behaviours. By increasing the severity of lesions in this way, the consumption of the novel analgesic creep feed should result in the relief of pain that is more significant to the lambs. In order to ensure that lambs had the best opportunity to learn the association between consumption of the analgesic feed and the relief of pain and to retain what they had learned, training was continued throughout the study. Also, to avoid the development of an aversion to any one feed as a result of excessive consumption of feed during training or choice testing, a different commercially available creep feed was used as a test feed and was given at a separate time from the feed given for nutritive purposes. Thus, the feeding regime was more consistent and there was reduced conflict between the quantity of feed required for nutrition and the comparatively small amount of test feed required to administer the analgesic.

No evidence of toxicity was found as a result of consuming the NSAID FM over a six week period in the previous study and the schedule in which two days without analgesic in every seven was used again in the present study.

A change of breed of sheep had to be made because of precautions taken within the Moredun Institute to minimise and prevent disease within their experimental flocks. As a result Scottish Blackface lambs were used. This change was not expected to adversely affect the experiment, as SBF lambs had shown similar lesions and behavioural evidence of chronic pain in a previous study of the effects of RR castration. It was in this study by Kent *et al* (1999) that a possible relationship between the expression of abnormal 'pain' behaviours and the presence of castration lesions was first described. It is proposed that reduction of variation by these methods would permit confirmation of the self-administration phenomenon, inferred by the results of the previous study.

7.2 Methodology

7.2.1. Animals and management

28 six-week-old, single, male lambs (Scottish Blackface) were housed, with their dams, in adjacent, straw-bedded pens (1.6 x 3.2m) with a separate creep area, in accordance with management procedures outlined in Section 5.1. In keeping with general management procedures (see Section 2.2), the animals had *ad libitum* access to hay and fresh water, ewes were fed 500g of concentrated feed daily and lambs had access to their dam to suck milk. Between the hours of 17.00 and 08.00, the lambs had *ad libitum* access to creep feed (Nustart Lamb Creep Pellets, Pye-Frankland Balanced Feeds, protein 18%, fibre 8%) in a single hopper placed in the creep area. The layout of the pen was as described in section 6.4. Between 17.00 and 08.00 hours the two hoppers, represented in the diagram, were replaced with one feed hopper. This hopper was removed entirely from the pen at 08.00hrs.

7.2.3. *Experimental feeds*

Two experimental feeds were used in the self-administration protocol:

The un-drugged feed was a second brand of commercially available creep feed (Elite Lamb Pellets, Billington Agriculture, protein 10%, fibre 9%).

The analgesic feed was made by combining this creep feed with Finadyne® granules and repelleting the mixture as described in Section 5.2. The repelleted creep feed was slightly paler in colour and more brittle than the original creep feed and likely to have had a distinctive taste and smell.

7.2.4. *Treatments*

The 28 lambs were weighed and assigned to four groups of 7 lambs, so that live-weight was balanced across the groups. The location of the lambs within the barn was balanced to ensure that any variables resulting from the experimental environment had an equal effect across groups.

The daily consumption of creep feed was recorded for each lamb during the week prior to the beginning of the experiment so that the volume of creep feed available could be adjusted to account for appetite.

The treatment groups were as described in Section 5.3. and Table 5.1

7.2.5. *Self-administration protocol*

The protocol for self-administration of analgesic was carried out over a six-day cycle. On the first four days of each cycle a training programme was used. Here the lambs were given the opportunity to learn the association between the comparative benefits of consuming analgesic or un-drugged experimental feeds and the location and taste of these feeds.

On the fifth and sixth days of the cycle, lambs were offered a choice of the experimental feeds. The comparative volumes of analgesic and un-drugged feed consumed were measured on choice days, providing an estimate of preference.

The self-administration protocol (training and preference testing) was started five days after castration and tail-docking. Experimental feeds were offered to lambs for one hour only, between 12.00 and 13.00 daily. At this time lambs had had no access to concentrated feed for 4 hours and, although unlikely to be hungry, the lambs showed interest in the feed hoppers.

The lambs were weighed once during every six-day cycle to ensure that the correct dose of analgesic was offered to each lamb. The self-administration regime was continued for 6 weeks (7 cycles) while the chronic inflammatory lesions developed and then healed.

7.2.5.1. *Training regime*

The precise protocol for training was as follows: On the first day of training, lambs in the 'drugged' groups (CD and HD) were given two hoppers at the left and right sides of the back gate of the creep area, as illustrated in Figure 5.4. A quantity of analgesic feed (sufficient to provide a dose of 1.1mg/kg body weight) was placed in one hopper but the second hopper was left empty. On the second training day the hopper that had contained analgesic feed was left empty and a quantity of un-drugged feed (equivalent to the weight required to provide a dose of drugged feed) was placed in the second hopper. The hopper in which analgesic feed was placed did not change nor did the position of the hoppers within the pen.

The lambs in the 'non-drugged' groups (CN and HN) were also given two hoppers at the back of the creep area. A quantity of un-drugged feed (equivalent to the weight required to provide a dose of drugged feed) was placed in one hopper but again the

second hopper remained empty. On the second day of training the first hopper was left empty and a quantity of un-drugged feed (equivalent to the weight required to provide a dose of drugged feed) was placed in the second hopper. Again the position of the hoppers within the pen was not changed. At 13.00hours the experimental feeds hoppers were removed from the pen and the remaining feed was weighed.

The hopper containing feed was thus alternated daily for four training days.

In order to show preference the lambs must learn the associations between the benefits of either consuming analgesic or un-drugged experimental feeds and characteristics (taste, smell, texture and location) of these feeds. To facilitate learning, it was essential that the lambs consumed the entire dose of analgesic feed during training. Throughout training the quantity of feed given at 17.00hours for nutritive purposes was restricted to half the estimated appetite of each lamb. This mild restriction ensured that lambs consumed the entire dose of drugged feed during the hour that experimental feeds were available during training.

7.2.5.2. *Preference testing*

On the fifth and sixth day of each cycle lambs were offered a choice of both feeds in the designated hoppers. Again the single hopper containing nutritive feed was removed at 08.00. The experimental feed hoppers were introduced to the pen at 12.00. Each hopper contained a dose quantity of feed (analgesic or un-drugged). The hoppers were removed at 13.00 (for the first three cycles) and the feeds weighed.

On the nights before preference testing, it was intended that no restriction should be made on the volume of feed consumed overnight and the quantity of feed made available was tailored to the appetite each lamb. However, as the lambs grew older the weight of feed they would consume increased. From the fourth cycle of self administration until the end of the study a maximum of 600g was provided overnight because in the previous study lambs that ate more than this showed signs of discomfort. In order to accommodate this change in nutritive feeding, the time for which experimental feeds were made available during preference testing was reduced to 30 minutes during the fourth self-administration cycle until the end of the experiment. The experimental feeds were thus introduced at 12.00, but removed and weighed at 12.30.

The side (left or right) of the pen at which analgesic feed could be found for each lamb was selected at random using random number generation (Excel, Microsoft). For the purposes of analysis an 'analgesic' hopper was nominated at random for lambs in the un-drugged groups (CN and HN). The proportion of the total feed consumed that was consumed that was taken from the nominated analgesic hopper was calculated for analysis. The latency to approach the hoppers, frequency and duration of visits to the hoppers were also recorded.

7.2.6. *Lesion assessment*

The chronic inflammatory lesions resulting from castration and tail-docking were examined and assessed twice in each six-day cycle. Each lamb was caught and inverted. The presence or absence of the tail and scrotum was noted. The width of the lesion was measured, using callipers, to the nearest millimetre. Subjective assessment of the severity of the lesion was made using the scale described by Kent *et al* (2000) as described in Section 2.7. Using this system, the lesion was scored for severity on a scale of 0-5 in increments of 0.5.

7.2.7. *Behavioural analysis*

Observations of behaviour were made, once in each cycle, on the second day of preference testing, for 3 hours between 14.00 and 17.00. The methods used are explained fully in Section 2.8. Three experienced observers were spaced around the penned area on raised platforms to ensure that every animal could be observed from at least two angles at all times. The experimental animals were divided into 3 manageable batches for observation (two groups of 10 lambs and one group of 8). Each batch included an equal number of animals from each treatment group as far as possible. Observations were recorded directly onto computers (Dell Latitude Laptops) using The Observer behavioural analysis software (Noldus Information Technology). All observations were made according to the ethogram shown in Table 2.2. The behaviours 'chew' and 'horn' were added to the original ethogram (Molony *et al*, 1995) as these behaviours occurred frequently in the penned SBF lambs.

The frequency of each active behaviour was recorded continuously throughout the observation time. Postures of lambs were recorded during scan samples every 6

minutes throughout the observation time. At each scan the lamb's posture and behaviour was recorded. Two of the observers were the normal handlers of the animals. During the week prior to the experiment beginning these handlers spent as much time as possible in the barn with the animals in an effort to minimise the effect of the observers on the sheep. Observers were seated on the platforms for at least 5 minutes before observations started to allow the animals time to settle.

7.2.8. *Blood samples*

4ml blood samples were taken (according to the methods described in Section 2.5) within 30 minutes after the hoppers were removed on the second day of preference testing in each cycle. The samples were immediately cooled to <4°C on ice and were centrifuged at 3000rpm for ten minutes within ten minutes. Plasma was transferred to labelled plastic tubes and stored at -20°C until required for analysis.

7.2.9. *Colorimetric determination of total protein and albumin in plasma*

The concentration of total plasma protein and albumin were determined using quantitative colorimetric assays as described in Section 5.5.

7.2.10. *High performance liquid chromatography for extraction and quantification of flunixin meglumine in plasma.*

The plasma concentration of flunixin meglumine in all animals receiving drugged feed, was measured using isocratic, reverse-phase high performance liquid chromatography (HPLC) as described in section 2.6.

7.2.11. *Statistical analysis*

General linear models (GLM) were used to determine differences between groups in the weight of lambs, the proportion of test feeds eaten during training (after log-tan transformation), the total weight of creep feed eaten during choice, the proportion of feed eaten from the analgesic hopper (first transformed using log-tan transformation), the frequency of visits and proportion of total duration spent at the analgesic hopper (transformed using log-tan transformation) during choice and the concentrations of flunixin meglumine (after square root transformation) and total protein and albumin

in plasma. GLMs were carried out for each sample point for each measure during the study. If effects of treatment were found, Fisher's approach was used *post-hoc* to determine where the differences lay. GLMs of total plasma protein and albumin were carried out using the plasma concentration of FM as a covariate to determine any relationship between the protein concentrations and the quantity of FM in the blood.

Student's t-tests were used to compare the width of castration lesions and Mann-Whitney U tests to compare the severity scores for castration and tail-docking lesions. Regression analysis was used to identify any relationship between the size of the lesion and the proportion of feed consumed from the analgesic hopper (no transformation). Lesion width was also used as a covariate in a GLM analysis of the weight of lambs to determine whether the size of the lesion was affected by the weight of the lamb. Regression analysis was also used to determine any relationship between the proportion of feed taken from that analgesic hopper and the width of castration lesions.

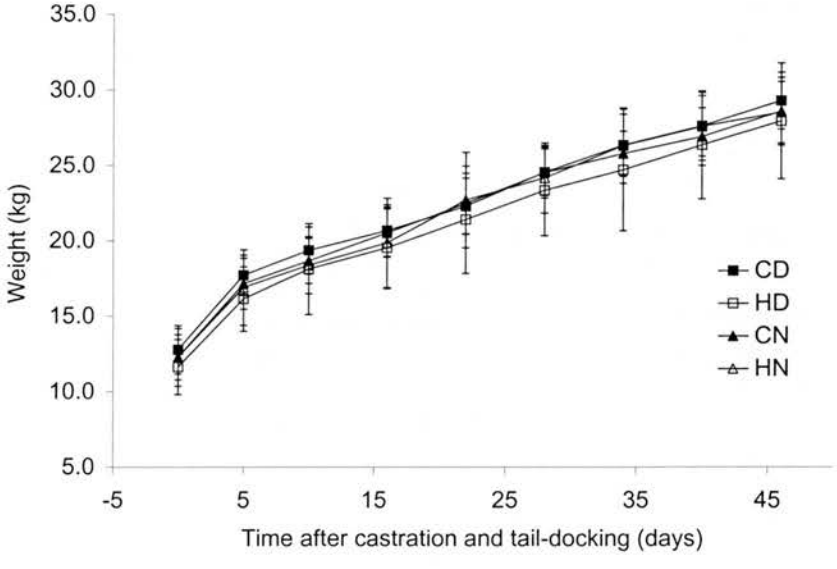
Genstat (version 5) was used to carry out generalised linear models on behavioural data. Differences identified using generalised linear models were examined more closely *post-hoc* using Fisher's approach to determine where differences lay. Data on active behaviours were analysed using poisson distribution and the logarithmic link function, whilst data on posture were analysed using the binomial proportions model and logit transformation provided in the Genstat package.

7.3. Results

7.3.1. *Weight of lambs*

As the lambs were initially divided into groups in which the weight of lambs was approximately equal, there was no initial statistically significant difference in the weight of lambs between treatments at $P<0.05$. Over the study period the average weight of lambs more than doubled from approximately 12kg to 28kg over the six weeks of the trial. There were no statistically significant differences in weight of lambs between treatment groups at any time during the study at $P<0.05$. The mean weight (±sd) of lambs in each group are shown in figure 7.1.

Figure 7.1. Change in live-weight (kg) of lambs over the duration of the trial, expressed as mean (±sd), n=7.

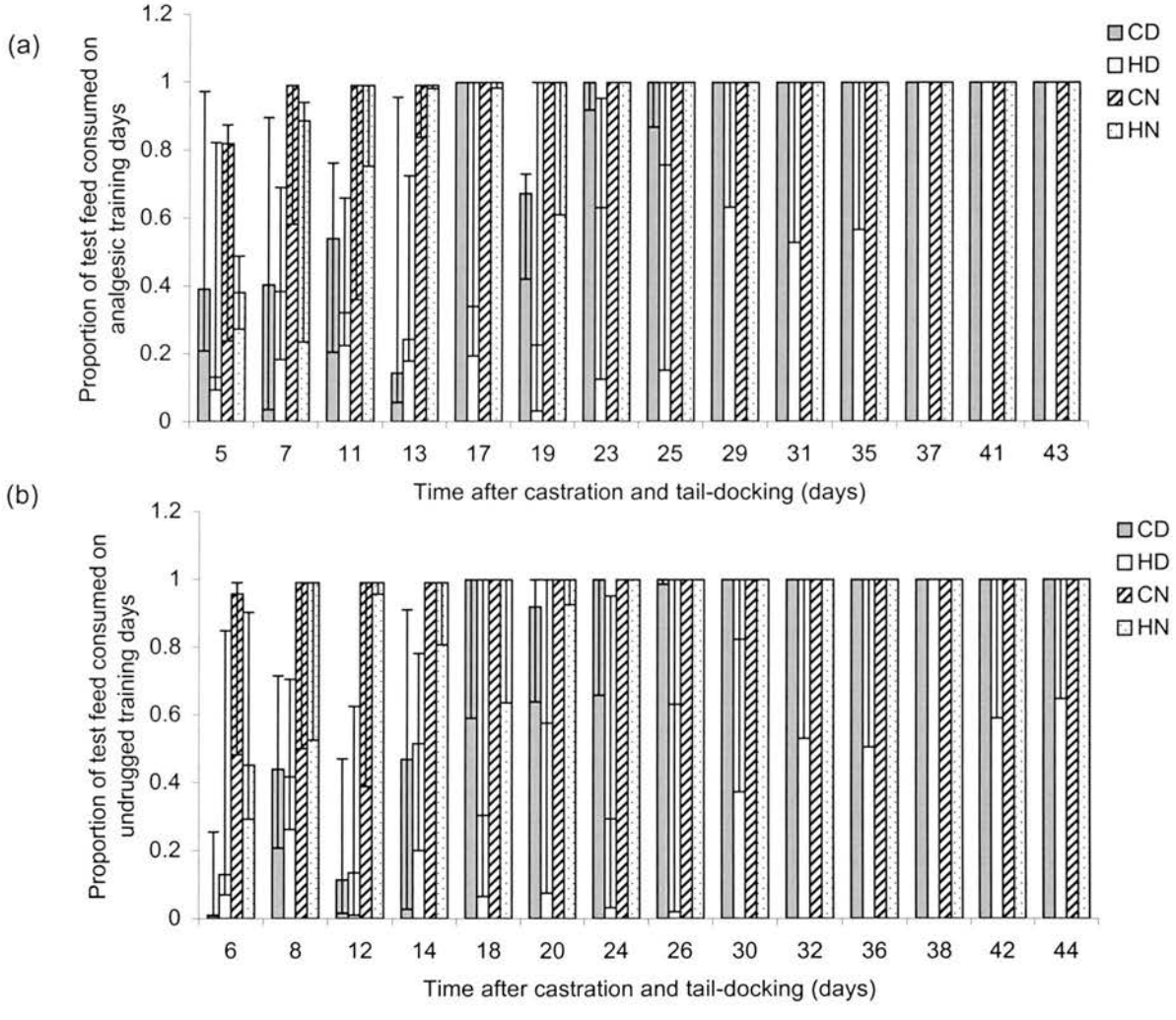


7.3.2. *Training*

The proportion (median (Q1-Q3)) of test feed consumed on training days are shown in Figures 7.2 a and b for 'analgesic' and undrugged training days respectively. Lambs in groups CD and HD initially ate less feed during training, irrespective of the type of test feed provided (i.e. analgesic or undrugged feed). This difference was found to be statistically significant during the second cycle of testing on days 12, 13 and 14 ($F_{3,24}=11.97$, $P=0.002$; $F_{3,24}=8.62$, $P=0.007$; $F_{3,24}=9.68$, $P=0.005$). However by day 23 only HD lambs were eating less than the other lambs ($F_{3,24}=9.45$,

P=0.005) and consumption by CD lambs was as high as that found in lambs without access the analgesic creep. The difference between HD lambs and the other groups occurred on two more occasions before the end of the trial (days 30-$F_{3,24}$=4.75, P=0.039; day 32-$F_{3,24}$=4.58, P=0.042).These results were confirmed post-hoc using Fisher's approach. Subsequently, almost all the lambs ate all the training feeds and no differences were found between groups.

Figure 7.2. Proportion (±sd) of test feed consumed on (a) analgesic and (b) un-drugged training days (n=7).



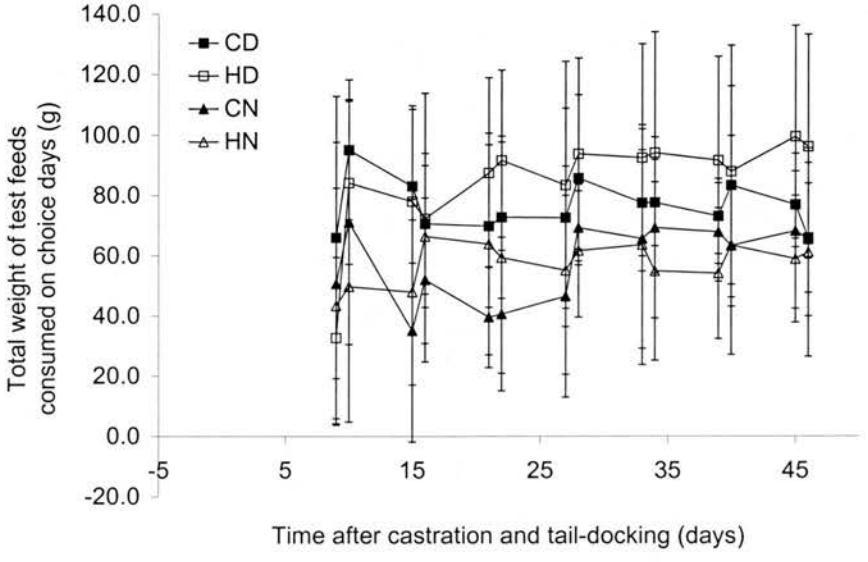
7.3.3. *Preference test*

Total weight of feed consumed

The change in the weight of test feed consumed during the trial is shown in figure 7.3. During choice testing lambs in groups CD and HD consistently ate more of the

test feeds than their undrugged counterparts. This difference was significant on days 10, 15, 21, 22, 28, 34, 39 and 45 after castration and tail-docking treatment. These results were confirmed post-hoc using Fisher's approach.

Figure 7.3. Mean (±sd) total weight (g) of test feed consumed during choice testing throughout the trial.

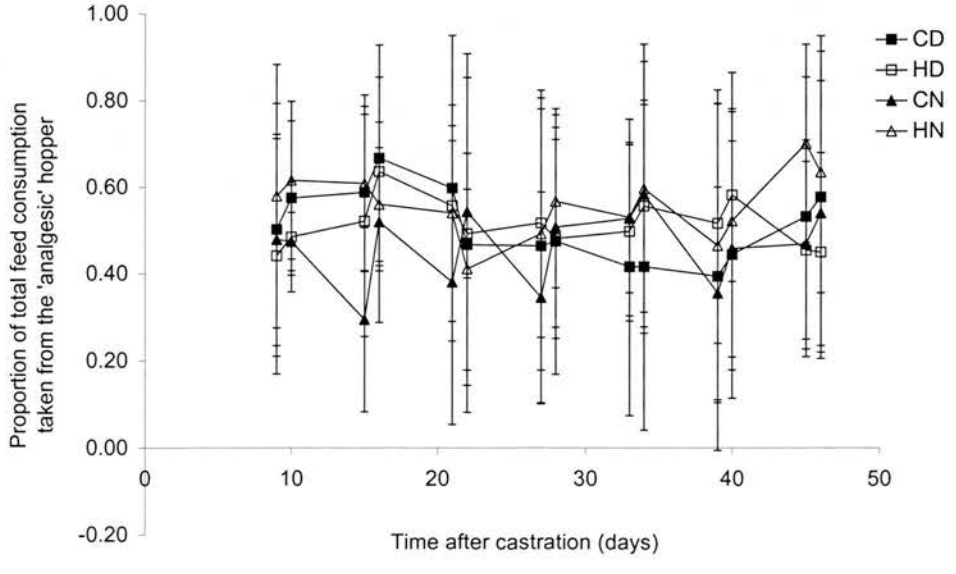


Self administration analysis

As can be seen in figure 7.4, there appeared to be no difference in the proportion of feed that was taken from the 'analgesic' hopper between groups at any point during the trial. This result was confirmed statistically using GLMs.

Regression analysis showed that there was no relationship between the size of the lesion and the proportion of feed taken from the 'analgesic' hopper.

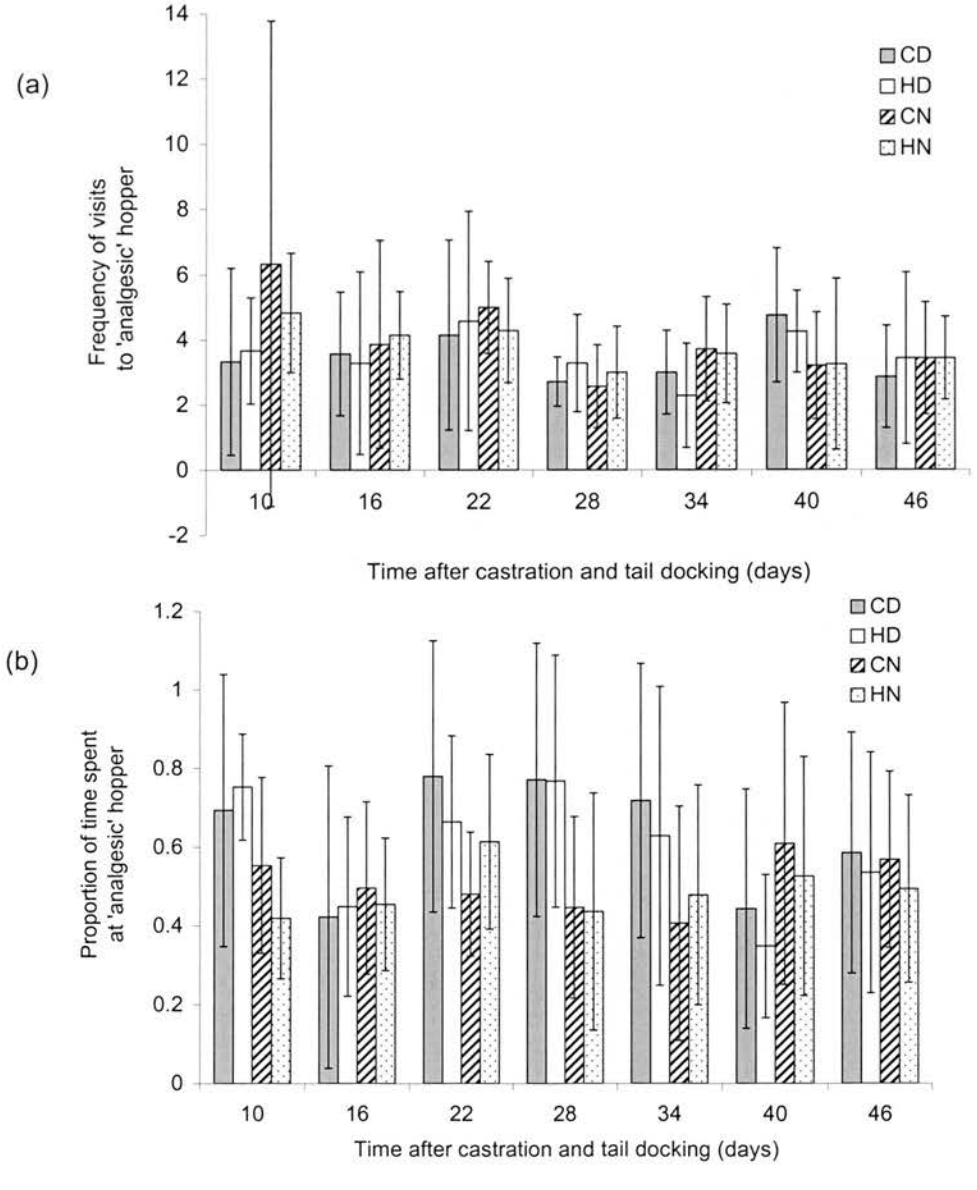
Figure 7.4. Mean (±sd) proportion of test feed consumed from the analgesic hopper during choice testing throughout the trial, n=7.



7.3.4. *Behaviour at the hoppers*

On days 28 and 34 an effect of analgesic treatment on the duration of visits to the analgesic hopper was found ($F_{3,24}$=6.05, P=0.021, and $F_{3,24}$=4.40, P=0.039 respectively). Post-hoc testing using Fisher's approach revealed that on both days CD and HD lambs spent longer at the hopper than CN and HN lambs, but that there were no differences between CD and HD lambs or between CN and HN lambs on either day. There was no difference either in the frequency of visits to the hopper between groups at any time during the study, or in the latency to approach the hoppers. The data on frequency and durations of hopper visits are presented in figure 7.5.

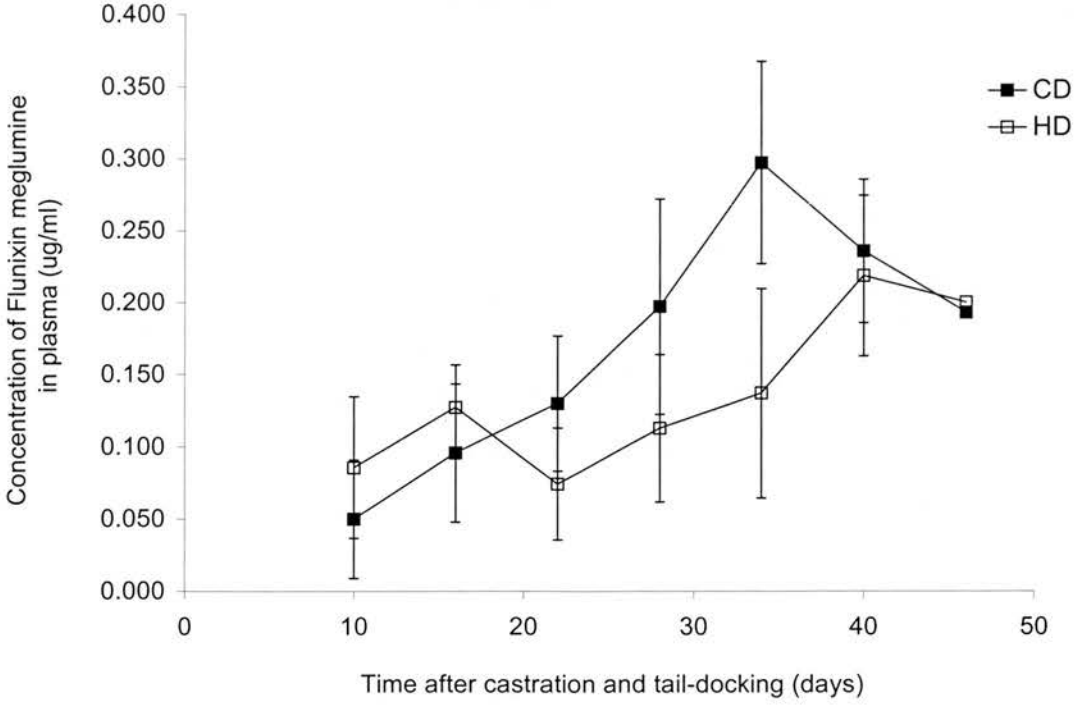
Figure 7.5. Behaviour of lambs at the test hoppers during the first three hours after the hoppers were placed in the pen, n=7. (a) Mean (±sd) frequency of visits to the 'analgesic' hopper. (b) Mean (±sd) proportion of the total duration of time spent feeding that was spent at the 'analgesic' hopper.



7.3.5. *Plasma concentration of flunixin meglumine*

The mean (±SEM) plasma concentrations of flunixin meglumine in lambs in groups CD and HD are presented in figure 7.6. There was a general increase in the plasma concentration of flunixin meglumine in both groups over the time of the trial. However, in CD lambs the concentration rose more quickly and reached a peak 34 days after c+td (0.297 ±0.075μg/ml). Subsequently the plasma concentration of FM in CD lambs declined to the same level as that in HD lambs. The plasma concentration of FM in HD lambs reached a maximum 40 days after c+td (0.219 ±0.070μg/ml). The difference between the plasma concentration of FM measured in CD and HD lambs neared significance at $P<0.05$ on day 34 when the concentration reached a peak in CD lambs ($F_{1,12}=3.46$, $P=0.088$). However there were no statistically significant differences in plasma concentration of FM at $P<0.05$ between CD and HD lambs at any time during the trial. Regression analysis indicated that there was no significant predictive relationship between the width of castration lesions and the plasma concentration of FM in CD lambs.

Figure 7.6. Mean (SEM) concentration (μg/ml) of FM in plasma 3-4hours after test feeds were given, in CD and HD lambs for the duration of the trial, n=7.

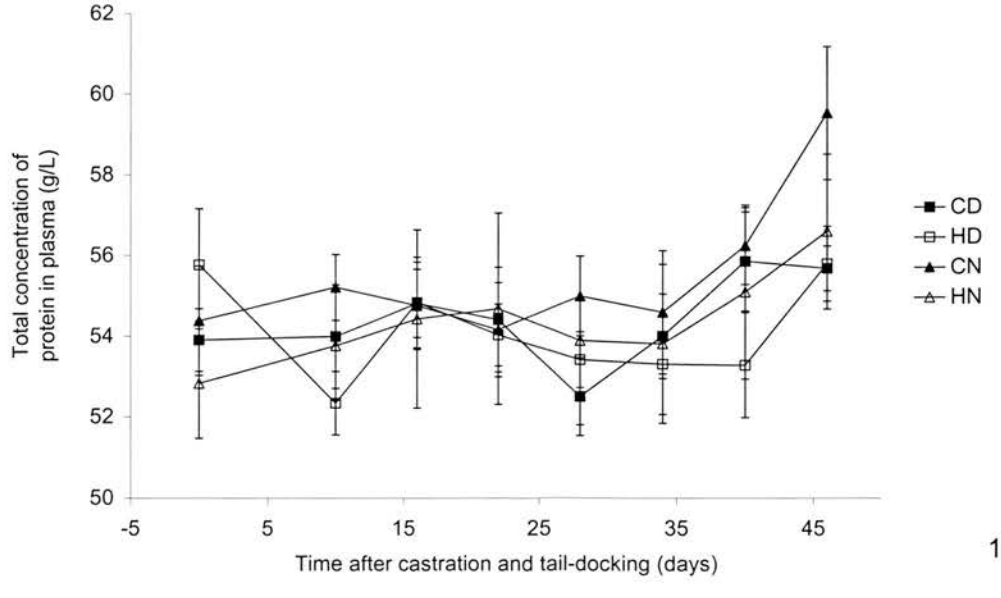


7.3.6. *Plasma protein analysis*

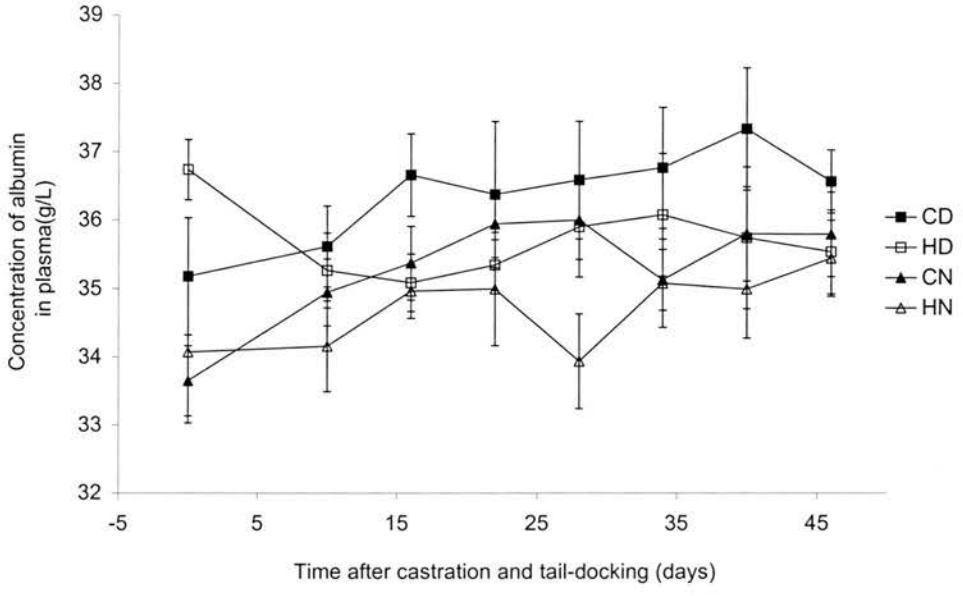
The mean (±SEM) concentrations of protein and albumin in plasma are shown in figures 7.7a and b. Both figures show that the concentration of protein and albumin in the plasma varied very little during the trial. There was an increase in the total concentration of protein towards the end of the trial, which appeared to be most important in group CN. In an initial analysis of this data, the effect of treatment was assessed using a GLM in which no covariates were included. This analysis revealed no differences in the total concentration of protein in plasma between treatments at any time during the trial. Interestingly lambs that were to receive 'analgesic' treatment showed significantly higher concentrations of albumin in plasma before the treatments had been given, but no further differences in the concentration of albumin were found. Further analysis was carried out in groups CD and HD to determine whether variations in the concentration of FM in plasma might have some effect on the total concentration of protein and the concentration of albumin in plasma. The concentration of FM found in plasma was added as a covariate to the analysis of the effect of castration between these groups. A slightly significant negative relationship between the concentration of FM and the concentration of albumin in plasma at $P<0.05$ was found 16 days after c+td (the second cycle of choice testing) ($F_{3,10}= 5.01$, $P= 0.049$). Analysis of total protein data revealed no effect of the concentration of FM but that CD lambs had a higher total concentration of protein in plasma 40 days after c+td than HD lambs. No further significant differences were found.

Figure 7.7(a) Mean (SEM) total concentration (g/L) of protein in plasma in CD and HD lambs over the duration of the trial, n=7. (b) Mean (SEM) plasma concentration (g/L) of albumin in CD and HD lambs over the duration of the trial, n=7.

(a)



(b)



7.3.7. *Severity of lesions*

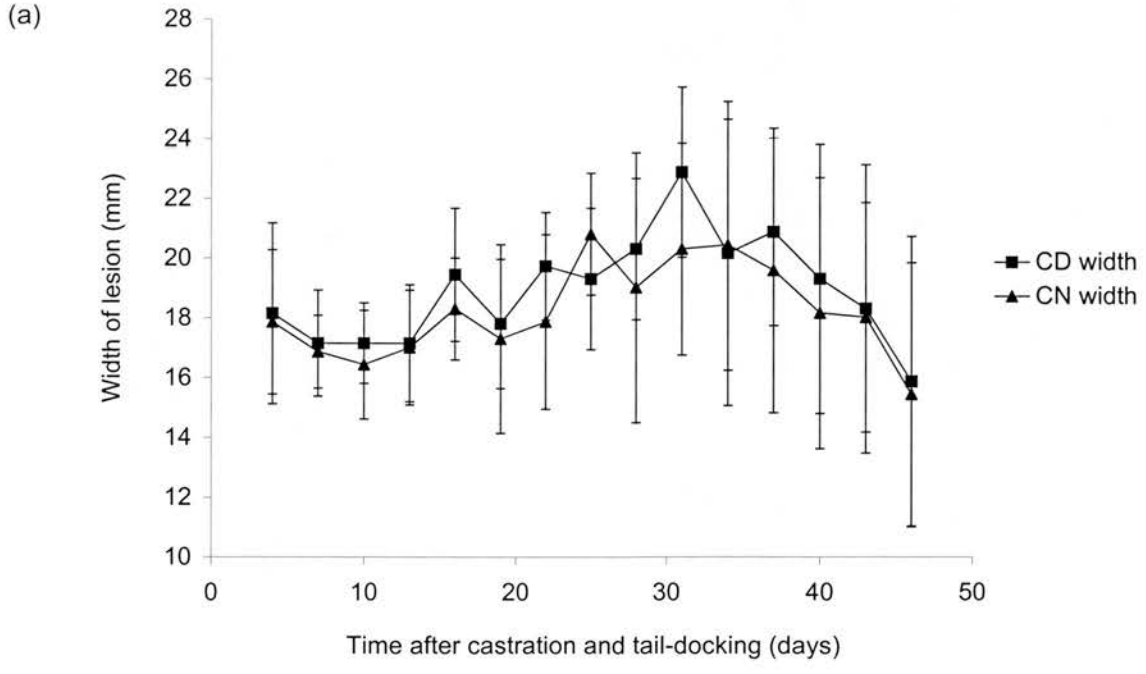
The width and score of scrotal lesions and the score of tail lesions for lambs in groups CD and CN are presented in figure 7.8a, b and c. As can be seen from figure 7.8a that the mean (±sd) width of the lesions observed in CD lambs rose to a peak of 22.9±2.9mm on day 31. The maximum lesion width observed in CN lambs was observed slightly later on day 34, but reached a lower peak of 20.4±4.2mm. The severity scores of scrotal lesions in CD and CN lambs (see figure 7.8b) rose to a median plateau of 3.5. This plateau was reached slightly sooner in CN lambs (day 19) than in CD lambs (day 22). The lesions in CD lambs did not exceed a median (Q1-Q3) of 3.5 (3.5-3.5) during the trial, but lesions in CN reached a peak in severity of 4.0 (3.125-4.0) on day 40. The severity of lesion in CN lambs subsequently dropped off faster than in CD lambs. The severity of tail lesions reached a median (Q1-Q3) maximum of 1.0 (0.5-1.25) in CD lambs, whilst reaching only 0.75 (0.25-1) in CN lambs (see figure 7.8c). Only four lambs in group CD and three lambs in group CN lost their scrotums before the end of the trial. The median time taken for the scrotums to drop off was 46 (38.5-49) and 46 (43-46) days for CD and CN lambs respectively. The tails of all the tail-docked lambs had dropped off by day 49 (3 days after the end of the trial). The mean time taken for tails to drop off was 28.00 (±5.74)

and 30.14 (±5.67) days for CD and CN lambs respectively. This difference was not significant at $P<0.05$ (Student's T test $T_{11}=0.70$, $P=0.500$).

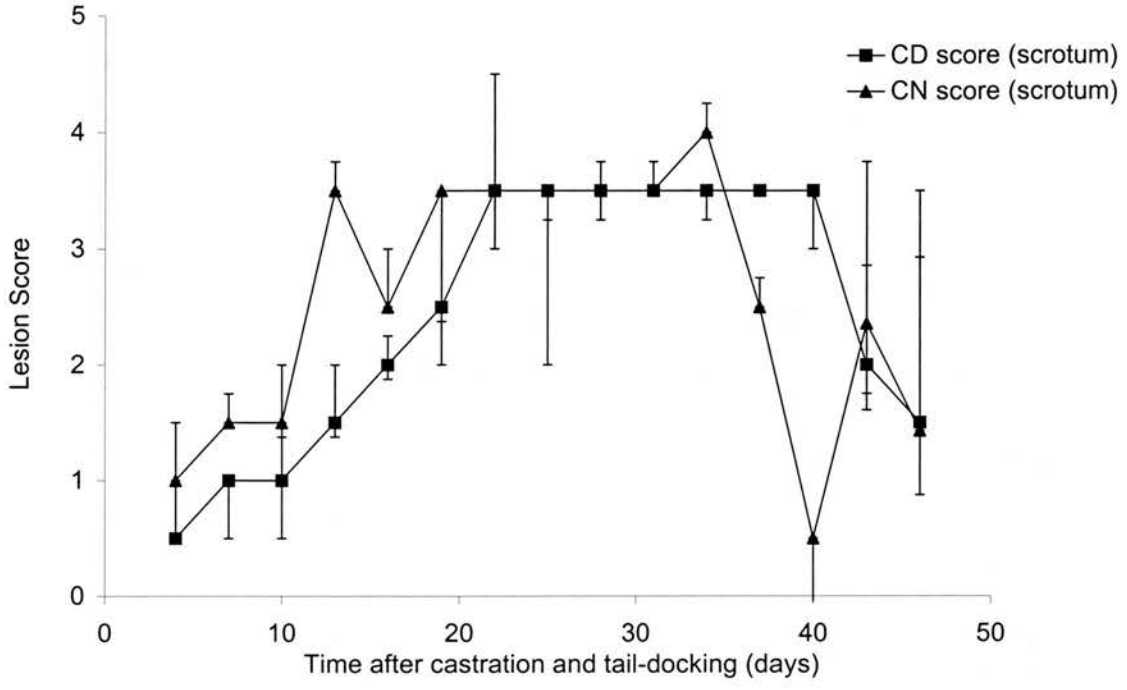
There were no statistically significant differences in the width and severity of lesions observed in lambs with and without access to analgesic. In order to distinguish between the effect of the drug on the lesion and possible extraneous variation as a result of the variations in the size of lambs within groups, the weight of lambs was used as a covariate in a GLM to test for differences in lesion width between CD and CN lambs. This analysis revealed that for the majority of the study, the weight of the lamb had no effect on the width of the lesion observed. However, on day 34 after c+td there was a significant negative relationship between the weight of lambs and the width of the lesion ($F_{3,10}=6.41$, $P=0.030$). As in the initial analysis 'analgesic' treatment was found to have no significant effect on lesion width at any time.

Pictures of the changing severity of a chronic inflammatory lesion over the 6-weeks after ring application in a representative lamb are shown in Appendix D.

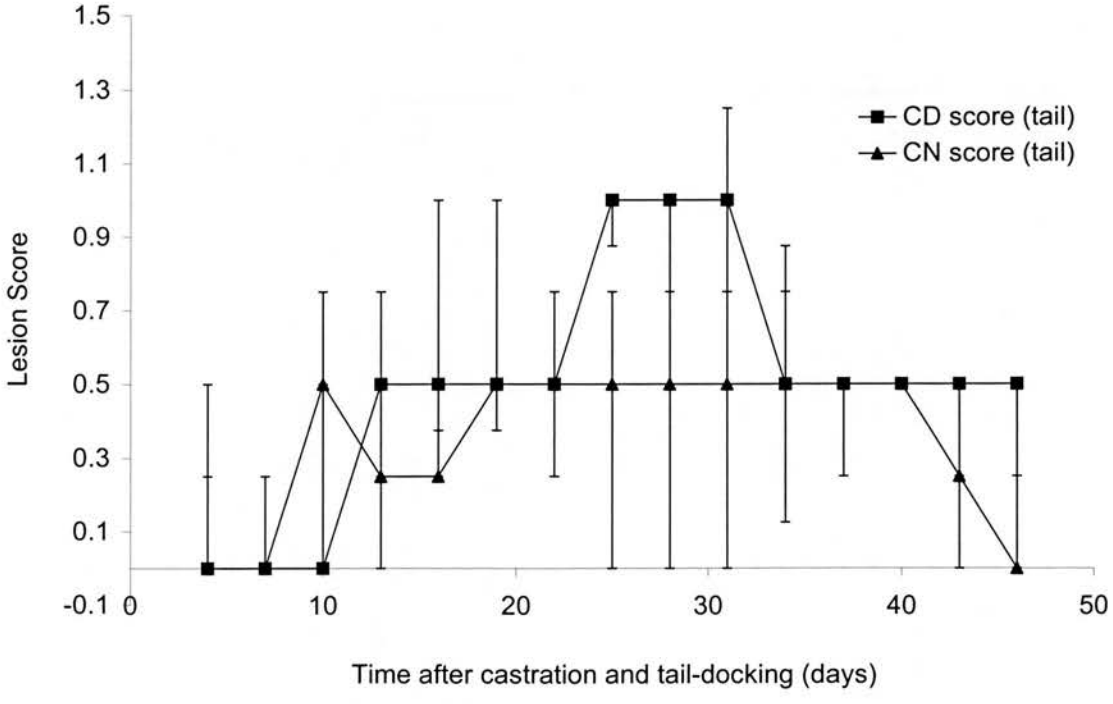
Figure 7.8. Severity of lesions resulting from c+td of CD and CN lambs (n=7). (a) Mean (±sd) width of lesions (mm). (b) Median (Q1-Q3) score of scrotal lesions. (c) Median (Q1-Q3) score of tail lesions.



(b)


CD score (scrotum)
CN score (scrotum)
Lesion Score
Time after castration and tail-docking (days)
0
1
2
3
4
5
0
10
20
30
40
50

(c)


CD score (tail)
CN score (tail)
Lesion Score
Time after castration and tail-docking (days)
-0.1
0.1
0.3
0.5
0.7
0.9
1.1
1.3
1.5
0
10
20
30
40
50

7.3.8. *Behaviour*

Postures

HN, CD and HD lambs were recorded in S1 posture in a slightly decreasing number of observations over the course of the trial. This pattern was most evident in HN lambs and is shown in figure 7.9a. Lambs in group CN spent fewer observations in S1 standing posture during the first four observation periods (days 10-28). On days 10 and 22 a significant effect of c+td was found ($t_{3,24}$=-2.67, P=0.008 and $t_{3,24}$=-2.09, P=0.047 respectively). Post-hoc analysis using Fisher's approach showed that this result reflected a statistically significant difference between HN and CN lambs. On both days CN lambs showed significantly less S1 standing than HN lambs ($t_{3,24}$=-2.67, P=0.0131 and $t_{3,24}$=-2.09, P=0.046 on days 10 and 22 respectively). After day 28 there was no difference in the number of observation spent S1 standing, nor was there any difference in the number of observations spent S1 standing overall (see figure 7.9b).

A statistically significant effect of c+td and of the interaction between c+td and analgesic treatment was found on day 16 of the study ($t_{3,24}$=2.80, P=0.050, $t_{3,24}$=-2.98, P=0.003). Post-hoc testing revealed that HN lambs spent fewer observations in V1 lying posture than both CN and HD lambs ($t_{3,24}$=2.80, P=0.0096 and $t_{3,24}$=2.40, P=0.0237 respectively). However, the number of observations in which lambs were observed to be in V1 lying posture was low overall and no other differences in the expression of the behaviour were found. The mean total number of observations spent in V1 posture was also not significantly different between groups although there appeared to be a slight tendency for castrated animals to spend more time in this posture. Data on V1 posture are presented in figures 7.9c and d.

There was no pattern of variation in the number of observations spent in V2 lying posture during the trial (see figure 7.9e and f). No statistically significant differences were found in the occurrence of V2 lying posture between groups.

When S1, V1 and V2 postures were summed to provide a measure of the expression of normal postures (see figure 7.9g and h), there was a tendency for CN lambs to express less normal posture over the first 4 observations (days 10-28), however this trend did not reach significance. When the mean total number of observations of normal behaviour was examined for the trial a statistically significant effect of c+td

and the interaction was found. Post-hoc testing showed that CN lambs spent significantly less time in normal postures than HN lambs ($t_{3,24}$=-2.68, P=0.0130). CD lambs spent as much time in normal postures as handled lambs throughout the trial and access to analgesic made no difference to the expression of normal postures.

CN lambs showed a tendency to express more V3 lying posture during days 10 – 28 of the trial (see figure 7.9i). In this group, the expression of V3 posture rose to a peak at day 28, coinciding with the peak in the severity of castration and tail-docking lesions. Subsequently the expression of V3 posture declined. Differences in the expression of V3 posture between groups did not reach significance at P<0.05 during days 10-28. When the mean total number of observations spent in V3 lying posture were examined for the whole trial however, a significant effect of c+td and the interaction was found ($t_{3,24}$=2.51, P=0.019 and $t_{3,24}$=-2.34, P=0.028 respectively) (see figure 7.9j). CN lambs were found to spend significantly more time in V3 posture than HN lambs ($t_{3,24}$=2.51, P=0.0189). On day 40 a significant effect of the interaction between c+td and analgesic treatment was found and post-hoc analysis showed that CD lambs were observed in V3 posture significantly less often than CN lambs although this difference did not reach significance at P<0.05 ($t_{3,24}$=-1.70, P=0.0996).

SS posture contributed very little to the overall expression of abnormal behaviour and no significant differences in the expression of this behaviour between groups were found. However, when abnormal posture (the sum of V3 and SS postures) was examined, an effect of the interaction was found on days 10 and 40 and when the behaviour was summed for the duration of the study. Post-hoc analysis showed that CN lambs expressed more abnormal behaviour than CD lambs on day 10 ($t_{3,24}$=-2.94, P=0.0069) and 40 (although this differences was not significant on day 40 ($t_{3,24}$=-1.70, P=0.0996)) and that CN lambs showed more abnormal postures than HN lambs overall ($t_{3,24}$=2.60, P=0.015).

Behavioural states

During observations on days 10-28 of the trial CN lambs tended to eat less than the other groups of lambs and HN lambs tended to eat more (see figure 7.9k). These differences were not significant, except on day 22 after c+td when CN lambs spent

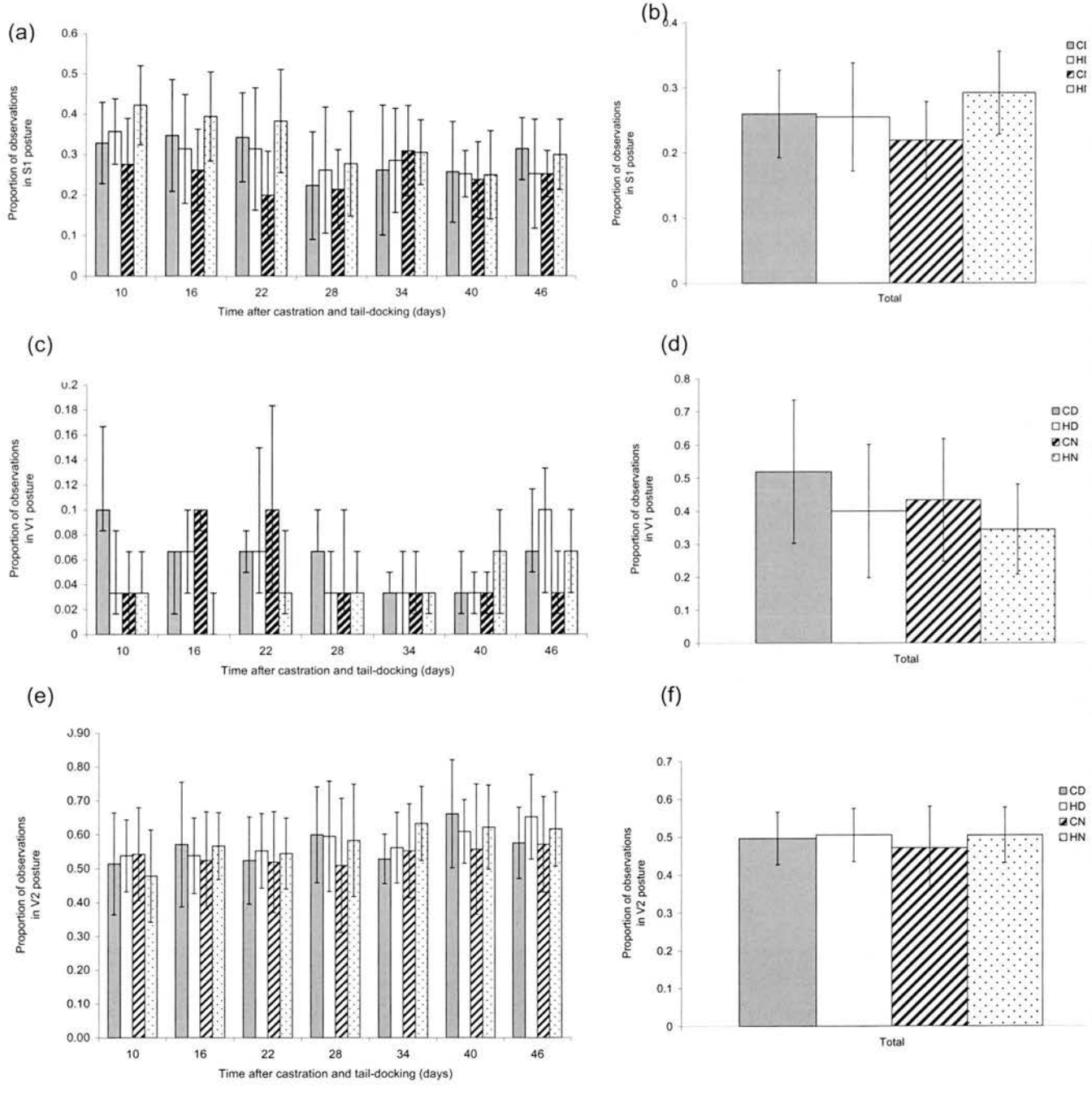
significantly less time eating than HN lambs ($t_{3,24}$=-2.65, P=0.014 post-hoc Fisher's approach). There was no overall difference in the mean total amount of time spent eating between groups (see figure 7.9l).

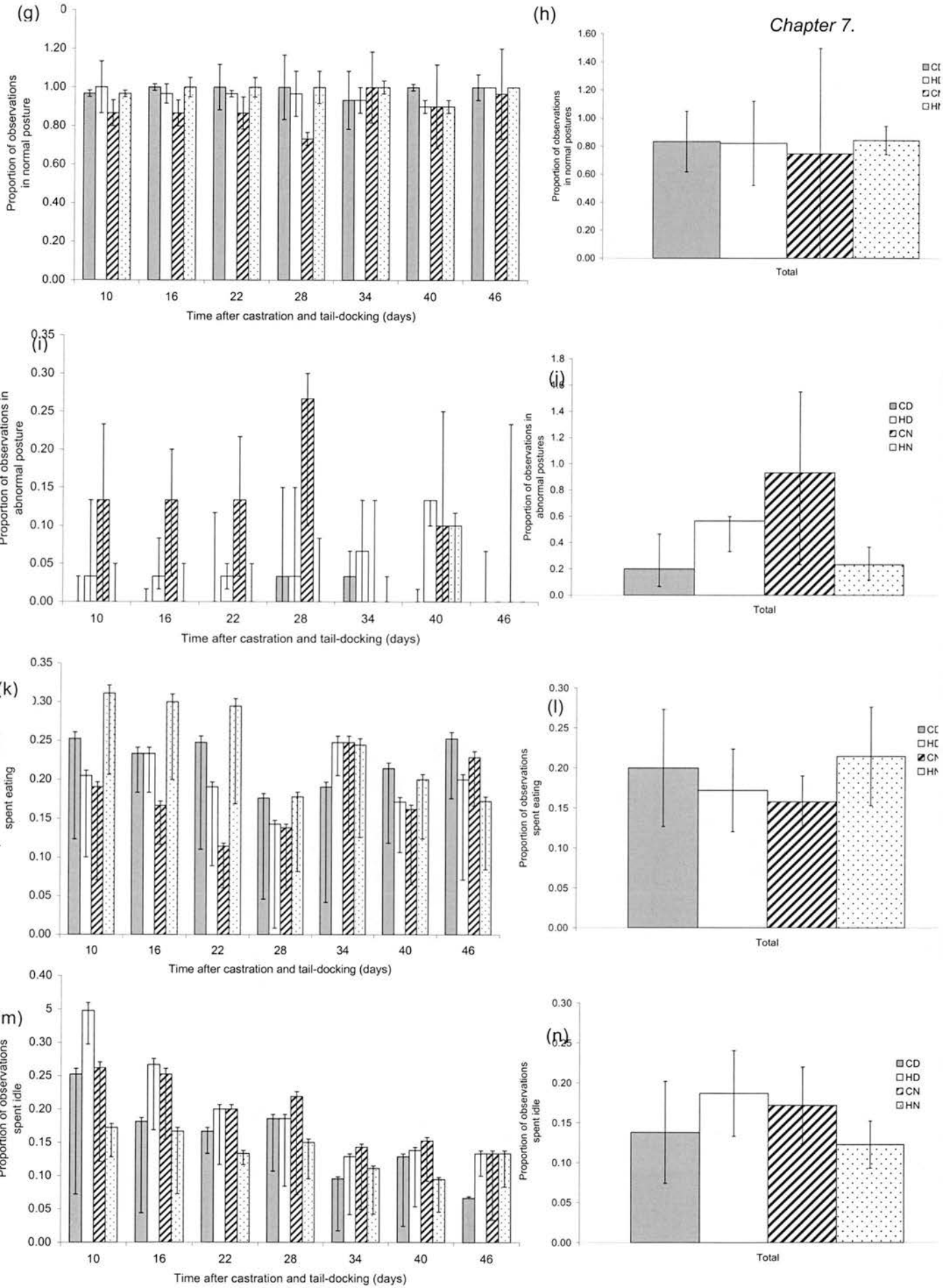
There was an overall decrease in the number of observations spent idling in all groups as the trial progressed (see figure 7.9m). A significant effect of analgesic treatment and the interaction factor was found overall ($t_{3,24}$=2.3, P=0.031 and $t_{3,24}$=-2.53, P=0.019 respectively) and post-hoc analysis showed that HD lambs spent significantly more time idling than HN lambs over the whole time of the trial ($t_{3,24}$=2.29, P=0.030) (see figure 7.9n). Significant effects of treatment were found on days 10, 16 and 22 after c+td and post-hoc analysis revealed that HD lambs showed more idling than HN lambs on day 10 ($t_{3,24}$=2.38, P=0.025)

Over the time of the trial there was a consistent increase in the number of observations spent ruminating until 34 days after c+td. On day 34 a significant effect of c+td and the interaction was found. After post-hoc tests HN lambs were found to spend more time ruminating than CN lambs ($t_{3,24}$=-1.97, P=0.060), although not significantly so. However no consistent differences in the expression of this behaviour were observed (see figure 7.9o and p) and no difference in the mean total expression of the behaviour during the trial were found.

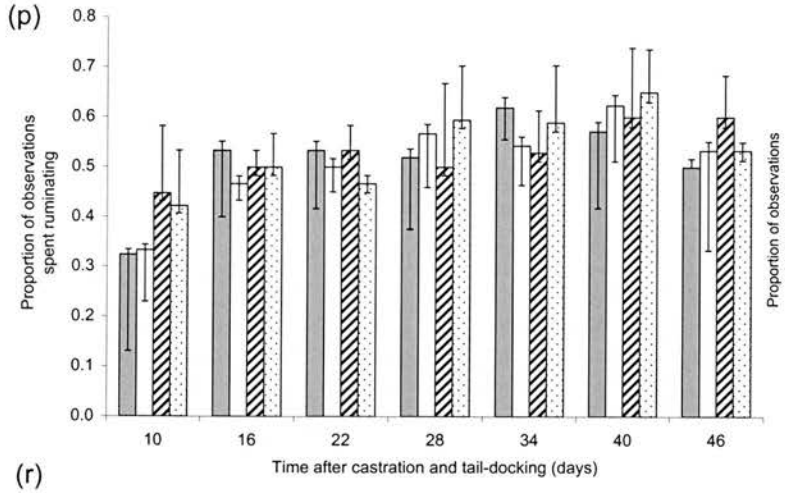
CD spent more time sleeping on day 10 after c+td but this difference was not significant at P<0.05. There was an increase in the amount of time spent sleeping by CN lambs during days 10-28. There was a significant effect of treatment on the amount of time spent sleeping on days 16, 22 and 28 after c+td ($t_{3,24}$=-2.22, P=0.026, $t_{3,24}$=-2.07, P=0.039 and $t_{3,24}$=2.89, P=0.004 respectively). Post-hoc testing showed that HN lambs spent less time sleeping than CN and CD lambs ($t_{3,24}$=2.88, P=0.008 and $t_{3,24}$=2.24, P=0.034 respectively) HN lambs spent less time sleeping during the trial than the other lambs but this difference did not reach significance at P<0.05. Data on sleeping are presented in figures 7.9q and r.

Figure 7.9. Proportions of observations of postures and behavioural states during two 2 hours observation periods; obtained in scan samples every 6 minutes. **(a)** Mean (±sd) proportion of observations in **S1** posture for each weekly observation throughout the trial. **(b)** Total mean (±sd) proportion of observations observed in **S1** as a summary for the whole study period. **(c)** Median (Q1-Q3) **V1 (d)** Total median (Q1-Q3) **V1 (e)** Mean (±sd) **V2 (f)** Total mean (±sd) **V2 (g)** Median (Q1-Q3) **normal (h)** Total median (Q1-Q3) **normal (i)** Median (Q1-Q3) **abnormal (j)** Total median (Q1-Q3) **abnormal (k)** Mean (±sd) **eating (l)** Total mean (±sd) **eating (m)** Mean (±sd) **idling (n)** Total mean (±sd) **idling (p)** Mean (±sd) **ruminating (q)** Total mean (±sd) **ruminating (r)** Median (Q1-Q3) **sleeping (s)** Total median (Q1-Q3) **sleeping**. CD=black bars, HD=open bars, CN=hashed bars, HN=spotted bars.

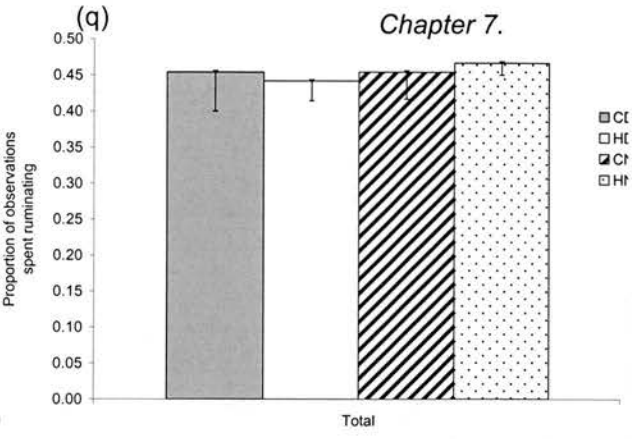



(g)
Proportion of observations in normal posture
Time after castration and tail-docking (days)
(h)
Proportion of observations in normal postures
Total
(i)
Proportion of observations in abnormal posture
Time after castration and tail-docking (days)
(j)
Proportion of observations in abnormal postures
Total
CD
HD
CN
HN
(k)
Proportion of observations spent eating
Time after castration and tail-docking (days)
(l)
Proportion of observations spent eating
Total
(m)
Proportion of observations spent idle
Time after castration and tail-docking (days)
(n)
Proportion of observations spent idle
Total

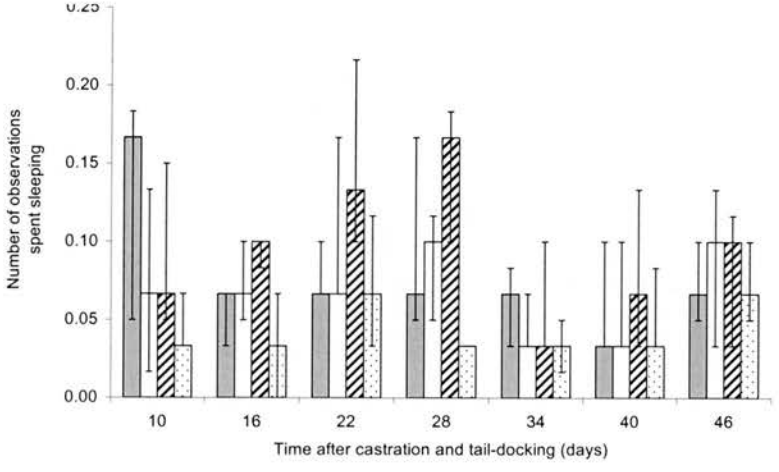
(p)


Proportion of observations spent ruminating
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
10
16
22
28
34
40
46
Time after castration and tail-docking (days)

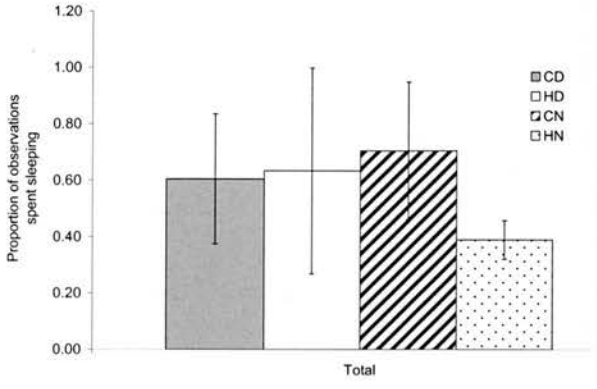
(q)


Proportion of observations spent ruminating
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Total

(r)


Number of observations spent sleeping
0.20
0.15
0.10
0.05
0.00
10
16
22
28
34
40
46
Time after castration and tail-docking (days)

(s)


Proportion of observations spent sleeping
1.20
1.00
0.80
0.60
0.40
0.20
0.00
CD
HD
CN
HN
Total

Frequency of active behaviour

For some behaviours the variance within the data was not balanced across groups because they occurred infrequently overall but were expressed frequently by few individuals. Where variance problems within the data were experienced statistical analyses using generalised linear models could not be carried out, as this analysis assumes approximately equal variance. The data are presented in graphical form in figure 7.10.

Examination of the frequency of active behaviours revealed few differences between treated animals and controls. As can be seen in figure 7.10a, there was no overall change in the frequency of restlessness during the trial in any group. On day 22 c+td was found to have a significant effect on the occurrence of restlessness $P<0.05$ ($t_{3,24}=1.98$, $P=0.048$). After post-hoc analysis using Fisher's approach the differences between groups was not found. The total mean frequency of restlessness for the whole time period did not differ between groups (see figure 7.10b).

The pattern of expression of easing quarters in CD and CN lambs varied over time (see figure 7.10c). CD lambs initially expressed easing quarters with the same frequency as HD and HN lambs, but their expression of this behaviour increased in frequency during the trial to reach a peak in frequency 34 days after c+td. This rise and peak coincides with the plateau of maximum severity of scrotal and tail lesions measured during the trial. However, because of the variation in the expression of the behaviour between individuals in the group, the difference between CD lambs and the other groups did not quite reach significance at $P<0.05$ on day 34 after c+td ($t_{3,24}=2.07$, $P=0.050$). Two peaks in the expression of the behaviour are observed in CN lambs, the first occurred 16 days after c+td and the second 40 days after c+td. However, the frequency of easing quarters in CN did not differ significantly from that in the other groups at any time during the trial.

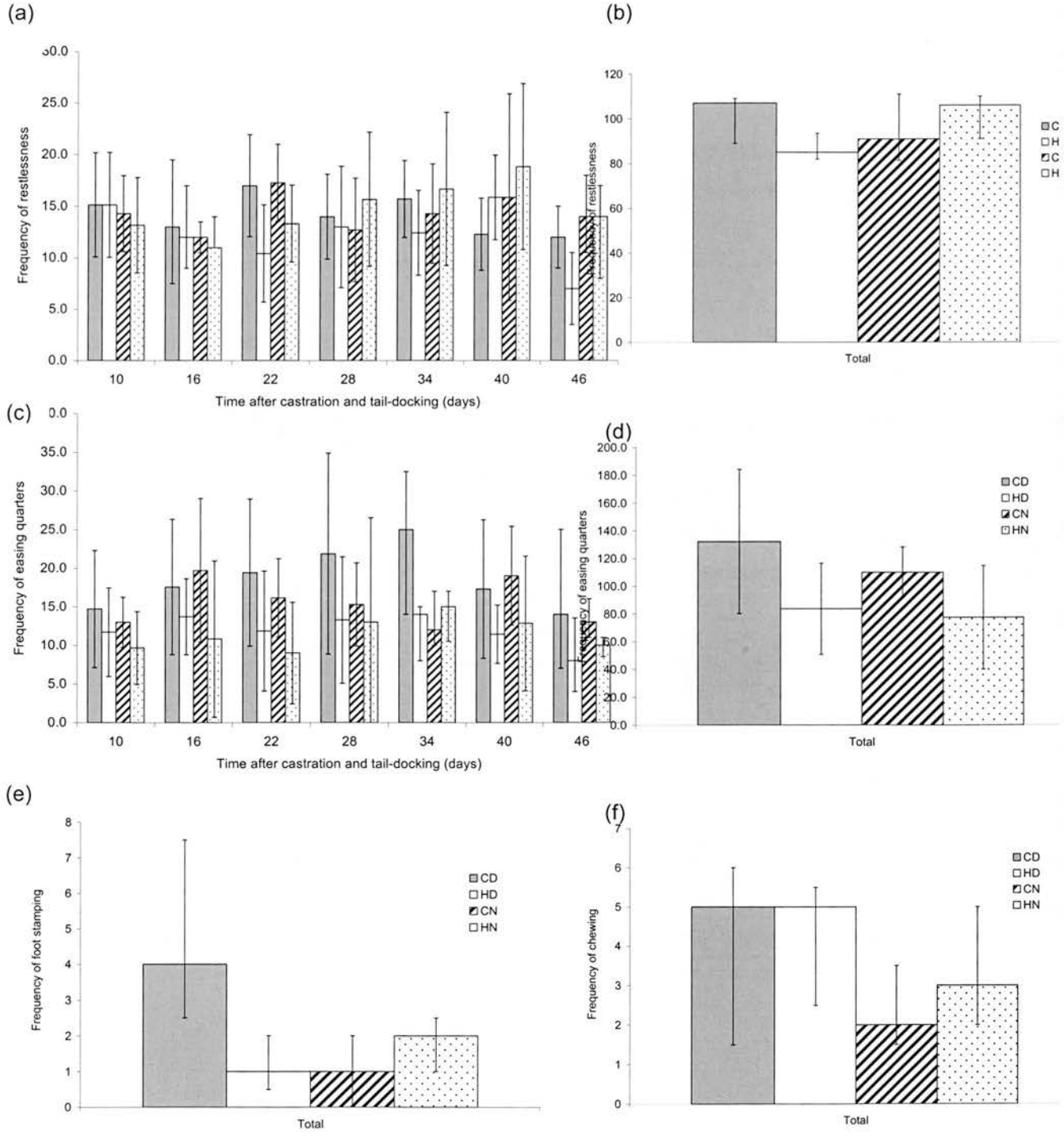
Much of the data on foot-stamping could not be analysed statistically for reasons described above. However, the mean total expression of the behaviour was analysed as was data for day 22 but no significant differences were found. Overall, CD lambs expressed the behaviour more frequently (see figure 7.10e), however the variation in frequency within the group was high and the difference did not reach significance at $P<0.05$.

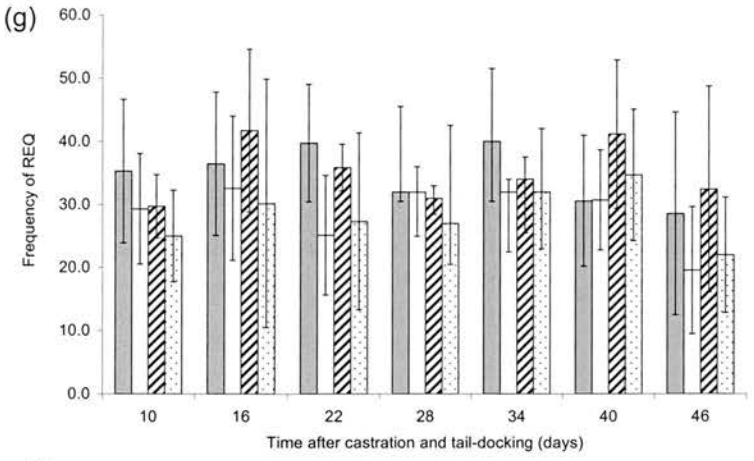
The REQ score (sum of rst, eq and fsk) verified patterns of easing quarters observed in CD and CN lambs but no differences in the frequency of expression of these behaviours occurred either during the trial or in the total mean expression for the whole period (see figure 7.10g and h).

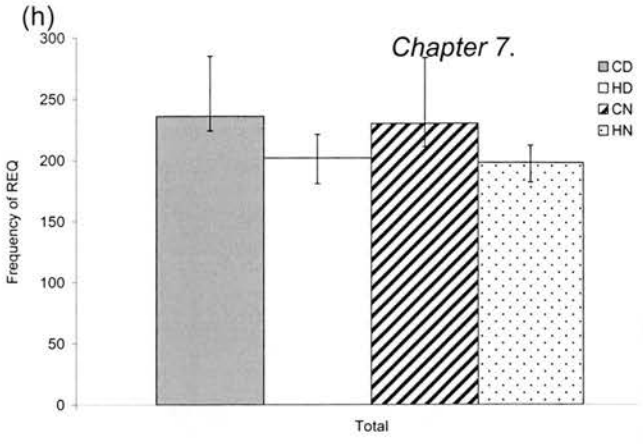
There was a decline in the frequency of teat-seeking over the six weeks of the trial (see figure 7.10i and j). This decline was observed in all groups and there were no significant differences between groups in the frequency of the behaviour. The frequency of tail-wagging in CN lambs peaked twice during the trial, at days 16 and 40 after c+td (see figure 7.10k). These peaks in expression occurred at the same time as peaks in the expression of easing quarters behaviour in the same lambs. The expression of tail-wagging in lambs in the other groups did not fluctuate, but showed an overall decline during the trial. Despite peaks in expression of tail-wagging in CN lambs on days 16 and 40, there were no significant differences in the frequency of tail-wagging between groups at any time point or in total for the whole study (see figure 7.10l).

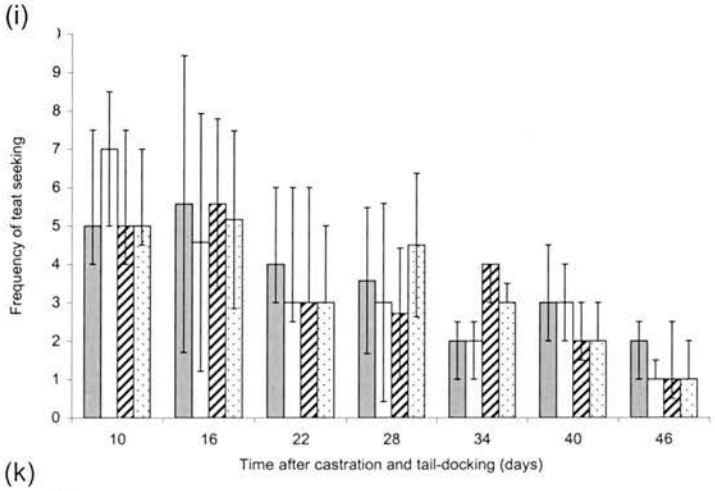
Head-turning behaviour occurred very infrequently and again much of this data could not be analysed statistically. However, there were no significant differences in the frequency with which this behaviour was expressed between treatment groups when considered for each target (i.e. head turn to flank, scrotum, back or outside hind leg) separately on the occasions when variances were equal between groups and data could be analysed. When head-turning to each target was summed to provide data on head-turning in general (see figure 7.10m) no differences in the frequency of expression of the behaviour between groups were found. The summary data for head turning is presented in figure 7.10n. Play behaviour also occurred very infrequently during the trial and on days 10, 28, 34 and 46 the data could not be analysed. On day 16, play was observed more frequently in handled than in castrated lambs ($t_{3,24}$=-2.08, P=0.037) although again the differences were not found to be significant with post-hoc testing. When the mean total frequency of behaviour was examined, no statistically significant differences were found although play appeared to occur more frequently in handled animals (see figure 7.10q). There were no differences in the frequency of expression of rubbing (see figure 7.10o and p), chewing (see figure 7.10f) or horning (see figure 7.10r) at any time during the trial.

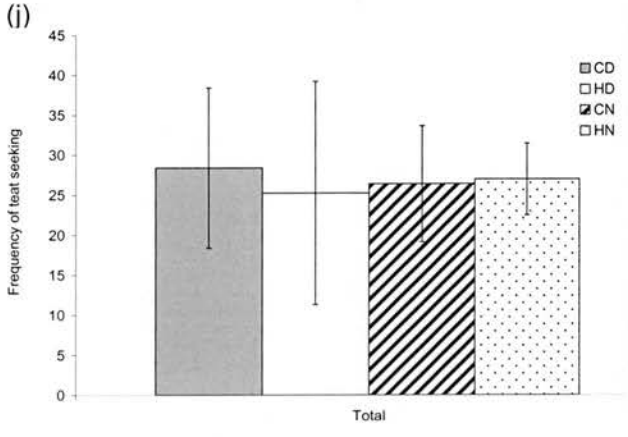
Figure 7.10. Frequency of active behaviours observed during two, 2 hour long observation periods. **(a)** Mean (±sd) frequency of **restlessness** for each weekly observation throughout the trial. **(b)** Total median (Q1-Q3) frequency of **restlessness** as a summary for the whole study period. **(c)** Mean (±sd) **easing quarters (d)** Total mean (±sd) **easing quarters (e)** Total median (Q1-Q3) **foot-stamping (f)** Total median (Q1-Q3) **chewing (g)** Mean (±sd) **REQ (h)** Total median (Q1-Q3) **REQ (i)** Median (Q1-Q3) **tail-wagging (j)** Total median (Q1-Q3) **tail-wagging (k)** Median (Q1-Q3) **teat-seeking (l)** Total mean (±sd) **teat-seeking (m)** Median (Q1-Q3) **head-turning (n)** Total median (Q1-Q3) **head-turning (o)** Total median (Q1-Q3) **play (p)** Median (Q1-Q3) **rubbing (q)** Total mean (±sd) **rubbing (r)** Total median (Q1-Q3) **horning**. CD=black bars, HD=open bars, CN=hashed bars, HN=spotted bars.

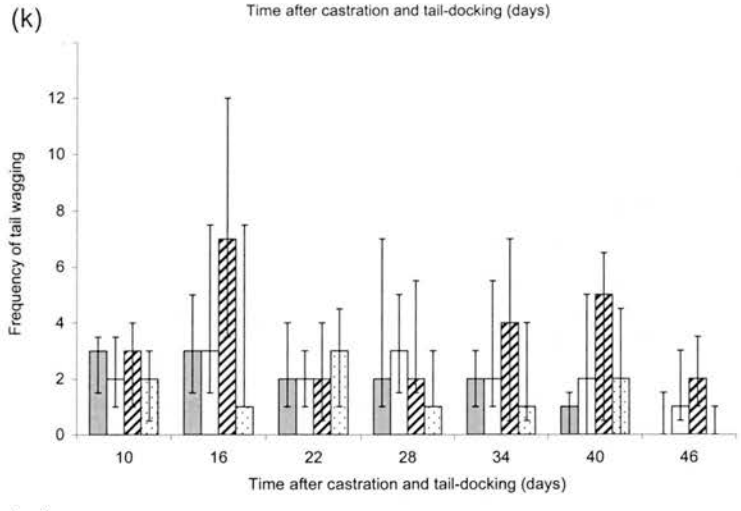


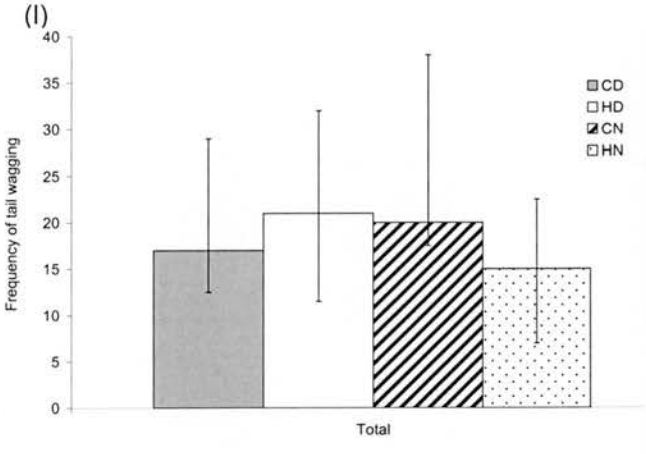

(g)
Frequency of REQ
60.0
50.0
40.0
30.0
20.0
10.0
0.0
10
16
22
28
34
40
46
Time after castration and tail-docking (days)

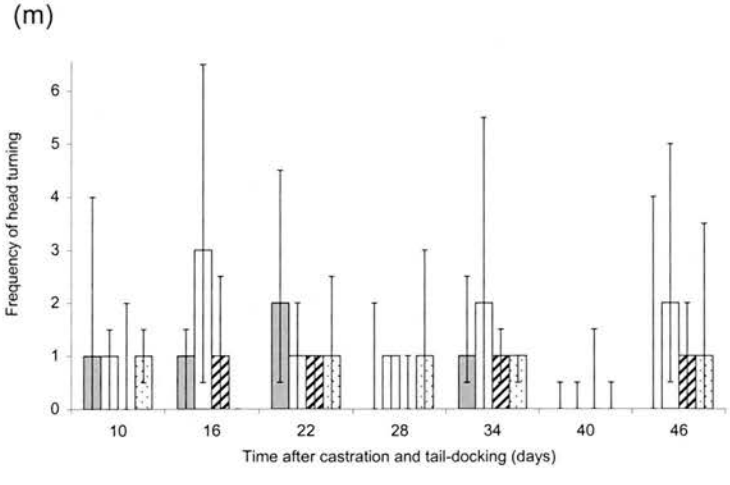

(h)
Frequency of REQ
300
250
200
150
100
50
0
Total
CD
HD
CN
HN

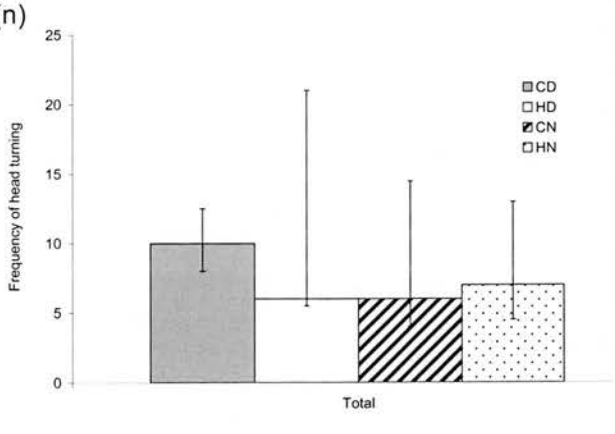

(i)
Frequency of teat seeking
9
8
7
6
5
4
3
2
1
0
10
16
22
28
34
40
46
Time after castration and tail-docking (days)


(j)
Frequency of teat seeking
45
40
35
30
25
20
15
10
5
0
Total
CD
HD
CN
HN

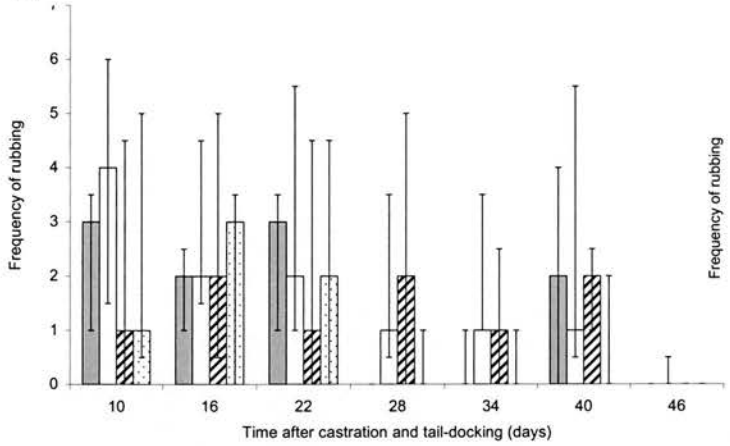

(k)
Frequency of tail wagging
12
10
8
6
4
2
0
10
16
22
28
34
40
46
Time after castration and tail-docking (days)


(l)
Frequency of tail wagging
40
35
30
25
20
15
10
5
0
Total
CD
HD
CN
HN

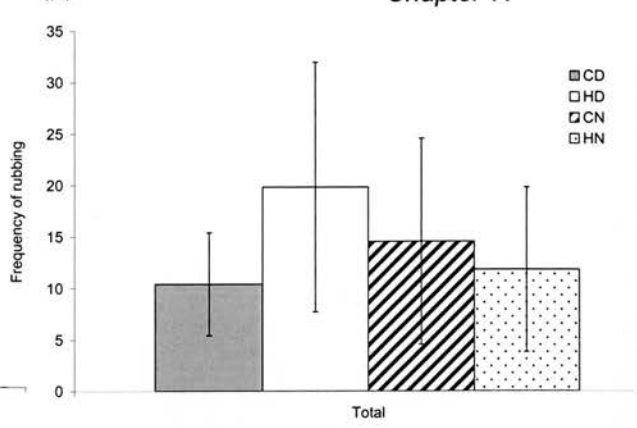

(m)
Frequency of head turning
6
5
4
3
2
1
0
10
16
22
28
34
40
46
Time after castration and tail-docking (days)


(n)
Frequency of head turning
25
20
15
10
5
0
Total
CD
HD
CN
HN

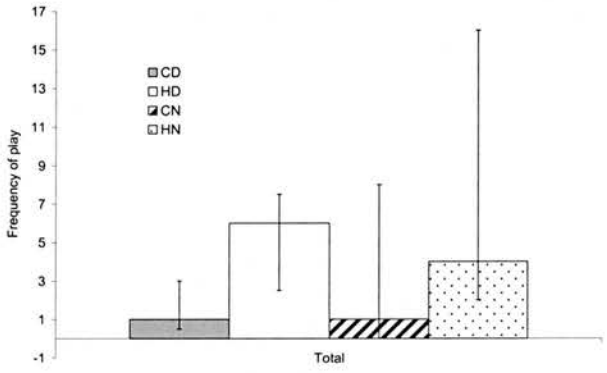
(o)


Frequency of rubbing
0
1
2
3
4
5
6
10
16
22
28
34
40
46
Time after castration and tail-docking (days)

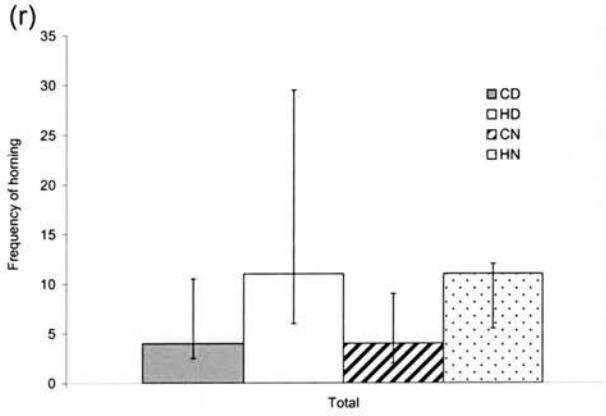
(p)


Frequency of rubbing
0
5
10
15
20
25
30
35
CD
HD
CN
HN
Total

(q)


Frequency of play
-1
1
3
5
7
9
11
13
15
17
CD
HD
CN
HN
Total

(r)


Frequency of horning
0
5
10
15
20
25
30
35
CD
HD
CN
HN
Total

7.4. Discussion

7.4.1. *Physical evidence of chronic inflammatory pain?*

Weight

In the present study no statistically significant differences in daily live-weight gain were found between treatment groups. This is in accordance with the study described in Chapter 6 and with previous studies in which c+td was found to have no effect on weight gain (Kent *et al*, 2000). However, in the study described in Chapter 6 a non-significant trend of reduced weight gain in CN lambs was found, whilst CD lambs showed the most rapid weight gain. CN lambs were also found to spend less time eating than the other lambs during the period when the lesions from c+td were most severe. Although these differences were not statistically significant, the fact that the slight differences occurred together and that the trend was not observed in CD lambs suggests that the changes may have been associated with pain of some degree. In the present study no such changes in weight were found although CN lambs were found to spend less time eating than the other lambs as was observed in the previous study. This difference will be discussed in more detail below.

Severity of lesions

In previous studies of the chronic effects of RR castration in six-week-old, Scottish Blackface lambs, lesions measuring 27mm across and with a severity score of 4.2 were observed (Kent *et al*, 2000). In the present study castration lesions with a width of 22.9± 2.9mm and a maximum severity of 3.5 (3.5-3.5) were found in CD lambs and a width of 20.4± 4.2mm and a maximum severity of 4.0 (3.125-4.0) were found in CN lambs. These lesions were both smaller and less severe than those found by Kent *et al* (2000). Lesions of limited severity were also found on the tails of these lambs. Although the peak in lesion severity occurred at a similar time to that previously reported (3-4weeks after RR application), the scrotums and tails also took longer to drop off than in previous studies, a fact likely to be the result of the reduced severity of lesions. The lesions found in this study were no more severe than those found in the previous study, described in Chapter 6.

One of the most important changes in the experimental protocol for this study was the increase in the age of lambs at the time of c+td. This change was included in an

attempt to increase the severity and consistency of the lesions observed, thus theoretically increasing the pain experience of the lambs. It was hypothesised that such an increase in pain severity would increase the probability that lambs would learn the association between analgesic effects and the consumption of a novel feed. The severity of lesions that developed during this trial was less than expected, thus reducing the potential effect of increasing the age of lambs at c+td. No definite explanation can be given for these lesions being unusually mild. However it is proposed that fresh bedding may have been provided more frequently in this study than in previous studies, thus reducing the opportunity for bacterial contamination of developing lesions. This aspect of the study was not controlled with respect to previous studies. However, in this study the positioning of the lambs in different groups was balanced across the study environment and therefore there should have been no differences in the quantity of bedding provided to lambs in different treatment groups. In order to ensure that consistently severe lesions were obtained in studies of the chronic effects of RR c+td, it might be considered necessary to control the quantity of bedding provided and to regulate the level of bacterial infection of the lesions, by deliberate, controlled contamination of the ring or lesion.

7.4.2. *Behavioural evidence of chronic inflammatory pain?*

As was observed in the previous study, CN lambs in the present study spent less time eating than the other lambs. The amount of time CN lambs spent eating declined over the first 3 weeks after c+td and the difference between CN lambs and the other groups reached significance 22 days after c+td and neared significance at $P<0.05$ when the data was summed for the whole study period. The difference is consistent with appetite suppression associated with chronic inflammatory pain, reaching significance as the c+td lesions neared their maximum severity. This proposal is supported by the fact that CD lambs showed no such time-dependent changes in the amount of time they spent eating suggesting that the NSAID FM reduced the experience of pain and thus prevented the appetite inhibition observed in CN lambs. Suppression of appetite is widely accepted to be a symptom of chronic pain and is usually associated with weight loss or reduced weight gain (Kitchell and Johnson, 1985; Zimmerman, 1986). However as was discussed above, the reduction in

appetite in CN lambs was insufficient to induce any statistically significant change in live-weight gain in CN lambs.

Differences in the amount of time spent in other behavioural states were also observed. In contrast however, these differences were not consistent with changes in behaviour that would be expected as a result of chronic inflammatory pain. It is commonly accepted that chronic pain can result in interrupted sleep patterns (Kitchell and Johnson, 1985; Zimmermann, 1986), but in this study CN lambs spent significantly more time sleeping than other lambs. This result differs from that in the previous study in which c+td lambs were found to sleep less than other lambs and there are few reasonable explanations for these differences. One possible explanation is that as the lambs got older they became less inclined to move around their relatively restricted environment and therefore when they were not eating showed a greater tendency to lie down and sleep than the younger lambs used in the previous trial. The restriction on movement and behaviour may therefore have increased the effect of dependence in scan sampling data, which is an unavoidable error in the analysis.

The inconsistencies in behavioural states were less evident in the analysis of posture. CN lambs showed more abnormal posture than the other lambs and the amount of time spent in abnormal postures rose to a peak 28 days after c+td, coinciding with the peak in lesion severity. In this study, V3 posture was the most common abnormal posture observed, with very few occurrences of SS contributing to the data slightly. Changes in posture have been considered indicative of the experience of avoidable pain (Molony *et al*, 1993). Lambs are thought to adopt these postures to minimise aggravation of sensitised tissue. The adoption of such posture during a period of chronic inflammatory pain is likely to be particularly effective, as chronic inflammation has been reported to result in general tenderness and the experience of severe pain when the region is manipulated (Levine and Taiwo, 1994). The changing expression of abnormal postures was not observed in CD lambs and the time spent in abnormal postures was consistently lower in these lambs. Thus, it may be concluded that the consumption of the NSAID FM reduced inflammation sufficiently to reduce generalised inflammatory pain and therefore the appearance of abnormal postures used to minimise harm to the affected region. This indicates that analysis of posture

has the potential to provide a useful means of assessment of chronic inflammatory pain.

Evidence of an effect of c+td was also found in analysis of the frequency of active behaviours. In studies of acute pain from RR c+td, incidences of abnormal active behaviour were considered to be indicative of inescapable pain (Molony *et al*, 1993). In this study, there were few consistent differences in the occurrence of active behaviour over time. However, c+td lambs showed more restlessness, easing quarters and foot-stamping than handled lambs. In CD lambs, a peak in the expression of easing quarters coincided with the time at which c+td lesions reached the maximum of severity. In CN lambs, two coinciding but transient peaks were observed in the frequency of both easing quarters and tail-wagging. Play behaviour was observed more frequently in handled lambs, although not significantly so. Overall the differences observed were not highly significant and in isolation, could not be considered strong evidence of an effect of c+td. However, in combination their occurrence can be considered to provide more substantial evidence of pain.

It is important to note that access to analgesic did not appear to reduce the frequency of active behaviour in c+td lambs, with higher frequencies of behaviour found in both CN and CD lambs. There are two possible conclusions that can be drawn from this anomaly. Firstly, as has been discussed in relation to acute pain form RR c+td (Molony *et al*, 1993), it might be proposed that two types of pain were present in these lambs. Lambs may have experienced a general background level of chronic inflammatory pain and were able to minimise their experience of this pain by adopting subtle changes in posture. The consumption of FM reduced inflammation and thus the degree of sensitisation of damaged tissue and therefore chronic inflammatory pain and resulted in a reduced need to assume these abnormal postures. However, movement or irritation from bedding or insects for example, may have induced more isolated episodes of acute unavoidable pain, inducing higher than normal frequencies of active behaviours. The presence of inflammation is also known to increase the occurrence of spontaneous activity in nociceptors (Levine and Taiwo, 1994), which could again result in the expression of active 'pain' behaviours. The occurrence of this active behaviour and associated bouts of unavoidable pain, was unaffected by analgesic in CD lambs. A second possible conclusion is that, as

before, the changes in posture seen here are indicative of chronic inflammatory pain that can be relieved by the consumption of the NSAID FM. The increases of active behaviours, however may have occurred in response to transient irritation of the wound and were not associated with pain and thus the frequency of their occurrence was unaffected by the consumption of analgesic and was high in both groups of castrated lambs.

7.4.3. *Evidence of self-administration of analgesic?*

During training both CD and HD lambs initially ate less feed in total than undrugged lambs. However, by day 23 only HD lambs consumed less feed overall, a trend that continued until day 30 of the study. These differences occurred irrespective of which feed was available on each training day. As the initial intake of the test feed was different in only those groups with access to analgesic test feed (although not only associated with days when analgesic feed was offered) it appears reasonable to conclude that it was some aspect of the analgesic feed that caused neophobia or aversion. The fact that CD lambs appeared to overcome neophobia more quickly than HD lambs suggests that CD perceived the beneficial effects of consuming the previously aversive feed, although the evidence is far from conclusive.

In contrast, during choice testing no difference in the total consumption of test feeds between CD and HD lambs was found and both groups of lambs ate consistently more than undrugged lambs. This result suggests that FM stimulated appetite in both CD and HD lambs, although no reports of appetite stimulation by the NSAID could be found in the literature.

Although CD and HD lambs spent longer at the 'analgesic' hopper than control lambs, there was no difference in the proportion of test feed that was taken from the this hopper. Consequently, despite there being no significant differences in the proportion of feed taken, the analgesic did change the pattern with which the lambs ate from the hopper.

Despite the lack of evidence that CD lambs consumed more analgesic feed than other lambs, HPLC analysis to measure the plasma concentration of FM revealed that the slight differences in the weight of feed taken were sufficient to result in differences in the concentration of FM in plasma. There was a general increase in the amount of

FM that reached the plasma of CD and HD lambs. This trend is likely to be a reflection of the fact that the dose of FM was increased as the weight of the lambs increased. Normally it would be expected that the plasma concentration of FM should remain relatively stable using this dose regime. However, in this study the lambs were fed a relatively large quantity of concentrated feed and had a restricted space in which to move around, thus a greater than normal proportion of their body weight was likely to have been fat (although this was not measured). As the distribution of FM into the fat partition is minimal, a higher concentration of FM in plasma is to be expected. Furthermore, castration is known to increase the proportion of body mass that is made up of fat, this argument could be used to explain the more rapid rate at which the plasma concentration of FM increased in CD lambs. However, this argument is refuted if the rapid decline in plasma concentration of FM 34 days after c+td is taken into account. This change coincided with lesion healing and there was no corresponding reduction in the weight of lambs. Thus, there is some evidence that CD lambs did select from the choice of hoppers, consuming more analgesic than HD lambs. The difference between CD and HD lambs was close to significance despite very high levels of individual variation within groups.

7.4.4. *Evidence of toxicity*

In this study no evidence of an effect of FM on the plasma concentration of albumin or total plasma protein was found. The results of this study therefore suggest that the NSAID FM administered orally at 1.1mg/kg, had no adverse effects on the integrity of the GI tract despite long-term administration of the drug, although it is noted that the NSAID could have hepatic and renal effects which were not investigated. In previous studies there was a suggestion that the incidence of rubbing might be indicative of adverse effects of FM as the behaviour was shown more frequently in animals with access to the drug (Chapter 6 and Rutherford 1999). No such behavioural differences were found in this present study.

7.4.5. *Alternative protocol for self-administration*

The adaptation of the original protocol is considered to have improved the study, both by reducing variation and strengthening the opportunity lambs had to learn the

association between consuming the drugged feed and a reduction in pain. However, it was also considered that the protocol has limitations. In the self-administration protocol used in both studies in this project, the dose of analgesic was dependent upon the quantity of feed consumed. Although studies of the pharmacokinetics of FM after oral administration in lambs (Chapter 3) indicated that the quantity of FM reaching the plasma compartment should be sufficient to result in significant inhibition of prostaglandins, the route of administration still resulted in variation in the dose administered. Further it was difficult to ensure that enough analgesic feed was consumed, quickly enough to obtain a full dose of analgesic, but that sufficient feed was available so that lambs could not eat the selected food too quickly and move onto the alternative feed before the choice could be assessed. This protocol was based on that described by Colpaert *et al* (1980; 1982). It is considered that administration of FM by a route that was independent of feed intake would eliminate analgesic dose as a source of variation. As a result the risk of overdosing would be eliminated and the quantity of feed available to the lambs need not be limited.

Other authors using the self-administration paradigm took this approach. In a study to examine the development of conditioned flavour aversions in lambs, Kyriazakis *et al* (1998) paired flavoured feeds of similar hedonic value with oxalic acid, a naturally occurring plant toxin. The toxin was administered orally, at different doses (or placebo) in a gelatine capsule. Thus the dose of toxin was independent of the weight of feed consumed and was easily controlled. In another study to examine the effects of changes in rate of fermentation on diet selection, Kyriazakis and Oldham (1993) administered infusions of water, urea or fructose into the rumen, through a rumen cannula. Again the effect was independent of the quantity of food consumed. Alternatively, the dose of analgesic administered during analgesic self-administration studies could be given through an i.v. cannula at the same time as or shortly after first introducing a flavoured feed to the pen. In this way variation in the dose of FM received by lambs in the analgesic treatment group could eliminated. It should be noted that the mean plasma concentration of FM measured in both self-administration studies was considered sufficient to provide some degree of analgesia, although from available data the efficacy of the dose cannot be absolutely confirmed (see chapter 3).

A second source of variation in these studies was the highly variable feed intake and diet selection in individual lambs in all treatment groups. It is known that in lambs, diet selection is influenced by social environment (Thornhallsdottir *et al*, 1987) and it has been suggested that individual variation in diet selection can be greatly reduced by housing animals in pairs (Forbes and Kyriazakis, 1995). It is proposed that twin male lambs should be used in future studies in order to reduce variation in feed intake. Both twins should undergo the same treatment and be considered as a single sample in analysis. It should be noted that this would potentially double the number of individuals used in the study and the justification for the work would have to be carefully considered. However, housing the lambs with siblings is likely to also likely to be beneficial as pen size would be increased and the lambs would have more opportunity for social interaction.

7.4.6. *Conclusions*

The lesions resulting from RR castration and tail-docking of six-week-old lambs in this study were smaller and less severe than expected in lambs of this age and thus the physical indications of the presence of chronic inflammatory pain was limited. Changes in posture and in feeding motivation as a result of c+td were identified and their appearance was reduced by the consumption of analgesic indicating that the plasma concentrations of FM achieved were efficacious and that such changes in behaviour may be indicative of the experience of chronic inflammatory pain. The increase in frequency of active behaviour in CN lambs was not reduced by the consumption of FM in CD lambs indicating either that these behaviours are not indicative of chronic inflammatory pain or that FM is not efficacious in the treatment of the type of pain that induced these behaviours. Although no differences were found in the proportion of feed that was taken from the analgesic hopper, the plasma concentration of FM was higher in CD than in HD lambs during the 3rd, 4th and 5th cycles of the protocol. This difference neared significance during the 5th cycle when c+td lesions were at their maximum severity. Subsequently the plasma concentration of FM declined. The combination of this difference in FM concentration and the differences in posture and feeding motivation are considered the most compelling evidence of the experience of chronic inflammatory pain in c+td lambs.

Apart from the limited severity of the lesions observed in this study, it is considered that the protocol used was such that variation was minimised and that the opportunity to learn the association between consumption of the distinctive test feed and the relief of pain was provided. Constraints of time and personnel limited the possibility of increasing the power of the experimental protocol by increasing the numbers in each group and the time for which behaviour was recorded. Increasing the severity of lesions by controlled, bacterial contamination is a possibility. In a commercial environment however, the appearance of particularly severe lesions is unusual because most lambs are c+td at less than a week of age. Because the aim of this research is to investigate whether the routine husbandry procedure of RR castration and tail-docking is associated with chronic inflammatory pain, inducing lesions that are more severe than would normally occur in order to validate a model is considered ethically unacceptable.

Finally, it is considered that the use of a protocol in which the administration of analgesic was independent upon the quantity of feed consumed, as described in a study of plant toxins by Kyriazakis *et al* (1998), would provide a more robust basis for analgesic self-administration studies in lambs. This would allow a consistent dose of analgesic to be administered and reduce the need for limitation of feed intake, increasing the chance of consistency of selections made. It is also proposed that housing of lambs in pairs (twins) would reduce variation in feed consumption, whilst reducing the cost of the study on the individual, Although it is noted that careful justification of the increase in numbers would be necessary.

Chapter 8

Hypothalamo-Pituitary-Adrenal Axis: modulation to produce stressor-specific responses

Chapter 8. The Hypothalamo-Pituitary-Adrenal Axis: Modulation to produce Stressor-Specific Responses.

8.1. *The central nervous and endocrine systems interact to control the stress response.*

The normal functioning of an animal's body requires that it is maintained in a state of dynamic equilibrium or homeostasis. If some external or internal challenge, or stressor, upsets this equilibrium, it is essential that the body can mount an appropriate behavioural, autonomic and endocrine response to restore the system to within the bounds of equilibrium. The hypothalamo-pituitary-adrenal (HPA) axis is the point at which the central nervous system (CNS) and the neuroendocrine system interact (Griffin and Ojeda, 2000). Stimulation from the CNS activates the HPA axis resulting in endocrine responses to stress. These endocrine responses include stimulation of specific regions of the CNS resulting in the co-ordination of behavioural and autonomic responses with endocrine responses to stress. Stressor-specific HPA responses are stimulated by an immense array of internal and external stressors.

8.2. T*he basic HPA response in acute stress.*

Following stimulation by an acute stressor, the basic mechanisms by which endocrine responses are mounted are well established. The stress system receives information about the body and environment through sensory receptors, via neural pathways and in the blood. The information is processed and relayed to the hypothalamus principally via the limbic system and brain stem (Aguilera, 1998). If this information indicates that homeostasis, against a background of diurnal variation, has been disrupted, the HPA axis is activated. Axons from parvocellular neurones in the paraventricular nucleus (PVN) of the hypothalamus project to the external zone of the median eminence and terminate at the portal capillary plexus. Upon activation, corticotrophin-releasing hormone (CRH), synthesised in these neurosecretory cells, is released into the hypophysial portal blood (HPB) (Berkenbosch *et al*, 1989). CRH is transported to the anterior pituitary where it acts

in synergy with other hormones (principally arginine vasopressin (AVP)), via specific receptors, to induce the release of adrenocorticotrophic hormone (ACTH) from anterior pituitary corticotrophs into the blood (Swanson *et al*, 1983). CRH also stimulates the synthesis of proopiomelanocortin (POMC), the precursor of ACTH (Levin *et al*, 1989), in anterior pituitary corticotrophs. Once released, ACTH is carried in circulation to the cortex of the adrenal gland where it acts on its receptor to activate protein kinases, resulting in the metabolism of cholesterol. The end products of this breakdown cascade are the glucocorticoids (for example deoxycorticosterone, cortisol, corticosterone and aldosterone), which are released into circulation (Plotsky, 1991).

Glucocorticoids act via specific receptors at many target sites to inhibit, for example, reproduction, growth and immune responses. This provides energy for the necessary responses to the stressor. ACTH and glucocorticoids regulate their own release by negative feedback at all levels of the HPA axis, thus minimising potential negative effects of their continued release.

The basic stress response mechanism does not explain the occurrence of distinct reactions to specific types of stressor. Nor does it explain changes in regulation of the stress response that occur when exposure to a stressor is prolonged. The regulation of the HPA axis at all levels, the mechanisms that allow specific responses to a variety of stressors and the specific HPA responses to chronic inflammatory pain are discussed in this chapter.

8.3. *Development of experimental stress paradigms*

In order to investigate the regulation of hypothalamic pathways in response to different stressors various experimental stress paradigms, both acute and chronic, have been developed and used to model aspects of stress-specific responses. These may be broadly categorised into four overlapping groups: Psychological stressors include isolation, immobilisation, restraint, white noise, swim stress and forced inhalation of ether. Physiological stressors include hypernatraemia, dehydration, insulin induced hypoglycaemia, heat stress, haemorrhage, and cold stress. Physical stressors, often incorporating inescapable pain, include electric foot shock, intra-peritoneal hypertonic saline, castration and tail-docking. Finally, immunological

stress paradigms including experimentally induced arthritis caused by injection of an adjuvant containing heat-killed *Mycobacterium butyricum*. Adrenalectomy (ADX) is also used to examine the effects of chronic hyper-activation of the HPA axis, by the removal of negative feedback from circulating glucocorticoids.

8.4. *CRH and AVP: hypothalamic mediators of the stress response*

In early studies, molecular sieve chromatography was used to separate hypothalamic extracts. This revealed high and low molecular weight proteins with corticotrophin-releasing activity (Antoni, 1987). The low molecular weight, nonapetide, arginine vasopressin (AVP) was first structurally elucidated by Turner *et al* in 1951. In this molecule disulfide bonds link two cystine residues giving the protein a characteristic ring structure that is essential to its biological activity (Samson, 2000). AVP is a potent stimulus for ACTH release *in vitro* (Gillies and Lowry, 1979). Corticotrophin-releasing hormone (CRH) was characterised by Vale *et al* (1981) and is a high molecular weight, 41-amino acid peptide derived from a 196-amino acid precursor molecule (Smith *et al*, 1987). Following the isolation of CRH from ovine hypothalamic extracts, the hormone was found to stimulate the secretion of ACTH and β-endorphin from the anterior pituitary of the rat with much higher potency than AVP. However, the potency of CRH was increased four-fold when CRH was administered with AVP (Gillies *et al*, 1982). The structures of CRH and AVP are shown in figure 8.1.

Figure 8.1 The structure of CRH

a. The structure of corticotrophin-releasing hormone (CRH)

H-Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Thr-Lys-Ala-Asp-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Asp-Ile-Ala-NH_2 (Vale *et al*, 1981)

b. The structure of arginine vasopressin (AVP)

H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 (Turner *et al*, 1951)

It has since been accepted that CRH acts synergistically with arginine vasopressin (AVP) to control the release of ACTH from the anterior pituitary following activation of the HPA axis (Antoni, 1993). This synergistic control of the HPA axis is consistent across species, in rats, mice and sheep.

However there are species-specific differences in the degree to which each hormone stimulates the release of ACTH. In the mouse, AVP has a more potent ACTH-releasing effect than in the rat, although not exceeding the response to CRH (Castro *et al*, 1989). Also, in *in vitro* studies in the sheep, AVP was found to strongly stimulate the release of ACTH from ovine anterior pituitary corticotrophs (Familiari *et al*, 1989) in a dose dependent manner and was found to be a more potent stimulus for ACTH release than CRH (Liu *et al*, 1990; Canny *et al*, 1999).

8.5. *Functional distribution of CRH and AVP*

The rat (Ivell and Richter, 1984) and bovine (Land *et al*, 1982) AVP neurophysin II precursor and the human (Shibahara *et al*, 1983), rat (Jingami *et al*, 1985) and sheep (Furutani *et al*, 1983) CRH precursors were cloned in the eighties. This facilitated study of the mechanisms regulating CRH and AVP synthesis at both cellular and molecular levels.

Immunocytochemical and *in situ* hybridisation studies in both rats (Paull *et al*, 1982; Swanson *et al*, 1983; Bloch *et al*, 1990) and sheep (Matthews *et al*, 1991; 1993) have shown that immunoreactivity and mRNA for CRH and AVP are widespread in the CNS. The wide distribution of CRH and AVP containing neurones and behavioural and autonomic effects induced by central administration of AVP (Parkes *et al*, 1988) and CRH (Scoggins *et al*, 1984), provide some evidence that these neuropeptides have neurotransmitter as well as hormonal functions. The simultaneous activation of central CRH and AVP pathways is considered to be responsible for the co-ordination of the behavioural and autonomic as well as the endocrine responses to homeostatic challenge (Swanson *et al*, 1983; Riphagen and Pittman, 1986).

8.6. *Pathways for hypothalamic control of hypothalamo-pituitary-adrenal axis*

The pituitary response to stress is controlled by hormones released from four neural pathways in the hypothalamus into HPB from the median eminence. These are i) the

magnocellular CRH and oxytocin expressing neurones, ii) magnocellullar AVP expressing neurones, iii) parvocellular CRH expressing neurones and iv) parvocellular CRH and AVP expressing neurones. Evidence that these pathways are selectively activated during different stress paradigms is outlined below.

8.6.1. *The role of magnocellular CRH in response to stress*

CRH is co-localised with oxytocin (OT) in about a third of magnocellular neurones in the SON and PVN (Sawchenko *et al*, 1984) and it has been proposed that CRH produced in these cells modulates OT secretion during stress. Expression of CRH mRNA in magnocellular neurones increases following stimulation by osmotic stressors. However, parallel expression of CRH mRNA in parvocellular neurones is reduced (Lightman and Young, 1987). Because the level of expression of CRH mRNA in magnocellular neurones is at least one order of magnitude lower than the expression of CRH mRNA in the parvocellular division (Holmes, 1986), there is a net reduction in the expression of CRH mRNA overall (Dohanics *et al*, 1990). This is reflected in the corresponding reduction in the release of ACTH from the anterior pituitary (Chowdrey *et al*, 1991). No change in the expression of CRH in magnocellular hypothalamic pathways is observed in response to non-osmotic stressors (Aguilera *et al*, 1994)

It is thought that less than 10% of the CRH in HPB (Palkovits *et al*, 1991; Antoni, 1993) originates in both the magnocellular CRH neurones and from dispersed parvocellular neurones in the perifornical region just outside the PVN (Palkovits *et al*, 1991). The remaining 90+% is thought to originate in parvocellular CRH neurones (Holmes *et al*, 1986).

8.6.2. *The role of magnocellular AVP in response to stress*

The principle and best-known function of AVP is as the anti-diuretic hormone (ADH). This anti-diuretic AVP is synthesised in the magnocellular division of the paraventricular nucleus (mPVN) and in the SON of the hypothalamus. Magnocellular neurones in these nuclei, project to the posterior pituitary gland terminating next to blood vessels within the gland and release hormones into circulation. AVP is carried in the blood to the kidney where it acts on collecting ducts and distal convoluted

tubules, increasing their permeability to water, thus reducing the excretion of water in urine.

Magnocellular neurones in the SON and PVN express the highest levels of AVP mRNA in the sheep and rat (Nojiri *et al*, 1985; Matthews *et al*, 1993). AVP expressing magnocellular neurones in the ventral region of the SON also express type II glucocorticoids receptors (Kiss *et al*, 1988). They are therefore a target for glucocorticoid regulation and are likely to have a role in the control of the stress response. Magnocellular AVP neurones in the PVN however, do not express these receptors.

Axons from magnocellular neurones in the SON pass through the lateral retrochiasmatic area and into the internal zone of the median eminence, before arriving at the posterior pituitary (Antoni, 1993). Morphological studies indicate that magnocellular neurones do not terminate next to the portal capillary plexus but that this AVP is released from swellings (Herring bodies) that occur along the length of the axon (Holmes *et al*, 1986). Portal capillaries penetrate the internal zone of the median eminence and there are no major barriers that would prevent AVP released from these magnocellular neurones reaching the hypthalamo-pituitary blood (HPB) (Antoni, 1993). Antoni *et al* (1990) reported that lesioning of the PVN in Wistar rats resulted in only a 10% reduction in AVP in HPB supporting the hypothesis that magnocellular neurones in the SON are an important source of AVP in HPB. However, collection of portal blood from the rat involves a severe operating procedure that is likely to induce the release of AVP as an artifact, thus results from such portal blood studies in rats have been called into question (Antoni, 1993).

The magnocellular vasopressinergic system is strongly activated in response to osmotic stress. In a study of chronically hyponatraemic rats, a reduced ACTH response was exhibited compared with controls (Dohanics *et al*, 1991). As magnocellular but not parvocellular AVP neurones were inhibited in these studies, it was concluded that magnocellular AVP expressing neurones are of importance in the control of ACTH release in osmotic stress paradigms (Dohanics *et al*, 1991). However, chronic water deprivation (Aguilera *et al*, 1994) and hypernatraemia (Dohanics *et al*, 1990) result in a sustained decline in ACTH release despite continued elevation of AVP synthesis in magnocellular neurones. This decline in

ACTH in response to chronic osmotic stress also occurs in response to psychological stress and is thought to be caused by receptor-mediated changes in pituitary responsiveness (Aguilera, 1994) as will be discussed later. No change in the expression of AVP in magnocellular pathways has been found in response to non-osmotic stressors including psychological, immunological and physical stress paradigms (Harbuz *et al*, 1994).

8.6.3. *Parvocellular CRH and AVP neurones: the main source of hypothalamic mediators in HPB*

The highest density of expression of irCRH and CRH mRNA has been found in the parvocellular PVN (pPVN) of the rat (Lightman and Young, 1987) and sheep (Matthews *et al*, 1991). In the rat, there are two subpopulations of parvocellular neurones that express CRH. Approximately 50% of these neurones express only CRH mRNA, whilst the other half synthesise both CRH and AVP (Whitnall, 1988; 1990). Labelling for AVP mRNA is strong in CRH/AVP parvocellular neurones, but not as strong as that found in the magnocellular division of the PVN (Matthews *et al*, 1993). The two subtypes of CRH expressing cells are differentially distributed within pPVN. CRH/AVP expressing neurones are found in the dorso-lateral region while cells expressing CRH alone are found more medially and ventrally. A small proportion of parvocellular CRH neurones are also found scattered within the magnocellular division of the PVN (Swanson *et al*, 1983). In mice only parvocellular neurones expressing both CRH and AVP have been found (Whitnall *et al*, 1992) and it is likely that variations in the ratio of parvocellular CRH and CRH/AVP expressing neurones exist in other species.

90% of CRH and CRH/AVP expressing parvocellular neurones in the PVN project to the external zone of the median eminence, terminate at the portal capillary plexus and release CRH and AVP into HPB (Whitnall, 1993). More than 90% of the CRH found in portal blood originates from parvocellular neurones in the PVN (Holmes *et al*, 1986) and it is accepted that the PVN is the main source of CRH controlling the stress response (Swanson *et al*, 1983). CRH and AVP are co-packaged into neurosecretory vesicles in the terminals of these neurones for release into the HPB (Whitnall *et al*, 1985). Type II glucocorticoid receptors are found on parvocellular

CRH and CRH/AVP neurones in the PVN, showing that they are also a target for glucocorticoid feedback.

8.7. *Central control of CRH and AVP release in response to stress*

The HPA axis is controlled centrally by neurones in regions of the brain stem, the locus coeruleus (medulla), limbic structures including the amygdala, hippocampus, and bed nucleus of the stria terminalis (BNST) and in hypothalamic nuclei. This control constitutes both excitation, in response to stress, and inhibition, preventing a response to non-noxious stimuli and reducing the damaging effects of glucocorticoids. There is evidence that excitation and inhibition are controlled by distinct nuclei and that responses to different types of stress are also controlled by different brain regions. These topics are reviewed and discussed in detail by Herman and Cullinan (1997). Evidence of the roles of these nuclei in the modulation of the HPA response to diverse stressors is summarised below.

8.7.1. *The brain stem*

The pPVN receives dense innervation from the brain stem, by neurones containing adrenaline and noradrenaline (from regions A1 and A2 and C1 and C2 respectively) (Sawchenko and Swanson, 1982; Cunningham *et al*, 1990). Studies of immediate early gene expression (*cfos* and NGF1-B) have shown that these areas are activated by acute haemorrhage, respiratory distress and immune challenge (Plotsky *et al*, 1989; Ericsson *et al*, 1994). Deafferentation of brain stem pathways inhibits the HPA response to acute stress (Li *et al*, 1996). Studies have shown that the effects of adrenaline and noradrenaline on the HPA axis vary depending on the nature of the stressor (Harbuz *et al*, 1991). However, both are thought to have excitatory and inhibitory roles (Mezey *et al*, 1984; Spinedi *et al*, 1988; Suda *et al*, 1987).

8.7.2. *The locus coeruleus*

The locus coeruleus contains approximately half of all the noradrenergic neurones in the brain (Nestler *et al*, 1999) and is extremely responsive to many stressors (Smith *et al*, 1991; Cullinan *et al*, 1995). It shows a massive increase in activity in response to chronic stress (Nestler *et al*, 1999). Innervation of the HPA directly from the locus

coeruleus is limited (Smith *et al*, 1991; Cullinan *et al*, 1995), however, projections from the locus coeruleus are received by limbic structures. The locus coeruleus also receives innervation from the brainstem and PVN (Valentino *et al*, 1998). Therefore, it has been proposed that the effects of this nucleus are indirect, through effects on other stress responsive nuclei (Herman and Cullinan, 1997).

CRH mRNA and irCRH are found in the locus coeruleus (Swanson *et al*, 1983; Matthews *et al*, 1991) indicating that CRH has a critical role in activating the nucleus during stress. Indeed intracerebroventricular (i.c.v.) administration of CRH at biologically relevant levels induces an increase in the rate of firing of locus coeruleus neurones (Valentino *et al*, 1983). This is associated with an increase in vigilant behaviour, which is appropriate in response to stress (Nestler *et al*, 1999).

All neurones in the locus coeruleus fire simultaneously and this pulsatility is extremely important to the function of the nucleus (Nestler *et al*, 1997). The response of the HPA axis to catecholaminergic stimuli from any nucleus is dependant on the exact rate and magnitude of catecholaminergic pulse (Haisenleder *et al*, 2000). Slower pulses of low magnitude favour increased expression of CRH mRNA in the PVN, whilst higher frequency and greater magnitude pulsatility favours the expression of AVP mRNA (Haisenleder *et al*, 2000). This provides another route by which the expression of AVP and CRH can be differentially modulated to induce stress specific responses.

8.7.3. *The limbic system*

The amygdala is thought to have a generally excitatory role in the control of the HPA axis. It is also considered to have a role in behavioural and cardiovascular responses to stress (Davis *et al*, 1992). Lesions of the amygdala reduce the HPA response to stress and to ADX (Herman and Cullinan, 1997). Also the expression of *cfos* is strongly up-regulated in this nucleus in response to swim and restraint stress (Cullinan *et al*, 1995).

The hippocampus is considered to have a strong inhibitory effect on the pPVN. The pPVN shows a very high density of glucocorticoid receptors (Jacobson and Sapolsky, 1991; Herman, 1993). Hippocampal lesions result in an increase in glucocorticoid release and an increase in CRH and AVP mRNA expression in the

pPVN (Herman *et al*, 1989; Jacobson and Sapolsky, 1991; Sapolsky *et al*, 1991; Herman *et al*, 1995).

The BNST provides a neuronal bridge connecting limbic structures to the HPA and HPA to brain stem (Moga *et al*, 1989; Herman *et al*, 1992; Cullinan *et al*, 1993; Herman *et al*, 1994). BNST has excitatory and inhibitory effects on pPVN neurones. Neurones controlling the excitatory and inhibitory effects of the nucleus are spatially separated within the nucleus. Lesioning of specific regions mimics the effects of lesions of the amygdala or hippocampus (Herman *et al*, 1994; Herman *et al*, 1996; Herman and Cullinan, 1997). This suggests that the BNST is simply responsible for relaying the actions of the neural structures that it connects.

8.7.4. *Hypothalamic nuclei*

Local inhibition of the PVN by other hypothalamic nuclei, including the SCN, ventromedial nucleus, and arcuate nucleus, has also been found. Studies have shown that the response to stress increases in magnitude and duration after lesioning of each of these nuclei (Buijs *et al*, 1993; Larsen *et al*, 1994; Suemaru *et al*, 1995).

8.7.5. *Stressor-specific activation of different central control pathways*

Herman and Cullinan (1997) discuss the hypothesis that 'processive' and 'systemic' stressors activate different central controls. According to this hypothesis, those stressors that constitute a direct threat to the integrity of physiological homeostasis, i.e. systemic stressors, for example respiratory distress, activate brain stem regions (Sawchenko *et al*, 1996). This allows direct activation of the HPA axis and appropriate responses, with only one synaptic connection between the brainstem and the PVN (Swanson and Sawchenko, 1983).

Those stressors requiring some mental processing (processive stressors), for example novelty or restraint, activate the nuclei of the limbic system. These stressors require a perception of the present situation, as well as the use of previous experience and do not necessarily represent an immediate threat to the integrity of the system. They therefore activate systems whose connections to the PVN are indirect, involving more than one synaptic connection (Herman and Cullinan, 1997).

While this hypothesis provides a general rule by which the expected central response to a specific stressor could be estimated, many exceptions to this rule must exist. For example foot-shock, would appear to constitute both an immediate threat (pain) as well as psychological stress (inescapability), however deafferentation of the brain stem has no effect on the HPA activity in response to foot-shock (Li *et al*, 1996).

8.8. *Effects of acute stress on AVP and CRH in the pPVN*

The expression of CRH and AVP mRNA also varies in response to different types of stressor. When stimulated by an acute stressor, CRH and AVP are rapidly released from parvocellular neurones in the PVN and an increase in CRH synthesis is induced within 30 minutes of the onset of exposure to stress (Bartanuz *et al*, 1993). However the ratio of secretion of CRH and AVP is dependant on the nature of the stressor. pPVN responses to the 'same' stressor may also vary between researchers, reflecting both the sensitivity of the system and the difficulty of reproducing stress paradigms.

8.8.1. *Effects of acute psychological stressors*

8.8.1.1. *Restraint*

Psychological stress can be induced in rats by restraint in a Perspex restraining device (Harbuz *et al*, 1994) or restraint cage (Imaki *et al*, 1993) in which movement is restricted. CRH mRNA in the pPVN was strongly up-regulated in response to 60 minutes of restraint stress (Harbuz *et al*, 1994). Depending on the strain of rat used, acute restraint induced either a small up-regulation of AVP mRNA or no change in its expression. In all strains, the AVP response was of a lower magnitude than the CRH response (Harbuz *et al*, 1994). Restraint has also been used to study the effects of stress on sheep (Mears and Brown, 1997). 14 week-old animals were inverted and held at the head or neck for 1 minute, as is done while shearing. No attempt was made to examine the effects of this stress on the synthesis of CRH and AVP, but plasma cortisol was found to be elevated for the first 30 minutes after treatment. This stressor must, in theory, induce some elevation in the release of CRH from the pPVN. However, the plasma cortisol concentration was low in this study, suggesting that the stressor may have been insufficient to stimulate CRH synthesis and that the

release of CRH stored in nerve terminals alone was sufficient to produce the observed rise in plasma cortisol.

8.8.1.2. *Immobilisation*

Immobilisation is a similar psychological stressor to restraint, but produces different hypothalamic responses. Experimental immobilisation of the rat is achieved by taping the feet to a platform, with or without head restraint, for varying lengths of time. In a study by Bartanuz *et al* (1993), rats were exposed to 30 and 150 minutes of immobilisation. They found that the expression of both CRH and AVP mRNA were up-regulated and that this up-regulation was independent of the duration of immobilisation.

In a similar study, Makino *et al* (1995) found that immobilisation for 60 minutes, with the head restrained between two stainless steel loops, induced an increase in CRH mRNA expression but, surprisingly, no concurrent rise in the expression of AVP mRNA. Apart from head restraint, the only difference between the studies appeared to be the strain of rat, Makino *et al* (1995) used Sprague-Dawley rats whilst Bartanuz *et al* (1993) used Wistar rats. In a study by Harbuz *et al* (1994) the responses of Wistar, Sprague Dawley and CFY rats to the similar stress of restraint were compared. The difference between basal and stressed levels of plasma corticosterone was higher in the Wistar strain than in Sprague-Dawley rats. Also the change in expression of CRH mRNA in response to restraint was greater in Wistar rats. The change in plasma corticosterone and expression of CRH mRNA was greatest in CFY rats. Wistar rats appear to show a higher HPA response to stress than Sprague-Dawley rats, which may explain the differences found in response to immobilisation in different studies. The HPA response to acute immobilisation is attentuated after the application of CRH antagonists (Jezova *et al*, 1998), showing that CRH is critical to the regulation of this acute stress response.

8.8.1.3. *Isolation*

A third psychological stressor and one considered to be of special relevance in the sheep, which is particularly social species, is isolation. In a study by Mears and Brown (1997), isolation from flockmates, for one hour, induced a transient rise in

plasma cortisol that peaked at around 30 minutes after initial isolation. Matthews *et al* (1993) examined the effects of 1-hour of isolation on the expression of CRH and AVP in the pPVN. In this study isolation induced a small, but non-significant rise in CRH mRNA in the pPVN, which appears to be in agreement with the cortisol data from the Mears and Brown (1997) study of restraint. No changes in AVP mRNA expression were found. It should be noted however that Matthews *et al* (1993) used only 3 sheep in this study.

8.8.2. *The effects of physical and physiological stressors*

8.8.2.1. *Heat Stress*

Heat stress (24 hours at 35°C) has been found to induce a three-fold increase in plasma cortisol in sheep (Minton and Blecha, 1990). Plasma cortisol returned to basal levels within 12 hours of the onset of this stress, but no attempt was made to assess its effects on the synthesis and release of hormones in the pPVN.

8.8.2.2. *Cold stress*

Cold stress has been reported to induce an increase in AVP mRNA (Wu and Childs, 1990), but this group did not distinguish between AVP mRNA in parvocellular or magnocellular neurones in the PVN. A slight, but statistically insignificant, increase in the expression of CRH mRNA and a corresponding rise in POMC mRNA in the anterior pituitary was found in response to cold stress by Harbuz and Lightman (1989).

8.8.2.3. *Intra-peritoneal hypertonic saline*

Intra-peritoneal injection of hypertonic saline (5ml of 1.5M NaCl (Aguilera, 1994)) has been described as a physical stressor (e.g. Harbuz *et al*, 1994), but it incorporates pain, as well as osmotic challenge and the psychological element of restraint (Matthews *et al*, 1993). This stressor induced an increase in the expression of CRH mRNA and stimulated AVP mRNA expression in pPVN (Harbuz *et al*, 1994). The AVP response to i.p. hypertonic saline was less than that of CRH although of the same order of magnitude (Lightman and Young, 1988).

8.8.2.4. *Insulin induced hypoglycaemia*

CRH and AVP release during insulin-induced hypoglycaemia (IIH), was examined by Plotsky *et al* (1985) who reported significant increases in portal AVP during IIH in the rat. However, collection of portal blood from the rat involves a severe operating procedure that is likely to induce the release of AVP as an artefact. CRH release was not stimulated in this study. In freely-moving, conscious rams (Caraty *et al*, 1990), the concentration of CRH and AVP in portal blood was increased in response to IIH when the dose of insulin used was 0.2IU/kg (sufficient to induce a significant drop in plasma glucose). Prior to insulin injection, the portal blood concentration of AVP was approximately half that of CRH, corresponding with the synthesis of AVP in approximately half of pPVN neurones (Whitnall, 1988). This ratio of CRH to AVP was maintained following increases in the concentrations of the hormones after IIH. However, following administration of insulin at the much higher dose of 2.0IU/kg the concentration of CRH rose much more dramatically, to approximately six times basal levels. The concentration of AVP exceeded that of CRH, reaching 140pg/ml/min, approximately ten times the basal concentration of AVP. Because this study examines only the concentration of CRH and AVP in HPB, the source of these hormones cannot be determined.

Berkenbosch *et al* (1989) studied the rate of depletion of CRH and AVP in the external zone of the median eminence of the rat after IIH. Colchicine was used to block rapid axonal transport of newly synthesised hormones, thus preventing replenishment of stores of CRH and AVP, and the rate of depletion was estimated using quantitative immunocytochemistry. The rate of depletion of CRH and AVP was similar for the first hour. At later time points the rate of AVP depletion increased more rapidly than that of CRH. In both these studies a dramatic rise in plasma ACTH was concomitant with increases in release of hypothalamic releasing factors.

8.8.2.5. *Experimental haemorrhage*

Experimental haemorrhage of 15ml/kg over three hours in the rat induced a rise in CRH mRNA for at least 4 hours and a transient rise in ACTH, which returned to normal within 4 hours. The expression of AVP mRNA was not elevated, indicating

that while a haemorrhage of this order results in stress, the CRH response is sufficient to generate an appropriate HPA response (Darlington *et al*, 1992).

8.8.2.6. *Inescapable foot-shock*

Imaki *et al* (1993) and Harbuz *et al* (1991) demonstrated an increase in the expression of CRH mRNA in the pPVN in response to acute inescapable electric foot-shock. However, the synthesis of AVP mRNA was not measured in these studies.

8.8.3. *Summary of HPA responses to acute stress*

The HPA response to acute stress varies depending on the exact nature and duration of the stressor. Overall the parvocellular PVN response to acute stress is characterised by an increase in CRH mRNA and release of CRH and AVP. In response to some acute stressors AVP synthesis may also be stimulated but in general the expression of AVP mRNA and AVP release is lower than that of CRH in parvocellular neurones.

8.9. *Effects of adrenalectomy on AVP and CRH in the pPVN*

Adrenalectomy (ADX) is widely used to model of the effects of chronic activation of the HPA axis. The removal of glucocorticoid feedback results in a generalised activation of the HPA axis, including up-regulation of the synthesis of CRH in the pPVN. The synthesis of AVP in the pPVN also increases dramatically and one of the major characteristics of ADX is that the number of parvocellular neurones expressing AVP mRNA increases until very few neurones expressing only CRH remain (Kiss *et al*, 1984; Sawchenko *et al*, 1984; Marti, 1999). De Goeij *et al* (1993) found that ADX did not influence the level of irCRH stored in parvocellular nerve terminals in the median eminence of Wistar rats. However, irAVP accumulated dramatically in parvocellular CRH/AVP expressing neurones. Makino *et al* (1995) found that standardised glucocorticoid replacement in ADX rats resulted in slightly lower plasma cortisol levels than those found in sham-operated rats. These rats showed up-regulation of AVP mRNA in response to immobilisation, while sham-operated rats did not. There was no change in the expression of CRH mRNA in glucocorticoid-

replacement ADX rats when compared with sham-operated controls. This suggests that AVP is more sensitive to glucocorticoid negative feedback than CRH and that the small reduction in corticosterone in glucocorticoid-replaced ADX rats is sufficient to allow escape from inhibition by AVP. The dramatic increase in AVP mRNA expression in the pPVN and the massive accumulation of AVP in nerve terminals after ADX supports this hypothesis (De Goeij *et al*, 1993).

Quantitative immunohistochemistry measuring AVP and CRH mRNA in the PVN, measurement of the peptide content of the median eminence and of the concentration of hormones in portal blood (Plotsky and Sawchenko, 1987) revealed much higher secretion of AVP and CRH by 72 hours after adrenalectomy. By using colchicine to block rapid axonal transport in ADX rats and therefore removing the effect of the differential synthesis of the two hormones, De Goeij *et al* (1993) found that AVP stores were depleted at a much faster rate than those of CRH (8.1% vs 6.9% per hour after 1 week and 7.8% vs 3.4% per hour after 4 weeks respectively). The rate at which CRH is synthesised in the pPVN is approximately equal to the rate at which the hormone is secreted from the nerve terminals. In contrast, the progressive accumulation of ir AVP in nerve terminals indicates that the rate of synthesis of AVP in pPVN is higher than the rate at which the hormone is secreted. Also, as stores of AVP in the external zone of the median eminence are depleted at a much faster rate than those of CRH, it is concluded that AVP is preferentially released from nerve terminals.

CRH and AVP are co-packaged in vesicles in nerve terminals and, according to Whitnall *et al* (1985), the labelling of AVP and CRH in nerve terminals is uniform. However, Bertini and Kiss (1991) conducted a study to determine if the ratio of co-packaged neurohormones could be modulated by ADX. They found that the ratio of AVP to CRH is dynamic and that the neurosecretory vesicles increased in size to accommodate the increase in the rate of synthesis of AVP and CRH and achieve their release into the pericapillary space.

8.9.1 *Summary of HPA response to ADX*

ADX results in up-regulation of CRH mRNA in the PVN and release of CRH into HPB. An increase in the expression of AVP mRNA and the release of AVP also

results, but is of greater magnitude than the CRH response. These changes are characterised by the recruitment of almost all CRH expressing parvocellular neurones to synthesise AVP. POMC mRNA expression and ACTH release also increase in response to ADX. The effects of ADX can be reversed by the administration of exogenous glucocorticoids. AVP appears to be much more sensitive to glucocorticoid regulation than CRH. The proportion of AVP and CRH released from the same neurones can be modulated with respect to their relative rates of synthesis.

8.10. *Effects of chronic stress on AVP and CRH in the pPVN*

Chronic and repeated stress paradigms result in similar changes in the HPA axis to those observed after ADX, but are lower in magnitude. As in acute stress paradigms the response varies depending on the nature of the stressor. In chronic stress, as with ADX, there is a tendency for the increased synthesis and release of AVP to exceed that of CRH. This higher magnitude AVP response is conserved across chronic stress paradigms. In contrast, the level of synthesis and release of CRH varies depending on the type of stressor (Ma and Aguilera, 1999).

8.10.1 *Chronic psychological stress*

Generally, in response to the chronic or repeated application of psychological stressors, there is an initial activation of the HPA axis, as might be observed with acute application of the stressor. However, as exposure to the stressor continues, there is a progressive decline or desensitisation of the stress response (Aguilera, 1994). However, when a novel stressor is introduced, after desensitisation, HPA hyper-responsiveness is observed.

8.10.1.1. *Restraint*

A study by Ma and Lightman (1998) demonstrates this desensitisation. In this study the effects of repeated restraint stress of varying frequency and duration were examined. It was found that CRH mRNA expression was significantly higher than basal expression in response to a single acute restraint stress and was also increased in response to intermittent repeated immobilisation stress for 3 hours every 7 days.

However as the frequency and duration of the stressor was increased so the CRH mRNA response declined, indicating that, in response to a homotypic stressor, the HPA axis can habituate. In contrast, the expression of AVP mRNA increased significantly as the duration and frequency of stress increased. This demonstrates that the synthesis of AVP can be specifically facilitated in chronic stress, however, the mechanism by which this is achieved is uncertain (Ma and Lightman, 1998).

8.10.1.2. *Immobilisation*

A similar increase in the expression of CRH mRNA and AVP mRNA in the pPVN is observed in response to repeated immobilisation stress (2 hours/ day for 14 days) (Makino *et al*, 1995). The magnitude of the increase in AVP mRNA is much greater than that of CRH mRNA, as is seen ADX rats. Generalised activation of the HPA is demonstrated by a sustained increase in POMC mRNA in the pituitary (Makino *et al*, 2002). Despite this, the concentration of ACTH in the peripheral circulation was the same as in control rats indicating that the release of ACTH was inhibited and desensitisation had occurred.

An initial increase in the release of ACTH was observed, in Sprague-Dawley rats, in response to intermittent immobilisation. This response declined over 16 days until the plasma concentration of ACTH in stressed rats did not differ from that in control rats. However, subsequent exposure to ether stress induced a massive increase in the concentration of ACTH, reaching more than 10 times basal levels and 3 times the concentration observed in response to the initial immobilisation (Hauger *et al*, 1990).

8.10.2. *Chronic physical stress*

In chronic physical stress paradigms, repeated stimulation does not result in desensitisation of the HPA axis (Aguilera, 1994).

8.10.2.1. *Intra-peritoneal Hypertonic Saline*

I.p. hypertonic saline injections were repeated every day, for 14 days, by Ma and Aguilera (1999), resulting in increased CRH and AVP expression in the pPVN. Despite up-regulation of both hormones, the magnitude of the AVP mRNA response was twice that of CRH (400% higher than controls and 250% higher than controls

respectively). Over the 14 days no decline in CRH and AVP expression or plasma ACTH levels were found.

8.10.2.2. *Chronic inflammatory stress*

Experimental arthritis can be induced in Sprague-Dawley (SD) and Piebald-Viral-Glaxo (PVG) rats by the intra-dermal injection of an adjuvant containing heat-killed *Mycobacterium butyricum.* Adjuvant induced arthritis (AA) is a model of rheumatoid arthritis, inducing chronic inflammatory pain in joints. Joint inflammation develops within 12 days of adjuvant injection and peaks after approximately 21 days (Sarlis *et al*, 1992). In this chronic stress paradigm, CRH mRNA is not up-regulated in the pPVN and in one study CRH synthesis and release has been shown to decline (Harbuz *et al*, 1992). AVP mRNA expression however, is increased and is associated with a rise in the concentration of AVP in HPB. The number of parvocellular neurones in the paraventricular nucleus that express AVP mRNA increases, as does the density of expression of AVP within these neurones (Chowdrey *et al*, 1995). Expression of POMC mRNA in the anterior pituitary is increased (Stephanou *et al*, 1992), as are the concentrations of ACTH and corticosterone in the peripheral circulation, despite the apparent decline in the synthesis and release of CRH (Chowdrey *et al*, 1995). In this model the daily rhythmic changes in ACTH and corticosterone release are abolished (Sarlis *et al*, 1992).

8.10.3. *Summary of HPA responses to chronic stress*

In chronic stress, heightened expression of AVP is conserved across types of stress paradigm. This suggests that AVP has a specific and important role in chronic stress. This is particularly well illustrated in adjuvant-induced arthritis in rats, where no change in CRH expression is found, but the release of ACTH still increases. Hyper-responsiveness of the HPA axis to a novel stressor is conserved across chronic stress paradigms and high levels of AVP mRNA and irAVP may ensure that the chronically activated HPA axis is able to respond to acute heterotypic stress (Aguilera, 1994).

8.11. *Regulation of CRH and AVP receptors contributes to stress-specificity of HPA response*

The actions of CRH and AVP throughout the body are mediated through specific membrane receptors. Binding of the hormone to the receptors in cell membranes of tissues induces the activation of intracellular mechanisms resulting in responses to stress. Differential regulation of these receptors represents another route by which specific responses to different types of stress may be produced.

8.11.1. *CRH receptors*

There are two subtypes of receptor that modulate the effects of CRH around the body. Type 1 (CRH-1R) and type 2 (CRH-2R) receptors have pharmacologically different properties and their distribution is distinct (Aguilera *et al*, 2001). The differential distribution, structure and pharmacological action of the CRH receptor subtypes indicates that they mediate distinct roles of CRH in disparate tissues (Perrin *et al*, 1995). After cloning CRH-1R from human pituitary tumour cells (AtT20 cells), Chen *et al* (1993) found that the cDNA of CRH-1R encodes a 415-amino-acid protein with seven trans-membrane domains. This structure is characteristic of G-protein coupled membrane receptors. Binding of CRH to the receptor activates a G-protein pathway. The subsequent cascade of responses is as follows: Binding of CRH activates G-protein, which in turn activates adenylate cyclase. Adenylate cyclase catalyses the formation of cyclic AMP (cAMP) and cAMP stimulates phosphorylation of intracellular proteins by protein kinase A. These mechanisms result in the opening of ion channels, influx of Ca^{2+} and initiation of the exocytosis of secretory vesicles (Imaki *et al*, 1996).

There are two splice variants of CRH-2R (Perrin *et al*, 1995). CRH-R2α is expressed in neural structures including the hypothalamus, lateral septum and olfactory bulb. CRH-R2β is expressed mainly in the periphery, in lung, heart and skeletal muscle and the smooth muscle of the cardiovascular system (Lovenberg *et al*, 1995).

CRH-1R has higher binding affinity for CRH than CRH-R2α (Lovenberg *et al*, 1995). It is found in the pituitary, cortex, limbic system, cerebellum and in some sensory nuclei. It is by interaction with CRH-1R that the effects of CRH on anterior pituitary corticotrophs are mediated (Abou-Samra *et al*, 1987).

8.11.2. *AVP receptors*

The effects of AVP are mediated through three pharmacologically different receptor subtypes (Rabadan-Diehl *et al*, 2000). All are G-protein coupled receptors, like those for CRH. The V2 receptor mediates the effects of AVP in the kidney and, in mammals, is found exclusively in the renal medulla (Antoni, 1987). Like CRH receptors, the V2 receptor is coupled to adenylate cyclase (Jard, 1983). The V1a receptor or V1 receptor is found in vascular smooth muscle, liver, testis, the adrenal gland and the brain (Antoni, 1987). This receptor is coupled to phospholipase C (PLC) and is known to have pressor functions. The V1b or V3 receptor is also coupled to PLC (Sugimoto *et al*, 1994) but is considered to be pharmacologically different from the V1a receptor as it is not inhibited by V1a receptor antagonists (Antoni, 1987). It is found in the majority of pituitary corticotrophs and is thought to play an important part in corticotroph responsiveness (Rabadan-Diehl *et al*, 1997). The binding of AVP to the receptor activates G-protein, which in turn activates PLC. PLC catalyses the hydrolysis of phosphotidyl-inositol 4,5-biphosphate producing the second messengers inositol triphosphate and 1,2-diacylglycerol. Inositol triphosphate releases Ca^{2+} from intracellular stores and activates ion channels allowing an influx of Ca^{2+} into cytoplasm (Antoni, 1993). There is evidence that this change in membrane potential is partially responsible for the stimulation of exocytosis from secretory vesicles (Antoni, 1993). The other second messenger, 1,2-diacylglycerol also mediates cellular changes that lead to exocytosis and is thought to maintain secretion over a longer period of time (Griffin and Ojeda, 2000). V1b receptors are also found in many brain regions including the cerebral cortex, cerebellum, hippocampus and hypothalamus, and in peripheral structures including the heart, lung, thymus, uterus, kidney and breast (Lolait *et al*, 1995).

8.11.3. *Sensitivity of tissues to CRH and AVP*

The regulation of their membrane receptors represents another means by which the effects of hormones can be modulated in response to different stressors. CRH, AVP and glucocorticoids interact to regulate receptor mRNAs. The number of receptors expressed and their affinity for their agonist (Aguilera *et al*, 2001) contribute to the overall sensitivity of the tissue to stimulation during HPA activation.

8.11.4. *Regulation of pituitary CRH receptors in Response to stress*

The CRH-1R is the principle receptor mediating the effects of CRH in the anterior pituitary. The regulation of these receptors varies in response to different stressors.

In acute stress the concentration of CRH and AVP in HPB increases. This activates the rest of the HPA axis resulting in an increase in the synthesis of POMC and the release of ACTH and subsequently of glucocorticoids. However, acute activation of the HPA axis is initially (first 2 hours) associated with a decline in the expression of CRH-1R mRNA followed by recovery to basal levels (4hours) (Rabadan-Diehl *et al*, 1996).

ADX is a preparation associated with chronic release of CRH and AVP and a lack of glucocorticoids. Following ADX, CRH-1Rs are down-regulated (Wynn *et al*, 1998) as is the level of CRH-stimulated adenylate cyclase activity (Aguilera *et al*, 1987). The synthesis of CRH is also transiently reduced (Rabadan-Diehl *et al*, 1997). Paradoxically down-regulation is accompanied by an increase in the expression on POMC mRNA and the release of ACTH (Marti *et al*, 1999).

In chronic stress paradigms there is no correlation between the number of CRH-1Rs and the magnitude of the pituitary response (Aguilera *et al*, 2001). Despite this, reduced ACTH and glucocorticoid release is observed in CRH knock-out mice, suggesting that some CRH must be present to allow normal HPA activation (Muglia *et al*, 2001). The occupation of 50% of CRH-1Rs in anterior pituitary corticotrophs is sufficient to produce a maximal ACTH response (Aguilera *et al*, 1987). This explains why high levels of secretion of ACTH can still be induced after ADX when the number of CRH-1Rs available is reduced. Together this evidence suggests that CRH is necessary for the HPA response to take place, but the concentration of CRH released and number of receptors that are occupied is not important (Aguilera *et al*, 2001). Thus, CRH has a permissive role in HPA activation during chronic stress.

The effect of AVP on the expression of CRH-1Rs appears to be the opposite of CRH, i.e. upregulating the receptor mRNA after acute and chronic administration (Rabadan-Diehl *et al*, 1996). However AVP also potentiates the down-regulating effect of CRH on the number of CRH-1Rs (Aguilera *et al*, 1987)

Glucocorticoids inhibit binding of CRH to CRH-1R. They have also been found to reduce CRH-1R mRNA, but mRNA levels recover after a short time (Rabadan-Diehl

et al, 1997). Small, but physiologically significant changes in glucocorticoid concentration have been found to be sufficient to inhibit CRH-1R mRNA expression and reduce plasma ACTH (Hauger *et al*, 1987). Thus, the immediate decline in CRH-1R binding and mRNA after onset of stress may be caused by the rapid release of glucocorticoids (Ochedalski *et al*, 1998). It is likely that the action of glucocorticoids on CRH-1R is one route by which glucocorticoid negative feedback occurs (Aguilera *et al*, 1987).

8.11.5. *Regulation of pituitary AVP receptors in response to stress*

In contrast to CRH-1Rs, there is a positive correlation between the number of pituitary AVP (V1b) receptors and ACTH responsiveness to stress (Aguilera, 1994). The number of V1b receptors increases in response to stressors associated with an increase in ACTH release (Rabadan-Diehl *et al*, 1995).

Aguilera *et al* (1994) studied V1b receptor activity in response to a range of stressors. They found that the affinity of binding sites for AVP remained the same for all stressors studied, but maximum binding varied depending on the stressor. In response to water deprivation and hypernatremia, where ACTH release declines after chronic stimulation, binding was markedly reduced. Whereas in response to i.p. hypertonic saline, a paradigm associated with prolonged elevation of ACTH release, binding was markedly increased. The correlation between the number of available receptors and ACTH responsiveness suggests that V1b receptor regulation is critical to the maintenance of pituitary responsiveness despite chronic HPA activation and HPA down-regulation in chronic stress (Aguilera *et al*, 2001). In ADX, there is a huge rise in AVP mRNA expression and release, inducing a down-regulation of V1b receptor binding and a transient, 50% reduction in V1b receptor mRNA (Rabadan-Diehl *et al*, 1997). Glucocorticoid treatment of control rats induced a 50% increase in the expression of V1b receptor mRNA.

In some chronic stress paradigms (typically physical stressors), where glucocorticoid concentration is high, it is thought that the ability of AVP to maintain corticotroph responsiveness in the face of a novel stressor is facilitated by the release of glucocorticoids (Aguilera *et al*, 2001). Glucocorticoids can increase the coupling efficiency of the V1b receptor to phospholipase C. Thus, there is an increase AVP-

stimulated inositol phosphate formation despite receptor down-regulation (Aguilera and Rabadan-Diehl, 2000). This could be the mechanism by which AVP maintains corticotroph responsiveness despite elevated glucocorticoids during chronic stress.

8.12. *Actions of CRH and AVP on the anterior pituitary*

CRH has three known effects on anterior pituitary corticotrophs. It stimulates proopiomelanocortin (POMC) transcription, the release of ACTH, and cell mitosis. As was discussed is section 8.4, these effects of CRH are potentiated by AVP and the release of CRH and AVP is modulated in response to different types of stressor. This results in subtle variations in the synthesis of POMC, release of ACTH and the subsequent release of glucocorticoids.

8.12.1. *Synthesis of POMC*

POMC is the precursor hormone for several derived proteins including melanocyte stimulating hormone (MSH) ACTH and β-endorphin. It is expressed throughout the brain and in the anterior and intermediate lobes of the pituitary. The products of POMC breakdown are dependent on the cellular location of the hormone. In the corticotrophs of the anterior pituitary the products of POMC breakdown are ACTH and β-endorphin (Ojeda and McCann, 2000). In anterior pituitary corticotrophs CRH stimulates the transcription of POMC both *in vivo* and *in vitro* (Bruhn *et al*, 1984; Wand *et al*, 1987; Levin and Roberts, 1991). *In vivo* studies of administration of exogenous CRH in the rat revealed an increase in the rate of POMC gene transcription and an increase in levels of primary nuclear transcript in the cytoplasm (Autelitano *et al*, 1989). It is thought that CRH acts directly to increase the rate of transcription of the POMC gene (Levin *et al*, 1989). POMC synthesis is up-regulated by the activation of adenylate cyclase pathways (Reisine *et al*, 1985; Dave *et al*, 1987; Levin *et al*, 1989) and increases in intracellular Ca^{2+} (Dave *et al*, 1987; von Dreden *et al*, 1988; Kuryshev *et al*, 1996).

CRH is not the only factor to influence POMC gene expression. These effects of CRH are again potentiated by AVP (Wand *et al*, 1987). The mechanisms by which AVP acts are complex. AVP binding increases intracellular calcium, inducing POMC gene transcription (von Dreden *et al*, 1988) and potentiating CRH-stimulated

cAMP production (Abou-Samra *et al*, 1987). AVP is also thought to influence POMC gene expression at a stage after transcription, by increasing the rate of turnover of the primary nuclear transcript (Wand *et al*, 1987).

In studies of the effects of ADX, the removal of glucocorticoid feedback induces a transient rise in POMC mRNA in cytoplasm (Rabadan-Diehl *et al*, 1997) suggesting that POMC gene expression is inhibited by glucocorticoids. This rise in POMC transcription is still observed in CRH knockout mice, although of a lower magnitude than in intact animals (Muglia *et al*, 1996) and in rats with lesions of the PVN (Rabadan-Diehl *et al*, 1997). This indicates that POMC transcription is not dependent on the presence of hypothalamic factors. Indeed basal levels of POMC transcription are not influenced by PVN lesions, median deafferentation or CRH antagonists (Rabadan-Diehl *et al*, 1997), suggesting that the stimulatory effects of CRH and AVP are only relevant when their concentration in the median eminence is markedly increased during stress (Aguilera *et al*, 2001).

8.12.2. *ACTH release*

The binding of CRH and AVP to their membrane receptors activates adenylate cyclase and phopholipase C signal transduction pathways respectively, resulting in changes in membrane potential and the exocytosis of secretory vesicles containing ACTH. The effects of CRH are potentiated by the enhancement of cAMP production by AVP. This is achieved either by the direct activation of adenylate cyclase or by inhibiting the factors that inhibit cAMP production (Abou-Samra *et al*, 1987)

8.12.3. *Cell mitosis*

ADX produces an increase in the number of corticotrophs in the anterior pituitary (Childs *et al*, 1995). Administration of CRH *in vivo* also induces an increase in the number of corticotrophs and *in vitro* the mitogenic activity of cultured corticotrophs is increased. However the mitogenic activity of CRH occurs over a very narrow dose range (0-0.5nM). At concentrations above this range CRH may in fact retard cell growth (Childs *et al*, 1995).

8.12.4. *Glucocorticoids: feedback inhibition on the HPA axis*

Circulating glucocorticoids are critical to the modulation of PVN responsiveness to stress (Ma and Aguilera, 1999a). They act at all levels of the HPA axis inhibiting HPA responsiveness to stress (Ma and Aguilera, 1999b). Their function is protective, providing a mechanism that prevents HPA activation in response to minor deviations from homeostasis, effectively broadening the range of acceptable stimulation of the stress axis.

Removal of glucocorticoids by ADX is characterised by hyper-responsiveness of the HPA axis (Plotsky and Sawchenko, 1987). However, there is evidence to suggest that AVP is more sensitive to glucocorticoid regulation than CRH. For instance, in ADX rats, the administration of exogenous glucocorticoids inhibits the synthesis of AVP much more than it does CRH (Ma and Aguilera, 1999) and CRH mRNA was not found to be inhibited at all by glucocorticoid administration to ADX rats (Ma *et al*, 1997). However, the administration of exogenous glucocorticoids strongly inhibited AVP mRNA (Ma *et al*, 1997). The ability of parvocellular AVP neurones to overcome glucocorticoid inhibition depends on the nature and severity of exposure to stress and may involve the interaction of glucorticoids with other neuropeptides released in response to stress (Herman *et al*, 1997).

Two types of glucocorticoid receptor are recognised. Type I receptors have a lower distribution and preferentially bind naturally occurring corticoids (often referred to as mineralcorticoid receptors). These receptors have a very high affinity for their agonists and under normal circumstances most (89.5%) of these receptors are occupied (Reul and de Kloet, 1985). It is thought that type I receptors control basal levels of glucocorticoids (de Kloet *et al*, 1987). Type II receptors preferentially bind dexamethasone, a synthetic glucocoticoid (often referred to as glucocorticoid receptors). These receptors are more widely distributed in the limbic system, hypothalamus and pituitary. They have a lower affinity for naturally occurring glucocorticoids than type I receptors and basal levels of occupancy are low in comparison with type I receptors (Reul and de Kloet, 1985). However, under stressful circumstances, when the concentration of glucocorticoids in the blood is high, binding to type II receptors increases. For this reason type II receptors are

thought to mediate glucocorticoid action during stressful episodes (de Kloet *et al*, 1987).

8.13. *Conclusion*

The basic mechanisms by which the endocrine responses to stress are mounted are well-known. However the mechanisms by which specific responses to diverse stressors are mounted are less well understood. Currently the balance of evidence indicates that different types of stressor activate different sets of stress-regulatory neurones in areas of the brain including the limbic system, medulla and brainstem. Stressors representing an immediate threat to the integrity of the animal activate brainstem neurones, which rapidly, after only one synaptic connection, activate the PVN. Other stressors, requiring mental processing and constituting a less immediate threat to the animal, activate the PVN through multi-synaptic pathways, including limbic nuclei (Swanson and Sawchenko, 1983; Sawchenko *et al*, 1996; Herman and Cullinan, 1997).

In the resting state, approximately 50% of parvocellular neurones in the PVN express both CRH and AVP (Whitnall, 1988; 1990) although this varies in different species (Whitnall *et al*, 1992). In response to acute stress, the synthesis and release of CRH in parvocellular neurones is increased. The synthesis and release of AVP may also be stimulated but the magnitude of the AVP response rarely exceeds that of CRH.

After exposure to all types of chronic stressor, the proportion of parvocellular neurones that express both CRH and AVP increases. The expression of AVP mRNA increases dramatically after exposure to all types of chronic stress. Its expression remains high until exposure to the stressor ceases. AVP mRNA expression far exceeds that of CRH, and AVP is preferentially released from nerve terminals in the median eminence during chronic stress (Bertini and Kiss, 1991).

The level of synthesis and release of CRH varies between chronic stress paradigms and may be desensitised during a period of prolonged psychological stress (Ma and Lightman, 1998). After prolonged exposure to a homotypic stressor, ACTH hyper-responsiveness to a novel stressor is observed (Aguilera, 1994). Receptor studies and studies of CRH knock-out mice indicate that the presence of some CRH is necessary for the HPA response to chronic stress but that CRH must bind to only 50% of

receptors to allow a full ACTH response (Aguilera *et al*, 1987). This evidence suggests that the presence of CRH is permissive for the HPA response to chronic stress and that elevated levels of AVP may help maintain anterior pituitary responsiveness and mediate hyper-responsiveness to novel stressors (Aguilera *et al*, 2001).

Subtle differences in the HPA response to stress have been found between different strains of rats (Harbuz *et al*, 1994). More fundamental differences exist between animals of different species (Familiari *et al*, 1989; Liu *et al*, 1990; Whitnall *et al*, 1992; Canny *et al*, 1999). Since much of the accepted theory of the HPA axis response to stress results from studies in the rat, care must be taken to ensure that these theories apply to other species.

Chapter 9

In-situ Hybridisation Histochemistry to determine Chronic Inflammatory Pain

Chapter 9. *In-situ* hybridisation histochemistry to validate the behavioural assessment of chronic inflammatory pain after rubber ring castration and tail-docking.

9.1. *Introduction*

9.1.1. *Chronic inflammatory lesions from castration and tail-docking*

Lesions begin to form 3-7 days after c+td by application of a RR to the neck of the scrotum and to the tail. These lesions reach a peak in size and severity after approximately 21 days (Kent *et al*, 1999) and can measure 4cm across. The tissue becomes inflamed and often infected, sometimes severely as the skin breaks down at the site of the RR.

9.1.2. *Expression of 'pain' behaviours*

Intermittent expression of the behaviours used to assess acute pain from c+td have been observed in lambs with c+td lesions (Kent *et al*, 1999). The changes in behaviour observed are often very subtle, but their occurrence suggests that the lesions are painful (Kent *et al*, 1999). Before these behaviours can be confidently used to assess pain from these chronic inflammatory lesions, it must be determined whether determine whether expression of these behaviours is associated with pain and if so, how consistent the relationship is.

In association with behaviour and self-administration studies, it is proposed that changes in the synthesis and release of the neuropeptides arginine vasopressin (AVP) and corticotrophin-releasing hormone (CRH) from the hypothalamic paraventricular nucleus (PVN) will provide further evidence of the significance of these c+td lesions to the lambs.

9.1.3. *The HPA response to chronic inflammatory pain*

CRH and AVP act in synergy to mediate the hypothalamo-pituitary-adrenal (HPA) axis response to stress (Gillies *et al*, 1982; Antoni, 1993). Acute stressful challenge results in an increase in the synthesis and release of CRH from the parvocellular division of the paraventricular nucleus (pPVN) (Bartanuz *et al*, 1993). An increase in

the release of AVP is also stimulated, but up-regulation of the synthesis of AVP depends on the nature of the stressor (Bartanuz *et al*, 1993; Harbuz *et al*, 1994). The expression of AVP mRNA does not generally exceed that of CRH mRNA (Lightman and Young, 1988).

During and after chronic exposure to stress in rats, the rate of synthesis and release of AVP and CRH from parvocellular paraventricular neurones changes. The number of neurones expressing AVP mRNA in the pPVN and the density of expression of AVP mRNA within these neurones increases dramatically. The concentration of AVP in the hypothalamo-pituitary blood (HPB) also increases (Chowdrey *et al*, 1995; Ma and Aguilera, 1999). However, in chronic stress, the synthesis and release of CRH from parvocellular neurones varies depending on the nature of the stressor and is generally exceeded by that of AVP (Ma and Aguilera, 1999).

In adjuvant-induced arthritis in rats, an experimental model of chronic inflammatory pain, these changes are particularly pronounced. The synthesis and release of AVP is strongly up-regulated (Chowdrey *et al*, 1995), whist the synthesis of CRH does not change at all or is slightly reduced (Harbuz *et al*, 1992)

These changes in the way the HPA axis is regulated in response chronic inflammatory pain show that the roles of AVP and CRH differ when exposure to the stressor is prolonged. AVP is considered to be essential for the maintenance of pituitary responsiveness during chronic stress (Aguilera, 1994). However, the presence of some CRH in HPB is also necessary for the induction of a maximal ACTH response (Muglia *et al*, 2001).

9.1.4. *HPA responses to removal of sex steroids*

It is also well established that sex related variations exist in the expression, regulation and release of hormones at all levels of the HPA axis. This includes differences between males and females in the expression of CRH and AVP in the parvocellular PVN (Paulmyer-Lacroix *et al*, 1996; Viau and Meaney, 1996). These differences are considered to reflect the differential expression of gonadal steroids in males and females. While these sex differences are accepted, few direct comparisons of the male and female HPA axis have been made (Canny *et al*, 1999).

The effects of sex steroids are principally mediated centrally through the PVN and through their action on glucocorticoid receptors. Although receptors for gonadal steroids are found in the PVN, they are not found in cells that regulate the HPA axis (Simerly *et al*, 1990).

In studies in the rat, variable effects of gonadal hormones on the synthesis and release of CRH have been reported. *In vitro* studies have shown that oestrodiol can increase CRH gene expression, by its action on a putative oestrogen promoter region in the CRH gene. However, in *in vivo* studies oestrogen has an inhibitory effect on CRH mRNA levels in the pPVN. Through studies of ADX, it has been shown that the inhibitory action of oestrogen is mediated through the stimulatory effect of oestrogen on glucocorticoid release, which in turn inhibits CRH synthesis (Paulmyer-Lacroix *et al*, 1996). In sheep, pregnancy, lactation and oestradiol and progesterone treatment were found to have no effect on the level of expression of CRH mRNA in the PVN (Broad *et al*, 1995). In studies of GDX with testosterone replacement, no differences were found in the concentration of irCRH in the median eminence between GDX rats with and without testosterone replacement (Viau and Meany, 1996). In sheep, the concentration of irCRH in the median eminence doubled after GDX in both rams and ewes (Canny *et al*, 1999).

The concentration of ir AVP was lower in GDX male rats with testosterone replacement than in vehicle treated GDX animals (Viau and Meany, 1996). However, the number of irAVP neurones in the PVN did not change after GDX in male rats (Bingaman *et al*, 1994). Testosterone has been found to have a stimulatory effect on the number of neurones expressing AVP mRNA in the PVN in the pig (van Eerdenberg *et al*, 1991) and the rat (de Vries *et al*, 1994). In sheep, the concentration of irAVP in the median eminence of 3-year old rams has been found to be significantly higher than that in 3-year old ewes, although no differences were found in the concentration of irCRH (Canny *et al*, 1999). The concentration of irAVP in the median eminence doubled in rams after GDX, but no change was found in ewes. Together this evidence suggests that testosterone is particularly important in the regulation of AVP synthesis and release, but does not appear to be as important in the regulation of CRH synthesis and release. However, some evidence suggests that oestrogen may have an important influence on the expression of the CRH gene.

Sex steroids, particularly testosterone, have an inhibitory influence on the HPA response to inflammatory stress (Hadid *et al*, 1995). Changes in expression of immunoreactivity for AVP and CRH in the median eminence do not reflect changes in the synthesis of AVP and CRH in the parvocellular paraventricular nucleus (pPVN) (Bingaman *et al*, 1994). However, the possibility that, in sheep, castration induces changes in the expression of AVP and CRH mRNA as a result of the removal of gonadal steroids cannot be ruled out. Therefore any changes in the expression of AVP and CRH mRNA in the median eminence may be the result of chronic inflammatory pain from the lesion or of removing testosterone and oestrogen by castration, or both.

9.1.5. *Use of* in situ *hybridisation histochemistry to detect changes in AVP and CRH mRNA expression in the pPVN.*

The aim of this study was to use *in situ* hybridisation histochemistry to test the hypothesis that rubber-ring c+td of lambs, resulting in the formation of chronic inflammatory lesions and associated 'pain' behaviours, causes chronic inflammatory pain and that such pain induces changes in the expression of AVP and CRH mRNAs in the pPVN indicative of chronic stress. A second *in situ* hybridisation study, in which lambs were tail-docked but not castrated, was designed to determine whether any changes in the expression of AVP and CRH mRNAs were associated with the removal of testosterone by castration, rather than in response to the chronic inflammatory lesions.

9.2. Methodology

9.2.1. *Animals and management*

Twin male lambs were housed together in a large (5x20m) straw-bedded pen with their dams. All animals were subject to normal management procedures as outlined in Chapter 2 part 2.2. Briefly, animals had continuous access to fresh water and hay. The ewes were fed 500g of ESCA ewe nuts daily. The lambs were given constant access to lamb creep feed (Pye-Frankland Balanced Feeds, Lamb Creep Pellets) and had constant access to their dam to suck milk. The animals were brought into the experimental environment one week prior to treatment. The lambs were weighed and one of each pair of twins was allocated to one of the two treatments, with weight balanced across treatment groups.

9.2.2.1. *Experiment 1: Animals and treatments*

Sixteen four-week-old twin male lambs (Grey Face x Suffolk) were divided between two treatments as follows:

1. C+td by the application of tight RRs to the neck of the scrotum and tail. n=8 lambs.
2. Handled as required to c+td but without the application of the RRs. n=8 lambs.

9.2.2.2. *Experiment 1: Lesion assessment*

The chronic inflammatory lesions resulting from c+td were examined and assessed twice weekly according to the lesion assessment protocol (see Section 2.7). The lambs were humanely killed (Euthatal, sodium pentabarbitone) 28 days after castration, when the chronic inflammatory lesions from c+td reached a peak in severity.

9.2.3.1. *Experiment 2: Animals and treatments*

Fourteen, six-week-old, twin, male lambs (Scottish Blackface) were divided between two treatments as follows:

1. Tail-docked by the application of tight RR to the tail. n=7 lambs.
2. Handled as required to tail-dock but without the application of the RR. n=7 lambs.

9.2.3.2. *Experiment 2: Lesion assessment*

The chronic inflammatory lesions resulting from tail-docking were examined and assessed, according to the lesion assessment protocol (see Chapter 2 part 2.7), 21 days after treatment, when the lambs were humanely killed (Euthatal, sodium pentabarbitone).

9.2.4. *Behavioural observation*

In both experiments, behavioural observations were recorded directly onto The Observer behavioural analysis software whilst watching the lambs. All the lambs were observed, simultaneously in the group pen with their dams, by 3 experienced observers.

In the first experiment observations were made for 2 hours between 13.00 and 16.00 hours on days 18 and 25 after castration, according to the ethogram described in Section 2.8. The behaviours 'chew' and 'horn' were not recorded on this occasion as Greyface x Suffolk lambs have very small horns and no bars were available in the pen. Lambs could not therefore perform these behaviours. The presence of three straw bales in the pen increased the likelihood of play behaviours in this group of lambs.

In the second experiment observations were made for 3 hours between 13.00 and 16.00 hours on days 12 and 18 after treatment, according to the ethogram described in Chapter 2 part 2.8. The behaviour 'chew' was not recorded on this occasion as no bars were available in the pen and lambs could not therefore perform this behaviour.

9.2.5. *Tissue collection and sectioning*

When the severity of lesions peaked, the lambs were humanely killed by overdose of anaesthetic ('Euthatal', Sodium Pentabarbitone). From this point onwards the methods used in both experiments were the same. Following decapitation the brains were rapidly (<5min) removed and cut into six coronal slices. Counting from the rostral pole, the third slice contained the paraventricular nucleus (PVN). This slice was taken between the optic chiasma (rostrally) to a point just caudal to the median eminence (as carried out by Matthews *et* al 1993). Each slice was rapidly frozen by placing on a flat Teflon-coated metal tray pre-cooled to -85°C using dry ice. The slices were covered with powdered dry ice. Full freezing occurred within 5 minutes. Once frozen the slices were transferred to individually labelled polythene bags and stored at -85°C until required for further processing.

The brain slices containing the hypothalamus were removed from the freezer and brought to -16°C in a cryostat. A cube of tissue, measuring approximately 2x2x2cm, was cut from the centre of the slice so that the hypothalamus and the third ventricle were contained entirely within it. From this cube 15μm coronal sections were cut. Sectioning began at the level of the subfornical organ and was continued serially to the median eminence. The sections were thaw-mounted on to positively charged, nuclease free microscope slides and stored at -85°C in sealed plastic boxes until required for *in situ* hybridisation histochemistry to seek AVP and CRH mRNA in the parvocellular division of the PVN.

9.2.6. *Labelling of oligonucleotide probes*

For the detection of AVP mRNA an antisense 45-mer oligonucleotide probe was used, as designed by Matthews *et al* (1993). This probe is complementary to bases 397-441 of the bovine AVP neurophysin II gene, a G-C rich (86%) area of the gene (see figure 9.1a).

For the detection of CRH mRNA an antisense 45-mer oligonucleotide probe was used, as described by Matthews *et al* (1991). This probe is complementary to bases 1424-1468 of the ovine CRH gene and is also G-C rich (51.1%) (see figure 9.1b)

Fig 9.1. (a) Sequence of the antisense, 45-mer, oligonucleotide probe to detect AVP mRNA in PVN. This probe is complementary to bases 397-441 of the bovine AVP neurophysin II gene, as described by Matthews *et al* (1993). (b) Sequence of the antisense, 45-mer, oligonucleotide probe to detect CRH mRNA in PVN. This probe is complementary to bases 1424-1468 of the ovine CRH gene, as described by Matthews *et al* (1991).

(a) $^{5'}{}_{441}$ GTA GAC GCC GGG CTG GGC GGG CTC CGC GGG CTC CGG CGC CGC CGC $_{397}{}^{3'}$

(b) $^{5'}{}_{1468}$ CCT ATT GCT ATG AGC TTG CTG CGC TAA CTG ATC GGC CTT GGT CAT$_{1424}{}^{3'}$

The oligonucleotide probes were 3'-end-labelled with ^{35}S deoxyadenosine triphosphate ([^{35}S]dATP, specific activity 1250Ci or 46.2TBq/mmole). 1µl of probe and 125µCi (4.62MBq) [^{35}S]dATP were incubated for 1hour at 37°C with 25U terminal deoxynucleotidyl transferase (TdT) (2µl) in tailing buffer (5µl) and sterile water (17µl), making a total volume of 25µl. 75µl of ice-cold 12mM EDTA was added to stop the reaction and the reaction mixture was placed into crushed ice for 15minutes. The labelled probe was then purified through Microspin G25 columns, by centrifugation, following the manufacturer's instructions. A 1µl sample of the probe mixture was taken for scintillation counting before and after purification in order to calculate the percentage incorporation of [^{35}S]dATP into the AVP and CRH oligonucleotide probes. The mean (±SEM) percentage incorporations of [^{35}S]dATP into the AVP and CRH oligonucleotide probes were 32.8%(±1.40) and 30.7%(±2.16) respectively. The purified probe was diluted with hybridisation buffer for storage overnight at 20 000cpm/µl in a -20°C freezer.

9.2.7. *Pre-hybridisation buffer*

Pre-hybridisation buffer was made up under sterile conditions. The following volumes of chemicals were mixed; 10.67ml diethyl pyrocarbonate water (DEP water), 4.80ml 5M NaCl, 400µl 1M trisodium-HCl, pH7.5, 132µl 6% FICOLL, 132µl 6% polyvinylpyrrolidone (PVP), 668µl 6% bovine serum albumin (BSA),

160µl 250mM ethylene diamine tetra-acetic acid (EDTA), 2ml 10mg/ml sonicated salmon sperm DNA, 1ml 2mg/ml glycogen and 40µl 50mg/ml yeast tRNA.

9.2.8. *Hybridisation buffer*

Hybridisation buffer was also prepared under sterile conditions. The following volumes of chemicals were mixed; 4.0g dextran sulphate, dissolved in 13.17ml of DEP water, 4.80ml 5M NaCl, 400µl 1M Tris-HCl, pH7.5, 132µl 6% FICOLL, 132µl 6% PVP, 668µl 6% BSA, 160µl 250mM EDTA, 400µl 10mg/ml sonicated salmon sperm DNA, 100µl 2mg/ml glycogen and 40µl 50mg/ml yeast tRNA.

Pre-hybridisation and hybridisation buffers were stored at twice the working concentration and were diluted with an equal volume of 50% deionised formamide before use.

9.2.9. *Box buffer*

Box buffer was made to provide a suitable background environment in which to facilitate pre-hybridisation and hybridisation. Box buffer is 4x SSC, diluted from 20x SSC using DEP water, mixed with an equal volume of deionised formamide.

9.2.10. *Fixation, prehybridisation and hybridisation*

Before hybridisation the sections were fixed for 10 minutes in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.5. The sections were then immersed in two consecutive washes of 2x SSC for 5 minutes each. The slides were then drained and laid on foam, soaked in box buffer, in hybridisation boxes. 75µl of pre-hybridisation buffer was dispensed on to each section and the sections were incubated for 1-3 hours at 37°C. Prehybridisation buffer was then drained from the slides and 75µl of hybridisation buffer was applied to each section. The hybridisation buffer contained 10 000cpm of ^{35}S-labelled antisense oligonucleotide and 10µl of dithiothreitol (DTT) was added to each millilitre of buffer in order to keep the ^{35}S if reduced form. Hybridisation incubation of the sections at 37°C for 18-24 hours was carried out in the sealed hybridisation boxes.

Following hybridisation the slides were washed in three successive washes of increasing stringency (2x, 1x and 0.5x SSC), for one hour each, at 37°C. The slides

were then dehydrated, for two minutes each, in three graded ethanol solutions (50%, 70% and 90%) containing 0.3M ammonium acetate. The slides were air-dried overnight.

9.2.11. *Autoradiography and staining*

Ilford G5 emulsion was melted in a water bath at 42°C and diluted with distilled water in a 1:1 dilution by volume. The dry, hybridised slides were dip-coated in the diluted emulsion and left to dry overnight in the darkroom. The following day the slides were packed and sealed into light-tight plastic boxes and stored at 4°C for 3 weeks to expose. The exposed slides were developed at 19°C for 4 minutes in Ilford Phenisol developer diluted 1:4 with distilled water. The slides were then washed briefly in distilled water before being immersed for 4 minutes each in three successive troughs of Ilford Hypam fixative diluted 1:4 with distilled water, before a final 10 minute wash in a large basin of distilled water.

The slides were then stained for 30 seconds in a 1% aqueous solution of Pyronine, followed by four successive washes in distilled water and a final soak in distilled water for 10 minutes. The slides were then dehydrated and cleared by immersion in acetone for two minutes, 1:1 acteone:Histoclear II for two minutes and finally two troughs of Histoclear II for two minutes each. The slides were then mounted in Histomount.

9.2.12. *Microscopy and image analysis*

The tissue sections were examined microscopically using bright-field illumination with 10x and 20x objectives (Nikon, UK Ltd.). The total number of labelled, parvocelluar neurones in the paraventricular nucleus was counted using a 21mm graticule and a hand-held tally counter. The density of silver grains within labelled parvocellular neurones was measured using NIH Image (image analysis software made available by Roslin Institute). In order to reduce error arising from clumping of grains, the area of silver deposit was measured rather than the numbers of individual silver grains in each cell. Silver grain density within thirty representative cells for each animal was used. This sample size has previously proven to be optimal, combining ease of counting without reduced accuracy. Cells were considered

labelled if the density of grains within the cell was at least twice that of background. Cells were only used for quantified measurement if they contained a nucleus.

9.2.13. *Comparison of AVP and CRH mRNA content and distribution*

To compare the content and distribution of AVP and CRH mRNA expressing cells, both AVP and CRH oligonucelotide probes were used on sections from each animal. In order to match the sections anatomically, every fifth slide was removed from the series cut for each animal. The sections were fixed in ethanol and stained in a 1% aqueous solution of Toluidine blue followed by four successive washes in distilled water and a final soak for 10 minutes in distilled water. Examination of these slides allowed adjacent and anatomically matched slides in the series to be probed with each probe. Sections from each pair of twin animals were also matched anatomically as far as possible to aid between treatment comparisons.

9.2.14. *Controls*

To ensure that positive signals of probe in this study represented RNA, the *in situ* hybridisation process was carried out on sections using the AVP and CRH oligonucleotide probes. However the sections were incubated for 1 hour at 37°C in a solution containing RNaseA, after fixing and before pre-hybridisation. The preparation of the RNase solution was as follows; RNase was reconstituted in distilled water at a concentration of 10mg/ml. This solution was boiled for 10 minutes to destroy DNase. The cooled solution was diluted to 30μg/ml in 10mM Tris-HCl, pH 7.5, containing 0.5M NaCl. Pre-incubation in RNase solution removes all signals due to RNA.

In order to ensure that any positive signals obtained represented specifically AVP and CRH mRNAs, the method was also carried out using ^{35}S-labelled oligonucelotide probes for CRH and AVP with scrambled sequence (see fig 9.2). Such scrambled probes should produce no specific signal. Following labelling, the mean (±SEM) percentage incorporations of [^{35}S]dATP into the AVP and CRH scrambled probes were 23.6%±(0.95) and 43.5%(±1.5) respectively.

Figure 9.2. (a) Sequence of scrambled antisense, 45-mer, AVP mRNA oligonucleotide probe. (b) Sequence of scrambled antisense, 45-mer, CRH mRNA oligonucleotide probe.

(a) $^{5'}$ GCC GGG GGG CTC GTA GAC CTG GGC CGC GGG CGC CGC CTC CGG CGC $^{3'}$

(b) $^{5'}$ CTA GTC CAG GCT ATA CTA TCT ACT TTA GTA GTT CCG GTC CGG TGG $^{3'}$

9.2.15. *Statistical analyses*

All 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 in the distribution and density of expression of AVP and CRH mRNA in the PVN of treated (c+td or tail-docked alone) lambs and their untreated siblings.

The behavioural data were not always normally distributed. Square root and Log_{10} transformation were attempted but were not sufficient to normalise data with skewed distribution in any case. Statistical comparisons of behaviour were therefore carried out using paired *t*-tests or Wilcoxon/Sign rank depending on the distribution of the data.

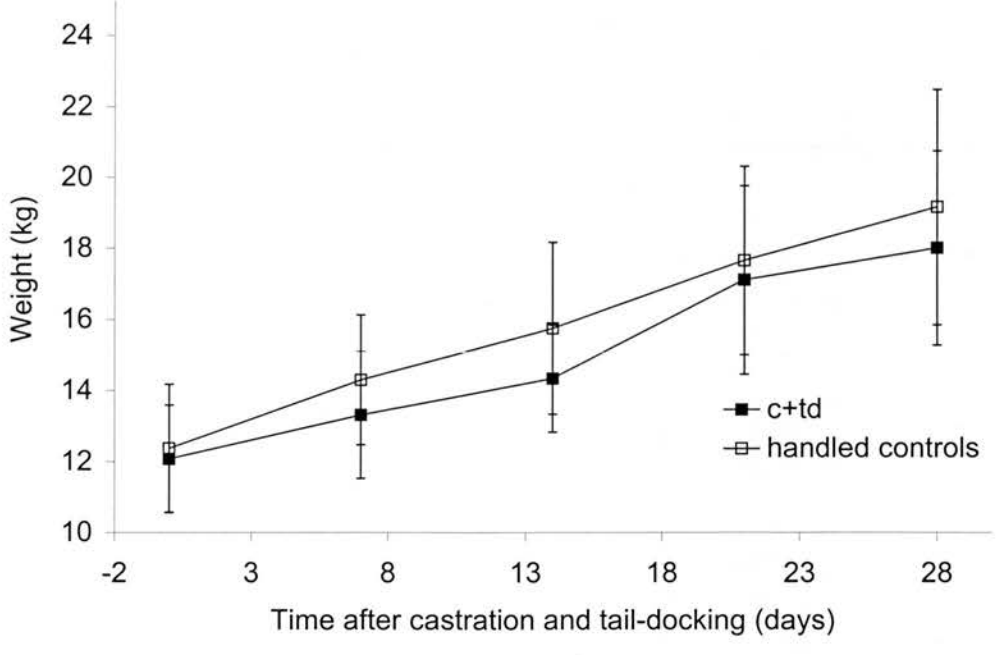
9.3 Results

9.3.1. *Experiment 1*: *Castration and tail-docking*

9.3.1.1. *Lamb weight*

No effect of treatment on the daily weight gain of lambs was found during the trial. The weights of lambs were balanced at the start of the trial to reduce any effect of weight on treatment. Therefore, prior to treatment there was no difference between the weights of lambs in the two treatment groups ($T_{1,7}$=0.49, P=0.64). After castration and tail-docking, the weights of c+td lambs and their handled control siblings diverged slightly, with control lambs being consistently heavier. However, the differences between groups did not reach significance at any time between 7 and 28 days after treatment when the lambs were killed. These results are shown in figure 9.3.

Figure 9.3. Change in weight 4-week-old lambs (n=8/group) over the 28 days following either castration and tail-docking or handling as controls. (mean± s.d. are presented).



9.3.1.2. *Lesions*

Inflamed lesions developed at the neck of the scrotum and on the tail where the RRs were applied in all c+td lambs. The severity of lesions was scored using the systems

described in Section 2.7. The width of the lesion (mm) was measured. The lesion was also subjectively assessed using the eleven-point scale described in table 2.1.

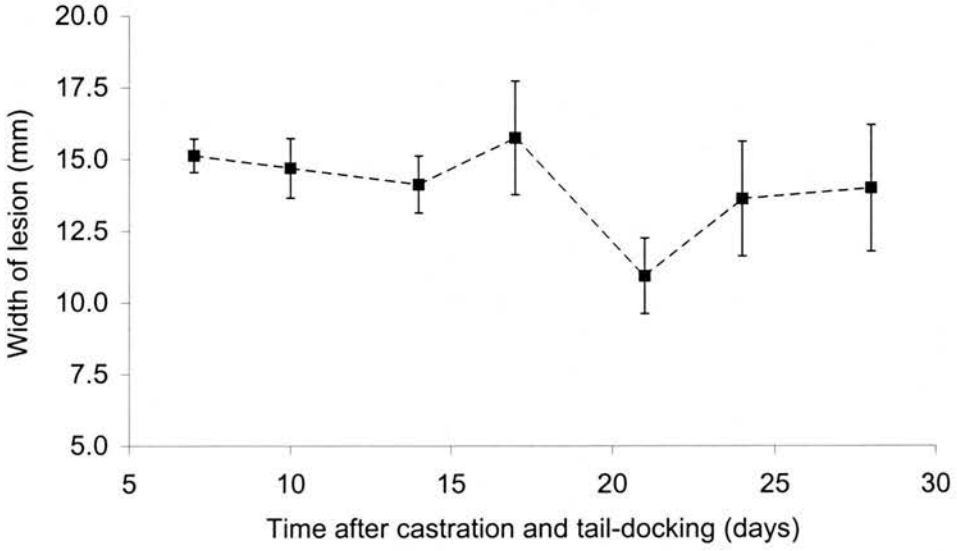
Lesion Scores: All c+td lambs developed lesions with pus (score 3.5) during the trial. The maximum mean (±sd) lesion score from castration was 3.25 (±0.25) (figure 9.3), reaching this peak, on average, 17 days after castration and remaining high until 28 days after treatment when the lambs were killed.

Lesion Width: The mean width of castration lesions reached a slight peak of 15.75 (±0.70)mm, 17 days after castration. At 21 days the mean width dropped to a low of 10.94 (±3.2)mm, but did not reduce further in size before the lambs were killed 28 days after treatment. None of the lambs lost their scrotums before being killed.

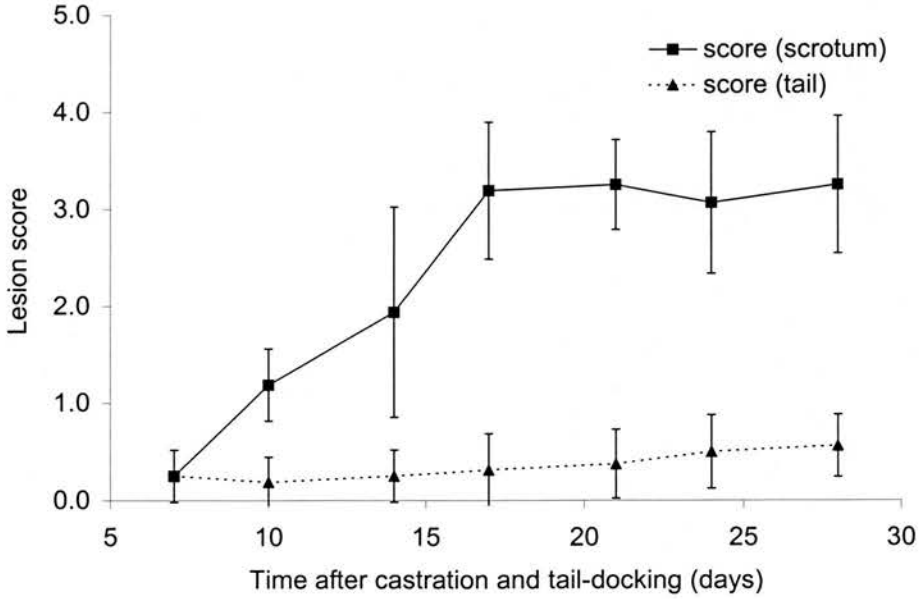
The tail lesions became progressively more severe throughout the trial but the mean score did not exceed 0.56 (±0.11) recorded 28 days after treatment on the day of kill. No pus was present in tail lesions in any of the lambs. No tails were lost before 21 days, but half of the c+td lambs had lost their tails 28 days after treatment. These results are presented in figure 9.4.

Figure 9.4. Change in the (a) width and (b) score of lesions produced by c+td of 4-week-old lambs (n=8/group) over the 28 days following treatment. (mean± s.d. are presented)

(a)



(b)



9.3.1.3. *Behavioural observations*

Behaviour was observed on days 18 and 25 after castration, for two hours. There was considerable variation in the expression of all behaviours within both groups during both observation periods. Normal and abnormal behaviours and postures were expressed by both groups of lambs.

Frequency of active behaviour

18 days after treatment, c+td lambs and handled control lambs expressed restless behaviour, easing quarters, foot-stamping, head turning, and rubbing quarters. However, no differences were found in the frequency of expression of these behaviours between groups. C+td lambs expressed more tail-wagging than handled lambs, although this difference did not reach significance at $P<0.05$. C+td lambs also showed more teat-seeking than handled controls ($T_{1,7}$=-2.60, P=0.035). Regression analysis was performed on these results to determine whether the number of times lambs were observed teat-seeking could be used to predict the frequency of tail-wagging. There was found to be a significant relationship between tail-wagging and teat-seeking in handled lambs ($T_{1,7}$=-0.71, P=0.047) but the relationship did not reach significance in c+td lambs ($T_{1,7}$=0.55, P=0.062).

25 days after treatment c+td lambs eased-quarters more frequently than handled lambs ($T_{1,7}$=-3.90, P=0.006). C+td lambs also showed more restlessness than handled controls but this difference was not statistically significant ($T_{1,7}$=-1.62, P=0.150). The more frequent expression of active behaviours by c+td lambs is reflected in a significant difference in the overall frequency of active behaviour (REQ) between control and treated lambs ($T_{1,7}$=-3.45, P=0.011). No other differences between groups in the expression of active behaviours were found 25 days after castration. These results are presented in tables 9.1 and 9.2.

Postures and behavioural states

On the 18th day after treatment, c+td and handled lambs stood and lay in normal and abnormal postures for a similar number observations. C+td lambs appeared to spend more time eating (straw, hay or creep feed) than handled lambs although this difference did not reach significance at $p<0.05$ ($T_{1,7}$=-2.04, P=0.080). No other

differences in the occupation of lambs were found. Data on the number of observations spent ruminating and sleeping on day 18 were lost because of a computer error.

25 days after castration, no differences were found in the postures expressed by c+td lambs and handled controls. These results are presented in tables 9.3a and b and 9.4a and b.

Table 9.1. The frequency of active behaviours expressed by lambs 18 days after either c+td by RR or handling as controls (n=8). Mean (±sd) or median (Q1-Q3) frequency of expression for each group and each behaviour is presented. The statistical test used and the level of statistical probability are also shown.

	Restlessness		Ease quarters		Foot-stamp/kick		REQ		head-turn		play		tail-wag		teat-seek		rub	
Twin	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled
1	5	6	2	3	0	1	7	10	3	3	4	7	19	10	9	4	0	0
2	8	4	1	10	0	0	9	14	0	4	3	7	11	1	2	0	0	0
3	9	7	8	5	0	0	17	12	1	1	1	5	1	1	4	1	2	0
4	5	6	7	7	0	0	12	13	3	3	0	6	2	0	1	1	0	0
5	11	13	6	4	1	1	18	18	6	2	0	2	9	6	5	3	0	2
6	7	9	4	1	0	0	11	10	5	4	3	4	0	1	3	3	0	0
7	3	6	0	2	0	0	3	8	0	1	1	7	2	1	3	2	0	0
9	6	5	3	4	2	0	11	9	3	7	14	1	1	0	0	0	0	0
Mean/Median	6.8	7.0	3.9	4.5	0.0	0.0	11.0	11.8	2.6	3.1	2.0	5.5	5.6	2.5	3.4	1.8	0.0	0.0
sd/Q1-Q3	2.55	2.83	2.90	2.88	0-0.25	0-0.25	4.93	3.24	2.20	1.96	0.75-3.25	3.5-7	6.76	3.59	2.77	1.49	0-0	0-0
TEST	T-test T=0.30 *P*=0.77		T-test T=0.45 *P*=0.66		Wilcoxon T=1.0 *P*=1.00		T-test T=0.61 *P*=0.56		T-test T=0.54 *P*=0.61		Wilcoxon T=28.0 *P*=0.183		T-test * T=-2.14 *P*=0.069		T-test ** T=-2.60 *P*=0.035		Wilcoxon T=1.5 *P*=1.00	

Table 9.2. The frequency of active behaviours expressed by lambs 25 days after either c+td by RR or handling as controls (n=8). Mean (±sd) or median (Q1-Q3) frequency of expression for each group and each behaviour is presented. The statistical test used and the level of statistical probability are also shown.

	Restlessness		Ease quarters		Foot-stamp/kick		REQ		head-turn		play		tail-wag		teat-seek		rub	
Twin	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled
1	9	4	9	1	0	1	18	6	4	1	5	10	0	8	3	3	0	0
2	9	7	6	5	0	0	15	12	1	2	1	4	3	1	1	1	3	0
3	13	4	2	0	0	0	15	4	6	3	10	0	0	1	1	1	0	0
4	9	6	2	2	0	0	11	8	3	1	1	4	4	1	2	2	0	0
5	5	8	4	0	0	0	9	8	3	3	1	0	0	2	2	2	0	2
6	11	5	6	2	0	0	17	7	3	4	16	6	0	1	1	1	0	0
7	5	9	7	3	0	0	12	12	4	3	0	17	0	0	1	1	0	0
9	5	3	5	1	0	0	10	4	0	2	1	0	1	0	1	0	0	0
Mean/Median	8.25	5.75	5.13	1.75	0.0	0.0	13.38	7.63	3.00	2.38	4.38	5.13	1.00	1.75	1.00	1.00	0.0	0.0
sd/Q1-Q3	3.01	2.12	2.42	1.67	0-0	0-0	3.34	3.11	1.85	1.06	5.76	5.94	1.60	2.60	1.0-2.0	1.0-2.0	0-0	0-0
TEST	T-test T=-1.62 *P*=0.15		T-test T=-3.90 *P*=0.006		Wilcoxon T=1.0 *P*=1.00		T-test T=-3.45 *P*=0.011		T-test T=-0.92 *P*=0.39		T-test T=0.24 *P*=0.81		T-test T=0.63 *P*=0.55		Wilcoxon T=0.00 *P*=1.00		Wilcoxon T=0.00 *P*=1.00	

Table 9.3a. The number of observations spent in each posture by lambs 18 days after either c+td by RR or handling as controls (n=8). Table 9.3b The number of observations spent in each behavioural state by lambs 18 days after either c+td by RR or handling as controls (n=8). The mean (±sd) or median (Q1-Q3) number of observations spent in each posture or behaviour for each group and each posture is presented. The statistical test used and the level of statistical probability is also shown.

18 days	Normal		S1		V1		V2		Abnormal		SS		V3		LL	
lamb	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled
1	20	20	12	8	0	3	8	9	1	1	0	1	1	0	0	0
2	20	20	16	5	0	6	5	10	0	0	0	0	0	0	0	0
3	18	15	8	11	0	0	10	4	3	6	0	0	3	6	0	0
4	20	14	8	6	0	0	13	8	1	7	1	1	0	5	0	1
5	20	20	11	6	1	1	8	14	1	0	1	0	0	0	0	0
6	20	20	10	13	2	0	8	8	1	0	0	0	1	0	0	0
7	20	20	17	14	0	0	3	7	1	0	0	0	1	0	0	0
9	20	20	13	12	0	0	7	8	0	1	0	1	0	0	0	0
mean	20.00	20.00	11.88	9.38	0.00	0.00	7.75	8.50	1.00	0.50	0.00	0.00	0.50	0.00	0.00	0.00
sd	20.0-20.0	18.75-20	3.36	3.54	0-0.25	0-1.5	3.01	2.83	0.75-1	0-2.25	0-0.25	0-1	0-1	0-1.25	0-0	0-0
Test	Wilcoxon T=6.0 P=1.00		T-test T=-1.56 P=0.16		Wilcoxon T=5.0 P=0.423		T-test T=0.48 P=0.64		Wilcoxon T=13.5 P=0.60		Wilcoxon T=4.0 P=0.789		Wilcoxon T=9.0 P=0.787		Wilcoxon T=1.0 P=1.00	

18 days	Eat		Idle		Teat-seek		Ruminate		Play		Sleep	
lamb	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled
1	6	5	4	2	1	1	*	*	1	0	*	*
2	12	3	3	1	1	0	*	*	0	1	*	*
3	5	5	2	2	0	0	*	*	1	4	*	*
4	7	2	1	2	0	1	*	*	0	0	*	*
5	8	2	2	3	1	0	*	*	0	1	*	*
6	7	10	2	3	1	0	*	*	0	0	*	*
7	13	10	4	4	0	0	*	*	0	0	*	*
9	8	7	4	5	0	0	*	*	1	0	*	*
mean/median	8.25	5.50	2.50	2.50	0.50	0.25			0.38	0.75		
sd/Q1-Q3	2.82	3.25	2.0-4.0	2.0-3.25	0.0-1.0	0-0.25			0.52	1.39		
Test	T-test T=-2.04 P=0.080		Wilcoxon T=10.0 P=1.00		Wilcoxon T=2.5 P=0.465				T-test T=0.81 P=0.44			

Table 9.4a. The number of observations spent in each posture by lambs 25 days after either c+td by RR or handling as controls (n=8). Table 9.4b The number of observations spent in each behavioural state by lambs 25 days after either c+td by RR or handling as controls (n=8). Mean (±sd) or median (Q1-Q3) number of observations spent in each posture for each group and each posture is presented. The statistical test used and the level of statistical probability is also shown.

25 days	Normal		S1		V1		V2		Abnormal		SS		V3		LL	
lamb	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled
1	20	20	11	13	0	1	9	6	0	0	0	0	0	0	0	0
2	17	20	10	10	0	0	7	10	3	0	0	0	3	0	0	0
3	20	20	10	14	1	0	9	6	0	0	0	0	0	0	0	0
4	20	20	11	10	0	0	9	10	0	0	0	0	0	0	0	0
5	20	15	13	10	0	0	7	5	0	3	0	0	0	3	0	0
6	20	20	11	13	0	2	9	7	0	0	0	0	0	0	0	0
7	20	20	6	13	0	0	14	7	0	0	0	0	0	0	0	0
9	20	20	10	8	2	6	8	6	0	0	0	0	0	0	0	0
Mean/Median	20.00	20.00	10.25	11.38	0.00	0.00	9.00	7.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
sd/Q1-Q3	20-20	20-20	1.98	2.13	0-0.25	0-1.25	2.20	1.89	0-0	0-0	0.00	0.00	0-0	0-0	0.00	0.00
Test	Wilcoxon T=3.0 *P*=1.00		T-test T=0.96 *P*=0.37		Wilcoxon T=0.85 *P*=0.273		T-test T=-1.80 *P*=0.12		Wilcoxon T=1.5 *P*=1.00				Wilcoxon T=1.5 *P*=1.00			

25 days	Eat		Idle		Teat-seek		Ruminate		Play		Sleep	
lamb	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled
1	7	6	2	5	0	0	4	6	1	2	0	1
2	9	7	4	4	0	0	7	9	0	0	0	0
3	1	13	11	2	0	0	7	5	0	0	1	0
4	9	7	3	8	0	0	8	5	0	0	0	0
5	10	7	5	7	0	0	5	4	0	0	0	2
6	10	12	8	1	0	0	2	7	0	0	0	0
7	4	10	4	2	0	0	10	6	1	2	1	0
9	10	6	4	2	0	0	10	4	0	0	2	2
Mean/Median	7.50	8.50	5.13	3.88	0.00	0.00	6.63	5.75	0.25	0.50	0.50	0.63
sd/Q1-Q3	3.34	2.78	2.95	2.59	0,0	0,0	2.83	1.67	0.46	0.93	0.76	0.92
Test	T-test T=-0.63 *P*=0.55		T-test T=-0.74 *P*=0.48		Wilcoxon T=2.5 *P*=0.465		T-test T=-0.68 *P*=0.52		T-test T=1.49 *P*=0.18		T-test T=0.36 *P*=0.73	

9.3.1.4. *Neurohistochemistry*

Examination of the control tissue sections (labelled with AVP or CRH oligonucleotide probes after RNase pre-treatment, or labelled with scrambled AVP or CRH probes), using bright-field illumination at low magnification, revealed that no cells contained labelling and there was very little or no randomly distributed background labelling.

After 3 weeks of incubation at 4°C, the AVP oligonucleotide probe produced clear and consistent labelling concentrated within the PVN. Expression of CRH mRNA was much lower than that of AVP. This may reflect problems with the *in-situ* methodology using the CRH oligonucleotide probe. Very little or no randomly distributed background labelling was present and never exceeded a density of 1.5 (±0.5) square pixels.

On visual inspection, using bright-field illumination at low magnification (x10), AVP-labelling was more dense in the magnocellular than parvocellular division of the PVN. In contrast, little CRH-labelling was observed in the magnocellular region. CRH labelled cells were found mainly in the parvocellular division of the PVN.

Counting labelled cells revealed that, on average (±SEM), 206.5 (± 19.2) parvocellular neurones in the PVN were labelled with AVP mRNA in c+td lambs. Handled lambs showed labelling in an average of 183.8 (±9.6) parvocellular neurones. This difference was not statistically significant at $P<0.05$ ($T_{1,7}=0.91$, $P=0.395$).

The mean (±SEM) density of silver grains within AVP labelled parvocellular neurones was 321.4 (±90.2) and 287.2 (±32.1) square pixels in c+td and handled lambs respectively, but again this difference was not significant ($T_{1,7}=0.66$, $P=0.532$).

No differences were found in the number of parvocellular neurones expressing CRH mRNA in the PVN (67.1(±30.5)) and 69.5 (±21.3) in treated and control animal respectively, ($T_{1,7}=0.21$, $P=0.840$). Likewise no difference was found in the density of silver grains within CRH labelled cells 22.8(±10.6) and 21.3 (±8.6) in treated and control animal respectively, ($T_{1,7}=0.47$, $P=0.654$).

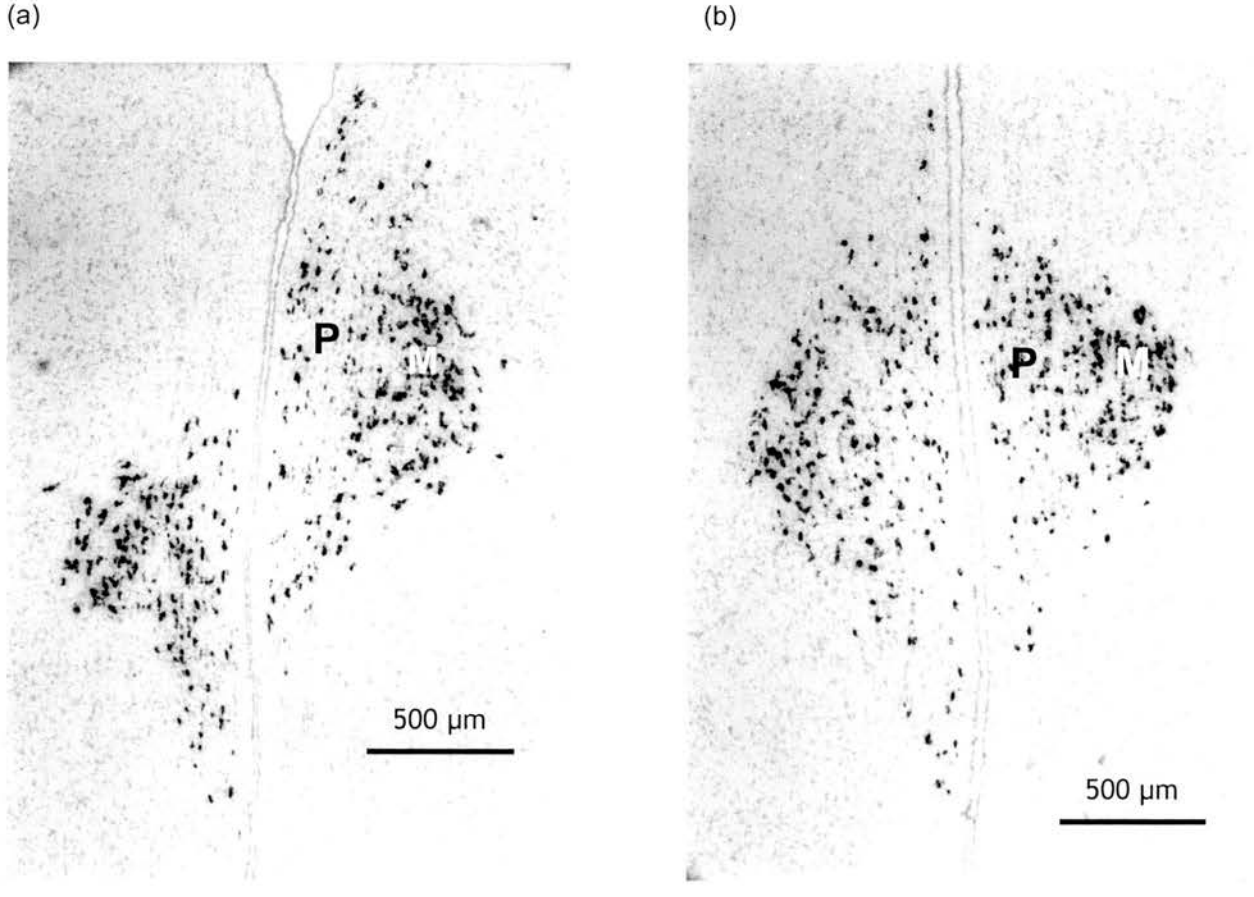
There was also no difference between groups in either the ratio of AVP to CRH expressing neurones 3.2(±1.2) in c+td, 3.4 (±1.7) in handled controls ($T_{1,7}=0.29$,

p=0.781), nor in the ratio of the density of expression within these cells (17.7(±9.7)) in c+td and 15.3(±.3.9) in handled control lambs ($T_{1,7}$=0.77, p=0.465). These results and statistical analyses are presented in table 9.5. Bright-field photomicrographs showing examples of the distribution and density of AVP labelling within the PVN are presented in figure 9.5.

Table 9.5. The mean (±SEM) number of cells expressing AVP or CRH mRNA, the mean (±SEM) density of expression of AVP or CRH within these cells and the mean ratio of AVP:CRH (±SEM) expression are shown. The statistical test used to analyse the significance of these results and the level of significance is also presented.

mRNA	Mean no. of cells (±SEM)		Mean density of grains (sq pixels ±SEM)		Statistical difference (Paired Student's T-test n=8)	
	C+td	Control	C+td	Control	Cells	Density
AVP	206.5(±19.2)	183.8(±9.6)	321.4(±90.2)	287.2(±32.1)	T=0.91	T=0.66
					P=0.395	*P*=0.532
CRH	67.1(±30.5)	69.5(±21.3)	22.8(±10.6)	21.3(±8.6)	T=0.21	T=0.47
					P=0.840	*P*=0.654
AVP:CRH	3.2(±1.2)	3.4(±1.7)	17.7(±9.7)	15.3(±3.9)	T=-0.29	T=0.77
					P=0.781	*P*=0.465

Figure 9.5. Bright-field photomicrographs (using Ilford G5 emulsion) of; (a) an example of AVP mRNA expression in the pPVN of c+td lambs, (b) an example of AVP mRNA expression in the pPVN of handled control lambs. The greatest concentration of AVP mRNA was located in the magnocellular division of the PVN (M), but strong labelling was also found in the parvocellular division (P), the area of interest in this study.



9.3.2. *Experiment 2*: *Tail-docking*

9.3.2.1. *Lamb weight*

Pairs of lambs were selected for each treatment group on the basis of weight to ensure that weight was balanced across groups. There was therefore no difference in the mean weight of lambs in each group at the start of the trial ($T_{1,6}$=0.17, p=0.87). There was also no difference in the weight of lambs in each group at the end of the trial ($T_{1,6}$=0.32, P=0.72).

9.3.2.2. *Lesions*

The median (Q1-Q3) severity of tail lesions when lambs were euthanised for tissue collection was 1.0 (0.0-1.5).

9.3.2.3. *Behavioural observations*

Behaviour was observed on two occasions, for three hours each, on days 12 and 18 after treatment. There was a great deal of variation within each group in the expression of both normal and abnormal behaviours. Both treated and control animals performed normal and abnormal behaviours.

Frequency of active behaviour

12 days after treatment, all active behaviours were expressed by lambs in both groups. Handled lambs appeared to show some more head-turning behaviour than tail-docked lambs but this difference did not reach significance at $P<0.05$ ($T_{1,6}=2.38$, $P=0.063$). When then different elements (head turn to back, flank or outside of hind leg) of the combined head-turning score were examined, no differences in the individual types of head turning were observed ($W=6.0$, $P=0.181$, $T_{1,6}=1.20$, $P=0.29$ and $T_{1,6}=1.00$, $P=0.36$ respectively). No further differences were found between groups in expression of active behaviours.

18 days after treatment tail-docked lambs eased quarters more frequently than control lambs. This difference did not reach significance at $p<0.05$ ($W=0.00$, $P=0.059$) and was not reflected in the overall frequency score of active behaviour (REQ, $T_{1,6}=-1.22$, $P=0.28$). No further differences were found between the groups. These results are presented in tables 9.6 and 9.7.

Postures and behavioural states

Very few abnormal postures were observed in any of the lambs. Ventral lying with extended legs (V3) was observed on a few occasions in lambs in both groups and there was no difference between the groups ($W=2.0$, $P=1.00$ and $W=0.0$, $P=0.371$ for 12 and 18 days after treatment respectively). No differences were found in the postures expressed by the lambs at 12 days or at 18 days after treatment. These results are presented in tables 9.8a and b and 9.9a and b.

Table 9.6. The frequency of active behaviours expressed by lambs 18 days after either c+td by RR or handling as controls (n=8). Mean (±sd) or median (Q1-Q3) frequency of expression for each group and each behaviour is presented. The statistical test used and the level of statistical probability is also shown.

	Restlessness		Ease quarters		Foot-stamp/kick		REQ		head-turn		play		tail-wag		teat-seek		rub		horn	
Twin	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	
1	8	8	5	1	0	1	13	10	0	2	0	1	2	2	5	8	2	0	4	
2	32	14	1	5	2	0	35	19	5	5	2	1	10	6	4	4	17	0	3	
3	11	6	2	0	3	0	16	6	0	0	4	1	1	2	6	4	0	1	3	
4	24	41	5	5	2	0	31	46	2	5	5	1	4	0	5	1	2	16	5	
6	19	24	1	3	0	4	20	31	1	3	1	1	0	4	6	6	0	1	0	
7	7	5	1	3	0	0	8	8	0	6	1	1	0	3	5	3	0	0	0	
Mean	16.83	16.33	2.50	2.83	1.17	0.83	20.50	20.00	1.33	3.50	2.17	1.00	2.83	2.83	5.17	4.33	3.50	3.00	2.50	
sd	9.95	13.98	1.97	2.04	1.33	1.60	10.52	15.74	1.97	2.26	1.94	0.00	3.82	2.04	0.75	2.42	6.69	6.39	2.07	
TEST	T-test T=-0.11 *P*=0.92		T-test T=0.28 *P*=0.79		T-test T=-0.32 *P*=0.76		T-test T=-0.10 *P*=0.92		T-test T=2.38 *P*=0.063		T-test T=-0.32 *P*=0.76		T-test T=0.00 *P*=1.00		T-test T=-0.85 *P*=0.43		T-test T=-0.12 *P*=0.91		T-test T=0.00 *P*=1	

Table 9.7. The frequency of active behaviours expressed by lambs 25 days after either c+td by RR or handling as controls (n=8). Mean (±sd) or median (Q1-Q3) frequency of expression for each group and each behaviour is presented. The statistical test used and the level of statistical probability is also shown.

	Restlessness		Ease quarters		Foot-stamp/kick		REQ		head-turn		play		tail-wag		teat-seek		rub		horn
Twin	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td
1	12	18	4	2	0	2	16	22	0	2	0	2	0	4	3	6	4	1	0
2	43	18	6	4	3	3	52	25	0	2	1	2	42	14	8	4	4	5	1
3	19	11	3	2	3	1	25	14	0	0	2	0	17	1	5	3	3	2	7
4	20	27	11	8	7	7	38	42	0	1	6	0	18	20	3	3	5	5	1
6	31	24	14	1	3	0	48	25	5	2	0	0	5	2	3	3	6	3	9
7	6	8	2	2	0	4	8	14	0	1	0	1	5	1	7	8	0	1	0
Mean/Median	21.83	17.67	5.00	2.00	2.67	2.83	31.17	23.67	0.83	1.33	1.50	0.83	14.50	7.00	4.83	4.50	3.67	2.83	3.00
sd/Q1-Q3	13.35	7.28	4.80	2.56	2.58	2.48	17.71	10.29	2.04	0.82	2.35	0.98	15.27	8.05	2.23	2.07	2.07	1.83	3.95
TEST	T-test T=-0.85 *P*=0.43		Wilcoxon T=0.0 *P*=0.059		T-test T=0.16 *P*=0.88		T-test T=-1.22 *P*=0.28		T-test T=0.65 *P*=0.540		T-test T=0.16 *P*=0.88		T-test T=-1.50 *P*=0.19		T-test T=-0.34 *P*=0.75		T-test T=-1.11 *P*=0.32		T-test T=-0.64 *P*=(

Table 9.8. (a) The number of observations spent in each posture by lambs 18 days after either c+td by RR or handling as controls (n=8). (b) The number of observations spent in each behavioural state by lambs 18 days after either c+td by RR or handling as controls (n=8). The mean (±sd) or median (Q1-Q3) number of observations spent in each posture or behaviour for each group and each posture is presented. The statistical test used and the level of statistical probability is also shown.

	Normal		S1		V1		V2		V3	
12 days	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled
1	30	30	21	20	0	5	8	5	1	0
2	30	30	15	20	2	0	13	10	0	0
3	30	29	21	20	3	1	6	9	0	0
4	28	30	20	17	1	0	9	13	0	0
6	30	30	26	21	0	0	8	7	0	2
7	29	28	19	24	4	0	6	6	0	0
Mean/Median	30.00	30.00	20.33	20.33	1.50	0.00	8.33	8.33	0.00	0.00
sd/Q1-Q3	29.25-30.0	29.25-30.0	3.56	2.25	1.63	2.00	2.58	2.94	0-0	0-0
Test	Wilcoxon T=3.0 *P*=1.00		T-test T=0.00 *P*=1.00		T-test T=-2.09 *P*=0.091		T-test T=0.00 *P*=1.00		Wilcoxon T=2.0 *P*=1.00	

12 days	Eat		Idle		Teat-seek		Ruminate		Play		Sleep	
	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled
1	11	5	11	14	0	1	8	5	0	0	0	5
2	8	8	15	17	0	0	5	5	0	0	2	0
3	12	8	10	13	0	0	1	8	2	0	5	1
4	7	10	16	13	1	0	4	3	1	0	0	0
6	10	6	19	20	0	0	0	4	0	0	0	0
7	6	15	15	9	2	2	0	3	0	0	6	1
Mean/Median	9.00	8.67	14.33	14.33	0.00	0.00	3.00	4.67	0.50	0.00	2.17	1.17
sd/Q1-Q3	2.37	3.56	3.33	3.78	0-0.75	0-0.75	3.22	1.86	0-0.75	0-0	2.71	1.94
Test	T-test T=-0.15 *P*=0.89		T-test T=0.00 *P*=1.00		Wilcoxon T=1.5 *P*=1.00		T-test T=1.11 *P*=0.32		Wilcoxon T=0.0 *P*=0.371		T-test T=-0.68 *P*=0.52	

Table 9.9. (a) The number of observations spent in each posture by lambs 25 days after either c+td by RR or handling as controls (n=8). (b) The number of observations spent in each behavioural state by lambs 25 days after either c+td by RR or handling as controls (n=8). Mean (±sd) or median (Q1-Q3) number of observations spent in each posture for each group and each posture is presented. The statistical test used and the level of statistical probability is also shown.

18 days	Normal		S1		V1		V2		V3	
	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled
1	28	30	13	17	10	3	5	10	2	0
2	30	30	9	17	8	1	13	12	0	0
3	30	30	17	22	2	2	11	6	0	0
4	29	29	19	17	1	2	9	10	1	1
6	29	30	15	21	2	1	12	8	1	0
7	30	30	22	22	5	1	3	7	0	0
Mean/Median	29.50	30.00	15.83	19.33	3.50	1.50	8.83	8.83	0.50	0.00
sd/Q1-Q3	29.0-30.0	30.0-30.0	4.58	2.58	2.0-7.25	1.0-2.0	4.02	2.23	0-1.00	0-0
Test	Wilcoxon T=3.0 *P*=0.371		T-test T=2.27 *P*=0.073		T-test T=-0.53 *P*=0.62		T-test T=0.00 *P*=1.00		Wilcoxon T=0.0 *P*=0.371	

18 days	Eat		Idle		Teat-seek		Ruminate		Play		Sleep	
	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled	C+td	handled
1	6	5	13	14	1	1	0	5	0	0	10	5
2	6	10	15	18	0	0	1	1	0	0	8	1
3	8	14	13	17	1	0	4	5	0	0	4	2
4	6	10	17	9	0	0	6	4	0	0	1	1
6	7	10	18	15	0	0	3	4	0	0	2	1
7	14	17	10	11	1	0	0	0	0	0	5	2
Mean/Median	7.83	11.00	14.33	14.00	0.50	0.00	2.33	3.17	0.00	0.00	5.00	2.00
sd/Q1-Q3	3.13	4.10	2.94	3.46	0.0-1.0	0-0	2.42	2.14	0.00	0.00	3.46	1.55
Test	T-test T=3.35 *P*=0.89		T-test T=-0.18 *P*=0.86		Wilcoxon T=0.0 *P*=0.371		T-test T=0.88 *P*=0.42				T-test T=-2.82 *P*=0.037	

9.3.2.4. *Neurohistochemistry*

Control tissue sections (labelled with AVP or CRH oligonucleotide probes after RNase pre-treatment, or labelled with scrambled AVP or CRH probes), showed no cells containing labelling and very little or no randomly distributed silver grains representing background labelling.

Again, after 3 weeks of incubation at 4°C, the AVP oligonucleotide probe produced clear and consistent labelling concentrated within the PVN. Expression of CRH mRNA was again much lower than that of AVP. Very little or no randomly distributed background labelling was present and never exceeded a density of 2.0(±0.4) square pixels.

Visual inspection, using bright-field illumination at low magnification (x10), revealed that AVP-labelling was again more dense in the magnocellular than parvocellular division of the PVN and that little CRH-labelling was observed in the magnocellular region.

After quantified analysis, no differences were found between groups either in the number of cells expressing AVP or CRH mRNA, the density of silver grains with cells expressing CRH mRNA or in the ratio of AVP to CRH expressing cells.

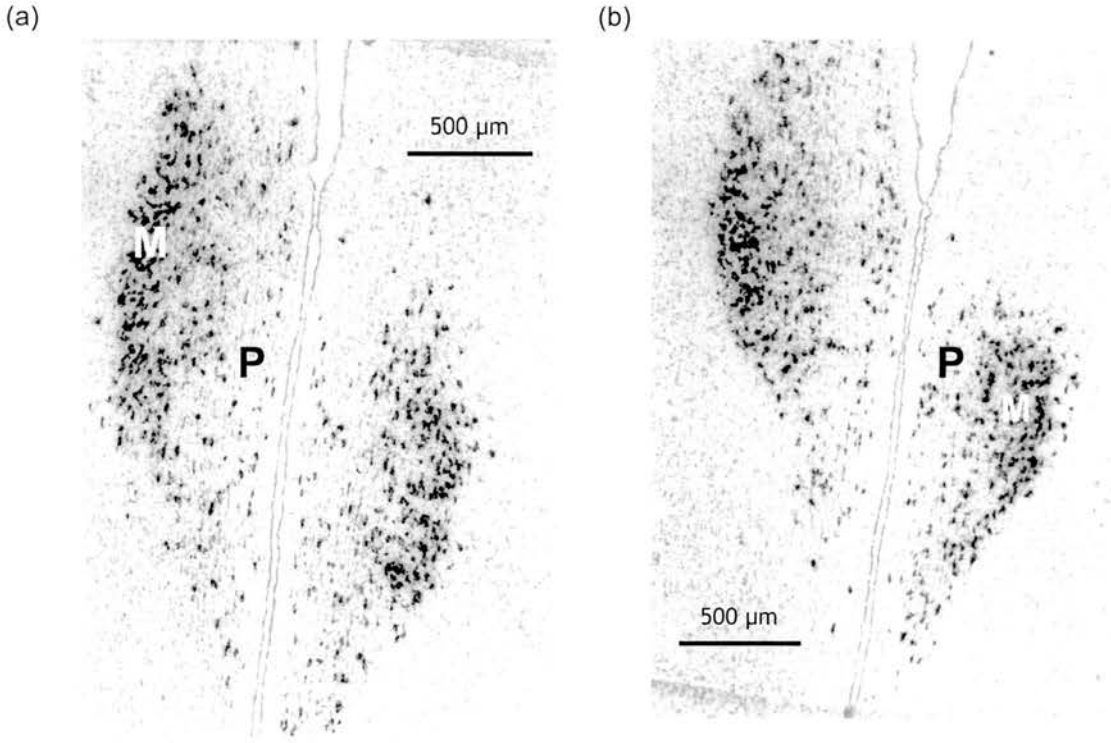
There was a difference in the density of silver grains within cells labelled with AVP. The mean density of silver grains within these parvocellular neurones was 211.9 (±25.2) and 260.7 (34.1) square pixels in tail-docked and control lambs respectively, however this difference did not reach significance at $p<0.05$ ($T_{1,6}$=-2.47, P=0.069).

These results and statistical analyses are presented in table 9.10. Bright-field photomicrographs showing examples of the distribution and density of AVP and CRH labelling within the PVN are presented in figure 9.6.

Table 9.10. The mean (±SEM) number of cells expressing AVP or CRH mRNA , the mean (±SEM) density of expression of AVP or CRH within these cells and the mean ratio of AVP:CRH (±SEM) expression are shown. The statistical test used to analyse the significance of these results and the level of significance is also presented.

mRNA	Mean no. of cells (±SEM)		Mean density of grains (sq pixels ±SEM)		Statistical difference Paired Student's T-test (n=6)	
	td	Control	td	Control	Cells	Density
AVP	287.2 (±41.8)	268.6 (±35.4)	211.9 (±25.2)	260.7 (34.1)	T=0.33	T=-2.47
					P=0.758	*P*=0.069
CRH	48.5 (±6.68)	42.4 (±3.02)	22.6 (±4.85)	22.6 (±3.27)	T=0.91	T=-0.01
					P=0.412	*P*=0.994
AVP:CRH	6.0 (±0.56)	6.6 (±1.25)	10.9 (±2.01)	12.0 (±1.39)	T=-0.44	T=-0.34
					P=0.684	*P*=0.753

Figure 9.6. Bright-field photomicrographs (using Ilford G5 emulsion) of; (a) an example of AVP mRNA expression in the pPVN of tail-docked lambs, (b) an example of AVP mRNA expression in the pPVN of handled control lambs. The greatest concentration of AVP mRNA was located in the magnocellular division of the PVN (M), but strong labelling was also found in the parvocellular division (P), the area of interest in this study.



9.4.*Discussion*

9.4.1. *Weight*

The differences in weight between c+td lambs and handled control lambs were not significantly different at $P<0.05$. However, there was a non-significant trend towards lower weight gain between 7-14 days after treatment and for lower weight overall in c+td lambs. This trend is consistent with effects on weight that might be expected as an effect of the experience of chronic pain (Chapman, 1985; Kitchell and Johnson, 1985; Zimmermann, 1986; Short, 1998).

9.4.1. *Severity of lesions*

The average width and score of castration lesions in the experiment 1 was slightly lower than those observed previously in four-week-old lambs (Kent *et al,* 1999). This difference may be associated with breed differences in size. In the previous study, Dorset x Finnish Landrace lambs were used (Kent *et al*, 1999), these lambs are larger than Greyface x Suffolk lambs at four weeks of age (by comparison of two sets of lambs used in this project ($P=0.0069$)). An increase in size has previously been associated with more severe lesions (Kent *et al*, 1999). Variation in the severity of lesions between studies may also be attributed to differences in the level of bacterial contamination in the pen. Despite the slight reduction in the severity of castration lesions in comparison with previous studies, all lesions in experiment 1 were inflamed and showed evidence of infection.

9.4.2. *Behaviour following c+td*

The variation in behaviour in all lambs in experiment 1 was extremely high, making true differences between animals in each group more difficult to detect. Watching the lambs for longer during each observation may have helped to expose differences more clearly. Despite this, differences were found between c+td individuals and their handled siblings.

Teat-seeking and tail-wagging

15 days after castration, before lesions from c+td had reached a peak, c+td lambs showed a tendency to teak-seek more frequently than their handled sibling. This may

indicate that the treated lamb sought comfort or distraction from pain from the lesion. It is considered particularly surprising and important, as twin lambs normally suckle simultaneously in response to a signal from the ewe (Ewbank, 1967).

C+td lambs also showed a tendency to tail-wag more frequently than their handled siblings, although this difference was not statistically significant. Two explanations for this result may be acceptable. Tail-wagging may indicate pain or discomfort in the tail region of c+td animals. Alternatively, as lambs wag their tails during attempts to suckle and during suckling, the increase in tail-wagging in c+td lambs may also be a reflection of the increased the frequency of teat-seeking observed in these animals. Although care was taken not to record instances of tail wagging that were clearly associated with teat seeking, closer consideration of results showed that, in handled lambs, there was a significant predictive relationship between the number of suckling attempts and the number of times the lambs wagged their tails. However, in c+td lambs this relationship was slightly deviated suggesting that on some occasions, something other than the motivation to suckle, induced c+td lambs to tail-wag. This could have been pain. Studies in children and rats have shown that suckling and drinking of certain components of milk, prinicipally sucrose, have some pain-relieving properties (Blass and Watt, 1999; Mukherjee *et al*, 2001; Anseloni *et al*, 2002; Gray *et al*, 2002). Similar increases in teat-seeking were observed in RR c+td lambs after naloxone pre-treatment in comparison with lambs receiving RR c+td alone (Wood, 1991), although Landa (2000) found that suckling of sucrose or from the dam did not influence behavioural responses to tail-docking.

Easing quarters

25 days after castration, when lesions had reached a peak, c+td lambs showed active behaviours more frequently than handled lambs. This was reflected in a significant difference in REQ scores (combined score of restlessness, foot stamping and easing quarters). This score is used to determine whether there is an overall difference in active behaviour, when individual behaviours show no difference between groups. In this case, the difference in the REQ score was primarily the result of the highly significant increase in the frequency of easing quarters shown by c+td lambs. Restlessness also occurred more frequently in c+td animals, but this difference was

small and was not significant at $P>0.05$. These behaviours are also suggestive of the experience of pain in c+td lambs.

Posture and behavioural states

Although there was no change in the posture of the lambs at either 15 or 25 days after treatment, there was a non-significantly increased tendency to spend more time eating in c+td lambs. Feeding behaviour, seen as teat-seeking at 15 days and eating at 25 days, may simply act as a distraction from pain, but as suggested above it may also be a means of reducing pain or distress.

Significance of changes in behaviour

The detection of these subtle changes in behaviour is important and possible despite the problem of wide variations between individuals in the same treatment group. Studies of acute pain from c+td have shown that these behaviours are sensitive indicators of the severity of acute pain suffered by an individual (Molony *et al*, 2002). This study confirms that changes in behaviour also occur in association with long-lasting lesions produced by castration and docking and suggests that these lambs experience some chronic inflammatory pain.

9.4.3. *Behaviour following tail-docking*

In experiment 2, lambs were watched for one hour longer than in the experiment 1, however as the variation in behaviour between individuals in the same group was high, differences remained difficult to detect. 12 days after treatment, there was some evidence that tail-docked lambs showed more head-turning in total, than their untreated siblings, although the target to which the head was turned was not important. After 18 days, tail-docked lambs eased quarters more frequently than their untreated siblings, although again this difference was not statistically significant.

There was no difference in the overall frequency of active behaviours between tail-docked and control lambs at either time point, nor was there any difference in the postures of lambs. Lesions from tail-docking are not as severe as those from castration (peak score usually 1-2 compared to 3.5-5 from castration), and are

therefore likely to be less painful than that from castration. Also, the lesion from tail-docking is less likely to be aggravated during locomotion.

No significant behavioural indications of pain were observed in these lambs. It is possible that the lesions produced by tail-docking in this study were insufficiently severe to produce significant chronic inflammatory pain.

9.4.4. *Neurohistochemistry*

9.4.4.1. *General observations*

Initial examination of the tissue sections in both studies showed that AVP mRNA was expressed more strongly in the magnocellular division than in the parvocellular division. This distribution of labelling is consistent with that in previous studies in both sheep and rats (Nojiri *et al*, 1985; Matthews *et al*, 1993). Expression of CRH mRNA in both studies was much lower than that of AVP and lower than that observed in other *in-situ* hybridisation studies using ovine oligonucleotide CRH probes (Matthews *et al*, 1991). This may reflect problems with the *in-situ* methodology using the CRH oligonucleotide probe. For example, the stringency of washes following hybridisation may have been too high, or the length of exposure too short. Many other components of the procedure may also have been at fault and more extensive experience with the method may have improved the labelling. As changes in AVP mRNA expression are the principle characteristic of chronic stress, improvement of the method for CRH labelling was not attempted. Analysis was carried out on CRH results for both experiments, but no major conclusions have been drawn from CRH data.

Despite measures to minimise error in the counting and density of labelling of cells during *in situ* hybridisation histochemistry, some level of error remains. Some magnocellular neurones are present in the parvocellular division and discrimination between cells types is difficult. Also, in AVP mRNA studies, labelling was strong and therefore determination of the numbers of cells that overlapped in a cluster was difficult. This is an intrinsic difficulty with the *in situ* hybridisation method and does not apply to this project alone.

9.4.4.1. *No neurohistochemical evidence of chronic inflammatory pain*

In the first study, despite inflammation and infection in lesions and the presence of 'pain' behaviours, no evidence of chronic pain was found in tissue sections containing the PVN of c+td lambs. There was no change in the expression of AVP or CRH mRNA in the pPVN of treated lambs, when compared to handled controls. The number of cells expressing mRNA, the density of silver grains within each cell and the ratio of AVP:CRH expression did not change.

In the second study there was again no evidence of changes in the expression of AVP and CRH mRNA in the pPVN that are characteristic of chronic inflammatory pain. There was however, evidence that AVP mRNA was expressed more densely in the pPVN of untreated lambs than in tail-docked individuals. This result is the opposite of that expected if tail-docked lambs were experiencing chronic pain and may reflect differences in the control of the stress response in sheep (Familiari *et al*, 1989, Liu *et al*, 1990, Canny *et al*, 1999)) as discussed in section 8.4.

9.4.4.3. *No effect of the removal of gonadal steroids on CRH and AVP mRNA expression.*

The results of neurohistochemical analyses showed no change in the expression of AVP and CRH mRNA, which also indicates that the removal of sex steroids by castration had no effect on the expression of these neuropeptides in the pPVN. The plasma concentration of testosterone in the blood of ram lambs between 4 and 17 weeks of age fluctuates, but remains approximately four times lower than that in adult rams (Lee *et al*, 1976). The plasma concentrations of lutenising hormone (LH) and follicle stimulating hormone (FSH) also vary in lambs in this age range. Peaks in the concentration of FSH between 5 and 7 weeks and LH at 5 weeks of age exceed the plasma concentration found in adult rams. In their study of the effects of castration on the expression of hormones in the pPVN, Canny *et al* (1999) used 3-year old adult rams. The sudden removal of a high concentration of testosterone is likely to have had a greater influence on the expression of hormones in the pPVN, than the removal of the lower concentration of testosterone in 4-6 week old lambs. Also, Canny *et al* (1999) castrated the rams 10 months before brain tissue was collected and examined for expression of hormones in the median eminence. The

comparatively short-term effects of the removal of testosterone, such as those in the present study of c+td are likely to be different from the long-term effects of castration found by Canny *et al* (1999).

9.4.4.4. *Do chronic inflammatory lesions from castration and tail-docking cause chronic pain?*

The lack of neurohistochemical evidence of chronic inflammatory pain may be explained in more than one way. Firstly, the lack of changes in the expression of AVP and CRH mRNA may indicate that, despite some behavioural evidence of pain or discomfort, the lesions present in this study did not cause sufficient pain to induce changes in the PVN that were indicative of chronic stress.

9.4.4.5. *Species differences in HPA control: positive controls*

Significant species differences exist in the potency of action of AVP and CRH and in the number of parvocellular neurones that express AVP mRNA in non-stressed animals (Familiari *et al*, 1989, Liu *et al*, 1990, Whitnall *et al*, 1992). Therefore, an alternative explanation for the lack of neurohistochemical evidence of chronic pain is that the PVN response to chronic stress may be different in sheep, or responds at a different threshold from that in the rat. In order to demonstrate that changes in the expression of AVP and CRH mRNA are characteristic of chronic inflammatory pain in the sheep, positive controls for this response should have been included in the study.

Experimental models of inflammatory pain in animals include carrageenan injections, and adjuvant induced arthritis in rodents. Other frequently used animal models include those for neuropathic pain and central pain. These models are less appropriate as controls for this study as they either do not last long enough or involve limited inflammation (Walker *et al*, 1999).

Carrageenan

Injection of carrageenan has been used to model inflammatory pain in sheep (e.g. Dolan *et al*, 1999; Dolan and Nolan, 2002) and rats (Xu *et al*, 1995; Willoughby *et al*, 2000). Carrageenan injections produce local inflammation of the joint and soft

tissue, with substantial swelling, for up to seven days (Walker *et al*, 1999). Injection of carrageenan intra-dermally into the intra-digital skin of sheep has been found to produce significant hyperalgesia (Dolan *et al*, 1999). Repeated injections of carrageenan, such that inflammation and hyperalgesia were maintained over weeks rather than days, could provide a suitable chronic model of chronic inflammatory pain in the sheep, but would be difficult to justify ethically.

Arthritis

Arthritis, induced by injection of heat-killed *Mycobacterium butyricum* has been used to model chronic inflammatory pain in rats and is known to induce changes in the expression of CRH and AVP mRNAs in the PVN that are characteristic of chronic inflammatory pain. Lambs of all ages are susceptible to arthritis of different types, collectively known as 'joint ill'. Arthritis in lambs is caused by both streptococcal and/or staphylococcal bacteria, and in older lambs by *Erysipelothrix rhusiopathiae*. Bacteria gain access to, mainly synovial, joints either by local damage or in the blood (Watkins 2000). Susceptibility to the different types of arthritis is determined principally by the immune status and thus the age of the lamb. If arthritis could be experimentally induced in immuno-compromised lambs, an arthritis model similar to that produced in rats might be developed.

Foot-rot

Naturally occurring foot-rot has been used as models of chronic inflammatory pain in sheep (Welsh and Nolan, 1995; Dolan *et al*, 2000). Foot-rot is caused by bacterial infection of the inter-digital skin and laminae of the hoof. The feet become more susceptible to infection when the integrity of the skin in compromised, either by continuous exposure to wet and dirty underfoot conditions, or by injury. Two bacteria are involved, prior infection with *Fusobacterium necroforum* facilitates invasion of the epidermis by *Dichilobacter nodosus.* Lesions of the interdigital skin develop and in severe cases lesions also develop within the hoof, which may result in separation of the sole from the wall and toe of the hoof. The condition is highly contagious and, in warm and damp conditions, has an incubation period of 10-14

days. After this, the condition may persist and become chronic, or resolve and heal naturally (Egerton, 2000).

The use of naturally occurring disease to model chronic inflammation has significant disadvantages over experimentally induced models. The severity of clinical conditions varies dramatically between individuals and therefore some means of assessing severity must be established in order to minimise this variation. The severity of foot-rot may be assessed using lameness scores like that defined by (Welsh *et al*, 1993). The size and severity of the lesion may also be estimated, but this may prove difficult if the lesion is contained within the hoof. Also, in order to select a group of animals in which the onset, severity and duration of disease is similar, access to a large flock is essential. Another problem with the use of foot-rot as a model of chronic inflammation for this study, is that foot-rot is less common in lambs than in the heavier ewes or rams (Egerton, 2000).

Foot-rot has been induced experimentally in Merino sheep by housing the animals in dirty conditions after damaging the inter-digital skin (Raadsma *et al*, 1993). Foot-rot induced in standardised conditions, producing standardised lesions, in susceptible individuals might have provided a suitable positive control model of chronic inflammatory pain in lambs and provide a positive control for this study, but would also be difficult to justify ethically.

9.4.6. *Indications of chronic inflammatory pain at other levels of the HPA axis*

As species specific differences in the mechanisms of control of the HPA axis exist, it is possible that these mechanisms are different in sheep at the level of the pPVN. Evidence of chronic activation should therefore be sought at other levels of the HPA axis. In rats the HPA axis responds to the chronic inflammatory pain of adjuvant induced arthritis at all levels. The increase in AVP mRNA expression in the pPVN is associated with a rise in the concentration of AVP in HPB (Chowdrey *et al*, 1995). The decline in CRH synthesis is associated with a decline in CRH release (Harbuz *et al*, 1992). Expression of POMC mRNA in the anterior pituitary (Stephanou *et al*, 1992), and the concentrations of ACTH and corticosterone in the peripheral circulation (Chowdrey *et al*, 1995) are increased in response to stress. The weight of the adrenal gland has also been found to increase during chronic activation of the

HPA axis (Harbuz *et al*, 1992). Thus this study would have benefited from the measurement of AVP and CRH in HPB, ACTH and cortisol in the systemic blood and from measurement of changes in the weight of the adrenal gland.

9.4.7. *Local endogenous analgesia.*

A second explanation for the lack of neurohistochemical evidence of chronic pain, as well as the limited behavioural evidence of pain, is that lambs are able to reduce the intensity of pain produced by the lesions. Endogenous opioids inhibit the hypothalamic stress response (Suda *et al*, 1992). Enkephalins are co-localised with CRH in a proportion of parvocellular neurones in the PVN (Sankanaka *et al*, 1989; Merchenthaler, 1992). These neurones project to the median eminence (Merchenthaler, 1992) and secretory granules containing enkephalins along with CRH and AVP are found in terminals in the median eminence (Hisano *et al*, 1987). CRH induces the secretion of β-endorphin as a product of the breakdown of POMC. The expression of enkephalin mRNA in the PVN (Harbuz and Lightman, 1989) and the concentration of β-endorphin in peripheral circulation (Engler *et al*, 1989) has been found to increase after exposure to stress. The level of expression and release of these opioids is determined by the nature of the stressor.

I.c.v. administration of β-endorphin results in a reduction in the expression of CRH mRNA in the pPVN, irCRH in the median eminence and expression of POMC mRNA in the anterior pituitary (Suda *et al*, 1992). This was accompanied by a reduction in basal and stress-induced levels of ACTH release (Suda *et al*, 1992). These mechanisms depend on the activation of central mechanisms and are thus likely to occur in association with activation of central stress responses. However, peripheral mechanisms are also present that may minimise pain from c+td lesions, without any activation of the PVN and other central mechanisms.

For example, CRH has autocrine and paracrine effects in the periphery. It was found to be present in inflamed tissue at the site of immune challenge (Hargreaves *et al*, 1989; Karalis *et al*, 1991), but not simultaneously in surrounding tissue or in general circulation (Karalis *et al*, 1991). In *in vitro* studies, CRH has been found to stimulate immune cells, enhancing their function and their release of immune mediators (Karalis *et al*, 1991). CRH also simulates the release of ACTH and β-endorphin from

leucocytes (Karalis *et al*, 1991; Cabot *et al*, 1997). ACTH and β-endorphin have anti-inflammatory and anti-nociceptive function. Thus, CRH has potent anti-nociceptive effects, but its actions locally are dependent on the presence of an inflammatory response in the tissue (Lariviere and Melzack, 2000).

It is proposed that if c+td lesions are below an as yet undefined 'threshold' of severity, peripheral antinociceptive mechanisms, which may include locally released CRH, could be sufficient to control any resulting pain. Thus, no changes in the expression of AVP and CRH mRNA in the PVN indicating chronic inflammatory pain would occur. This hypothesis could be tested by the local administration of CRH antagonists (Jezova *et al*, 1999) or naloxone to inhibit endogenous opioids.

9.4.8. *Conclusion*

In conclusion, chronic inflammatory lesions from c+td resulted in some changes in behaviour that may be indicative of chronic pain. However, no neurohistochemical evidence of chronic inflammatory pain was found. It is proposed that this may be because the lesions in this study were insufficient to cause significant pain and that behaviours expressed were indicative of discomfort or irritation rather than pain. Alternatively, the hypothalamic response to chronic inflammatory pain in the sheep may differ from that observed in the rat.

Investigation of other models of chronic inflammatory pain in the sheep, for example subcutaneous injection of carrageenan, adjuvant induced arthritis or experimentally induced foot-rot, could provide positive controls and would help to establish the detailed characteristics of the HPA response to chronic inflammatory pain in this species. However, it is considered ethically unacceptable to induce such severe chronic pain in order to provide positive controls for the study of the pain from c+td lesions. Another possibility is that local endogenous anti-inflammatory and analgesic responses induced by progressive inflammation and infection of the lesion, were sufficient to control any pain locally and that central hypothalamic responses were not activated. This hypothesis could be tested by the local administration of CRH and opioid antagonists.

Chapter 10

General Discussion

Chapter 10. General Discussion.

10.1. *Introduction*

The studies reported in this thesis were carried out to test the following hypotheses;

1. Lambs undergoing castration and tail-docking by tight rubber ring experience chronic inflammatory pain for up to six weeks, in association with chronically inflamed lesions.
2. The chronic inflammatory pain experienced by lambs is sufficient to induce changes in their behaviour.
3. Quantification of these behavioural changes constitutes a valid measure of the chronic inflammatory pain experienced by rubber ring castrated and tail-docked lambs.

The analgesic self-administration paradigm has been used as a means of investigating the experience of chronic inflammatory pain in rats suffering from AA. Evidence of analgesic selection was used to investigate that validity of AA as a model of chronic inflammatory pain. Similarly, in the studies reported in this thesis, the ability of RR c+td lambs to self-administer the NSAID FM, was used to determine the presence of chronic inflammatory pain.

Up-regulation of the synthesis of AVP and reduction in the synthesis of CRH in the parvocellular portion of the hypothalamic paraventricular nucleus was detected in rats with AA. Using *in situ* hybridisation histochemistry, such changes in the control of the HPA axis were sought in the present studies, to confirm the presence of chronic inflammatory pain in RR c+td lambs.

Throughout the self-administration and neurohistochemical studies, the severity of chronic inflammatory lesions and the expression of potential 'pain' behaviours were recorded to determine the significance of these measures with respect to chronic inflammatory pain. Evidence of a relationship between lesion severity and the magnitude of the change in behaviour was also sought.

10.2. *Evidence of chronic inflammatory pain?*

Some evidence of chronic inflammatory pain from RR c+td was found in the studies reported in this thesis. This evidence is summarised and discussed below.

10.2.1. *Weight.*

Anorexia and reduced weight gain have been associated with the experience of chronic pain in both humans and animals (Chapman, 1985; Kitchell and Johnson, 1985; Zimmermann, 1986; Short, 1998). The weight of c+td lambs appeared to be consistently less than that recorded in handled control groups and than c+td lambs with access to analgesic in the first self administration study and in lambs used in the first neurohistochemical study (chapters 6 and 9). Further, in both studies of self-administration of analgesic, CD lambs consistently appeared to eat more than CN lambs, indicating that analgesia reduced the effects of pain on appetite. However, on analysis, no statistically significant differences in weight or food intake between c+td and handled control lambs were found. It is considered that if differences occurred, they were small in comparison with the degree of variation within groups. This result is in accordance with that of Kent *et al* (2000).

10.2.2. *Severity of lesions*

The severity of c+td lesions reached a peak at between 3 and 4 weeks after application of the RR in all the studies reported in this thesis. This is in accordance with previous studies of the chronic effects of RR c+td (Kent *et al*, 1997; 1999; 2000). The severity of lesions recorded in the first of the self-administration studies was similar to that previously recorded in four-week-old lambs (Kent *et al*, 1999). However, in the neurohistochemical studies and in the second of the self-administration studies, the lesions that developed as a result of RR c+td were not as severe as those previously recorded in lambs of the same age (Kent *et al*, 1999; 2000). Despite these inconsistencies in lesion severity, the presence of inflammation and infection was recorded in all castration lesions and inflammation was recorded in all tail lesions.

In the first self-administration study, the severity of lesions in CD lambs was found to be greater than that in CN lambs. Further analysis revealed that administration of

the NSAID did not have a negative impact on healing, but that the slight, non-significant differences in weight between CD and CN lambs was sufficient to produce a difference in lesion severity. This effect of size is important for the use of RR c+td in the field, where particularly large lambs could be singled out for more careful monitoring, and for treatment if required. It is proposed that good hygiene, aided by the frequent provision of fresh bedding, may have limited the degree of infection and therefore the severity of lesions in the second self-administration study and in the two groups of lambs used in neurohistochemical studies. It is also considered likely that the imposed change of breed, from Greyface x Suffolk to Scottish Blackface lambs, influenced the severity of lesions.

Lesion severity is a factor that must be controlled for, if c+td lesions are to be used as a model of chronic inflammatory pain. Controlled contamination of the rubber rings, applied to lambs housed in clean-bedded pens, is considered likely to help standardise the lesions, reduce variation and to allow the severity of the treatment to be properly assessed for regulatory purposes.

10.2.3. *Behavioural evidence of chronic inflammatory pain.*

In both self-administration studies, CN lambs showed a transient decline in the time spent eating when the lesions reached peak in severity. The time CN lambs spent eating then returned to normal as the lesions healed. Whilst this change was not reflected in a change in DLWG, it is in accordance with the assertion that anorexia is indicative of chronic pain (Kitchell and Johnson, 1985; Zimmerman, 1986). This feeding pattern was not evident in CD lambs, showing that this change in eating behaviour could be reduced or eliminated by the administration of analgesic. This provides some evidence that the transient decrease in time spent eating by CN lambs was caused by the presence of chronic inflammatory pain.

In contrast, the lambs c+td for neurohistochemical studies spent more time eating and showed teat-seeking more frequently than controls. An increase in suckling may be explained if related to studies in human infants and in rats which suggest that suckling and the consumption of milk can reduce the perception of pain (Blass and Watt, 1999; Mukherjee *et al*, 2001; Anseloni *et al*, 2002; Gray *et al*, 2002). This is consistent with the findings of Wood (1991) who showed that routinely c+td lambs

showed less teat-seeking than lambs receiving the opioid antagonist naloxone prior to c+td.

The inconsistencies between the eating behaviour of lambs in the self-administration studies and the neurohistochemical studies reported in this thesis are difficult to explain. The only recorded difference in the treatment of lambs in the different studies was the housing condition used. Lambs in neurohistochemical studies were housed together, with their ewes, in a group whilst, in self-administration studies, the lambs were housed singly with their ewes. It is possible that the influence of other lambs in the group may have increased feeding motivation in c+td lambs. This hypothesis is supported by the studies of Thornhallsdottir *et al* (1987), who found that lambs housed in groups consistently ate more than those housed separately with their ewes.

In all studies, c+td lambs showed more active behaviours than handled controls, although the exact behaviours expressed varied between studies and few behaviours showed consistent changes over time after the application of the rings. Differences in the expression of active behaviour were most apparent in the second study of self-administration. However, in neither self-administration study, was the expression of active behaviour consistently affected by the consumption of analgesic creep feed. Two explanations for this result are proposed. Firstly, active behaviour may be indicative of the intermittent experience of uncontrollable pain, associated with aggravation of the sensitised tissue at the site of the rings and which was not relieved by the administration of FM. Secondly, the expression of active behaviour may not be indicative of the experience of pain in association with chronic inflammatory lesions, but is associated with transient irritation or discomfort that is unaffected by NSAID treatment.

The expression of abnormal postures was most apparent in the second self-administration study and this behaviour was affected by the administration of FM. The postures adopted by CD lambs were indistinguishable from those shown by handled control lambs. In contrast, CN lambs spent consistently more time in abnormal postures, a difference that was significant on three occasions and was directly related to the severity of the chronic inflammatory lesions. It has been suggested that subtle changes in posture can reduce the experience of pain by

minimising aggravation of sensitised tissues (Molony *et al*, 1993). These postural changes may therefore be indicative of the experience of pain over which the lambs have some control. However, their experience of pain may still be severe (Molony *et al*, 1993). This evidence supports the conclusion that the expression of abnormal postures is directly related to the experience of chronic inflammatory pain in RR c+td lambs.

10.2.4. *Evidence of self-administration?*

Investigation of the pharmacokinetics of FM after oral administration in 6 week-old lambs (reported in Chapter 3) indicated that, at a dose of 1mg/kg, the reached a concentration in the blood, sufficient to inhibit prostaglandins, within 30 minutes of administration and probably sooner. Previous studies of feed selection have shown that lambs are able to learn associations between positive and negative experiences and the consumption of novel feeds, even with a delay between feed consumption and perception of consequences of up to eight hours (Burritt and Provenza, 1991; Arsenos *et al*, 2000). It is therefore supposed that the delay between consumption of analgesic feed and the positive pain relieving consequences of its consumption should not represent a barrier to learning the association. In the first study of self-administration of analgesic, a higher plasma concentration of FM was found in CD lambs during the first 3 weeks of preference testing. This difference was significant only on the first week of choice, but coincided with the occurrence of a significant association between the width of the lesions and the plasma concentration of FM in these lambs. The plasma concentration of FM consistently declined from the first week of testing onwards and did not follow the changing severity of chronic inflammatory lesions. This result suggests that the severity of the experience of chronic inflammatory pain peaks before the lesions reach their maximum severity score. As changes in the expression of abnormal behaviour appeared to be positively associated with the changing severity of lesions, it must either be concluded that behavioural assessment of chronic inflammatory pain is invalid or that something other than the decline in the experience of pain was responsible for the decline in the plasma concentration of FM in CD lambs. Several confounding factors were identified that could have resulted in a decline in self-administration of FM despite

continuing experience of chronic inflammatory pain. These included; i) the feeding schedule which encouraged over-consumption of feed on the first day of choice and may have resulted in aversion to the test feeds, ii) the possibility of aversion as a result of adverse gastro-intestinal effects of long term administration of the NSAID and iii) the possibility that lambs were unable to remember what they learned during training if, for example, they ate too little analgesic to reinforce their preference each time they consumed some analgesic feed. Analysis of (total) protein in plasma suggested that adverse gastrointestinal effects of NSAID administration were not a factor.

In the second experiment, the confounding influence of the feeding regime and the single training session were eliminated by separating test feeding from nutritive feeding and by introducing a cycle which incorporated training days and testing days throughout the study. In this study of self-administration, CD lambs overcame food neophobia more quickly than HD lambs, consuming more of the analgesic feed more quickly than HD lambs. Again there was no significant difference in the quantity of feed consumed from the 'analgesic' hopper between groups. However, the plasma concentration of FM increased more quickly in CD lambs, reaching a peak that coincided with maximum lesion severity (34 days after c+td) and showing a decline which coincided with declining lesion severity. The difference in plasma concentration of FM in CD and HD lambs approached significance on day 34 (P=0.088). Thus, although the differences were consistent with the suggestion that the lambs learned to self-administer FM to relieve chronic inflammatory pain from castration and tail-docking lesions, these differences were not statistically significant at P<0.05.

10.2.5. *Neurohistochemical evidence of chronic inflammatory pain?*

In-situ hybridisation histochemistry to seek changes in the expression of AVP and CRH mRNAs in the pPVN, consistent with the presence of chronic inflammatory pain, showed no evidence of an effect of c+td. There were no differences in the number of cells expressing AVP and CRH mRNA, the density of their expression, or in the ratio of AVP: CRH expressing cells between c+td and handled control lambs. Paradoxically, in the study of the neurohistochemical effects of tail-docking, the

density of expression of AVP mRNA was significantly higher in lambs that had not been tail-docked than in their tail-docked siblings.

The results suggest that either; i) lambs do not experience chronic inflammatory pain as a result of RR c+td, ii) that their experience of pain as a result of RR c+td is insufficient to result in changes in the rate of synthesis of AVP or CRH or iii) that the HPA axis of the lamb responds differently in response to chronic inflammatory pain to that of the rat (Harbuz *et al*, 1992; Chowdrey *et al*, 1995). Evidence of significant species differences in the control of the HPA axis in response to acute stress already exists in rats, mice and sheep (Familiari *et al*, 1989, Liu *et al*, 1990, Whitnall *et al*, 1992).

10.3. *Do lambs experience chronic inflammatory pain from rubber ring castration and tail-docking?*

Evidence of chronic inflammatory pain as a result of RR c+td from self-administration and neurohistochemical studies is limited. It is therefore considered insufficient to validate the use of measures of changes in behaviour and of lesion severity for the recognition and assessment of chronic inflammatory pain. On the basis of this evidence, it might be concluded that the degree of inflammation and tissue damage is not directly associated with the experience of pain. Similarly, the changing expression of abnormal behaviours associated with the formation and healing of these lesions could be associated with the experience of mild discomfort or irritation or transient experiences of recurrent acute pain. However, this conclusion would be in conflict with studies of acute pain resulting from RR c+td, in which a reliable relationship between the degree of tissue damage caused by the RR and the magnitude of the quantified behavioural response was found (Molony *et al*, 2002). It is noted that exact parallels between the experience of acute pain and putative chronic inflammatory pain from RR c+td cannot be drawn, because of differences in the peripheral and central mechanisms involved in these two pain states.

It is proposed that the presence of a relationship between the magnitude of the behavioural change and the severity of lesions is indicative of chronic inflammatory pain, the significance of which remains to be determined. This suggestion is

supported by the fact that the administration of the NSAID (FM) eliminated the expression of abnormal postures and the reduced motivation to eat. If this is the case, the self-administration paradigm and neurohistochemical investigation, used to confirm the interpretation of these indices were either ineffective or inappropriate.

In the case of neurohistochemical studies it is possible that the response of the HPA axis to chronic inflammatory pain is different in the sheep from that reported in the rat. In the case of self-administration studies it is concluded that, either the studies were not sufficiently well controlled or the lambs were unable to learn the association between consuming the analgesic feed and the subsequent relief of pain. This may either be because lambs do not have the cognitive capacity to learn the association or because of unidentified problems with the design of the experiments.

The effects of chronic inflammatory pain on the synthesis of AVP and CRH in the pPVN of sheep might be determined using alternative models of chronic inflammatory pain, including chronic administration of carrageenan, experimentally induced arthritis and experimentally induced foot rot. Further evidence of HPA activation at the level of the median eminence, HPB, anterior pituitary and the adrenal gland should also be sought to support the results. Several changes to the self-administration protocol that are considered likely to reduce variation were proposed. These include; separation of the actual administration of the analgesic from the consumption of feed, thus reducing variation in the dose of analgesic administered, the use of twin lambs to minimise variation in feed intake and the controlled contamination of rubber rings in order to standardise lesion severity.

A final conclusion that could be drawn from the results of these studies combines ideas from previous conclusions. It is possible that RR c+td lambs experience some degree of chronic inflammatory pain in association with chronic inflammatory lesions, but below a certain threshold of nociceptive activity, lambs are able to control the pain by adopting changes in posture and through activation of local endogenous analgesic mechanisms, thus avoiding the need for activation of the pPVN or other central mechanisms. In inflamed tissue, CRH is thought to stimulate immune cells, enhancing their release of immune mediators (Karalis *et al*, 1991) and to stimulate the release of ACTH and β-endorphin from leucocytes (Karalis *et al*, 1991; Cabot *et al*, 1997). ACTH and β-endorphin have anti-inflammatory and anti-

nociceptive function. These mediators are not necessarily found simultaneously in surrounding tissue or in general circulation (Hargreaves *et al*, 1989; Karalis *et al*, 1991). Thus, no changes in the central control of the chronically stimulated HPA axis would necessarily be apparent and the experience of pain may be insufficient to enable lambs to discriminate any beneficial effect of consuming analgesic food. This hypothesis could be tested by determining whether the local or systemic administration of CRH antagonists (Jezova *et al*, 1998), naloxone or other antagonists to inhibit endogenous anti-nociceptive systems, are accompanied by increases in the expression of abnormal behaviours.

10.4 *Conclusions and implications for animal welfare*

The studies reported in this thesis further support the relationship between assessments of lesion severity and quantified changes in behaviour that have been associated with acute pain. The validity of the use of subtle changes in posture (including extension of the hind legs during ventral lying and statue standing) and changes in feeding motivation to quantify chronic inflammatory pain was supported as the consumption of analgesic creep feed resulted in elimination of the abnormal expression of these behaviours. Some evidence that c+td lambs select more analgesic feed was also present in these studies, although the responses were not reliable within and between individuals.

No evidence was found of changes in the control of the HPA axis that are characteristic of chronic inflammatory pain in rats, but it is considered possible that this is a reflection of species differences in the control of the chronically activated HPA axis. Definitive evidence of the experience of chronic inflammatory pain was not provided by either the self-administration paradigm or the neurohistochemical studies. The use of changes in behaviour to assess chronic inflammatory pain must still be validated, but it is proposed that the incidence of abnormal lying and changes in feeding motivation are reliably associated with the severity of chronic inflammatory pain. The results of these studies also indicate that flunixin meglumine, administered orally to lambs at a dose of 1mg/kg, effectively reduces inflammation and the associated experience of chronic inflammatory pain without evidence gastrointestinal toxicity following long-term administration.

From the perspective of the welfare of commercially reared lambs, these results should be considered important. The evidence supports the assertion that chronic inflammatory lesions from RR c+td are painful, a conclusion that can be used to oppose of the use of RRs commercially in older lambs, as suggested by FAWC (1994), particularly as the need for c+td is subject to debate.

The potential use of RR c+td lesions as a model of chronic inflammatory pain was demonstrated in self-administration studies, where administration of FM reduced the expression of abnormal behaviour. The c+td of lambs by RR reliably produces chronic inflammatory lesions that form and heal over a period of 6-7 weeks, with a peak in severity at 3-4 weeks after ring application. If a reliable association between the severity of chronic inflammatory pain, the severity of chronic inflammatory lesions and changes in the expression of 'pain' behaviours could be established, RR c+td lesions in male lambs might provide a useful, ethically more acceptable and inexpensive model in which to test the therapeutic effects of anti-inflammatory agents and analgesics for use in sheep, for studies of antinociceptive mechanisms and for more fundamental studies.

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Appendix A

Appendix A.

15-keto-13,14-dihydro Prostaglandin $F_{2\alpha}$ EIA Protocol

Preparation of solutions

All water used in preparations of all EIA reagents and buffers must be 'Ultrapure' i.e. free from all trace organic contaminants. Activated carbon filter cartridges or other organic scavengers should be used.

Solution preparations are described in appendix B.

Method for EIA for 13,14-dihydro,15-keto prostaglandin $F_{2\alpha}$

Precautions

As many of the chemicals used in this procedure are volatile, highly flammable and irritant on inhalation it is recommended that the whole procedure be carried out in a fume cupboard. Latex rubber gloves and a lab coat should also be used at all times. Following evaporation of methyl and ethyl acetate in the vacuum desiccator the vacuum should only be released within the fume cupboard so that any vapour is not inhaled.

Procedure

1. Remove required plasma samples from the freezer and leave to thaw at room temperature.
2. Take one 10µl aliquot of prostaglandin out of the freezer and pulse microfuge to gather all the solution into the bottom of the Eppendorf.
3. Remove lid and place in tube rack in vacuum desiccator. Desiccate for approx. 1 hour until all methyl acetate has evaporated.
4. Meanwhile begin purification of the plasma samples. Samples should be divided between two columns to ensure that columns are purifying the sample with sufficient repeatability.
5. Acidify the sample for purification by adding 50µl of concentrated hydrochloric acid per ml of plasma. Leave to sit at 4°C for 15 mins.

6. Microfuge for 2 mins at 3000rpm to remove any precipitate. Pour off the supernatant into a fresh Eppendorf, use a dropping pipette if necessary to ensure all of the sample is taken.
7. Prepare the C18 reverse phase column by washing with 2ml ethanol followed by 2ml UHP water. Apply 500µl at a time and then use the adapter and 5ml syringe to gently push the liquid through the column (should take approx. 30 secs to push through each aliquot) Allow the liquid coming through the column to drip into a trough marked waste.
8. Apply 500µl of acidified sample to the column. Allow the sample to drip through the column without pressure. Allow the liquid coming through the column to drip into the waste beaker.
9. Wash the column with 2ml UHP water, 2ml ethanol, 2ml hexane in aliquots as before using the adapter and syringe to apply gentle pressure as before. Again allow the washes to drip through into the 'waste' beaker.
10. Elute the sample using 1.5ml of ethyl acetate in two 500µl lots. Apply the first 500µl and allow to drip through the column into a fresh Eppendorf, this should take about 5 mins. The second 500µl of ethyl acetate can then be applied. This should be pushed gently through the column as before using pressure from the syringe taking approx. 30 secs.
11. Dispose of columns and any waste from plasma samples in a clinical waste bag (including columns). The contents of the 'waste' beaker can be evaporated off in the fume cupboard over night.
12. Samples should be analysed at 2 dilutions initially to ensure that the concentrations fall within the standard curve and can be calculated. Once the range of concentrations of PG in plasma have been determined, only the appropriate dilution should be necessary.
13. Take the vacuum desiccator, still under vacuum, to the fume cupboard and release the vacuum, thus allowing fumes to disperse safely.
14. Dilutions of the PG can now be carried out to produce a standard curve for assay. Care must be taken during these dilutions that the measurement of each quantity is absolutely accurate or the concentrations calculated during the assay will not be reliable.

15. Reconstitute the PG using 1ml of EIA buffer to make a stock solution of 100μg/ml. Pulse microfuge and then whirlimix to ensure proper dissolution.
16. Dilute a 1μl sample of the stock solution to 1μg/ml by whirlimixing with 99μl EIA buffer.
17. From the 1μg/ml solution prepare a 1ng/ml solution by whirlimixing a 1μl sample with 999μl EIA buffer.
18. The standard curve should have eight point made by serial 1:1 dilutions from the 1ng/ml PG solution with EIA buffer.
19. Pipette 500μl of the 1ng/ml solution into a clean Eppendorf and mix with 500μl of EIA buffer. Whirlimix to ensure proper mixing. This will provide the next point on the curve at a concentration of 500pg/ml.
20. Pipette 500μl of this solution into a clean Eppendorf and mix with 500μl of EIA buffer. Whirlimix to ensure proper mixing. This will provide the next point on the curve at a concentration of 250pg/ml.
21. Repeat step 20 until all eight concentrations for the standard curve have been prepared.
22. PG solutions in EIA buffer should be stored on ice and used within 12hrs or frozen immediately, as PGs and used within a few months are not stable within aqueous buffers.
23. Meanwhile evaporate off the ethyl acetate from the samples in the vacuum dissector
24. Once fully evaporated, reconstitute in 10μl of ethanol and 490μl EIA buffer.
25. Assay samples at two dilutions to ensure that the concentration of PG lies within the concentration of the standard curve. (Assay at full concentration as above and at half this concentration ie take 250μl and dilute to 500μl using EIA buffer.)
26. The pre-coated plates may now be prepared for incubation. Take the required number of plates from the freezer and allow them to come up to room temperature.
27. Wash the plate using 250μl of pre-prepared wash buffer (see preparation protocol) in each well. Invert the plate and tap or shake the contents out.

Plate set up:

	1	2	3	4.....
A	Blank	S1	S5	*
B	Blank	S1	S5	*
C	NSB	S2	S6	*
D	NSB	S2	S6	*
E	Bo	S3	S7	*
F	Bo	S3	S7	*
G	TA	S4	S8	*
H	TA	S4	S8	*

NSB- Non-specific binding, Bo- Maximum binding, TA-Total activity, S1-S8-standards 1-8, *-sample

28. Add 100µl EIA buffer to NSB wells and 50µl EIA buffer to maximum binding wells.
29. Add 50µl of standard, in duplicate and in increasing order of concentration to each of the standard wells.
30. Add 50µl of each sample at two dilutions and in duplicate to each of the sample wells. After the first plate has been read it will become obvious that diluted or undiluted sample falls best within the curve and from this point on only one dilution may be required.
31. Add 50µl of tracer to each well except the TA and Blank wells.
32. Add 50µl of antiserum to each well except the TA, NSB and Blank wells.
33. Cover the plate and incubate at 4°C for between 18 and 24 hours
34. When ready to develop the plate reconstitute sufficient vials of Ellman's reagent (see separate protocol). Once reconstituted Ellman's reagent should protected from light and should be used within 24 hours.
35. Empty the plate by inverting and shaking or tapping out the contents of the wells.
36. Wash the wells 5 times using 250µl of wash buffer per well.
37. Ensure all wash buffer is removed from wells.
38. Add 200µl of Ellman's reagent to each well.

39. Add 5µl of tracer to the TA wells
40. Cover the plate and incubate at 4°C for 90 mins
41. When the incubation time is up ensure that the bottom of the plate is clean by wiping with a tissue.
42. The plate should then be read at 405-420nm using an endpoint programme, see attached programme sheet.

Appendix B

Appendix B.

Preparations for EIA for 15-keto-13,14-dihydro PGF2α in sheep plasma.

Products required, Storage and Stability.

Product	Supplier/Cat No:	State	Temp	Stability
Ellman's Reagent	Alexis Corp/Cayman Cat No: 400050	lyophilised	-20°C	1 year
		reconstituted	4°C	24 hours
Precoated 96 well plates	Alexis Corp/Cayman Cat No: 400007	unopened	-20°C	1 year
		opened packet	2-4°C	Short term
EIA Buffer Concentrate	Alexis Corp/Cayman Cat No: 400060	concentrate	4°C	1 year
		diluted	4°C	1 month
Wash Buffer	Alexis Corp/Cayman Cat No: 400062	concentrate	4°C	2 years
		Diluted	4°C	1 month
Tween 20	Alexis Corp/Cayman Cat No: 400035	concentrate	R temp	Indefinitely
15-keto-13,14-dihydro PGF2α Tracer	Alexis Corp/Cayman Cat No: 416670	lyophilised	-20°C	1 year
		reconstituted	4°C	1 week
15-keto-13,14-dihydro PGF2α Antiserum	Alexis Corp/Cayman Cat No: 416672	lyophilised	-20°C	1year
		reconstituted	-4°C	4 weeks
15-keto-13,14-dihydro PGF2α	Alexis Corp/Cayman Cat No: 16670	methyl acetate	-20°C	2 years
		Diluted water	-20°C	12 hours

All procedures must be carried out using fume cupboard, latex rubber gloves and a lab coat.

A. Ultrapure water

Water used to prepare all EIA reagents and buffers must be de-ionised and free from all trace contaminants, (UHP grade).

B. Ellman's Reagent- preparation from lyophilised reagent

(Alexis Corporation/ Cayman Chemical catalogue No: 400050)

Arrives lyophilised and needs to be reconstituted.

Reconstituted reagent should be protected from light.

Vials supplied containing 100 or 250 determinations (dtn).

100 dtn vial should be reconstituted using 20ml of UHP water.

250 dtn vial should be reconstituted using 50ml of UHP water.

Requirements

Lyophilised Ellman's Reagent

UHP water

Whirlimixer

Procedure

1. Take the lyophilised Ellman's reagent vials from freezer and allow to come up to room temperature.
2. Measure required volume of UHP water (for 100dtn vial add 20ml UHP H_2O, for 250dtn vial add 50ml UHP H_2O) in measuring cylinder and add to vial.
3. Leave vial for 2-3 ins before whirlimixing for a few seconds to reconstitute.
4. Store at 4°C and use within 24 hours.

C. EIA Buffer – Preparation from concentrated EIA Buffer

(Alexis Corporation/ Cayman Chemical catalogue No: 400060)

Requirements

10ml vial concentrated EIA buffer

UHP H_2O

100ml volumetric flask

funnel

Procedure

1. Pour contents of EIA buffer vial into 100ml volumetric flask using the funnel.
2. Rinse the vial with a small volume of UHP H_2O and pour washings into the volumetric flask.
3. Make volume up to 100ml mark including washing from the funnel.
4. Invert flask several times to mix.
5. Store at 4°C until use.

E. Tween20 – preparation from concentrated Tween20 solution.

(Alexis Corporation/ Cayman Chemical catalogue No: 400035)

Requirements

3ml vial Tween20

K_2HPO_4

KH_2PO_4

UHP H_2O

1ml syringe

1 litre volumetric flasks x2

1 litre conical flask x2

1 litre beaker x2

Magnetic hotplate stirrer and flea

Thermometer

Funnel

Stop clock

Procedure

1. Prepare a 1.0M phosphate buffer solution by combining 133g K_2HPO_4 and 32.15g KH_2PO_4 in the beaker

2. Add approx. 800ml UHP H_2O and set on the magnetic hotplate stirrer (if the phosphates are slow to dissolve heat the solution up to 40°C) until the phosphates have completely dissolved.
3. Decant into the volumetric flask using the funnel and make up to the 1litre mark including washings from the beaker and funnel.
4. Put the lid on the flask and invert several times to mix the solution.
5. Decant this solution into a conical flask and cover.
6. In a beaker combine 0.5ml (measured using 1ml syringe) of Tween20 with 10ml of the pre-prepared phosphate buffer.
7. Dilute this solution with 500ml of UHP H_2O and set to mix on the magnetic stirrer for 10 mins
8. Decant into a volumetric flask.
9. Make the solution up to the 1 litre mark with UHP H_2O to include the washings from the beaker and funnel.
10. Stopper the flask and invert several times to thoroughly mix the solution.
11. Decant into a 1 litre conical flask and cover.

D. Wash Buffer – preparation from concentrated wash buffer

(Alexis Corporation/ Cayman Chemical catalogue No: 400062)

Requirements

5ml vial of concentrated wash buffer

UHP H_2O

Tween 20

1000µl Gilson

volumetric flask

500ml beaker

1ml syringe

500ml conical flask

Parafilm

Procedure

1. Using the 1000µl Gilson take a 2.5ml sample of wash buffer concentrate and decant into the beaker.
2. Using the syringe measure 0.5ml the diluted solution of Tween20 and deposit that into the beaker.
3. Decant into a volumetric flask
4. Make this mixture up to 1L with UHP H_2O including washings from the beaker.
5. Invert the volumetric flask several time to mix the solution using parafilm to cover the top.

Decant into the conical flask, cover and store at 4°C before use.

15-keto-13,14-dihydro PGF2α Acetylcholinesterase EIA Tracer – Reconstitution
(Alexis Corporation/ Cayman Chemical catalogue No: 416670)

Requirements

100dtn or 500dtn vial of lyophilised 15-keto-13,14-dihydro PGF2α AChE EIA Tracer
EIA buffer (see protocol for dilution of concentrate above)
Whirlimixer
10ml syringe and needle

Procedure

1. Add 6ml to 100dtn vial (or 30ml for 500dtns) EIA buffer to the vial using the syringe and needle and leave to stand for 2-3 mins
2. Whirlimix the solution to ensure proper reconstitution
3. Store at 4°C until use
4. Whirlimix before use

15-keto-13,14-dihydro PGF2α EIA Antiserum – Reconstitution
(Alexis Corporation/ Cayman Chemical catalogue No: 416672)

Requirements

100dtn or 500dtn vial of lyophilised 15-keto-13,14-dihydro PGF2α AChE EIA Tracer

EIA buffer (see protocol for dilution of concentrate above)

Whirlimixer

10ml syringe

Procedure

1. Add 6ml to 100dtn vial (or 30ml for 500dtns) EIA buffer to the vial using the needle and syringe and leave to stand for 2-3 mins
2. Whirlimix the solution to ensure proper reconstitution
3. Store at 4°C until use
4. Whirlimix before use

Standard Curve using 15-keto-13,14-dihydro PGF2α

(Alexis Corporation/ Cayman Chemical catalogue No:16670)

Requirements

EIA buffer

15-keto-13,14-dihydro PGF2α 1mg/100µl in methyl acetate

Access to a fume cupboard

1.5ml snap top Eppendorfs

1.5ml screw cap Eppendorfs

Gilson pipettes

Pipette tips

Vacuum desiccator

Procedure

1. Aliquot contents of vial of 15-keto-13,14-dihydro PGF2α in 10µl lots into 1.5ml snap top Eppendorfs
2. Return excess Eppendorfs to the freezer for future use
3. Place one aliquot into vacuum desiccator and desiccate for 1 hour
4. Take the vacuum desiccator, still under vacuum, to the fume cupboard and release the vacuum.
5. Dilutions of the PG can now be carried out to produce a standard curve for assay. Care must be taken during these dilutions that all measuring of samples is

absolutely accurate or the concentrations calculated during the assay will not be reliable.

6. Reconstitute the PG using 1ml of EIA buffer to make a stock solution of 100μg/ml. Pulse microfuge and then whirlimix to ensure proper dissolution.
7. Dilute a 1μl sample of the stock solution to 1μg/ml by whirlimixing with 99μl EIA buffer.
8. From the 1μg/ml solution prepare a 1ng/ml solution by whirlimixing a 1μl sample with 999μl EIA buffer.
9. The standard curve should have eight point made by serial 1:1 dilutions from the 1ng/ml PG solution with EIA buffer.
10. Pipette 500μl of the 1ng/ml solution into a clean Eppendorf and mix with 500μl of EIA buffer. Whirlimix to ensure proper mixing. This will provide the next point on the curve at a concentration of 500pg/ml.
11. Pipette 500μl of this solution into a clean Eppendorf and mix with 500μl of EIA buffer. Whirlimix to ensure proper mixing. This will provide the next point on the curve at a concentration of 250pg/ml.
12. Repeat step 11 until all eight concentrations for the standard curve have been prepared.
13. All PG solutions in EIA buffer should be stored on ice and used within 12hrs as PGs are not stable within aqueous buffers.

Appendix C

Appendix C.

Functions of components of *in situ* protocol.

Component	Function
Prehybridisation incubation	Facilitates penetration of hybridisation buffer into the tissues, optimising blocking of non-specific binding sites before the probe is added to the buffer.
Hybridisation incubation	Facilitates hybridisation of the denatured probe to the target mRNA.
^{35}S-adenosine triphosphate (^{35}SdATP)	Radioactive nucleotide that will bind to oligonucleotide probe.
terminal deoxynucleotidyl transferase (TdT)	A DNA polymerase that catalyses the tailing of the radioactive nucleoide to the 3'-OH terminal of an oligonucleotide probe
EDTA	Deactivates Mg^{2+} ions that activate nucleases, thereby stopping the tailing reaction when required. Also acts as a buffer to provide a suitable environment for hybridisation
NaCl	A buffer ingredient for hybridisation, stabilises pH without affecting stringency
Tris HCl	A buffer ingredient for hybridisation, stabilises pH without affecting stringency
Ficoll	A non-ionic polymer of sucrose, increases the volume and the effective concentration of the probe without effecting ionic balance of hybridisation buffer. May also bind impurities.
PVP	Increases the volume and the effective concentration of the probe. May also bind impurities.
BSA	Protein used to out-compete non-specific binding of the probe to protein.
Salmon sperm DNA	Used to out-compete binding of the probe to proteins and other non-specific DNA binding sites.
Glycogen	Large polymer to deter non-specific binding.
Yeast tRNA	Used to out-compete binding of the probe to non-specific RNA binding sites.
Dextran sulphate	Increases the volume of hybridisation mixture without diluting the probe, increasing the effective probe concentration allowing effective coverage without excessive use of probe.
Formamide	Denatures the oligonucleotide allowing hybridisation at lower temperatures. Can be used to increase stringency of washes to remove non-specific binding but this is unnecessary for oligonucleotide probes.
Paraformaldehyde	Mild protein cross-linking agent providing preservation suitable for light microscopy, but also allowing penetration of probe.
SSC	Used as a buffer controlling the ionic concentration of washes, thus controlling stringency. Decreasing concentration of SSC increases the stringency of the wash therefore reducing non-specific binding of the probe.
37°C	Temperature of washes also important, any change in temperature has a large effect on stringency. Any significant increase in temperature during washes could cause loss of all binding.

Appendix D

Appendix D

Photographic images of the chronic inflammatory lesion caused by rubber ring castration in a representative lamb.